Development of Nanoelectroporation-based Biochips for Living Cell Interrogation and Extracellular Vesicle Engineering

DISSERTATION

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Abstract

Intracellular delivery of exogenous materials (nucleic acids, proteins, molecular probes, nanodevices, etc.) plays a key role in a diversity of biomedical and pharmaceutical applications ranging from gene editing, cell-based therapy, regenerative medicine, biomanufacturing of therapeutic molecules, to fundamental research of molecular mechanism in diseases. Precise, rapid and benign introduction of biomolecules into a large population of cells at single cell resolution has thus long fascinates the scientific community. To circumvent the safety concern raised by viral vectors, a variety of non-viral delivery approaches have been developed, including chemical carrier-mediated methods and physical membrane-penetrating methods. Although novel synthetic lipoplex and polypelex nanocarriers provide opportunities for targeted delivery both in vivo and in vitro, the delivery process is slow and inefficient -- only a small fraction of encapsulated materials is eventually delivered to cell interior after endocytosis followed by endosomal escape. Electroporation has been the leading physical delivery method since its invention about four decades ago. Bulk electroporation (BEP) is the commercially available electroporation system. While BEP offers the advantage of simplicity to use without any package of delivery materials, it suffers from low cell viability and significant cell-to-cell variation owing to the non-uniform electric field imposed on the cells randomly suspended in the bulk solution.
A rapid growth of microfluidics has been witnessed since 2000, as microfabrication techniques get mature. The spatial confinement offers an opportunity to accurately control over fluid transport as well as the electric field distribution.

In this work, a miniaturized version of electroporation -- nano-electroporation (NEP) was integrated in three biochip platforms, which could revitalize this classic physical delivery method. Large-scale single-clone-resolution living cell interrogation, including single-cell motility, drug resistance, and underlying molecular mechanisms, based on a 3D NEP-cell migration platform was demonstrated using a clinically relevant study of patient-derive glioma stem cells (GSCs) in the highly lethal brain tumor – glioblastoma. This platform was utilized to follow the dynamics of phenotypic shift of cancer stem cells and thus has potential to shed a new perspective for current solid tumor research and help develop novel therapeutic strategy specifically targeting a subpopulation with high plasticity within cancer stem cells.

To enhance the transfection efficiency and the versatility of transfected cell types (i.e., both adherent and suspension cells) in the 3D NEP platform, a scalable microfluidic device enabling rapid, massive and accurate hydrodynamic-based cell trapping critical for large-scale 3D NEP cell transfection was introduced. Various cargoes from large plasmids to small oligos have been shown to be uniformly NEP-delivered into the trapped cell array in a controllable and uniform manner. Such innovative micro-/nano-technology-enabled cell transfection platform could be valuable for cell reprogramming and ex vivo cancer adoptive immunotherapies.
For the first time, we reported that NEP can not only serve as a unique gene delivery tool, capable of deterministic and benign non-endocytic transfection, but also be a revolutionary extracellular vesicle (EV) engineering tool promising for exosomal mRNA/miRNA transfer in regenerative medicine and cancer therapy, by orchestrating a heat-shock-mediated cellular responses in NEP transfected cells. An integrated NEP- Tethered Lipoplex Nanoparticles (TLN) biochip was also developed for in situ characterization of exosomal content secreted from NEP-transfected cells at the single-exosome level.
Dedication

To my parents and my girlfriend Xiaoyang Leng
Acknowledgments

I would like to express my sincere appreciation to my advisor, Dr. L. James Lee, for his continuous support and inspiration during my PhD life in The Ohio State University. As a passionate senior researcher, his vision on scientific research has profoundly influenced me. I am deeply motivated by his words and now I have made my decision to continue my academic career after graduation. I also own my special thanks to Dr. Carlos E. Castro who is my co-advisor. I still remember his first class when he used himself as an example to show us how an engineer can make contribution to biology research. His valuable guidance and encouragement meant a lot to me as I struggled in the transition from a student to a researcher. I like to thank Dr. Jonathan W. Song for serving as my committee member and my PhD studies benefited greatly from his class and his advice.

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Fields of Study

Major Field: Mechanical Engineering
# Table of Contents

Abstract ........................................................................................................................................ ii

Dedication ................................................................................................................................... v

Acknowledgments ......................................................................................................................... vi

Vita .................................................................................................................................................. viii

Table of Contents ........................................................................................................................ xi

List of Tables ................................................................................................................................ xvi

List of Figures ................................................................................................................................. xix

Chapter 1 Introduction .................................................................................................................. 1

1.1 Background ............................................................................................................................ 1

1.2 Objective and Outline ............................................................................................................. 3

Chapter 2 Literature Review ........................................................................................................ 6

2.1 Fluidic Mechanics in Micro-/Nano-scale .............................................................................. 6

2.1.1 Governing Equations ........................................................................................................ 6

2.1.2 Surface Tension and Contact Angle .............................................................................. 8

2.2 Basic Biophysics of Deoxyribonucleic Acid (DNA) .............................................................. 10
2.2.1 Introduction to DNA Molecular Dynamics .................................................. 10
2.2.2 Entropy and Free Energy ............................................................................. 12
2.3 Electrokinetic Phenomena ............................................................................. 14
  2.3.1 Electrophoresis ......................................................................................... 14
  2.3.2 Electrical Double Layer (EDL): ................................................................. 14
  2.3.3 Zeta Potential (ζ) .................................................................................... 14
  2.3.4 Dielectrophoresis (DEP) .......................................................................... 14
2.4 Introduction to Electroporation ...................................................................... 15
  2.4.1 Physical Principles of Electroporation ...................................................... 16
  2.4.2 Bulk Electroporation ................................................................................ 19
  2.4.3 Microscale Electroporation (MEP) ............................................................ 21
  2.4.4 Nano-Electroporation, from 2D to 3D ....................................................... 24
  2.4.5 Cell Manipulation Techniques for Nano-Electroporation ...................... 28
2.5 Biomedical Applications of Miniaturized Electroporation ............................. 31
  2.5.1 Gene Therapy .......................................................................................... 31
  2.5.2 Regenerative Medicine and Cell Reprogramming .................................... 35
  2.5.3 In situ Intracellular Investigation .............................................................. 35
Chapter 3 Nanochannel Electroporation (NEP) – Cell Migration Platform for Living Cell Interrogation of Patient-derived Glioma Stem Cells (GSCs) ................................. 39
3.1 Introduction ..................................................................................................................... 39

3.1.1 Background of Glioblastoma .................................................................................... 39

3.1.2 Glioma Stem Cell (GSC) .......................................................................................... 40

3.1.3 Cell Migration Assay and Glioma Cell Motility ....................................................... 41

3.1.4 NEP-based Living Cell Interrogation of Patient-derived GSCs .................................. 44

3.2 Results and Discussions ................................................................................................ 46

3.2.1 High-Throughput NEP-based Intracellular Biomarker Screening of in
Heterogenous Patient-derived GSC Population via in situ Molecular Beacon (MB)
Hybridization ...................................................................................................................... 48

3.2.2 On-chip Drug Resistance Test .................................................................................. 50

3.2.3 Biomimetic-guided Single Cell Migration Assay Coupled with Drug Test.......... 53

3.2.4 Identification of Molecular Signatures of Drug-resistant Subgroup in
Heterogenous Patient-derived GSC157 Population ......................................................... 57

3.2.5 Real-time Monitor of PN-MES Transition post Ionizing Radiation (IR) by
NEP-delivered MBs against CD133 (PN marker) and CD109 (MES marker) .......... 60

3.3 Materials and Methods ................................................................................................. 64

3.3.1 Single Cell Motility Assay ....................................................................................... 64

3.3.2 In vitro mRNA Detection by NEP-delivered Molecular Beacon (MB) Probes 65

3.3.3 Cell Culture .............................................................................................................. 67
3.3.4 Real-time Measurement of CD133 (PN marker) and CD109 (MES marker) mRNA Expression Level post Ionizing Radiation (IR)................................. 68

3.4 Conclusion............................................................................................................. 69

Chapter 4 Microfluidic Cell Trapping for High Throughput 3D NEP-based Transfection .................................................................................................................. 72

4.1 Introduction........................................................................................................... 72

4.2 Results and Discussion.......................................................................................... 75

4.2.1 CFD Simulation of Flow Velocity Field in Microfluidic Cell Trap Designs... 75

4.2.2 Microfluidic-cell-trapping-assisted 3D High-throughput NEP Platform Design ........................................................................................................... 82

4.2.3 Fabrication of Microfluidic Cell Trap Array.................................................. 83

4.2.4 Fabrication and Characterization of Si-based Nanochannel Array Device..... 86

4.2.5 Optimizing the Cell Density for Microfluidic Cell Trapping....................... 91

4.2.6 Necessity of Precise Cell Positioning in NEP Process............................... 95

4.2.7 Microfluidic Cell Trapping Approach Significantly Improved NEP-based Delivery Efficiency and Uniformity....................................................... 97

4.2.8 Efficient Non-viral Reprogramming via Deterministic Transfection Based on Microfluidic Cell Trapping Assisted 3D High-throughput NEP Platform......... 101

4.3 Materials and Methods........................................................................................ 103

4.3.1 CFD Simulation of the Flow Velocity Field in the Microfluidic Cell Trap... 103
4.3.2 FEM Analysis of the Electric Field Near the Nanochannel During the NEP Process

4.3.3 Assembly of the Microfluidic Cell Trapping Assisted 3D NEP Platform

4.3.4 Operation of Microfluidic Cell Trapping Followed by 3D NEP Transfection

4.3.5 Cell Culture

4.3.6 Bulk Electroporation (BEP) of MEF

4.3.7 Biomolecules for Cell Transfection Experiments

4.3.8 Image Acquisition and Analysis

4.3.9 SEM Characterization

4.4 Conclusion

Chapter 5 Characterization of Therapeutic Extracellular Vesicles (tEVs) Produced by NEP-based Cell Transfection

5.1 Introduction

5.2 Results and Discussions

5.2.1 Comparison of EV Secretion Using Different Transfection Methods

5.2.2 Visualizing Exosome Production in NEP-stimulated Cells

5.2.3 Identification and Quantification of Endogenously-Loaded EV mRNA Content

5.2.4 Distribution of Endogenously-Loaded RNA Content in EV Subpopulation – Exosomes (Exo) and Microvesicles (MVs)
5.2.5 Induced Neurons by Exosomal mRNA Transfer

5.2.6 Endogenous Loading of Regulatory RNA microRNA (miR) in NEP-produced Exosomes

5.2.7 NEP Increases EV Secretion by Triggering Heat-shock-mediated Cellular Responses

5.2.8 Comparison EV Loading Methods: Endogenous RNA Loading by NEP Transfection of Donor Cells with Plasmids vs. Exogenous RNA Loading into Collected Blank EVs by BEP Post-insertion

5.2.9 Improvement of Multiple mRNAs Co-localized in the Same Secreted EVs by Sequential NEP Transfection of DNA Plasmids to MEF Cells

5.3 Materials and Methods

5.3.1 Cell Culture

5.3.2 Cell Transfection

5.3.3 Collection of EVs Secreted by MEF Cells

5.3.4 in vitro Protein Translation

5.3.5 Isolation of Exosome and Microvesicles from Total EVs

5.3.6 Whole-cell Patch Clamp for Induced Neuron (iN) Characterization

5.3.7 FEM Heat Transfer Simulation of the NEP Process

5.3.8 Dynamic Light Scattering (DLS) Goniometer

5.3.9 qRT-PCR Quantification of EV-containing RNA Target Expression Levels
5.3.10 Total Internal Reflection Fluorescence (TIRF) Imaging ......................... 148

5.3.11 TLN assays ............................................................................................ 148

5.4 Conclusion .................................................................................................. 149

Chapter 6 Conclusions and Recommendations ......................................... 150

6.1 Conclusions ................................................................................................ 150

6.2 Recommendations ..................................................................................... 153

6.2.1 Large-scale drug screening based on multi-well 3D NEP platform ........... 153

6.2.2 On-chip migration assay after cell trapping ........................................... 155

6.2.3 Integrated NEP - TLN assay for time lapse studies of EV secretion at the single EV level in response to NEP-based stimulations ...................................................... 159

6.2.4 Polymer-based soft 3D NEP devices ..................................................... 162

References ...................................................................................................... 165

Appendix A: Multi-gene detection by NEP-delivered MBs ......................... 173

Appendix B: Induced Endothelium by in vivo NEP transfection for Ectopic Osteogenesis post bone scaffold (BMP-2/MBG) Subcutaneous implantation ......................... 178

Appendix C: Therapeutic effect study for EV-mediated drug delivery to AML MV4 .. 182

....................................................................................................................... 184
List of Tables

Table 1 Percentage changes of CD44$^{\text{high}}$ and CD133$^{\text{low}}$ phenotypes after NEP-based anti-miR-363(AM363) transfection, revealed by in situ MB hybridization experiments. 59
List of Figures

Figure 2-1 Schematic sketch of laminar flow and turbulent flow ............................................. 7
Figure 2-2 Schematic of HMDS treatment of silicon substrate.................................................. 9
Figure 2-3 The schematic structure of dsDNA molecule at different length scales (a)
DNA coil in solution, (b) DNA model as semi-flexible rod, and (c) DNA double helix
structure and base sequence .................................................................................................. 10
Figure 2-4 Entropic barrier for DNA translocation through nanopores. “Mechanism of
DNA Transport Through Pores” (Muthukumar, 2007). Figure permission from Annual
Review of Biophysics and Biomolecular Structure ................................................................. 13
Figure 2-5 Molecular dynamics showing the progress of an aqueous pore forming within
the lipid bilayer during electroporation ................................................................................. 18
Figure 2-6 Schematic of a bulk electroporation (BEP) system. Figure from “Micro-
nanoscale electroporation”, 2016, Lab on a chip (Chang, Li, et al., 2016) ...................... 20
Figure 2-7 Representative studies of micro- electroporation (MEP) ..................................... 22
Figure 2-8 First-generation Nano-electroporation (NEP) device platform comprised of 2D
nanochannel array and optical tweezer (Boukany et al., 2011) ........................................... 23
Figure 2-9 Dosage control by NEP (Boukany et al., 2011) ..................................................... 26
Figure 2-10 Si-based 3D NEP platform for high-throughput cell transfection. (Chang,
Bertani, et al., 2016) ........................................................................................................... 27
Figure 2-11 Diagram of gene therapies by personalized immunotherapy (Rosenberg & Restifo, 2015). ................................................................. 33
Figure 2-12 In situ Intracellular Investigation by miniaturized electroporation .......... 37
Figure 2-13 Monitor intracellular biomarker within individual living cells by molecular beacon probes ................................................................. 38
Figure 3-1 Schematic of glioblastoma tumor dissemination and invasion: single glioma cells migrate along in vivo oriented fiber-like microstructures such as blood vessels. .... 43
Figure 3-2 Schematic of NEP-cell migration chip platform for performing live-cell interrogation of patient-derived GSCs with single-clone resolution. (Gallego-Perez, Chang, et al., 2016) ........................................................................................................ 43
Figure 3-3 Patient-derived PN GSCs show distinct tumor dissemination pattern and gene expression levels ............................................................................. 45
Figure 3-4 Schematic of NEP-based MB delivery for in situ mRNA hybridization experiment .................................................................................................................. 47
Figure 3-5 High-throughput intracellular biomarker screening of GBM 157 & GBM 528 ........................................................................................................................................ 49
Figure 3-6 Drug resistance test of GBM157 on 3D NEP platform .............................. 50
Figure 3-7 Therapy efficacy studies of different concentrations of Temozolomide and/or the doses of anti-miR363 delivered by 3D NEP at Day1, Day2, and Day4. .......... 52
Figure 3-8 Biomimetic-guided single-clone motility assay coupled with drug resistance test of GSCs by 3D NEP – migration platform ........................................................................ 54
Figure 3-9 Anti-miR-363 effects on migration of GSC 157 ........................................ 56
Figure 3-10 In situ MB hybridization screening by 3D NEP platform identified drug-resistant surviving GBM157 cell subpopulation

Figure 3-11 IR provokes direct transformation of CD133+/CD109- PNs into more aggressive CD133-/CD109+ MES in GSCs

Figure 3-12 Biomimetic-guided single cell motility assay of irradiated GSCs

Figure 4-1 Modeling of a “bottom” standing microfluidic cell trap (with a herringbone pattern) and FEM simulation results

Figure 4-2 Modeling of the microfluidic cell trap (no herringbone pattern) and FEM simulation result

Figure 4-3 Device design for microfluidic cell trapping and high-throughput 3D NEP transfection

Figure 4-4 Representation of soft-lithography-based fabrication of the PDMS microfluidic channel with a cell trap array

Figure 4-5 Schematic of different types of optical lithography

Figure 4-6 High-aspect ratio (>20:1) micro-/nano-structures achieved by Bosch Process etching

Figure 4-7 A nanochannel (ø500 nm) array fabricated by projection photolithography and deep reactive ion etching (DRIE)

Figure 4-8 Microfluidic cell trapping results of Hoechst® stained NK92 cells (blue) using optimized conditions

Figure 4-9 Microfluidic cell trapping improved NEP-based delivery

Figure 4-10 FEM analysis of the electric field near nanochannel during NEP process
Figure 4-11 The microfluidic cell trapping approach significantly improved NEP-based transfection efficiency................................................................. 99
Figure 4-12 NEP-based transfection results with microfluidic cell trapping ........... 100
Figure 4-13 Results of NEP-based transfection of DNA plasmids by microfluidic cell trapping-assisted NEP (microfluidic NEP) in comparison with BEP transfection........ 102
Figure 5-1 Background of extracellular vesicles (EVs) (EL Andaloussi et al. 2013) .... 113
Figure 5-2 Concept of in situ detection and characterization of EV RNA by TLN (Wu et al. 2013) ........................................................................................................... 114
Figure 5-3 Schematic of a 3D Nanochannel Electroporation (NEP) biochip for donor cell transfection ........................................................................................................... 117
Figure 5-4 Comparison of BEP and NEP on YOYO™-1 fluorescence labelled DNA plasmid delivery efficiency at 1 h post-transfection ........................................ 118
Figure 5-5 EV number comparison in different transfection methods. .................... 119
Figure 5-6 Size distribution of NEP-produced EVs .............................................. 120
Figure 5-7 EV production dynamics in NEP and BEP transfected MEF cells. Visualization of EV was achieved by transfection of CD63-GFP plasmids ............... 122
Figure 5-8 Identification and quantification of mRNA expression in EVs by qRT-PCR. ....................................................................................................................... 124
Figure 5-9 NEP-triggered cell-secreted exosomes containing functional mRNAs. .... 125
Figure 5-10 Locate endogenously-loaded ABM mRNA content in a subgroup of EVs exosomes ........................................................................................................... 127
Figure 5-11 Electrophysiological measurement of induced neurons by EV-mediated exogenous ABM delivery. ................................................................. 129
Figure 5-12 Quantification of microRNA expression in EVs by qRT-PCR. ............ 131
Figure 5-13 Schematic of a NEP-transfected cell increasing the secretion of EVs endogenously loaded with intact and functional overexpressed RNAs via a heat-shock-protein-involved chaperone mediated autophagy triggered by a transient yet drastic heat shock during the NEP process. ........................................................................ 132
Figure 5-14 EV production by NEP is heat-shock-mediated .................................. 134
Figure 5-15 Simulated temperature field during NEP from a 200 V and 10 ms pulse... 136
Figure 5-16 Comparison of secreted EVs containing miR-128 by NEP transfection of DNA plasmid to MEF cells vs. existing EVs loaded with pre-collected miR-128 by BEP post-insertion ........................................................................................................ 138
Figure 5-17 Comparison of secreted EVs containing Brn2 mRNA by NEP transfection of DNA plasmid to MEF cells vs. existing EVs loaded with pre-collected Brn2 mRNA by BEP post-insertion. ........................................................................................................ 140
Figure 5-18 EV-mRNAs secretion profiles from NEP transfected MEF cells .......... 143
Figure 5-19 Increased mRNA co-localization in the same EV by sequential-NEP .... 144
Figure 6-1 Schematic of multi-well 3D NEP platform for large-scale drug screening .. 154
Figure 6-2 Schematic of NEP-migration chip concept ........................................ 158
Figure 6-3 Schematic of NEP-TLN biochip system for in situ detection and characterization of EVs secreted from NEP-stimulated living cells ............................. 161
Figure 6-4 Fabrication of soft NEP patch by nanoimprinting ............................... 163
Figure 6-5 Design of a silicon master mold with a nano-pillar array .................. 164
Figure 6-6 Fabrication of nano-pillars using different deep etching methods .......... 164
Figure A-1 NEP/MB-based detection of AXL and PDGFRα in shPDGFRα-treated GBM157 population .......................................................... 175
Figure A-2 Fluorescence images showing MB signals in shPDGFRα-treated GBM157 population, in comparison to control .......................................................... 176
Figure A-3 NEP/MB-based detection of multi-gene expression and oligo RNA ...... 177
Figure B-1 in vivo NEP transfection system set-up .......................................... 179
Figure B-2 ETV2 mRNA expression determined by qRT-PCR 24h post NEP ....... 180
Figure B-3 Confocal images of NEP-transfected skin and muscle tissues ......... 181
Figure C-1 NEP-based transfection of CEPBα plasmids induces upregulation of miR-181a in transfected cells and cell-secreted EVs ................................. 183
Figure C-2 EVs rich in miR-181a can kill Raji cells in 24h .............................. 184
Chapter 1 Introduction

1.1 Background

Intracellular delivery of exogenous materials (nucleic acids, proteins, molecular probes, nanodevices, etc.) plays a key role in a diversity of biomedical and pharmaceutical applications ranging from gene editing, cell-based therapy, regenerative medicine, biomanufacturing of therapeutic molecules, to fundamental research of molecular mechanism in diseases. Precise, rapid and benign introduction of biomolecules into a large population of cells at single cell resolution has thus long fascinates the scientific community. To circumvent the safety concern raised by viral vectors, a variety of non-viral delivery approaches have been developed, including chemical carrier-mediated methods and physical membrane-penetrating methods. Although novel synthetic lipoplex and polyplex nanocarriers provide opportunities for targeted delivery both in vivo and in vitro, the delivery process is slow and inefficient -- only a small fraction of encapsulated materials is eventually delivered to cell interior after endocytosis followed by endosomal escape.

Electroporation has been the leading physical delivery method since its invention about four decades ago. Conventional bulk electroporation (BEP) is the commercially available system in which a mixed conductive buffer containing both suspended cells
and transfection reagents is loaded into the electroporation cuvette with anode and cathode from two ends that apply high-voltage electric pulses (>1000V) to facilitate cargo delivery in permeabilized cells. While BEP offers the advantage of simplicity to use without any package of delivery materials, it suffers from low cell viability and significant cell-to-cell variation owing to the non-uniform electric field imposed on the transfected cells.

A rapid growth of microfluidics based lab-on-a-chip devices has been witnessed since 2000, as microfabrication techniques get mature. The spatial confinement offers an opportunity to accurately control the fluid transport as well as electric field distribution. Microscale-electroporation (MEP), which localizes the electric field to the scale of the cell, provides the possibility of a more benign and uniform delivery method at a significantly reduced voltage (<10V) compared to BEP.

Recently, our lab introduced an innovative nano-electroporation (NEP) technology that is capable of precise dosage-controllable intracellular delivery, not achieved by any established methods. In NEP, a nanochannel (<100-500nm) focuses the electroporation on only an extremely small portion of the cell membrane in touch of the channel. Charged biomolecules, after accelerated inside the nanochannel with a very large local electric field strength, are rapidly injected into cell cytosol. This electrophoresis-based direct “injection” mechanism of NEP enables efficient intracellular delivery for large cargoes such as plasmids and quantum dots, which are otherwise internalized by cells via endocytosis in BEP and microscale-electroporation (MEP). The nanochannel also serves as a diffusion barrier to prevent further mass diffusion in and out of the
transfected cell to achieve dosage control and high cell viability, not achievable in BEP and MEP.

Nano-electroporation (NEP) technology, as a miniaturized version of electroporation, revitalizes this classic cell transfection method. Motivated by the relatively low throughput (i.e., <200 cells) of the first-generation proof-of-concept two-dimensional (2D) NEP system, a three-dimensional (3D) NEP platform, which features a nanochannel array in the z-direction on the solid-state material (i.e., silicon wafer) fabricated by a series of semiconductor cleanroom-based top-down approaches, was recently developed in our lab for a high-throughput NEP- based cell transfection.

1.2 Objective and Outline

In this study, we aim to develop three NEP-based biochip platforms, capable of living cell interrogation and cell/exosome engineering. The NEP-enabled biochips presented in this thesis have the potential to not only shield new insights into the molecular mechanisms modulating tumor dissemination and therapy resistance, but also provide the possibilities of translational cellular and exosome-based therapeutic strategies.

1. We first demonstrated the large-scale single-clone-resolution living cell interrogation capability of a 3D NEP- cell migration platform by a clinically relevant study of patient-derive glioma stem cells (GSCs) from the highly lethal brain tumor – glioblastoma.
2. To enhance the transfection efficiency and the versatility of transfected cell types (i.e., both adherent and suspension cells) of 3D NEP platform for in vitro cell reprogramming and ex vivo cancer adoptive immunotherapies, we next introduced a scalable microfluidics based lab-on-a-chip device enabling rapid and accurate hydrodynamic cell trapping critical for high throughput 3D NEP cell transfection.

3. For the first time, we reported that NEP can serve as not only a unique gene delivery tool with deterministic and benign non-endocytic transfection, but also a platform for producing extracellular vesicles (EVs) containing functional mRNA/miRNA promising for EV-mediated gene/drug delivery by orchestrating a strong heat-shock-mediated cellular responses in NEP transfected cells. An integrated NEP-Tethered Lipoplex Nanoparticles (TLN) biochip was also developed for in situ quantitative characterization of EVs secreted from NEP-transfected cells at single-EV level.

This thesis is presented in the following order.

In Chapter 1, the background and significance of this Ph.D. research work are introduced. The outline of content is also presented. In Chapter 2, relevant theoretical and experimental research from the recent literature is reviewed. In Chapter 3, living cell interrogation of Glioma Stem Cells based on a 3D NEP-cell migration biochip is discussed. In Chapter 4, the design, fabrication and characterization of microfluidic cell trapping in the 3D NEP platform is demonstrated. In Chapter 5, the characterization of NEP-triggered EV secretion is performed and the EV content is measured by the
integrated NEP-TLN platform. Conclusion and recommendations for the future work is presented in Chapter 6.
Chapter 2 Literature Review

2.1 Fluidic Mechanics in Micro-/Nano-scale

2.1.1 Governing Equations

As known for a long time in the field of microfluidics, the governing equation in classic fluidic mechanics -- Navier–Stokes (NS) equation, which originally developed in 19th century for the study of fluidic motion in macroscale, remains valid in submillimeter scale. In fact, NS equation is proven to be still unexpectedly accurate even in nanoscale, if the critical dimension of geometry constraining the fluid D >1nm(Bocquet & Charlaix, 2010). Kinematic Viscosity $v$, one of the most commonly used physical properties in fluidic mechanics, is validated to be the same in microscale as it is measured at bulk condition for most types of common fluidic such as water.

“Side-wall effect”, on the other hand, plays an increasingly dominant role, as fluid confinement approaches microscale and the “volume - to- surface ratio” decreases(Bocquet & Charlaix, 2010). The Reynold number (Re), a highly-important dimensionless parameter in Newtonian fluidics, is often used to predict the flow patterns, namely laminar or turbulent flow, in different situations. Re is determined by three factors: fluidic properties, boundary condition, and flow velocity. Re is often defined as:

$$Re = \frac{uL}{v}$$
where \( u \) is the velocity of the fluid, \( L \) is a characteristic linear dimension, and \( v \) is the kinematic viscosity of the fluid. Generally, when \( Re > 2000 \), turbulent flow happens in which the velocity field of the fluid tends to be stochastic. When \( Re < 1000 \), the fluidic system is in laminar flow, which is characteristic of the orderly and stable flow condition.

![Figure 2-1 Schematic sketch of laminar flow and turbulent flow](image)

For common microfluidic devices \((Re \ll 1)\), Reynolds number \( Re \) is below the threshold 1000 -2000 and therefore the flow can always to be treated as in the laminar flow regime. The advantages of laminar flow in the confined space like microchannels are the highly controllable and predictable hydrodynamics (Sackmann, Fulton, & Beebe, 2014), hardly achieved in bulk solution.

In microfluidic equilibrium condition, since inertial force term can be neglected, the NS equation has a simplified equation, or often referred as Poiseuille equation or Poiseuille law:

\[
- \nabla p + \eta \nabla^2 u = 0
\]

where \( p \) is the pressure, \( u \) is the fluid velocity and \( \eta \) is the dynamic viscosity.
Pressure drop essentially drives the micro-flows and balances the viscous force (friction). Since pressure exerted by capillary force dominates at the microscale, capillary action is ubiquitous in a microfluidic system, in which the fluid advances along the microchannel or micropore by itself, even at the direction against gravity.

2.1.2 Surface Tension and Contact Angle

One important phenomenon to understand the mechanics of microfluidics system is the droplet forming at the interface of liquid and other medium like air and solid. This can be explained by surface tension. In the scale of single molecule at the boundary, surface tension is the result of the imbalance between the attractive cohesion force acted by internal similar molecules and the other forces from external environment. This tendency of contracting the liquid surface is essentially to reduce the free energy.

The wettability of a solid surface is often represented by the measurable value of “contact angle” $\theta_c \in (0^\circ, 180^\circ)$. For a water contact angle $\theta_c$, when $\theta_c < 90^\circ$, the surface is called “hydrophilic” with high surface energy and when $\theta_c > 90^\circ$, it is considered “hydrophobic” with low surface energy.

Surface functionalization is often necessary to adapt the surface property to different applications. For example, a common material used in microfluidic device, untreated polydimethylsiloxane (PMDS) is hydrophobic with water contact angle 115°~120°; however, after oxygen plasma surface treatment or extracellular protein coating, the modified PDMS surface can be hydrophilic with contact angle 40~70° (Chuah et al., 2015; Tan, Nguyen, Chua, & Kang, 2010).
Hexamethyldisilazane (HMDS) is the widely used in silicon microfabrication as adhesion promotor to increase adhesion of common positive photoresist to silicon substrate. When exposed to air, the silicon wafer surface quickly oxidizes and become hydrophilic, absorbing water molecules from the air. Therefore, a thin layer of water forming on the silicon wafer surface compromises the adhesion between photoresist and substrate. HMDS applied by vapor prime changes the SiO2 surface from hydrophilic to hydrophobic.

(a) Chemical reaction of HMDS vapor prime and (b) wettability alteration of Si substrate.

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2.2 Basic Biophysics of Deoxyribonucleic Acid (DNA)

2.2.1 Introduction to DNA Molecular Dynamics

Nucleic acids, including DNA (deoxyribonucleic acid) and RNA (ribonucleic acid), are considered as one of the most important biomolecules, because they encode genetic information and thus control the development of all kinds of living organism and their next-generation. Nucleic acids are essentially biopolymers, consisting of monomers often noted by the names of their nitrogenous bases: A (adenine), T (thymine), G (guanine) if in DNA or U (uracil) if in RNA, and C (cytosine). A tremendous amount of research has been conducted to unveil the physics of nucleic acids, especially the DNA molecule structure in solution, which eventually will facilitate the understanding of their amazing biological functions.

Figure 2-3 The schematic structure of dsDNA molecule at different length scales (a) DNA coil in solution, (b) DNA model as semi-flexible rod, and (c) DNA double helix structure and base sequence
For DNA molecules, when forming the double helix structure, the diameter of DNA helix is \(~2\text{nm}\) and the distance per base pair is \(~0.34\text{nm}\). Contour length \(L\) is defined as the total length of the DNA molecule stretched at two ends. Persistence length \(p\), often utilized to quantify the mechanical property of biopolymers and thus related to local DNA stiffness, is the distance over which the correlation between two end-point vectors tangent to the DNA chain contour become negligible.

Many theoretical models have been used to estimate the spatial structure of DNA chain. Worm-Like Chain (WLC) model, first reported by Benoit and Doty in 1953, has been proved a more accurate structural description for small and intermediate size DNA, considering both short-range elasticity and overall flexibility of the DNA chain. In WLC model, a DNA molecule is regarded as semi-flexible biopolymer, which can be locally modelled as elastic rod in the length scale of persistence length \(p\), and modelled as freely jointed chain (FJC) (or named random walk in mathematical language) subject to thermal fluctuation in the range much longer than persistence length \(p\). WLC model estimates the radius of gyration of DNA chain, which is determined by both the contour length \(L\) and persistence length \(p\) of the DNA molecule.

\[
R_g = p \left\sqrt{\frac{L}{3p} - 1 + \frac{2p}{L} - 2 \left(\frac{p}{L}\right)^2 (1 - e^{-L/p})}\right\]

Where \(L\) is the contour length, and \(p\) the persistence length. In most cases, where \(L \gg p\), a much-simplified equation can be used: \(R_g \approx \sqrt{pL}\).
Based on the enormous computational simulation results and experimental measurement data, the DNA persistence length is widely accepted to be 50nm (i.e. ~0.1kbp) in most physiologically-relevant conditions, i.e., aqueous solutions with 100~200 mM ionic strength at room temperature.

2.2.2 Entropy and Free Energy

Most experiments in this thesis are involved with nucleic acids transfection, in which DNA molecules translocate through confined regions (dimension $D < \text{radius of gyration } R_g$), ranging from the nanochannels in the micro-/nanofluidics device, to the nanopores on the cell membrane of the cell undergoing electroporation. Entropy ($S$), a concept in statistical mechanics, is of necessity in the study of dynamics of DNA in such process.

For certain macroscopic state, the entropy of this system is solely related to the number of possible microscopic configurations at fixed temperature. In general, entropy is calculated by the equation below:

$$S = k_B \ln \Omega$$

Where $k_B$ is Boltzmann constant, $\Omega$ the number of possible microscopic configurations given the macroscopic variables.

Therefore, the confinement of DNA molecules, decreases the number of allowed DNA chain configurations and thus decreases entropy. When the temperature change of the system remains negligible, Helmholtz free energy $F$ will increase, according to the equation below.

$$F = U - TS$$

where $U$ is the internal energy, $T$ is the absolute temperature, and $S$ is the entropy.
The free energy change at the interface of confined region will act as “entropic force” which essentially drives the DNA chain in low entropy confined region towards high entropy free region, and prevents the DNA chain in free region from entering the confined region if no external force is applied. Phenomenon are often described as “entropic recoil” and “entropic barrier”, respectively. Because of the entropic force, a free DNA chain in solution tends to form a spherical coiled conformation, as this macroscopic “blob” state could have a highest number of microscopic configurations.

Figure 2-4 Entropic barrier for DNA translocation through nanopores. “Mechanism of DNA Transport Through Pores” (Muthukumar, 2007). Figure permission from Annual Review of Biophysics and Biomolecular Structure.
2.3 Electrokinetic Phenomena

Other concepts and terminologies from electrokinetic phenomena that will be frequently mentioned in this thesis are listed below:

2.3.1 Electrophoresis

Electrophoresis is the movement of charged particles and the ion “cloud” surrounding them. Related techniques such as gel electrophoresis, is widely used in the separation and analysis of the biomacromolecules such as proteins and DNAs.

2.3.2 Electrical Double Layer (EDL):

Most solids will have a surface charge in the solution. The theory of electrical double layer (EDL) describes the two layers of charged ions surrounding the solid objects. In the well-established classic Stern model of EDL, the inner layer, often referred as Stern Layer, includes the ions that are tightly bonded to the surface due to chemical reaction. And the outer layer, known as diffusion layer, consists of the ions that are loosely attracted to the surface by Coulomb force. EDL effect is more obvious in the systems with large surface area to volume ratio, such as particles or pores in the micron or nanometer scale.

2.3.3 Zeta Potential (ζ)

At the boundary of the diffusion layer is so-called “slipping” plane, an imaginary interface which separates the mobile fluid and the ions attached to the charged solid surface. The electric potential at this plane, relative to the bulk solution, is named as electrokinetic potential, or more often as Zeta potential ζ. Zeta potential is an important parameter in the study of electrokinetic phenomena, and it is experimentally measurable.

2.3.4 Dielectrophoresis (DEP)
Dielectrophoresis (DEP) is the motion of a particle with dielectric property in the solution caused by the force produced by a non-uniform electric field (Pethig, 2010). DEP force is often utilized to separate and characterize the biological particles like cells. Our group has developed a DEP system to massively manipulate the cells and precisely align the cells on top of nanochannel array (Chang, Gallego-Perez, et al., 2015), which is prerequisite to successful nanochannel-based electroporation.

2.4 Introduction to Electroporation

Current cell transfection techniques can be divided into three categories, as discussed below:

- Viral vectors, in which virus encapsulates gene materials of interest which are then delivered into target host cells. Though efficiency of viral transfection is usually higher compared to other non-viral methods, it raises safety concern due to its inherent potential to disrupt the immune response. Retrovirus and lentivirus are the most commonly used viral vectors.

- Chemical methods, in which cargoes are first enclosed in nanocarriers and then uptaken by cells via endocytosis process. A tremendous amount of studies in recent decades have been conducted to develop the novel nanoparticle formations and their therapeutic applications particularly in drug delivery. This method basically relies on bulk mixing, and thus the delivery process is highly stochastic resulting in cell-to-cell variation. Nanoparticle toxicity (De Jong & Borm, 2008) and low transfection efficiency for primary cells (Stewart et al., 2016) and for large plasmids.
(>10kbp) delivery are the current limitations for this method, which require further studies. Popular nanocarriers for drug delivery are lipoplexes and polyplexes.

- Physical methods, in which biomolecules without any “package” directly enter the membrane-permeabilized cells by external forces applied, including mechanical cell membrane penetration (e.g., nano-injection), microscale space constraint (e.g. “cell squeezing” (Szeto et al., 2015)), or transient electrical field (e.g. electroporation). Physical methods usually require the temporary disruption of the cell membrane, followed by the delivery of biomolecules of interest. Common commercialized physical transfection tools are Gene Gun, Bulk Electroporation, Microinjection Systems.

2.4.1 Physical Principles of Electroporation

Electroporation is the transient structure disturbance of cell membranes subject to the electric pulses. It has been one of the most popular non-viral cell transfection methods both in vitro and in vivo since it was first introduced in 1980s, thanks to the fact that it’s easy to implement and its versatility in terms of transfection cell types. It keeps playing an increasing important role in various emerging biological research areas and cell-based therapeutic strategies, such as genome editing (e.g. CRISPR-Cas9), T-cell immunotherapy, cell reprogramming, etc., as the intracellular delivery of exogenous materials is a crucial step and sometimes a prerequisite in those applications.

Cell membrane is known to be basically composed of a lipid bilayer with thickness ~5nm, and functions as a barrier to the cellular components from extracellular environment. Cell membrane is considered an electrical insulator exhibiting excellent dielectric property in
normal physiological conditions, as it maintains the electric potential (~0.07 V) across the membrane due to the significant difference of ion concentration between cytosol and the fluid in extracellular microenvironment.

However, the electric breakdown of cell membrane happens when the transmembrane potential across the lipid bilayer $\Delta m$ reaches a threshold, reportedly a critical value 1 V (Tsong, 1991), because the lipid molecules within the membrane re-orient to form small hydrophilic openings (“aqueous pathways”) on the cell membrane, which is otherwise hydrophobic in the undisturbed state. This breakdown can be either reversible or irreversible, depending on the electric pulse intensity and duration as well as the cell type.

A variety of factors have been studied to model the transmembrane potential $\Delta m$. Schwan equation is one of the most widely-used models to calculate $\Delta m$, as shown below.

$$\Delta m = -f \cdot E(t) \cdot R \cdot \cos \theta \cdot \left(1 - e^{-\frac{t}{\tau}}\right)$$

where $f$ is the cell-shape factor (1.5 for spherical cells),

$E$ is the applied external electric field,

$R$ is the radius of the cell,

$\theta$ is the polar angle between the direction of $E$ and the specific location on the cell membrane,

$t$ is the time, and $\tau$ is the time constant of the cell membrane “capacitor” (characteristic charging time $\sim 1$ µs). Therefore, in the steady-state condition, $\tau \ll t$, and the equation above can be simplified into:

$$\Delta m = 1.5 \cdot E \cdot R \cdot \cos \theta$$
This equation is very convenient to roughly calculate $\Delta m$. Recently, with the rapid development of numerical simulation, sophisticated models with a much larger set of parameters were developed to more accurately predict the electric field distribution and $\Delta m$ on single cells (Krassowska & Filev, 2007; Langus, Kranjc, Kos, Sustar, & Miklavcic, 2016; Zudans, Agarwal, Orwar, & Weber, 2007).

Figure 2-5 Molecular dynamics showing the progress of an aqueous pore forming within the lipid bilayer during electroporation.

From left to right (1) the intact bilayer, (2) a few water molecules enter the lipid regime, starting to form a “water path”, and (3) the neighbouring lipids reorient, stabilizing the “water pore” and allowing the ions to enter. Figure permission from “Cell membrane electroporation-Part 1: The phenomenon”, 2012 IEEE Electrical Insulation Magazine, IEEE (Kotnik, Kramar, Pucihar, Miklavcic, & Tarek, 2012)
2.4.2 Bulk Electroporation

Conventional electroporation technique is done in bulk solution and thus it is called bulk electroporation (BEP). BEP is still a very popular tool in molecular biology study today. Common BEP systems setup is shown in Figure 2.6.

The cells and exogenous molecules are mixed together and suspended in the buffer solution within a cuvette with built-in electrodes connected to the power supply. Then external transient electrical pulses (lasts only a few milliseconds) with high voltage (>1kV) are applied to create intense electric field across cell membrane and temporarily induce the permeabilization of the cell membrane. Meanwhile, the exogenous biomolecules (such as DNAs, RNAs, and proteins) are simultaneously delivered inside (or extracted from) the cell, through a combined effect of diffusion, electrophoresis, and endocytosis depending on the size of cargoes. The underlying biophysical details of membrane electroporation procedures remain elusive, which requires further experimental examinations and the development of more advanced measurement instrument with higher temporal and spatial resolution for real-time monitor.Once the electric field is no longer existed, cell membrane will start to recover. For a portion of cells, because they are inevitably exposed to an extremely high electric field due to the non-uniform electric field distribution in the BEP setup, the membrane disruption become irreversible which eventually leads to cell death. Common commercial BEP systems include Lonza nucleofector from Lonza Group, Gene Pulser® electroporation systems from Bio-Rad, and Neon® Transfection System from Thermo Fisher Scientific.
Figure 2-6 Schematic of a bulk electroporation (BEP) system. Figure from “Micro-/nanoscale electroporation”, 2016, Lab on a chip (Chang, Li, et al., 2016)
2.4.3 Microscale Electroporation (MEP)

Recent years have witnessed a rapid progress on the development of microfluidic-based platforms for achieving electroporation at single cell level. It has been reported that Micro-electroporation (MEP) showed significant advantages over BEP in terms of transfection efficiency, transfection uniformity and cell viability (Adamo, Arione, Sharei, & Jensen, 2013; Chang, Howdyshell, et al., 2015; Fei et al., 2010). In the MEP systems, since the electric field is precisely controlled and focused in the microscale channels, individual cells that are trapped within the micro-aperture or that are flowing through the microchannel can be electroporated using a much lower voltage (5-10 V).

Despite the several benefits of MEP systems mentioned, precision dosage control is still not achieved yet as it shares the similar delivery mechanism with BEP which is based on diffusion and endocytosis-like uptake.
Figure 2-7 Representative studies of micro-electroporation (MEP)

(a) Flow-through electroporation with comb-shaped microelectrode pattern in a microfluidic device (Adamo et al., 2013), (b) Micro-nozzle-based “Sandwich Electroporation” (Fei et al., 2010), and (c) Magnetic tweezers-enabled cell manipulation coupled with micro-pore array etched in silicon platform for high-throughput micro-electroporation (Chang, Howdyshell, et al., 2015).
Figure 2-8 First-generation Nano-electroporation (NEP) device platform comprised of 2D nanochannel array and optical tweezer. (Boukany et al., 2011)

(a) Master mold used for the fabrication of 2D NEP platforms. Illustration of (b) cell loading (via optical tweezers) and (c) nanoporation
2.4.4 Nano-Electroporation, from 2D to 3D

Nanochannel electroporation (NEP) is reported to provide a high precision and benign cell transfection platform, in which a focused and intense electric field is created in the nanochannel geometrically, and thus the biomolecules, after drastic electrophoretical acceleration within the nanochannel, are driven into the targeted cell across its locally nanoporated cell membrane juxtaposed with the nanochannel. In the equivalent electrical circuit of the nanoelectroporation system, the dominantly high-resistance nanochannel with equivalent resistance of 100~600 MΩ almost endures the entire voltage drop. Thus, enormously intense electric field is generated within the nanochannel, which only has an extremely limited length (1-10μm). Nanochannel, furthermore, prevents the uptake of exogenous reagents by the permeable cell via diffusion after electric pulses. Therefore, NEP technology features a unique electrophoresis-based cargo delivery mechanism, which theoretically enables precision control of delivery dosage by building a sophisticated nanochannel electrophoresis model. This simultaneous cargo injection pattern without the involvement of endocytosis, which dominates in either BEP or nanoparticle mediated delivery process, may trigger a series of cellular responses after NEP process, as discussed with more details in Chapter 5.

Since the major electric potential drop happens at the nanochannel because of its high electrical resistance, only the small area of cell membrane, which is exposed to the nanochannel outlet where the external applied electric potential exceeds the normal range of transmembrane potential (TMP), is electroporated. The nanochannel, as a diffusion barrier, also protects the transfected cell from the severe loss of endogenous molecules
after electroporation before the fully recovery of temporarily permeable cell membrane. Above all, localized membrane poration restrained by nanochannel, instead of whole cell membrane poration situation in BEP, significantly improves cell viability after cell transfection for nearly all cell types.

The first-generation NEP device platform (Boukany et al., 2011), as shown in Figure 2.8, was designed to transfet a small cell population (~100 cells), which is sufficient for proof-of-concept in vitro experiments, but not for in vivo animal studies and ultimately clinical trial, where at least one to ten million transfected cells are needed.

Recently a silicon-based 3D NEP device was developed for high-throughput precise and benign NEP cell transfection. This 3D NEP biochip is capable of simultaneous transfection of ~1 million cells per cm² for a single batch. Semiconductor cleanroom micro-/nano-fabrication techniques are used for engineering the nanochannel array in the z-direction of the silicon wafer. The potential biomedical applications of the high-throughput NEP platform have been demonstrated, such as cancer cell intracellular biomarker measurement (Gallego-Perez, Chang, et al., 2016), anti-tumor drug screening (Gao et al., 2016), cell reprogramming (Gallego-Perez, Otero, et al., 2016), etc.
Figure 2-9 Dosage control by NEP. (Boukany et al., 2011)

(a) Calibration curve: pulse length vs. delivered Cy3-ODN copies quantified by fluorescence intensity. Transfection uniformity in (b) NEP and (c) BEP
Figure 2-10 Si-based 3D NEP platform for high-throughput cell transfection. (Chang, Bertani, et al., 2016)

(A) Schematic of 3D NEP system. (B) Cross-section micrograph. (C) Fluorescence expression after electro-injection through nanochannel array. Reproduced with permission from RSC.
2.4.5 Cell Manipulation Techniques for Nano-Electroporation

Since the electric field, which accelerates the charged transfection agents and porates the cell membrane during NEP process, diminished exponentially outside nanochannels, it is critical to achieve close contact between the to-be-transfected individual cells and the corresponding nanochannels. A variety of cell manipulation techniques were integrated with 2D and 3D NEP platforms to achieve accurate cell trapping against nanochannel.

Optical tweezer is a precision instrument in which a focused laser beam can manipulate the dielectric microscopic particle by a piconewton-level force. Owing to the conservation of momentum, the refraction of light path across the particle which essentially is equivalent to the change of the momentum of photons, will in return provide a force on the particle. OT has been broadly used in biophysics research for force-extension measurement of biomolecules. As a particle manipulation tool, OT has been proven to be successfully implemented with the first-generation 2D NEP device and it can achieve position of the cell against the nanochannel under the microscope for NEP transfection in a precise manner. (Boukany et al., 2011) Even though OT can achieve uncoupled nanoscale displacement and positioning accuracy in all three x-y-z-directions, most OT systems provide only one laser beam and thus it can only manipulate one cell at a time. The cell loading time in the OT platform is proportional to the cell number, considering the operation of each cell is fixed. In addition, there is a upper limit of the cell number, because the previous loaded cells will gradually drift away from the nanochannel after a certain amount of time without exerted pushing force. Therefore, OT fits in the 2D NEP platform in which <20 cells can be transfected in one batch due to the throughput limit.
In addition to OT, a simple yet efficient centrifugation-based cell loading method was also developed for 2D NEP platform where a handful of cells can be parallelly loaded onto the nanochannel within a few minutes. (Gao et al., 2014)

To meet the cell number requirement for biological applications, the next generation high-throughput 3D NEP system was developed. Dielectrophoresis (DEP)-assisted cell loading method was integrated with 3D NEP platform. It has been demonstrated that positive dielectrophoresis (pDEP) can parallelly translocate single cells to the nanochannel outlets in a high-throughput manner (>60,000 cells/cm²). (Chang, Gallego-Perez, et al., 2015).

Dielectrophoresis happens whenever a dielectric particle is suspended in a non-uniform electric field. DEP force is the result of dipole interactions of the polarized charges in the dielectric particle and the direction of the force is dependent on the electric field gradient. DEP has been widely used in cell separation and cell characterization. Although DEP occurs in either direct current (DC) or Alternating current (AC) fields, in the DEP-NEP platform, an AC field was used for DEP force generation which is uncoupled with AC-induced electrophoresis in NEP process.

Magnetic tweezer-based cell manipulation was also reported to efficiently position the single cells in 3D microchannel electroporation (MEP) chip. (Chang, Howdyshell, et al., 2015) The cells labelled with magnetic micro-beads via antibody-antigen bonding can be moved to the microchannel outlet by controlled the magnetic field ready for electroporation.

In addition to the electromagnetic forces, cell trapping can also be achieved by combined effort of microstructure and hydrodynamic flow, without the sophisticated design of
electric circuit and the analysis of electromagnetic fields. Recently, a simple “dipping-trap” approach has been developed for rapid and massive cell trapping and high-efficiency NEP transfection, in which individual cells were mechanically trapped within the cell trap unit. (Chang, Gallego-Perez, et al., 2016)
2.5 Biomedical Applications of Miniaturized Electroporation

2.5.1 Gene Therapy

Gene therapy is a simple yet revolutionary concept that treating a disease by modifying the problematic gene. Despite its potential of radically curing otherwise highly lethal disease at gene level, intracellular gene delivery strategy is one of the biggest hurdles. Micro/nano electroporation is a competitive physical gene delivery candidate, with the benefits of instantaneous delivery process, well-defined delivery dosage, high transfection efficiency, minimal cell damage and being viral-free, circumventing the inherited safety concerns posted by viral vectors (Thomas, Ehrhardt, & Kay, 2003).

2.5.1.1 Ex vivo Adoptive Immunotherapy

Genetic engineering of immune cells (e.g., T cells, NK cells) is prerequisite for adoptive immunotherapy which harnesses the patient’s own immune system to combat diseases (Grupp et al., 2013; Porter, Levine, Kalos, Bagg, & June, 2011). For instance, in CAR T therapy, patient’s T cells are transgenically engineered ex vivo, and thus express tumor-associated antigen receptors which enable the targeting of specific cancer cells in vivo (Rosenberg & Restifo, 2015), as illustrated in Figure 2-11. However, current technical challenge of such applications lies in the limited number of “trained” immune cells due to low yielding of plasmids transfection (Kalos & June, 2013).

2.5.1.2 RNA Interference (RNAi) – based Therapy

Specific knockdown of oncogene or proto-oncogene messenger RNA (mRNA) by small non-coding RNA, e.g. small interfering RNA (siRNA) and microRNA (miRNA) has been proven an effective and safe RNAi therapeutics by a set of clinical trials including the first
treatment of an RNAi therapeutic targeting VEGF and KSP in liver cancer patient (Tabernero et al., 2013). Delivery of siRNA or miRNA is key to successful RNAi (Tabernero et al., 2013). Lipid nanoparticles based delivery system has been widely adopted for its good transfection efficiency, biocompatibility and ease of production (Akinc et al., 2010; Wolfrum et al., 2007). However, it inevitably suffers from nonuniform delivery due to its stochastic process in nature. On the other hand, nanochannel electroporation (NEP)-based oligonucleotide delivery has been proven an unprecedented deterministic cell transfection method and thus a perfect technique for RNAi-based therapy which requires precision dosage control (Gao et al., 2014).
Second row: non-viral transfection of genes into T cells is prerequisite to CAR-T immunotherapy. Figures reproduced with permission from Science
2.5.1.3 Genome Editing

Manipulating the genome of the living cells by efficiently adding, deleting, or changing the DNA sequence has long fascinated biology community. In recent five years, the concept of “gene editing” has become a research “hot spot”, fueled by the breakthrough in the development of novel editing tools such as engineered nucleases. The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) - Cas9 (CRISPR -associate protein) technology is the most widely-used platform available for gene editing and it has enormous potential to boost the development of molecular therapeutics against a variety of diseases (Sander & Joung, 2014).

Electroporation, as a common membrane-disrupted intracellular cargo molecule delivery method, has been reported to be used for delivering the CRISPR/Cas9 gene editing materials and thus play a key role in gene editing(Chu et al., 2015; Dever et al., 2016; Maresch et al., 2016). Miniatured electroporation (e.g. nanoelectroporation), as next-generation electroporation with the advantages over conventional bulk electroporation, is believed to potentially further spur the development of genome editing, by improving the delivery efficiency of novel gene editing molecular agents into difficult- to- transfect cell types such as immune cells and stem cells.
2.5.2 Regenerative Medicine and Cell Reprogramming

Cell reprogramming can be performed on either readily available somatic cells (such as skin cells) or induced Pluripotent Stem Cells (iPSCs) (Takahashi & Yamanaka, 2006). Since the dosage of reprogramming factors delivered in individual cell is critical, development of the relevant tool capable of gene delivery in a deterministic manner is crucial. Our group reported an efficient reprogramming of mouse embryonic fibroblasts (MEFs) to functional neuron by a combination of three transcription factors: Ascl1, Brn2, and Myt1l (ABM) via a novel nanochannel electroporation (NEP)- based platform (Gallego-Perez, Otero, et al., 2016). The induced neuronal (iN) cells generated by NEP-delivered factors could have profound effect on the future of regenerative medicine, because NEP-based iN cells could be provided fast and efficiently in a namely unlimited source of patient-specific cells of interest without the introduction of tumorigenic viral vectors.

2.5.3 In situ Intracellular Investigation

Living cells are complex dynamic systems that constantly interact with both intracellular and extracellular environments (Chang, Hu, et al., 2016; R. Phillips, 2013). Development and progression of diseases such as cancer always involve disseminating cells undergoing a variety of physically, physiologically, or pharmacologically induced events (Visvader & Lindeman, 2008; Whiteside, 2008) that remains elusive using conventional cell analysis methods that are based on bulk assays and cell lysis. Below are the summarized novel micro/nano electroporation technologies that enabled real-time living cell measurement at single-cell level. They can perform either direct measurement for the physical signals (e.g.
electric signals, mechanical properties), or intracellular readouts of biomarkers (at RNA level) via molecular sensor probes.

2.5.3.1 Intracellular Recording of Physical Cues

Xie et al. developed a novel vertical nanopillar electroporation device (Fig. 7 A) which successfully recorded intracellular action potentials of living cardiomyocytes in vitro over days with high signal-to-noise ratio (Xie, Lin, Hanson, Cui, & Cui, 2012). As shown in Fig. 7 (B), intracellular recording of action potentials of an HL-1 cardiomyocytes in a developing culture over four consecutive days was achieved via a nanopillar electrode device. Hanson et al. used a similar vertical nanopillar device to characterize nuclear biomechanics in adherent cells (Fig. 7 C). Back in 2008, a patch-clamp nanoelectrode has been reported to achieve single-cell electroporation, in addition to targeted single neuron patch-clamp recording in vivo (Kitamura, Judkewitz, Kano, Denk, & Hausser, 2008) (Fig. 7 E).

2.5.3.2 Intracellular Gene Probing via Molecular Beacons

Molecular beacons (MB), pre-designed oligonucleotide hybridization probes, are frequently used in intracellular cell marker detection. A nanochannel electroporation-based MB delivery platform was introduced by Zhao et al. (Fig. 8 A), which was used to analyze the DNMT3A/B mRNA levels of acute myeloid leukemia (AML) cells following miR-29 upregulation (Zhao et al., 2015). Giraldo-Vela et al. also used MBs, transfected into living cells by nanofountain-based electroporation, for mRNA detection at the single-cell level (Fig. 8 B) (Giraldo-Vela et al., 2015)
Figure 2-12 *In situ* Intracellular Investigation by miniaturized electroporation

(a) schematic of nanopillar electroporation device (b) Long-term, benign and precision intracellular recording of action potentials of a single HL-1 cell over consecutive four days by nanopillar electroporation. (c) Vertical nanopillars for probing of nuclear biomechanics. (d) SEM images of nanopillar electrode device and nanopillar interaction with HL-1 cardiomyocytes. (e) *In vivo* single neuron patch-clamp recording 24 h after electroporation. Left, cells exhibiting GFP fluorescence were electroporated; arrowhead indicates the cell recorded. Middle, real-time measurement of membrane potential fluctuations and action potentials. Right, responses to current injections of 350 pA and -100 pA. Scale bars, 20 µm

Figures reproduced with permission from Nature.
Figure 2-13 Monitor intracellular biomarker within individual living cells by molecular beacon probes

(a) micrographs of wild-type Kasumi-1 AML cells transfected with DNMT3A/B MBs. Reproduced with permission from Wiley (b) HeLa cells transfected with MBs and imaged after 24 h of incubation showing that the electroporated cells divided. Reproduced with permission from ACS.
Chapter 3 Nanochannel Electroporation (NEP) – Cell Migration Platform for Living Cell Interrogation of Patient-derived Glioma Stem Cells (GSCs)

3.1 Introduction

3.1.1 Background of Glioblastoma

Glioblastoma (GBM) is an aggressive tumor arising from astrocytes that form the supportive tissue of the brain. GBM is the most common primary malignancy of brain in adults with poor prognosis and the median survival of patients is less than 2 years (Omuro & DeAngelis, 2013). Current standard of therapy involves surgical resection followed by local ionizing radiation (IR), and temozolomide chemotherapy. However, due to invasive nature of GBM tumor, conventional therapies are, in most cases, unable to fully eradicate the primary GBM tumors, which almost inevitably lead to highly lethal post-therapeutic recurrence (Chaichana, McGirt, Laterra, Olivi, & Quinones-Hinojosa, 2010). However, molecular mechanisms that protect a subgroup of GBM tumor cells from radiation treatment remains elusive. Furthermore, the blood–brain barrier (BBB) limits the drug delivery (Ballabh, Braun, & Nedergaard, 2004), thus making it difficult to completely kill the residual tumour cells by effectively applying chemotherapeutic agents after operation (Stern & Raizer, 2006).
3.1.2 Glioma Stem Cell (GSC)

Though GBM tumor cells share the same histologic origin, they are composed of heterogeneous cell populations with distinct biological hallmarks such as growth and motility. Proneural (PN) and mesenchymal (MES) are two most well recognized GBM phenotypes, which exhibit significantly variation in terms of molecular signatures. MES is a more aggressive and radio-resistant phenotype than PN (H. S. Phillips et al., 2006). Due to the high plasticity of GBM, in certain circumstances such as radiation therapy treatment (Bhat et al., 2013; Halliday et al., 2014; Mao et al., 2013; H. S. Phillips et al., 2006), a termed “PN-MES” transition could occur, similar to “epithelial–MES transition” EMT in other solid tumors (Nakano, 2014).

Recently a subgroup of GBM termed “glioma stem-like cells” (GSCs) have drawn a tremendous amount of attention because of their reported strong tumor-initiating ability (Hemmati et al., 2003; Singh et al., 2004). Experimental and clinically evidence suggests that GSCs greatly contribute to therapy resistance and are presumed to play a key role in recurrence and tumor metastasis (Bao et al., 2006; Beier, Schulz, & Beier, 2011; Capper et al., 2009; Vescovi, Galli, & Reynolds, 2006). However, GSCs’ behaviors vary considerably between individual clone of GSCs. Thus, development of nanotechnology-enabled living GSC interrogation platform for single-clone analysis is urgent, which is not achieved yet by conventional bulk biological assays.
3.1.3 Cell Migration Assay and Glioma Cell Motility

Cell migration is referred to any directed cell movement in biology. Cell migration is a highly coordinated multistep cell behavior which is mechanically controlled by the actin filaments constantly assembled and disassembled in the leading edges and lagging edge within the cell (Pollard & Borisy, 2003). Thus, dynamic cell morphology change, in most cases, can be observed in the motile cells. Cell migration crucially orchestrates a variety of biological processes, ranging from embryonic development (Dirks, 2001; Kurosaka & Kashina, 2008), cell differentiation (Janin, 2013), wound healing (Schneider et al., 2010), to tumor invasion and metastasis (Entschladen, Drell, Lang, Joseph, & Zaenker, 2004).

Although metastasis is the main cause of cancer-related mortality, accounting for 90% of deaths, thorough understanding of the underlying migratory mechanism of metastatic tumor cells and their role in critical steps in metastasis has been far from being reached by now. Since in vivo cell motility monitor requires the expensive imaging device and is often limited by the complex anatomical structures, in vitro cell migration assays were developed for evaluating the migratory potential and the invasive property of cells such as scratch assay, trans-well assay, fence assay (Liang, Park, & Guan, 2007; Poujade et al., 2007; Sagnella et al., 2004).

Rapid development of hardware like microscopes with sufficient optical magnification coupled to high-resolution charge coupled device (CCD) cameras for time-lapse living cell image and the advance of software containing sophisticated mathematical image analysis algorithms for high-throughput single cell tracking together enable direct record of detailed information of the positions and shapes of individual cells for the quantitation of single-
cell motility, also facilitate the *in vitro* single cell migration assays (Hilsenbeck et al., 2016).

Metastatic tumor cells tend to travel along anatomical fiber-like micro/nano topographical cues within the body, e.g. collagen architecture, blood vessels, lymphatic vessels, white-matter tracts, and other fiber network, in solid tumors such as breast cancer and glioma (Bellail, Hunter, Brat, Tan, & Van Meir, 2004; Gallego-Perez et al., 2012; Johnson et al., 2009). Migration behaviors of tumor cells are highly regulated by extracellular matrix (ECM) and tumor microenvironment.

GBM tumor features distinct intracranial distribution patterns due to unique migratory nature of individual glioma cells, as shown in Figure 3-1 below. Microfabricated structures which mimic the *in vivo* cell migration situation is highly demanding for *in vitro* single cell motility assay, which could potentially provide valuable insight into fundamental glioma single-clone analysis and screening of anti-tumor gene therapy such as irradiation and oligo nucleotides (e.g., siRNA, microRNA, anti-microRNA).
Figure 3-1 Schematic of glioblastoma tumor dissemination and invasion: single glioma cells migrate along *in vivo* oriented fiber-like microstructures such as blood vessels.

Figure 3-2 Schematic of NEP-cell migration chip platform for performing live-cell interrogation of patient-derived GSCs with single-clone resolution. (Gallego-Perez, Chang, et al., 2016)
3.1.4 NEP-based Living Cell Interrogation of Patient-derived GSCs

Commercial cell analysis assays (PCR, immunohistochemistry, flow cytometry, western blot, etc.) provide quantitative information on RNA/protein expression down to single-cell level. However, most assays are end-point and thus cell lysis or fixing is inevitable, which prevents further probe of the cancer cells of interest in their physiological conditions. Recent years see a rapid development of nanotechnology which has provided a variety of powerful tools for the living cancer cell interrogation assay with unprecedented spatial and temporal resolution. Herein, we introduce a novel 3D nanochannel electroporation (NEP) - cell migration platform (shown in Figure 14) for on-chip living glioma cell interrogation assay with single clone resolution, in which both intracellular biomarker screening through deterministic and benign NEP-based delivery for in situ molecular beacon (MB) hybridization, and real-time monitor of biomimetic-guided migration status of individual cells can be done on this biochip platform.

Focuses of the single – clone glioma stem cell interrogation on three clinically-relevant aspects, namely intracellular biomarker expression, cell motility and drug resistance, was pursued in two well-established patient-derived PN GBM samples: GBM157, GBM528. Our intracellular screening results showed the remarkable tumor cell heterogeneity between and within these 2 GBM populations, which was also confirmed by tumor-xenograft experiments and transcriptome gene profiling with microarrays, as shown in Figure 3-3.
Figure 3-3 Patient-derived PN GSCs show distinct tumor dissemination pattern and gene expression levels

(a) GBM528 form a well-defined tumor boundaries (b) GBM157 form diffuse tumor boundaries indicative of more active single-cell migration and invasion. (c) Microarray heatmap shows that GBM157 overexpress a set of migration-related genes compared to GBM528 (Gallego-Perez, Chang, et al., 2016)
3.2 Results and Discussions

This novel micro-/nanotechnology-enabled device provided valuable insight into the mechanisms of GSC motility and therapy resistance, which could lead to more efficacious treatments. A CD44-high subgroup of GSCs characterized by enhanced cell motility and drug resistance were revealed by our platform, which potentially motivates the development of novel glioma stem cell targeted therapeutical strategy.
Figure 3-4 Schematic of NEP-based MB delivery for *in situ* mRNA hybridization experiment

(a) Schematic of NEP-based MB delivery platform for single clone intracellular biomarker screening. Left: Cell loading on 3D NEP system set-up. Right: Zoom-in image of a single cell undergoing NEP-based transfection of molecular beacons (MBs) by applying a focused electric field through nanochannel. (Gallego-Perez, Chang, et al., 2016) (b) Schematic of Molecular beacon (MB) probe’s structure before and after hybridization with target.
3.2.1 High-Throughput NEP-based Intracellular Biomarker Screening of in Heterogenous Patient-derived GSC Population via *in situ* Molecular Beacon (MB) Hybridization

While transcriptome microarray data (Figure 15 c) qualitatively revealed the molecular differences between two patient-derived GSC neurosphere samples, this bulk assay failed to provide the gene expression profile at single cell resolution. To further quantify mRNA expressions of 2 migration-associated genes (i.e. Vimentin and CD44), and a novel PN signature gene CD133 (Nakano, 2015) in individual GSC clones, high-throughput NEP-based injection of molecular beacon probes for large-scale intracellular screening of both GBM 157 and GBM 528 samples were conducted. Schematic of NEP-based MB delivery for *in situ* intracellular mRNA detection is shown in Figure 16.

Experimental results of *in situ* molecular beacon (MB) hybridization showed although both GBM populations had similar CD133 expression pattern resulting from proneural GSC phenotypes, GBM157 exhibited significantly higher expression of CD44 and vimentin compared to GBM528 as GBM157 MB intensity distribution shifted to the right. Among three markers, CD44 showed the largest difference across the two GBM populations. The single-clonal fluorescence intensity distributions quantifying biomarker expressions in GBM 157 and GBM 528 are displayed in Figure 3-5. It is also worthwhile to notice that there existed a subpopulation in GBM157 with remarkably higher CD44 expression, as shown in the Figure 3-5 blue-color inset, which presumably contributed most to the aggressiveness and therapy resistance of GSCs. This result indicates GBM157 were a population of overall more invasive GSCs, featuring a subgroup of aggressive mesenchymal-like clones.
Figure 3-5 High-throughput intracellular biomarker screening of GBM 157 & GBM 528 GBM 157 (left) & GBM 528 (right). The results unveiled the variation between and within populations at single clone level. Single-cell fluorescence from hybridized MB probes is quantified by fluorescence imaging.
3.2.2 On-chip Drug Resistance Test

Attention of the following study was focused on the more aggressive GSC phenotype -- GBM157. Another powerful chip-based function, therapy efficacy test, was demonstrated by examining their therapy resistance against conventional GBM chemotherapeutic reagent -- Temozolomide (TMZ) (Friedman, Kerby, & Calvert, 2000), and a potential novel RNAi therapeutic agent, anti-miR363 (Babashah & Soleimani, 2011; Floyd et al., 2014; Sana, Hajduch, Michalek, Vyzula, & Slaby, 2011), respectively. The schematic diagrams of chip-based drug tests are in Figure 3-6 a-b.

![Figure 3-6 Drug resistance test of GBM157 on 3D NEP platform](image)

(a) Schematic of chemotherapy via treatment of temozolomide (TMZ) (b) Schematic of oligo-RNA interference (RNAi) therapy via NEP-based delivery of anti-miR-363. (c) NEP-based delivery of anti-miR-363 had a dose-dependent (two vs five pulses) effect on inducing cell apoptosis within 96h. (d) Additive exposure to both TMZ and anti-miR-363 further decreases the percentage of surviving cells.
Apoptotic effect of anti-miR363 was compared with chemo agent TMZ. The results were shown in Figure 18 c-d. Cell viability assay, via live/death staining followed by fluorescence imaging, was conducted to track the cell viability for up to 4 days. NEP-based injection of anti-miR363 (100nM) induced close to 80% of cell death at 96 h (Figure 18c), higher than ~60% in TMZ treatment group (5uM) (Figure 18d, control). The additive effect of both drugs was found to cause cell viability drop of nearly 90% after 4 days. (Figure 18d) Dosage-dependent apoptotic effect of anti-miR363 was also observed in 3D NEP delivery conditions (i.e., 5 pulses vs. 2 pulses): 5 pulses of NEP-based anti-miR363 injection resulted in more cell death compared to 2 pulses (p=0.014, t-test) at 48 h, but both NEP pulse conditions caused similar cell apoptosis at 96h. (Figure 18c). Complete therapy efficacy study results, under additive treatment of different TMZ concentrations ranging from 5nM to 5uM, and 2 dose conditions of NEP-based anti-miR-363 delivery at three checkpoints (i.e., 24h, 48h, and 96h), are presented in Figure 3-7.
Figure 3-7 Therapy efficacy studies of different concentrations of Temozolomide and/or the doses of anti-miR363 delivered by 3D NEP at Day1, Day2, and Day4.
3.2.3 Biomimetic-guided Single Cell Migration Assay Coupled with Drug Test

It was found that GBM157 clones exhibited remarkably higher motility when guided by substrate physical cues, i.e. parallel micro tracks on PDMS mimicking in vivo structural and mechanical properties (Figure 3-8 a-b), while GBM528 clones displayed negligible motility and seemed insensitive of substrate topography. (Gallego-Perez, Chang, et al., 2016) Interestingly, conventional neurosphere-spreading assays were unable to reveal such huge difference of the migration behaviors across GBM157 and GBM528 population. Moreover, biomimetic-guided single cell migration assay is more representative of actual in vivo GSC dissemination patterns at the single-cell level, as such in vitro motility assays correlated well with in vivo mouse-brain xenograft experiments (Figure 3-3 a-b).

To further analyze the effects of apoptosis-induced drugs on cell motility pattern of the drug-resistant survived GSCs at single-clone level, biomimetic-guided single clone motility assay was conducted for the GBM157 under TMZ and/or anti-miR363 treatment.
(a) Atomic Force Microscopy (AFM) image of micro-engineered substrate topography mimicking *in vivo* fiber-like structures. (Gallego-Perez et al. 2016) (b) Patient-derived GBM157 responded to topography orientation and exhibited guided polarization and motility (c) Anti-miR363 resulted in a significantly drop in cell motility, compared to TMZ treated group and control. (d) Drug-resistant cells that survived the AM363 injection exhibited a much higher cell migration speed than those died within 48 hours. *p<0.05*
As shown in Figure 3-8 c, it was interesting to see that despite both TMZ and NEP-based injection of anti-miR363 can induce significant cell death, only anti-miR363 caused a diminished cell motility within 48 h and there was no major shift of cell motility in the TMZ surviving cell population. Prolonging the on-stage cell imaging of single cell motility assay strikingly showed that survived drug--resistant GBM157 clones quickly recovered and even enhanced their motility 48 h after therapy treatment. (Figure 3-8 d) This result suggested that the drug-resistant subpopulation of GBM157 also exhibited the strong tumor dissemination ability based on the measured high single cell motility velocity. Additional biomimetic-guided single cell motility assay results are shown in Figure 3-9. Biomimetic-guided single-clone motility assay results showed a dosage-dependent effect of anti-miR363 delivered by 3D NEP (i.e., 1pulse and 5pulses) on single cell motility suppression, compared to TEM treatment (5uM).
Figure 3-9 Anti-miR-363 effects on migration of GSC 157
3.2.4 Identification of Molecular Signatures of Drug-resistant Subgroup in Heterogenous Patient-derived GSC157 Population

Large-scale 3D NEP-based injection of CD44/CD133 MB was subsequently conducted to identify the intracellular molecular signatures of this potential drug-resistant and tumorigenic subpopulation within GBM157. It was observed that CD44 MB fluorescence emission distribution shifted to the right (Figure 3-10 a), and CD133 distribution shifted to the left after anti-miR363 injection (Figure 3-10 b). The percentage of CD44/CD133 high and low defined by MB signal before and after NEP-based anti-miR-363 injection is listed in Table 1. Altogether, this CD44\textsuperscript{high} and CD133\textsuperscript{low} mesenchymal(MES)-like phenotypes which harbored strong therapy resistant properties was phenotypically unveiled.

In the future, with more thoroughly understanding of molecular mechanisms, therapeutic plans specifically targeting the MES subpopulation is promising to significantly lower the chance of glioma recurrence and ultimately eradicate it.
Figure 3-10 *In situ* MB hybridization screening by 3D NEP platform identified drug-resistant surviving GBM157 cell subpopulation

(a) CD44 MB fluorescence emission distribution shifted to the right and (b) CD133 distribution shifted to the left after anti-miR363 injection.
(a) CD44^high^ (MB signal >10) percentage jumped to more than 85% post anti-miR363 transfection, compared to only about 10% in control. (b) CD133^low^ (MB signal <20) percentage increased more than 1.5-fold, from less than 65% to 95% after anti-miR363 injection.

Table 1 Percentage changes of CD44^high^ and CD133^low^ phenotypes after NEP-based anti-miR-363(AM363) transfection, revealed by in situ MB hybridization experiments.

<table>
<thead>
<tr>
<th>CD44 MB signal</th>
<th>Control 157</th>
<th>2 days After AM363 transfection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low(&lt;10)</td>
<td>88.6%</td>
<td>12.5%</td>
</tr>
<tr>
<td>High (&gt;10)</td>
<td>11.4%</td>
<td>87.5%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CD133 MB signal</th>
<th>Control 157</th>
<th>2 days After AM363 transfection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low(&lt;20)</td>
<td>63.2%</td>
<td>95.8%</td>
</tr>
<tr>
<td>High(&gt;20)</td>
<td>36.8%</td>
<td>4.2%</td>
</tr>
</tbody>
</table>
3.2.5 Real-time Monitor of PN-MES Transition post Ionizing Radiation (IR) by NEP-delivered MBs against CD133 (PN marker) and CD109 (MES marker)

It was reported that IR treatment of PN GSCs up-regulated MES signature genes while down-regulated PN-related genes, (Mao et al., 2013) indicating a potential shift from proneural (PN) toward mesenchymal(MES) identity. CD133 is a novel PN GSC marker, which is absent in most patient-derived MES GSCs. Like CD44, a well-known MES maker as explained in previous context, CD109 was also a gene highly upregulated in MES and post-IR PN GSCs based on the data of conventional end-point assays, e.g. qRT-PCR, microarray, and immunostaining. However, a more solid evidence with direct observation of this PN-MES transformation is of great importance, ruling out the possibility that this CD109 enrichment was the result of selection in which irradiation kills eliminated most of CD133+/CD109- PN GSCs and thus radio-resistant CD109+/CD133- MES subgroup dominated in the survived GSCs. Herein, our unique 3D NEP-based living cell interrogation platform featuring *in situ* molecular beacon (MB) hybridization, was utilized for real-time continuous detection of potential increase of MES signature gene CD109 mRNA expression in CD133+ PN GSCs, following irradiation. (Figure 3-11) The image data revealed that IR treatment resulted in a gradual increase of CY5 fluorescence intensity in 70.2% ± 8.3% of FAM-emitting CD133+ tumor cells (n = 2.0x10³), indicating upregulation of CD109 expression in the same cells.
Figure 3-11 IR provokes direct transformation of CD133+/CD109- PNs into more aggressive CD133-/CD109+ MES in GSCs

(a) Snaps of single GBM84 cells at different timepoints post-IR indicated a gradual increase of CD109 maker expression in originally CD133 high GSCs by NEP-based co-transfection of CD133 (FAM) and CD109 (Cy5) molecular beacons. (b) Fluorescent images of large-scale NEP-based CD109 (cy5) molecular beacon screening for CD133 pre-sorted GBM157 cells, 24h after irradiation, CD133- cells (left) and CD133+ cells (right). (c) Time-lapse curve of CY5 fluorescence intensity (CD109 MB) within single cells after irradiation.
Snapts of single GSCs at 4h, 12h, and 24h timepoints post-IR after NEP-based co-transfection of CD133 (FAM) and CD109 (Cy5) molecular beacons are shown in Figure 3-11a. Large-scale intracellular screening of CD133-sorted GBM157 population (Figure 3-11b) also revealed that this significant increase of CD109 expression after IR treatment only is CD133-dependent (i.e., CD109 upregulation only happened in CD133+ PN GSCs). Fluorescence images of CD109 MB signals within single irradiated GBM157 PNs, corresponding to time-lapse curve of CY5 fluorescence intensity within single cells after irradiation, at 3h and 20h post-IR, respectively, are shown in Figure 3-11c.

Moreover, single cell motility assays of two patient-derived PN GSC populations, i.e., GSC157, and GSC84, showed that IR-PN cells showed significantly higher single cell motility, characteristic of MES tumor cells, than untreated control PN cells. (Figure 3-12) The data of chip-based experimental assays provided a strong evidence of the PN-MES shift in IR-treated PNs, by quantitatively monitor of both molecular biomarker expressions and cell migratory behaviors at the single-cell level. Together with other \textit{in vitro} and \textit{in vivo} experiments, these data suggest that IR provokes a direct transformation of CD133+/CD109- PN GSCs into a more aggressive CD133-/CD109+ MES phenotype.
Results showed a significant increased motility in irradiated GSCs, GSC 84 (top) and GSC157 (bottom), respectively. ***p < 0.001.
3.3 Materials and Methods

3.3.1 Single Cell Motility Assay

Single cell motility assay was conducted on the micropatterned polydimethylsiloxane (PDMS) surfaces and the guided cell motility was recorded by the time-lapse imaging for 24-72h. (Gallego-Perez et al., 2012; Gu et al., 2016; Petrie, Doyle, & Yamada, 2009)

The biomimetic surfaces resembling in vivo structural cues were fabricated by soft lithography technique. Si master wafer with the parallel micro-lines were patterned using Shipley1813 photoresist through photolithography (EV Group 620 Contact Aligner). In the replica-molding process, a 10:1 mixture of PDMS (Sylgard® 184, Dow Corning) was spin-coated on the pre-defined Si master wafer at 300-500 rpm for 1 minute, and then vacuumed for 30 min in a desiccator. After the mixed PDMS polymer solution cured at room temperature for at least 48h, demolding step was done by carefully peeling off the cured PDMS film from the Si substrate. Circular patches of finally patterned PDMS were cut out by a PDMS biopsy puncher (12 mm in diameter) and then attached to the bottom of 12-well plates as the substrates of single cell motility assay. The 12-well plate substrates were sterilized in 70% ethanol, before they were pre-soaked in Fetal Bovine Serum (FBS) for 1 day to improve the cell attachment.

During the living cell imaging experiments, the glioma stem cells cultured in neurosphere were first suspended in single cells and counted. 100k cells per sample was considered as optimal cell density. The cells were then plated and allowed to adhere and spread on the patterned PDMS surfaces after a few hours in 10% FBS-containing, heparin-free cell culture medium. Cell motility was then monitored and recorded via programmed
microscopy imaging fitted with an on-stage cell culture chamber (Okolab). Images were collected every 10 min by the inverted microscope (Eclipse Ti-E, Nikon) equipped with automated XY stage and EMCCD camera (Evolve, Photometrics) and quantitative cell migration analysis was through image processing using the manual tracker plugin in Fiji.

3.3.2 In vitro mRNA Detection by NEP-delivered Molecular Beacon (MB) Probes

The cells were then seeded as monolayer in 3D silicon NEP chip overnight in 10% FBS, heparin-free glioma cell culture medium before the NEP-based transfection to ensure a tight contact between cells and nanochannel array. Before the nano-electroporation for intracellular delivery of MBs, the MB solution was prepared in the optimal concentration from 200nM to 500nM, and then pipetted in the reservoir on the bottom gold-coated electrode slide with 500uL to 1mL volume. The MBs were then NEP-injected into the single glioma clones on the 3D NEP chip surface by applying an electric field pulse with conditions of 150-200 Votage, 20 -30 ms duration, across the nanochannel arrays.

After 1-hour incubator, MB hybridization with the specific target mRNA biomarker gradually occurred, and the marker expression was then quantified by fluorescence intensity, which came from the hybridized MB fluorescence emission, as the original hairpin-loop structure was open, separating fluorescent dye from the quencher. Cells were then transferred from incubator to on-stage cell culture chamber (Okolab) 1-2 hour post transfection, before starting time-lapse live cell imaging using fluorescence microscope system (Eclipse Ti-E, Nikon) equipped with motorized stage and EMCCD
camera (Evolve, Photometrics). Images were collected every 10 min and quantitative analysis of fluorescence intensity was through image processing using the software NIS-Elements Advanced Research.

All molecular beacon probes were purchased from Sigma-Aldrich, St. Louis, MO. The sequence information is below. LNA Bases: [+A], [+C], [+G], [+T]

The molecular beacons against GAPDH (GAPDH – MB):


TTGGATCGCG[BHQ3] – 3’

The molecular beacons against CD44 (CD44 – MB)


GATCGCG[BHQ-1] – 3’

The molecular beacons against CD109 (CD109 – MB):


+[G]GA-GATCGCG-BHQ3-3’

The molecular beacon against CD133 (CD133 – MB):


GATCGCG-BHQ1-3’

Control molecular beacons (scramble MBs) targeting cel-miR-39:


GATCGCG-BHQ3-3’


GATCGCG-BHQ1-3’
3.3.3 Cell Culture

Established GBM samples were collected from Dr. Ichiro Nakano’s group at The Ohio State University in accordance with an institutional review board–approved protocol. Freshly resected tumor samples were dissociated and formed a steady patient-derived GSC cell lines before they were cultured and passaged in neurosphere (NS) following the existing protocol (Nakano et al., 2011). Phenotypic characterization of GBM-derived neurosphere cultures was conducted by Dr. Ichiro Nakano’s lab at Department of Neurological Surgery, the Ohio State University.

Established GSCs were cultured in the medium DMEM/F12 (10565-042, Invitrogen, La Jolla, CA, Invitrogen) supplemented with B27 (1:50; GIBCO-Invitrogen), Glutamax (1:100; invitrogen-35050), heparin (5 mg/ml, Sigma-Aldrich, St. Louis, MO), penicillin/streptomycin (1:50, Gimini Bioproducts), bFGF (Peprotech, 20 ng/ml), and EGF (Peprotech, 20 ng/ml). bFGF and EGF were added twice a week.

Freeze spheres instead of single cells in 1 ml of Cell Banker (pre-chilled at 4 degree). Thaw spheres to restart culture by removing the Cell Banker medium and dissolving NSs into fresh neurosphere medium very gently, avoiding breaking NSs into individual cells.
3.3.4 Real-time Measurement of CD133 (PN marker) and CD109 (MES marker) mRNA Expression Level post Ionizing Radiation (IR)

PN GSCs were first exposed to ionizing radiation (IR) treatment (12Gy) for half an hour. Before cell experiment, 3D NEP chips were sterilized by 70% ethanol and under overnight UV exposure. CD133 - MB and CD109 - MB with optimized concentration (CD133 - MB: 200nM; CD109 - MB: 100nM) for monitoring CD133 and CD109 mRNA level via fluorescence imaging were pre-mixed and then ~ 100 μL mixed MB solution was pipetted into the electroporation reagent reservoir. Irradiated single PN cells (~ 100,000 cells per ml in suspension) were placed onto an engineered array of nanochannels that focuses the porating electric field (electroporation voltage 100 V, pulse duration 10 millisecond, 2 pulses) to a corresponding area on the cell membrane, leading to the uniform and benign NEP-based transfection of irradiated PN cells with MBs. Transfection efficiency of MB probes was in the range of 70% ~ 80%.

Cells were transferred from incubator to on-stage cell culture chamber (Okolab) one-hour post transfection before starting time-lapse in intro live cell imaging using inverted microscope system (Eclipse Ti-E, Nikon) equipped with motorized stage and EMCCD camera (Evolve, Photometrics). Dynamics of CD133&CD109 mRNAs were quantified by the fluorescence emission levels of FAM (green) and/or CY5 (red) imaged via subsequent time-lapse on-stage living cell fluorescence imaging.
3.4 Conclusion

Single-cell motility, drug resistance, and underlying molecular mechanisms are all considered to play key roles in tumor dissemination, progression, and recurrence. A novel nanotechnology-enabled chip platform that could quantify such cellular and subcellular behaviors would be of great importance for both biomedical evolution of clinical treatment and the fundamental biology research. In this chapter, a micro/nanofabrication-based biochip platform combing NEP component with cell migration platform was presented for multiplex in vitro living cell interrogation of patient-derived glioma stem cells (GSCs) at single-cell level, including cell migration monitoring, intracellular marker probing, and drug resistance test. Unlike conventional cell examining methods in which cell lysis or fixing is inevitable, our biochip-based assays have been demonstrated to have the ability to follow temporal changes of cellular/molecular activities of living cells subjected to specified stimulations (e.g. chemotherapy, irradiation (IR) treatment, and up-or down-regulation of genes by transfection of gene regulator such as anti-miR).

One-of-the-kind NEP technology is implemented in this platforms as a powerful physical cell transfection tool capable of deterministic and uniform delivery of either pre-designed molecular beacon probes for detecting target gene expression level associated with benign/aggressive phenotype, or oligo RNA such as anti-miR that could induce cell apoptosis, and downregulate cell motility. Controllable dosage delivery of nucleic acid cargos can be achieved by programed pulse duration and numbers of pulses, without affecting cell viability.
Engineered micro-features on the chip surface, which mimics the in vivo microstructures with parallel tracks, allows real-time monitoring of guided single-cell migration behaviors. Coupled with NEP’s unique functions, we for the first time achieved the screening of intracellular marker and monitoring guided single-cell motility in one single chip. The chip-based single-cell assays can easily distinguish relatively benign proneural (PN) phenotype from a much more aggressive mesenchymal-like (MES) phenotype within a highly heterogenous tumor population. Moreover, migration behaviors of single cells can be linked to their gene signatures screened by NEP-based transfection of MBs.

Our on-chip intracellular screening data revealed marked intertumoral differences in single-clone gene profiles between two populations of GSCs (i.e., GBM157 vs. GBM528), which correlated well with DNA microarray data. Chip-based therapy resistance tests on a conventional chemotherapeutic agent, temozolomide and a novel gene therapy candidate, oligo RNA anti-miR-363 revealed a subpopulation of CD44-high and CD133-low mesenchymal-like GSCs with strong drug-resistance capability and enhanced cell motility. This living-cell-interrogation chip platform also enabled the real-time direct monitoring of the potential proneural (PN)- mesenchymal (MES) shift post irradiation (IR) via NEP-based delivery of MBs against related biomarkers.

This biochip has the potential to quantitatively probe the gene expression in situ and detect the potential variation of cell migration speed of aggressive mesenchymal stem cells caused by NEP-based transfection of novel therapeutic gene under different NEP conditions for medical treatment evaluation. This platform can also be utilized to follow the dynamics of intracellular marker expression level and in vitro cell motility during induced phenotypic
shift of cancer stem cells such as EMT and thus has potential to shed a new perspective for current solid tumor research and help develop novel therapeutic strategy specifically targeting a subpopulation within cancer stem cells with high plasticity.
Chapter 4 Microfluidic Cell Trapping for High Throughput 3D NEP-based Transfection

4.1 Introduction

Efficient intracellular delivery of exogenous materials (e.g. nucleic acids, proteins, drugs, molecular probes, nanodevices, etc.) plays a key role in a diversity of biomedical and pharmaceutical applications ranging from gene editing (Chu et al., 2015; Dever et al., 2016; Maresch et al., 2016), cell-based therapy (Grupp et al., 2013; Kalos & June, 2013; Porter et al., 2011; Rosenberg & Restifo, 2015), regenerative medicine (Buganim, Faddah, & Jaenisch, 2013; Gurdon, 2016), production of therapeutic molecules by cell-based bioreactors, to fundamental biology research probing molecular mechanism in diseases such as cancer. Precise, rapid and benign introduction of biomolecules into a large population of cells at single cell resolution has thus long fascinates the scientific community. To circumvent the safety concerns raised by viral vectors, a variety of non-viral delivery approaches have been developed, including chemical carrier-mediated methods (e.g., synthetic lipoplex and polyplex nanocarriers, uptaken by cells via endocytosis and endosomal escape (Pack, Hoffman, Pun, & Stayton, 2005) and physical membrane-penetrating methods (such as micro-injection, biolistic gene gun, laser irradiation and sonoporation). Electroporation has been a popular physical delivery method
since its invention. Conventional bulk electroporation (BEP) is the commercially available system in which a mixed conductive buffer containing both suspended cells and transfection reagents is loaded into the electroporation cuvette with anode and cathode from two ends that apply high-voltage electric pulses (>1000V) to facilitate cargo delivery in permeabilized cells. While BEP offers the advantage of simplicity to use without any package of delivery materials, it suffers from low cell viability and significant cell-to-cell variation owing to the non-uniform electric field imposed on the large number of cells randomly suspended in the cuvette.

A rapid growth of miniaturized versions of electroporation integrated in micro-/nanofluidics-enabled lab-on-a-chip platforms has been witnessed since 2000. Microscale-electroporation (MEP), which confines the electric field to the scale of the cell, allows for a fine control over cell poration condition, i.e. creating a more uniform porating electric field by applying a significantly lower voltage (<10 V) which minimizes cell death. However, in both BEP and MEP, the process of cargo delivery is diffusion/endocytosis-based which is essentially stochastic. Recently, we introduced an innovative nano-electroporation (NEP) technology that is capable of dosage-controllable and benign intracellular delivery using electrophoresis-assisted cargo “injection” through a nanochannel aperture (Boukany et al., 2011). Motivated by the limited throughput (i.e., <200 cells) of the first-generation NEP system, a three-dimensional (3D) NEP platform, which features a massive nanochannel array in the z-direction (Chang, Bertani, et al., 2016; Chang, Gallego-Perez, et al., 2015) was developed in our lab. While the nanopores can be properly engineered by the mature semiconductor cleanroom-based fabrication techniques,
cell manipulation (i.e., position individual cells against nanochannel outlets), in a simple yet efficient manner, is a major technical hurdle that needs to be overcome. Since the electric field, which accelerates the charged biomolecules in the nanochannel, and porates the cell membrane during the NEP process, diminishes quickly outside nanochannels, it is critical to achieve close contact between the to-be-transfected cells and the corresponding nanochannels. Previous cell loading techniques coupled with NEP suffered from either low throughput (e.g., single-cell manipulation by an optical tweezer), excessive cell perturbation (e.g., cell labelling with magnetic beads and then cell manipulation by a magnetic tweezer) (Chang, Howdyshell, et al., 2015), or exposure to physiologically unfavorable low-conductivity buffer in the dielectrophoresis (DEP) based cell manipulation (Chang, Gallego-Perez, et al., 2015) which would compromise cell viability. It has been previously demonstrated that hydrodynamic weir-like microstructures could successfully immobilized cells (Chang, Gallego-Perez, et al., 2016; Di Carlo, Aghdam, & Lee, 2006; Skelley, Kirak, Suh, Jaenisch, & Voldman, 2009; Zhang, Chou, Xia, Hung, & Qin, 2014), however, those devices were unable to perform high-throughput nano-electroporation applications.

In this chapter, we introduce a scalable microfluidic cell-trapping and nano-electroporation (NEP) platform that offers high yield of NEP-based high-throughput intracellular delivery (i.e., >20,000 cells per cm² within minutes). Such new platform allows rapid cell loading, large-scale and uniform nano-electroporation with single-cell resolution, and fast post-transfection cell collection in one single chip. Unlike optical and electromagnetic cell trapping techniques (e.g., optical tweezers, magnetic tweezers, DEP) that require either
cumbersome instrumental setup and calibration procedures, or rely heavily on the expertise and/or experience of users, this microfluidic cell manipulation approach is easy to implement, cell-friendly and highly efficient. The precise cell positioning is achieved by a microfluidic cell trap array. By optimizing the cell density and flow rate, a capture efficiency >90% could be achieved within 2 minutes. The computational fluidic dynamics (CFD) simulation reveals that this cell trap structure not only creates a localized “safe harbor”, characteristic of low flow velocity, within the cell trap chamber to protect the trapped cells from shear stress, but also generates a downward flow velocity to push the trapped cell against the nanopores on the substrate. Therefore, this platform is good for both adherent (e.g., fibroblasts) and suspension cells (e.g., blood cells), regardless of cellular anchor properties. Our experimental results showed that the microfluidic cell trapping significantly improved the NEP-based transfection efficiency, achieving uniform and precise delivery of various cargos including a small fluorescently-labeled oligodeoxynucleotide (ODN) and a large ~9kbp plasmid.

4.2 Results and Discussion

4.2.1 CFD Simulation of Flow Velocity Field in Microfluidic Cell Trap Designs

The U-shaped micro-trap structure has been proven to be effective for trapping individual cells via a hydrodynamic force. (Skelley et al., 2009) Herein, two microfluidic cell trap array designs (i.e. “bottom” standing on substrate or “top” hanging from ceiling) were studied and compared using the FEM simulation with focus on the flow velocity field near the cell trap region. This analysis provided an insight on whether the local flow within the
cell trap chamber could facilitate a close contact of the trapped cell with the nanochannel outlet.

The geometry of the cell trap array was designed according to the size of mouse embryonic fibroblast (MEF) cells, with an average diameter of ~15 μm: the width and length of the cell trap were set to be $W = L = 15 \text{ μm}$, and the height of the cell trap was set to be $H = 20 \text{ μm}$. The cell trap array was arranged to be interleaved instead of parallel because this design could provide superior particle trapping efficiency compared to parallel array (Chang, Gallego-Perez, et al., 2016). The nanochannel array was neglected in this microfluidic modeling and simulation, as we assumed that the nanochannel located at the center of each cell trap would not affect the outlet of flow, considering the extremely high pressure needed to drive any fluid through the nanoscale channel.

We first analyzed the “bottom” standing micro trap array patterned on a silicon chip substrate. Not surprisingly, an upwards flow velocity was observed in the micro trap region. This is because the cell trap essentially functions as a “wall” near which the fluid cannot flow through but can only change the direction to go upwards and pass through the gap between the cell trap and the microfluidic channel ceiling. Although the flow velocity in the x-y plane will guide the individual cells towards the cell traps, the upwards flow velocity within the chamber of the cell trap will keep pushing the cell up away from the nanochannel.
Figure 4-1 Modeling of a “bottom” standing microfluidic cell trap (with a herringbone pattern) and FEM simulation results.
Figure 4-1 continued (a) a top-view shows model geometry (unit: μm) (b) CFD computation with meshed boundaries (c) FEM modeling results of the flow velocity field distribution in the micro-trap cross-section in the y-z plane and x-z plane.

To suppress the upward flow caused by the cell trap on the substrate and to ensure a tight contact between captured cells and nanochannels, a herringbone structure designed on the ceiling of the PDMS microfluidic device, aligned with the cell trap array is added to our modelling of a flow area of 600 μm x 400 μm (Figure 4.1 a-b). Such microfluidic herringbone structure designed on the ceiling of microfluidic devices has been found to be able to generate vortices in the microscale, thus increasing the cell capture efficiency (Stott et al., 2010). The FEM simulation result (Figure 4.1 c) shows that this herringbone pattern with the optimized geometry can indeed generate a downward flow velocity in the proximity of the micro-trap and thus can help guide the individual cells onto the micro-traps. However, within the micro trap chamber, the downward z component of the flow velocity is very small and there still exists an upward flow near the gap between the top of the micro trap and the ceiling (Figure 4.1c), which may lift the captured cell away from the nanochannel outlet on the bottom substrate. Thus, even though this “bottom” standing design is good for cell capture, its NEP transfection could be compromised due to the loose contact between the cell and the nanochannel.
Figure 4-2 Modeling of the microfluidic cell trap (no herringbone pattern) and FEM simulation result
In comparison, our FEM simulation shows that no additional herringbone structure is needed to secure cell capture within the cell trap if the cell trap array is to be built on the ceiling of the PDMS microfluidic device (Figure 4.2). The “top” hanging U-shaped cell trap structure can not only capture a cell with a proper size same as in the previous “bottom” standing design, but also function as a “wall” that re-orient the flow downwards to pass through the gap between the silicon NEP chip substrate and the PDMS cell trap (Figure 4.2 c-d). The FEM simulation results show that in the gap between the micro trap and the bottom substrate, the flow velocity magnitude is ~1 μm/s with a z component ~0.5 μm/s, which can generate a piconewton-level “pushing” force according to Stokes’ law. This hydrodynamic drag force exerted on the captured cells will secure their tight contact with their corresponding nanochannels underneath and thus lead to good NEP transfection.

As shown in Figure 4.2 e, another benefit of hydrodynamic weir-like microstructures for cell trap is that the cell capture chamber may serve as a “safe harbor”, within which the flow velocity is much lower (< 0.5 μm/s) compared to that in the region outside (> 5 μm/s).
This “safe harbor” could protect the captured cells from excessive drag force and shear stress during the hydrodynamic cell trapping process, even if a relatively large flow rate is applied for the rapid cell trapping.

While both microfluidic cell trap designs given in Figures 4.1 and 4.2 can position the cells in the traps, the “top” hanging cell trap design extruded from the PDMS ceiling is more advantageous because of its ability to exert an additional “pushing” force on the cells towards the nanochannel outlets during flow and its simplicity for fabrication and assembly.
4.2.2 Microfluidic-cell-trapping-assisted 3D High-throughput NEP Platform Design

As shown in Figure 4.3 a, this microfluidic-cell-trapping-assisted 3D NEP device is comprised of three main layers of solid-state materials from top to bottom: Layer1- a wide PDMS microfluidic channel with a patterned micro-trap array for capture of >20,000 cells, Layer 2- a silicon substrate with a dense nanochannel array, and Layer 3- a gold-coated glass slide as the bottom electrode.

Cell suspension in the buffer solution is added from the microfluidic channel inlet on the left and flows through the microfluidic channel driven by the pressure generated by a syringe pump. Cells are captured by the weir-like micro traps, ready for nano-electroporation (Figure 4.3 b). The cells that are not trapped will flow through and can be collected at the outlet and re-used in the next cell trapping cycle. Experimentally, an upright microscope (Leica Microsystems DM2500 MH) was used for real-time monitor of the cell capture process. When the cell trap array was nearly saturated (>90%), the NEP-based transfection was performed while the flow continued in order to keep holding the trapped cells against nanopores on the silicon substrate via a hydrodynamic force. Transfection reagents from the micro-reservoir between Layer 2 and Layer 3 were electrophoretically injected into nanoporated individual trapped cells by applying a focused electric field through the nanochannels (Figure 4.3 c). Square wave electric voltage pulses (voltage 220 V, pulse duration 10 ms, 1-5 pulses) for nano-electroporation were generated from a power supply (Gene Pulser Xcell™, Bio-Rad). An inverted microscope (Nikon Eclipse Ti) was used to check the fluorescence of the transfected cells post--NEP. After NEP, the flow
direction was simply reversed by a springe pump (PUMP 33, Harvard Apparatus) to release and collect the transfected cells from cell traps at the inlet (Figure 4.3d).

4.2.3 Fabrication of Microfluidic Cell Trap Array

According to previous FEM simulation results, the cell trap array was located on the microfluidic channel ceiling. Fabrication of the PDMS microfluidic channel with the cell trap array was based on soft lithography. The micro-fabricated cell trap array structure is shown in Figure 4.3 e and a detailed geometry of single cell trap is: 15 μm wide, 12 μm long and 15 μm in height, which is confirmed by zoom-in SEM micrographs showing in Figure 4.3 f-g. A gap in the middle of the trap is designed to let the fluid pass through.
Figure 4-3 Device design for microfluidic cell trapping and high-throughput 3D NEP transfection
(a) An exploded view of schematic of the microfluidic 3D NEP platform assembly showing the main components. (b-d) Schematics of the operation flow of the microfluidic NEP device. (b) Cell loading: individual cells are captured in micro-traps by a hydrodynamic force. (c) NEP-based transfection of target molecules by applying a focused electric field through a nanochannel. (d) Cell collection: transfected cells can be released from cell traps and collected from the inlet of the microfluidic channel, by simply reversing the flow direction. (e) SEM images of the micro-fabricated cell trap array (f-g) zoom-in SEM micrographs showing the detailed structure of a single cell trap. Measured cell trap geometry: 15 μm wide, 12 μm long and 15 μm in height. A designed gap in the middle of the trap to let the fluid pass through.
The fabrication procedure is illustrated in Figure 4.4. Briefly, the Si master wafer was patterned with the microscale cell trap array via contact photolithography (EV Group 620 Contact Aligner). In the replica-molding process, a 10:1 mixture of PDMS (Sylgard® 184, Dow Corning) was spin-coated on the pre-defined Si master wafer at 300-500 rpm for 1 min, and then vacuumed for 30 min in a desiccator. After the mixed PDMS polymer solution cured at room temperature for at least 48 h, a demolding step was carried out by carefully peeling off the cured PDMS film from the Si substrate.

Figure 4-4 Representation of soft-lithography-based fabrication of the PDMS microfluidic channel with a cell trap array.
4.2.4 Fabrication and Characterization of Si-based Nanochannel Array Device

Wafer-Scale Fabrication of High-throughput Silicon NEP chip

The nanochannel array on the silicon substrate as a 3D NEP platform was fabricated in a Class 100 cleanroom, after a series of optical lithography (including contact/proximity photolithography and projection photolithography, as illustrated in Figure 4.5) and Bosch-Process-based deep reactive ion etching (DRIE) steps (as shown in Figure 4.6) on both sides of <100> double-polished 4-inch silicon wafer (purchased from UniversityWafer Inc., 850 Summer St., Suite # 207, Boston, MA 02127, USA, Item# 2345). The detailed fabrication protocol is discussed below, in the manner of time order.

*Patterning nanochannel array by projection photolithography*

To pattern the nanopores with a diameter of 500nm, projection lithography was used to shrink (5x) and print the nanopore array from the pre-designed micropore pattern (2.5 µm in diameter) on a photomask. The schematics showing photolithography principles are given in Figure 4.3. Basically, contact/proximity lithography (Figure 4.3 a) imprinted 1:1 the exact size of the pattern on the photomask into the sample, and projection lithography (Figure 4.3 b) reduced the pattern on the photomask (or reticle) by adding an objective lens below and projecting the pattern on a small portion of the sample known as “die”. Multiple exposure is usually needed in projection lithography to cover the entire wafer with an array of dies. Projection lithography for the nanopore pattern was performed in the tool GCA 6100C Stepper (I-line) (STP 01, Nanotech West Lab) under optimized conditions: an exposure time of 15 sec (3 times of recommended value, to ensure full open of nano-pore feature with a 2:1 aspect ratio), and a focus offset 0. Post-bake was skipped, otherwise
AZ5214 would yield an opposite polarity of pattern as negative photoresist (PR). Lastly, the wafer was developed in MF-319 for 1 min for a good nanochannel array pattern on PR.

Figure 4-5 Schematic of different types of optical lithography

(a) Contact/proximity photolithography, in which a uniform light is exposed on the entire sample. (b) Projection photolithography, in which the mask (or reticle) is projected on a portion of the sample, known as “die”. The complete pattern is created by sequential exposure for multiple times.
**Etching the nanochannel array by Bosch Process**

Herein, a deep silicon structure etching method, deep RIE (DRIE) “Bosch Process”, was utilized to etch a high-aspect ratio (>20:1) nanochannel array (10µm nanochannel in depth). An alternating sequence of the etching processes (SF₆ gas) and the sidewall passivation steps (C₄F₈) enables fast etch rate, a nearly 90° sidewall profile, and high-aspect ratio features (Figure 4.6). Bosch process was carried out using the Oxford Inductively Coupled Plasma (ICP) -RIE system (Dreese lab, ECE Cleanroom) with optimized parameters (Chang, Bertani, et al., 2016; Chang, Gallego-Perez, et al., 2015), which led to a high-quality nanochannel array.

**Patterning and etching the microchannel array**

A backside microchannel array is needed to produce a through channel structure which allows for NEP function. A micropore array is patterned (50 µm diameter, 25µm spacing) by contact lithography (EV Group 620 Contact Aligner, ALGN02, Nanotech West Lab). Etching of the microchannel was performed using the same DRIE tool. Repeating the cycle for 350 times would result in a 250 µm deep microchannel. Characterization was done by SEM imaging of the nanopore surface and the cross-section of the silicon NEP device, as shown in Figure 4.7.
Figure 4-6 High-aspect ratio (>20:1) micro-/nano-structures achieved by Bosch Process etching

(a) Schematic of Bosch Process, deep reactive-ion etching (DRIE) progression: silicon sample is under repeated SF$_6$-based etch step and C$_4$F$_8$-based passivation step. Sidewall is protected by polymeric passivation (nCF$_2$). (b) SEM micrograph of the 3D silicon NEP chip cross-section showing smooth and straight channel sidewalls achieved by optimized conditions of deep reactive ion etching (DRIE) with repeated etching and passivation cycles.
Figure 4-7 A nanochannel (φ500 nm) array fabricated by projection photolithography and deep reactive ion etching (DRIE)

(a-b) SEM picture of top-view of uniform nanopore array with 5 µm pitch and a zoom-in image of a single nanochannel showing φ500 nm diameter. (c) A cross-section image showing multiple nanochannels connected with one microchannel.
4.2.5 Optimizing the Cell Density for Microfluidic Cell Trapping

A series of cell suspension buffers with different cell densities were used to find the best cell density condition for microfluidic cell trapping at the flow rate of ~100 µm/s suggested by the literature (Skelley et al., 2009). The cell trapping duration was fixed at 2 min. After trapping, fluorescence imaging was used to check the cell trapping efficiency (the ratio of the number of occupied cell traps to the number of total cell traps). As shown in Figure 4.8 a, the trapping efficiency kept increasing and reached ~90% when the cell density approached 0.5~1 million cells per 100 µL. It is worthwhile to notice that there seemed to be a linear relationship between cell density and capture efficiency in the range of 0.1 ~0.5 million cells per 100uL. When the cell density was higher than 1 million cells per 100 µL, the cell capture efficiency did not improve much (~90-95%); however, the probability of individual cells sticking together in the flow became larger and thus the “clogging effect” became significant with the percentage of “multiple trapping” cell traps increasing. This “clogging effect” is not desirable for uniform NEP transfection. Therefore, an optimal microfluidic cell trapping condition was selected for the remaining experiments in this chapter: cell density 0.5~1 million per 100 µL, flow rate ~100 µm/s, and trapping duration 2 min. A large-scale cell array (~1,000 cells) of captured Hoechst® stained NK92 cells (blue) is shown in Figure 4.8 b. A merged phase contrast and DAPI fluorescence image (Figure 4.8 c) showed that most of the cells (~95%) were captured by the cell trap array, as expected. To remove the un-trapped cells (especially adherent cells with sticky membrane) within the microfluidic channel, proper surface chemical modification such as
surface-grafted poly (ethylene glycol) (PEG) can be done in addition to PBS buffer washing after cell trapping.
Figure 4-8 Microfluidic cell trapping results of Hoechst® stained NK92 cells (blue) using optimized conditions

(a) The cell capture efficiency curve with different cell density conditions. Images taken after 2-min trapping. The flow rate was set at 100 µm/s. (b-c) Fluorescence images of the cell array after microfluidic cell trapping. Scale bars: 300 µm. (a) A large-scale cell array showing a high trapping efficiency >90%. (b) A merged image (phase contrast and DAPI) showing the positions of captured cells and micro-traps.
Cells captured by the weir-like cell traps got transfected by YOYO™-1-labelled plasmids showing both green and blue fluorescence (nucleus staining), while un-trapped cells only showing blue fluorescence. (a) GFP channel (b) merged GFP+DAPI channels

Figure 4-9 Microfluidic cell trapping improved NEP-based delivery.
4.2.6 Necessity of Precise Cell Positioning in NEP Process

As mentioned earlier, close contact between the to-be-transfected single cell and the nanochannel is critical to achieve good NEP cell transfection. As shown in Figure 4.9, only the captured “Trapped cells” which were pushed against the NEP silicon substrate by the hydrodynamic force generated by microfluidics were transfected as indicated by strong green fluorescence signals. On the other hand, unconstrained “Untrapped cells” showed no transfection (exhibiting only nucleus staining DAPI fluorescence).

This result can be physically explained by the spatial distribution of the electric field strength in the nanochannel region. The high-resistance nanochannel bears almost all the electric potential drop of applied voltage pulse for electroporation, which was confirmed by the FEM electrostatic simulation result (Figure 4.10 a). As illustrated in Figure 4.10 b-c, the electric field strength, which plays a key role in the NEP process, -- nanoporates cell membrane and drives charged biomolecules across the permeable membrane into cytosol via electrophoresis after intensely accelerating them within the nanochannel. This field strength, however, drops rapidly once the distance extends away from nanochannel interface (red line drawn in Figure 4.10 c). Therefore, the precise cell positioning against the nanochannel is essential for achieving successful NEP transfection.
(a) The high-resistance nanochannel bears almost all the electric potential drop of applied 200V voltage pulse for electroporation. (b) The electric field strength distribution. An extremely high electric field strength ~20 V/μm is created within and close to the nanochannel region which nanoporates cell membrane and drives charged biomolecules via electrophoresis. (c) The electric field strength drops drastically away from the nanochannel interface (red line). Nanochannel region defined \( z \in (-10,0) \)

Figure 4-10 FEM analysis of the electric field near nanochannel during NEP process
4.2.7 Microfluidic Cell Trapping Approach Significantly Improved NEP-based Delivery Efficiency and Uniformity

Our original high-throughput 3D NEP platform suffered from the lack of simple, rapid and efficient massive cell manipulation function, especially for difficult-to-attach suspension cells such as blood cells (e.g., T cells, NK cells, leukemia cells, etc.). As can be seen in Figure 4.11 a, randomly loaded suspension cells (NK-92, a cancer NK cell line) had an extremely low to no transfection efficiency of a DNA plasmid (7kbp), as they easily drifted away from the nanochannel surface in the buffer solution without any external forces. Adding a “spin down” step by centrifugation (1000 rpm for 5 min) slightly enhanced the NEP performance and resulted in the transfection efficiency to 25~30%. When adherent cells such as fibroblasts were used, close contact between cells and nanochannels can be easily achieved by the cellular anchor behavior. Usually, overnight cell culture of a single layer of cells on the silicon chip surface was done before NEP, so that the cells could attach and spread on the chip surface. By doing so, at least a portion of cells could be in contact with some nanochannels, and thus could be successfully NEP transfected (Figure 4.11 b). However, this cell attachment process is slow (at least 4-8 hours) and completely relies on the cellular behavior which is random and uncontrollable. For example, when mouse embryonic fibroblasts (MEF) were spread and attached onto the 3D NEP chip surface, some cells would be in contact with one or multiple nanochannels (Figure 4.11 b, inset) but others might seat in the space between nanochannels. As a result, the number of cells being transfected and transfection uniformity would depend on the relative position of cells and nanochannels. As shown in Figure 4.11 c, this method could only achieve 30~40%
transfection efficiency even after a prolonged cell anchor processes. To collect the transfected cells from the total cell population, an additional cell sorting step must be conducted, which adds operation difficulty and could compromise the cell viability.

On the other hand, our microfluidic cell trapping method can precisely position individual cells in a large array within 2 min without any cell anchor process, regardless of the cell attachment property. Our new method significantly increased the transfection efficiency to >80% by exerting a hydrodynamic “pushing” force on the captured individual cells for NEP-based transfection. Most of the un-transfected cells could be removed simply by flushing them through the microfluidic device because they were not captured by the micro traps. Compared to the centrifugation-based “spin down” random cell loading method, our method also enhanced the delivery dosage and improved the transfection uniformity, as shown in Figure 4.11 d. The quantified GFP fluorescence intensity distribution in the box plot shows the microfluidic cell trapping method had a stronger and more uniform YOYO™-1 labelled plasmid transfection. Mean fluorescence intensity, representing the delivery dosage jumped from 500 to >1500, with a much smaller scattering of fluorescent intensity indicating a more uniform delivery across the transfected cell population. Fluorescence micrographs of a living cell array after microfluidic cell trapping followed by uniform cell transfection by NEP is shown in Figure 4.12, with delivery cargos including a YOYO™ -1-labelled DNA plasmid (~7kbp) and a FAM-labelled 18-mer oligodeoxynucleotide (ODN, G3139).
Figure 4-11 The microfluidic cell trapping approach significantly improved NEP-based transfection efficiency.

(a) Suspension NK-92 cells suffered extremely low to no plasmid transfection efficiency. Only one cell showed green fluorescence indicated by the arrow. (b) Adherent cells could be transfected after cells attached on the NEP chip surface. But this cell anchor process took at least 4-8 h. Inset: a SEM image of a MEF cell spread on the NEP chip surface. (c) The transfection efficiency comparison of microfluidic cell trapping approach vs. other random cell loading methods. (d) Delivery dosage comparison of YOYO™-1 labelled plasmids to MEF cells quantified by fluorescence imaging.
Fluorescence micrographs of a living cell array after microfluidic cell trapping followed by NEP-based transfection of FAM-labelled ODN. Scale bar: 100 μm  
(b) Fluorescence images of a large-scale living cell array, after NEP-based delivery of YOYO™-1 labelled DNA plasmids (7kbp). Zoom-in images of single transfected cell located within the micro-trap. Scale bars: 300 μm (the large-scale cell array), 25 μm (single cell image)
4.2.8 Efficient Non-viral Reprogramming via Deterministic Transfection Based on Microfluidic Cell Trapping Assisted 3D High-throughput NEP Platform

Cell reprogramming can be performed on either readily available somatic cells (such as skin cells) or induced Pluripotent Stem Cells (iPSCs) (Takahashi & Yamanaka, 2006). Since the dosage of reprogramming factors delivered in individual cell is critical, development of a transfection tool capable of gene delivery in a deterministic manner is crucial. To demonstrate the potential clinical use of our microfluidic 3D high-throughput NEP platform (microfluidic -- NEP), a direct cell reprogramming model relevant to regenerative medicine was tested. Mouse embryonic fibroblasts (MEFs) were efficiently co-transfected with DNA plasmids encoding two induced neuron factors: a 7kbp Achaete-Scute Complex Like-1 (Ascl1) and a 9kbp Myelin Transcription Factor 1 Like (Myt1l) by microfluidic -- NEP, with conventional BEP transfection as comparison. Fluorescent micrographs were captured 24 h after transfection (Figure 4.13 a).

Quantification of images showed that the microfluidic -- NEP group had a higher and more uniform average expression level of both Ascl1 and Myt1l, indicated by reporter genes (Figure 4.13 b-c). GFP intensities from Ascl1 plasmid for NEP was $32,705 \pm 1,262$ (mean ± standard deviation), compared to $8,031 \pm 5,914.8$ for BEP. CFP intensities from Myt1l for NEP was $8,718 \pm 561.2$, compared to $3,488 \pm 1,656.6$ for BEP.
Figure 4-13 Results of NEP-based transfection of DNA plasmids by microfluidic cell trapping-assisted NEP (microfluidic NEP) in comparison with BEP transfection.

(a) Fluorescence micrographs of living MEF cells after microfluidic NEP (left) and BEP (right) transfection of Ascl1-GFP (top) and Myt1l-CFP (bottom) plasmids. (b-c) Box plots of expression levels quantified by GFP (b) and CFP (c) fluorescence intensity. The NEP group had significantly stronger and more uniform protein expressions than the BEP group. Plasmids encoding induced neuron factors: Achaete-Scute Complex Like-1 (Ascl1) (7kbp) and Myelin Transcription Factor 1 Like (Myt1l) (9kbp)
4.3 Materials and Methods

4.3.1 CFD Simulation of the Flow Velocity Field in the Microfluidic Cell Trap

Modeling and FEM simulation was conducted using the COMSOL® Multiphysics 5.0 (COMSOL Inc.). The geometry of the cell trap array was set based on previous experience in the “dip-trapping” NEP chip design. (Chang, Gallego-Perez, et al., 2016)

The flow rate at the inlet was approximately 1.5e-14 m³/s (equivalent to 50 μm/s).

The Reynolds number, characterizing the flow, is given by $Re = \frac{uL}{\nu} = 0.001$. where $\rho$ is the fluid density (1000 kg/m³), $u$ is a characteristic velocity of the flow (0.05 mm/s), $\nu$ is the fluid kinematic viscosity ($\nu = \frac{u}{\rho} = 1$ mPa·s/1000 kg/m³) and $L$ is a characteristic dimension of the device (20 μm).

Since $Re \ll 1$, the physical interface of Creeping Flow (microfluidic module) was used.

The governing equation in this model is Stoke equation, a linearized form of Navier-Stokes equation, as follows,

$$-\nabla p + \eta \nabla^2 u = 0$$

combined with the incompressible continuity equation:

$$\nabla \cdot u = 0$$

where $u$ is the flow velocity (m/s) and $p$ is the pressure (Pa).

4.3.2 FEM Analysis of the Electric Field Near the Nanochannel During the NEP Process

Due to symmetric conditions, modelling of the 3D NEP device was simplified to a row of nanochannels. The electric field near nanochannels was simulated using COMSOL® Multiphysics 5.0. (COMSOL Inc.), by solving the Laplace equation $\nabla^2 V = 0$, where $V$ is
the electrical potential. 200 V electroporation voltage was applied on the top surface of cell buffer and the ground was set to be the bottom surface of the transfection reagent buffer touching the bottom electrode.

In the electrostatics model, the electric field can be obtained by the governing equation \( \mathbf{E} = -\nabla V \). The electrical conductivity of buffer was set to be 1 S/m (according to PBS buffer property). Simulated data was exported to MATLAB (MathWorks, Inc.) for analysis and plotting.

4.3.3 Assembly of the Microfluidic Cell Trapping Assisted 3D NEP Platform

This lab-on-chip device was comprised of three main layers of solid-state materials from top to bottom: Layer 1- a PDMS microfluidic channel with patterned micro-trap array for cell capture, Layer 2- a silicon substrate with an engineered nanochannel array, and Layer 3- a gold-coated glass slide as the bottom electrode. Cells along with buffer solution were placed in the microfluidic channel between Layer 1 and Layer 2. The transfection reagent solution (with a concentration \( \sim 0.1 \mu g/\mu l \) measured by Nanodrop™) was held in the micro-reservoir defined by the PDMS spacer between Layer 2 and Layer 3, as shown in Figure 4. 3 a.

Before the assembly, each component was cleaned and sterilized: soaking in 70% ethanol, sonicating for 5 min, and \( N_2 \) blow dry. The fabricated PDMS microfluidic chamber with the cell trap array and the PDMS spacer were pre-treated with oxygen plasma (PTS oxygen plasma system) to secure their bonding to the substrate and make the PDMS cell trap microfluidic channel hydrophilic. The bottom electrode was a gold-coated glass slide by e-beam evaporation (Denton DV-502A) of Au on the glass.
substrate. An inserted gold needle was used as the top electrode. A PDMS spacer was added between the bottom electrode and the silicon substrate, in which the transfection reagents were added. An infuse/withdraw twin springe pump (PUMP 33, Harvard Apparatus), connected with microfluidic Tygon tubing, was used to drive the laminar flow in and out of the microfluidic channel.

4.3.4 Operation of Microfluidic Cell Trapping Followed by 3D NEP Transfection

Before use, the microfluidic channel was pre-wet by 70% ethanol, and gradually replaced by PBS infusion. 100~200 µL cell buffer solution with a cell population ~1 million cells was added from the inlet, and drawn from the outlet in a flow rate of 100~150 µm/s by an infuse/withdraw twin springe pump (PUMP 33, Harvard Apparatus) connected with microfluidic Tygon tubing for 2-5 min. The cells that were not trapped will flow through and could be re-collected from the outlet and used in the next cell trapping cycle. An upright microscope (Leica Microsystems DM2500 MH) was used for real-time monitoring of the cell capture process. When the cell trap array was nearly saturated (> 90%), the NEP-based transfection was performed while the flow continued to provide a hydrodynamic force on the trapped cells against nanochannels located on the Si substrate surface. Square wave electric voltage pulses (voltage 220 V, pulse duration 10 ms, 1-5 pulses) for nano-electroporation were generated from a power supply (Gene Pulser Xcell™, Bio-Rad). An inverted microscope (Nikon Eclipse Ti) was used to check the fluorescence of the transfected cells post-- NEP. To release and collect the transfected cells from cell traps at the inlet, the flow direction was simply reversed by changing the setting on the syringe pump.
4.3.5 Cell Culture

Mouse embryonic fibroblasts (MEFs) were purchase from Millipore. MEFs were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Thermo Fisher Scientific) containing 10% Heat-Inactivated Fetal Bovine Serum (FBS) (Catalog number: 10438034 Thermo Fisher Scientific) and 1% Non-Essential Amino acid (NEAA) (Catalog number: 11140050, Thermo Fisher Scientific). NK-92 cells were purchased from ATCC®. NK-92 cells were maintained in RPMI 1640 (Thermo Fisher Scientific) supplemented with 20% FBS and a 1 : 1000 dilution of IL-2 (Life Technologies).

4.3.6 Bulk Electroporation (BEP) of MEF

A commercial BEP system (Neon Transfection System, Thermo Fisher Scientific) was used for BEP as a control. Specific electric field conditions were implemented following the suppliers' protocol for mouse embryonic fibroblasts (MEF), i.e., pulse voltage: 1,350 V, pulse duration: 30ms, number of pulse: 1.

4.3.7 Biomolecules for Cell Transfection Experiments

FAM-labelled 18-mer oligodeoxynucleotides (ODN, G3139) were purchased from Alpha DNA. YOYO™-1 dyes were purchased from Thermo Fisher Scientific (Catalog no. Y3601). DNA plasmids were stained with YOYO™-1 following suppliers' protocol at a DNA base pairs to dye molecules ratio of 5:1 in 5x TBE. Protecting YOYO™-1 solution from light by wrapping tube in aluminum foil and store at -20 °C.

4.3.8 Image Acquisition and Analysis

Phase-contrast and fluorescence images were obtained using an inverted microscope (Eclipse Ti-E, Nikon) equipped with an EMCCD camera (Evolve, Photometrics). Videos
were filmed on the Nikon Eclipse Ti-E microscope. Image analysis was done using the Nikon software NIS-Elements Advanced Research (AR).

4.3.9 SEM Characterization

Characterization of the silicon NEP device was done by scanning electron microscopy (Hitachi S-3000H Scanning Electron Microscope).
4.4 Conclusion

A cell transfection tool capable of precise, rapid and benign delivery of biomolecules into a large population of cells at single cell resolution would be highly valuable in a variety of biomedical applications and fundamental biology studies. Chemical carrier-mediated methods (e.g., synthetic lipoplex and polyplex nanocarriers) and conventional physical membrane-penetrating methods such as bulk electroporation (BEP) are limited by low transfection efficiency due to the uptake pathway via endocytosis, large cell transfection heterogeneity, and excessive cell damage. In this chapter, a novel large-scale 3D NEP transfection platform, capable of deterministic, safe and uniform cell transfection, has been developed. Assisted by a microfluidic-based passive hydrodynamic weir-like cell trap approach, our NEP platform enables rapid, benign and efficient parallel cell manipulation with single-cell resolution, and high transfection efficiency with improved dosage uniformity, not achievable by any of the existing transfection tools. A simple micro-/nano-fabrication protocol was developed to engineer massive nanochannel arrays and the dense cell trap array. Using this unique platform, both adherent cells (i.e., mouse embryonic fibroblasts (MEFs)) and suspension cells (i.e., natural killer (NK) cells) can be massively trapped in the micro-cap array with a capture efficiency ~90% in a few minutes. Various cargoes from large plasmids to small oligos are able to be uniformly NEP-delivered into trapped cell array in a controllable manner, thus showing the versatility of this 3D microfluidic-NEP platform. This innovative micro/nanotechnology enabled cell transfection platform has a potential to boost the progress in many biomedical applications ranging from cell reprogramming, cell-based therapy, to gene editing.
Chapter 5 Characterization of Therapeutic Extracellular Vesicles (tEVs) Produced by NEP-based Cell Transfection

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Junfeng Shi carried out the silicon high-throughput NEP device design and fabrication. Junfeng Shi performed the simulations. Junfeng Shi performed the EV visualization experiments and imaging analysis, with contribution from Zhaogang Yang regarding the plasmid design. Junfeng Shi, Zhaogang Yang and Jingyao Sun conducted donor cell NEP transfection for EV production. Junfeng Shi and Zhaogang Yang performed the TLN biochip assays for EV analysis.

The following contents of this chapter will soon be submitted for future publishing. The method of production of tEV by NEP transfection has been submitted for patent application and is now under review.
5.1 Introduction

Extracellular vesicles (EVs), including exosomes, microvesicles and other vesicles, are secreted by numerous cell types. In human body, there are $>10^{12}$ EVs in 1 mL blood and they also exist in various body fluids. Exosomes are nano-vesicles (40–150 nm), while microvesicles have sizes varied from <100 nm to >1 micron. EVs encapsulate genetic and proteomic materials, including both coding and non-coding RNAs and their fragments, DNA fragments, proteins, and other cell related biomolecules that mirror the secreting cells. In the last decades, EVs have been found as a means of cell-to-cell communication (EL Andaloussi et al. 2013; Akers et al. 2013). EVs and their biomolecule contents have been recently proposed as biomarkers for disease diagnosis (Akers, Gonda, Kim, Carter, & Chen, 2013; Wu et al., 2013).

Moreover, cell-secreted vesicles (e.g. exosomes) have emerged as promising therapeutic agents and carriers for non-viral intracellular drug and/or gene delivery. While the exact delivery mechanism has not been fully unveiled yet, EVs loaded with functional RNAs and proteins have been proven as effective drugs and drug carriers for many therapeutic applications (Alvarez-Erviti et al., 2011; Kamerkar et al., 2017). Compared with synthetic carriers (e.g., synthetic lipoplex and polyplex nanocarriers), EV-based delivery, characteristic of membrane fusion and suppressed clearance in blood circulation (Kamerkar et al., 2017), is much more efficient with less cell toxicity and thus is promising as next-generation carrier-mediated intracellular delivery approach. (S, Mager, Breakefield, & Wood, 2013)
To deliver specific nucleic acids and/or proteins to target tissues or cell types *in vitro* and *in vivo* requires methods that can produce EVs with either endogenous or exogenous therapeutic cargos. Post-insertion of exogenous small interference RNA (siRNA) and shRNA plasmids into pre-existed exosomes by conventional bulk electroporation (BEP) has been developed in recent years (Alvarez-Erviti et al., 2011; Kamerkar et al., 2017). Although their therapeutic functions have been successfully demonstrated in several mouse models for cancer and non-cancer diseases, this approach faces many limitations. First, exogenous loading (e.g., post-insertion) of large biomolecules such as DNA plasmids, mRNAs, and proteins into nano-sized exosomes is technically challenging. Secondly, the strong electric field generated by BEP would break up many exosomes leading to a low yield of therapeutic exosome production. Furthermore, many large biomolecules such as mRNAs and proteins are difficult and expensive to be synthesized. Therefore, it would be highly desirable if new methods can be developed, which can efficiently produce therapeutic EVs containing specific therapeutic RNA and protein targets by endogenous loading via cell-based bioreactors (Figure 5-1).

In previous chapters, we have presented a nanochannel electroporation (NEP) biochip that can deliver DNA plasmids or other charged particulates and molecules into individual cells non-endocytically with good dosage control (Boukany et al., 2011). Herein, we demonstrated that NEP triggered heat-shock-mediated cellular responses that enable large-scale secretion of therapeutic exosomes, containing high copies of overexpressed functional mRNA and microRNA targets by NEP-based plasmid transfection, not achievable by any existing methods. For the first time, we report that NEP not only offers
a novel gene delivery tool characteristic of deterministic and benign non-endocytic cell
transfection, but can also serves as a unique platform for engineering exosomal
mRNA/miRNA promising for exosome-mediated intracellular delivery. This NEP-based
EV engineering platform could have an impact on biomedical applications such as
regenerative medicine and cancer therapy.

An integrated NEP - Tethered Lipoplex Nanoparticles (TLN) biochip is also developed for
*in situ* quantitative characterization of EVs secreted from NEP-transfected cells at the
single-EV level.
(a) Biogenesis of extracellular vesicles and their interactions with recipient cells

(b) Extracellular vesicles for gene and/or drug delivery
Figure 5-2 Concept of *in situ* detection and characterization of EV RNA by TLN (Wu et al. 2013)

a. EVs in serum are captured on the TLN biochip. miR-21 in EVs from lung cancer patient serum is identified using TIRF microscopy.

b. Preparation of specific MB-containing cationic lipoplex nanoparticle tethered on chip surface through biotin–avidin interactions

c. The fusion between an EV and a cationic lipoplex nanoparticle shown in Cryo-TEM images
5.2 Results and Discussions

5.2.1 Comparison of EV Secretion Using Different Transfection Methods

We first compared the number of EVs secreted from the equal number of transfected cells by different cell transfection techniques, i.e., our unique NEP-based transfection based on the 3D NEP platform (Figure 5.3), and commonly-used conventional transfection methods including lipofectamine (Lipo) and BEP. It has been previously reported that NEP-based cell transfection, unlike BEP and chemical nanoparticles (e.g., synthetic lipoplex and polyplex nanocarriers), is non-endocytic. As can be seen in Figure 5.4, the results of quantified delivery dosage by YOYO™-1 labelled plasmids showed that BEP could deliver nearly 3 folds more plasmids than NEP to the MEF cells. However, most plasmids were still near the cell surface 1 h after BEP transfection, while the injected plasmids by NEP have already been uniformly diffused within cytoplasm at the same time. In addition to this unique direct “injection” delivery characteristics, NEP was also found to trigger the EV production.

Figure 5.5 shows the NanoSight™ measurement results of EV numbers secreted from the same number of MEF cells (5.0 x 10⁶ cells) transfected with the combination of Ascl1, Brn2 and Myt1l plasmids at a weight ratio of 2/1/1 by either lipofectamine (Lipo), BEP or NEP. All EVs were collected from cell culture medium at 24 h post-transfection. The results show that lipofectamine (Lipo) based cell transfection did not change the EV secretion, with the EV concentration around 2.0 x 10⁹/ml close to non-transfected control group. Apparently, a slow plasmid endocytosis process by nanoparticle carriers would not stimulate the transfected cells much and, consequently, there was almost no change on EV
secretion. In comparison, BEP based cell transfection led to more EV secretion to ~6.0 x 10⁹/ml. A tremendous increase of EV secretion to >1.3 x 10¹¹/ml was observed by NEP cell transfection with or without adding plasmids. This implies that the transfected cells were somewhat stimulated by BEP, but highly stimulated by NEP leading to very significant increase of EVs in the latter case.

Analysis of particle size distributions measured by a dynamic light scattering (DLS) goniometer (Figure 5.6) showed that the EV size distribution for NEP stimulation did not change the larger EV (mostly microvesicles) distribution much, but substantially increased the secretion of exosomes with sizes ranging from 40 to 110 nm.
Mono layer of donor cells laid on the chip surface after overnight cell incubation.

DNA plasmids were injected into individual donor cells via nanochannels using a 220V electric pulse with 1-10 pulses and 10-15 ms pulse duration.

Figure 5-3 Schematic of a 3D Nanochannel Electroporation (NEP) biochip for donor cell transfection.

Monolayer of donor cells laid on the chip surface after overnight cell incubation. DNA plasmids were injected into individual donor cells via nanochannels using a 220V electric pulse with 1-10 pulses and 10-15 ms pulse duration.
Figure 5-4 Comparison of BEP and NEP on YOYO™-1 fluorescence labelled DNA plasmid delivery efficiency at 1 h post-transfection

(a) Representative epifluorescence images for the cells after BEP and NEP. (b) Comparison of fluorescence intensity in these two cases.
NEP cell transfection significantly stimulates the EV secretion from transfected mouse embryonic fibroblast (MEF) cells, with comparison to lipofectamine (Lipo) and BEP based cell transfection. Ctrl stands for non-transfected MEF cells; NEP stands for NEP cell transfection with DNA plasmids; NEP-PBS stands for NEP cell transfection with PBS buffer only. Same number of MEF cells were transfected with plasmids by various techniques, and cell culture mediums were collected 24 h post-transfection. The EV numbers were detected by NanoSight™. For the BEP, the transfection voltage was 1250 volts. For NEP, the transfection voltage was 220 volts with five 10 ms pulses.
Comparison of size distributions of EVs measured by DLS goniometry with (b) or without (a) NEP. Cell culture medium was collected 24 h post NEP transfection, and cell debris was removed by centrifugation at 1500 g for 10 min. EVs in supernatant were measured by DLS.

Figure 5-6 Size distribution of NEP-produced EVs
5.2.2 Visualizing Exosome Production in NEP-stimulated Cells

Live cell imaging using both epifluorescence and total internal reflection fluorescence (TIRF) was conducted to monitor the EV production dynamics in and out of the NEP transfected cells. CD63-GFP plasmids, encoding a widely-used exosome (a subtype of EVs) biomarker CD63 (Kowal et al., 2016) tagged with GFP, was transfected to visualize the exosomes. As shown in Figure 5.7, NEP-transfected cells showed a tremendous number of bright spots as early as 4 h post transfection, indicating active dynamics related to vesicle generation inside cytosol, while BEP-transfected cells only exhibited very weak green fluorescence. It corresponded well with previous EV number measurement results in which only NEP cell transfection could generate significantly more EVs in terms of particle number released into the cell culture medium.
Figure 5-7 EV production dynamics in NEP and BEP transfected MEF cells. Visualization of EV was achieved by transfection of CD63-GFP plasmids

(a) Snapshots of cell images and intensity surface profiles at 24 h post transfection. Scale bar: 25 μm

(b) TIRF images of BEP and NEP transfected cells at 4 h post transfection. Scale bars: 20 μm
5.2.3 Identification and Quantification of Endogenously-Loaded EV mRNA Content

It is naturally to ask whether the secreted EVs from NEP transfected MEF cells would contain corresponding overexpressed Ascl1, Brn2 and Myt11 mRNAs because of the transfection of Ascl1, Brn2 and Myt11 plasmids. Quantitative-Reverse Transcription Polymerase Chain Reaction (qRT-PCR) results supported our hypothesis that the secreted EVs from NEP cell transfection of A/B/M plasmids did contain a large amount of corresponding mRNAs or their fragments (Figure 5.8). Like the EV numbers, lipofectamine (Lipo) based cell transfection did not change the mRNA expression much, while the BEP based cell transfection could increase the mRNA expression several folds. In comparison, the NEP based cell transfection resulted in thousands folds increase of target mRNAs. The same amount of total RNA (20 ng) was used for each mRNA detection by qRT-PCR, according to manufacturer's instruction.

Furthermore, in vitro protein translation of mRNA using Rabbit Reticulocyte Lysate System (Promega) proved that some of the EV mRNAs were functional, capable of encoding polypeptides in support of protein synthesis, as shown in the Western blotting plot (Figure 5.9 a). The same amount of total RNAs (1 µg) from each transfection group was used for in vitro translation. These results confirmed that the NEP-produced EVs carry intact and functional mRNAs that can translate their protein products in the presence of functional protein machinery.
(a) CT values for Ascl1/Brn2/Myt1l mRNA using various cell transfection techniques at 24 h post-transfection. Total RNAs were obtained and reverse transcript according to manufacturer’s instruction. The same amount of total RNA (20 ng) for each mRNA was used mRNA detection by qRT-PCR. (b) fold changes of relative Ascl1/Brn2/Myt1l mRNA expression levels in EVs determined by qRT-PCR from MEF cells transfected by Ascl1/Brn2/Myt1l plasmids using various cell transfection techniques at 24 h post-transfection.
Figure 5-9 NEP-triggered cell-secreted exosomes containing functional mRNAs.

(a) Only NEP-triggered cells secreted EVs containing functional mRNAs determined by *in vitro* protein translation and Western blotting. (b) Separation of exosomes (Exo) and microvesicles (MV) by ultracentrifugation showed that most of functional mRNAs existed in Exo subpopulation. (c) Identities of Exo and MV were confirmed by their commonly-used biomarkers. Exo markers include CD63, CD9, and Tsg101 (Kowal et al., 2016), and MV marker is Arf6 (Muralidharan-Chari et al., 2009; Willms et al., 2016)
5.2.4 Distribution of Endogenously-Loaded RNA Content in EV Subpopulation –
Exosomes (Exo) and Microvesicles (MVs)

Exosomes and microvesicles are distinct EV subtypes with their own secretion pathways
and also biomarkers. Well-known exosomal markers include CD9, CD63 and Tsg101.
(Kowal et al., 2016) The larger microvesicles (MVs) carry the typical protein marker, Arf6.
(Muralidharan-Chari et al., 2009; Willms et al., 2016)

After size-based separation of EVs by ultracentrifugation, the total mRNA concentrations
in two groups were measured by Nanodrop™, while the A/B/M mRNA expressions and
their corresponding proteins were determined by qRT-PCR and in vitro protein translation
followed by Western plotting, respectively. The results showed that there was more than
twice RNA in exosomes than in microvesicles (Figure 5.10 a), and exosomal A/B/M
mRNA expression levels were 200-fold, 45-fold, and 125-fold more than the ones in MVs
(Figure 5.10 b-c), respectively.

Similar in vitro protein translation experiments were conducted and the results showed that
most functional ABM mRNAs were presented in exosomes instead of MVs (Figure 5.9 b-
c). This distribution pattern of mRNA content correlated well with previous DLS EV size
distribution data which showed that NEP based cell transfection would increase the
secretion of exosomes with sizes ranging from 40 to 110 nm. (Figure 5.6)
(a) The measurement of RNA amount by Nanodrop™ in EV subtypes: exosomes and microvesicles.

(b-c) The quantification of EV mRNA expressions by qRT-PCR in exosomes and microvesicles. (b) PCR CT value (c) Fold change. The results proved that EV-containing overexpressed A/B/M mRNAs from NEP group were found in the exosomes (Exo), instead of microvesicles (MV).

Figure 5-10 Locate endogenously-loaded ABM mRNA content in a subgroup of EVs exosomes.
5.2.5 Induced Neurons by Exosomal mRNA Transfer

Our previous identification and quantification of specific overexpressed RNAs being present in NEP-produced exosomes laid the foundation for a bold hypothesis that NEP-produced exosomes may represent a vehicle by which endogenously loaded RNA can be efficiently delivered into recipient cells and thus modulate protein production in recipient cells, as a way of cell-to-cell communication. To demonstrate that NEP-produced exosome-derived mRNAs have a potential therapeutic value in the fields such as regenerative medicine, a well-established direct nuclear reprogramming model in which fibroblast transdifferentiates into neurons by exogenous expression of ABM was tested. Using the exosome-based mRNA delivery strategy, treated MEFs showed clear induced neuron formation based on the electrophysiological measurement by patch clamp. At 24 days, the treated MEF cells showed typical neuron’s electrophysiological activities, i.e., a series of action potentials in response to current injections, as shown in Figure 5.11. A typical response to a 20 pA current injection is illustrated in red line. This induced neuron formation in the recipient cells indicated that transferred exosomal mRNA secreted from NEP-transfected donor cells can be functional after efficiently entering recipient cells via exosome-membrane fusion.
Action potential detection by patch clamp shows that MEF cells transfected every other day with Ascl1/Brn2/Myt1l mRNA containing EVs obtained from NEP could be reprogrammed into functional induced neurons (iNs) after 24 days. NEP transfected MEF cells were reprogrammed into iNs after 21 days.

Figure 5-11 Electrophysiological measurement of induced neurons by EV-mediated exogenous ABM delivery.

Action potential detection by patch clamp shows that MEF cells transfected every other day with Ascl1/Brn2/Myt1l mRNA containing EVs obtained from NEP could be reprogrammed into functional induced neurons (iNs) after 24 days. NEP transfected MEF cells were reprogrammed into iNs after 21 days.
5.2.6 Endogenous Loading of Regulatory RNA microRNA (miR) in NEP-produced Exosomes

Previously we mainly focused on the analysis of NEP-produced EVs loaded with large mRNAs (>1k nucleotide long). Here, we show that NEP could also produce EVs containing smaller regulatory RNAs such as microRNA and short hairpin RNA (shRNA) in the same manner as mRNA. The therapeutic applicability of BEP-loaded-exosomes was demonstrated by exogenous loading of small interference RNAs (siRNAs) in exosomes (Alvarez-Erviti et al., 2011; Kamerkar et al., 2017). Herein, one specific microRNA --miR-128-- was tested as an example. Like mRNA loading, donor MEF cells overexpressed the miR-128 via NEP-based transfection of the miR-128 plasmids. Lipofectamine and BEP transfection were used for comparison. Again, EVs were collected from cell culture medium at 24 h post-transfection. As expected, the qRT-PCR results showed that NEP-based transfection produced EVs containing a large amount of miR-128 (more than 4,500 folds increase), not achievable by BEP or lipofectamine based cell transfection (Figure 5.12).
EV miR-128 expression determined by qRT-PCR from MEF cells transfected by miR-128 DNA plasmid using various techniques at 24 h post-transfection. EVs were harvested from cell culture medium at 24 h post-transfection (miR-128 plasmid) by various techniques. The same amount of total RNA (30 ng) was used for miR-128 detection by qRT-PCR. (a) CT values for miR-128 using various cell transfection techniques at 24 h post-transfection. (b) Fold changes of relative miR-128 expression levels using various cell transfection techniques.

Figure 5-12 Quantification of microrna expression in EVs by qRT-PCR.
5.2.7 NEP Increases EV Secretion by Triggering Heat-shock-mediated Cellular Responses

During electroporation, Joule heating caused by the imposed electric field could tentatively increase the buffer temperature and, in turn, cause thermal shocking to the transfected cells. It is known that thermal shocking may increase cell secretion of EVs due to chaperone mediated autophagy caused by the increase of heat shock proteins (HSPs) in cells (Baixauli, Lopez-Otin, & Mittelbrunn, 2014; Lancaster & Febbraio, 2005; Takeuchi et al., 2015).

![Diagram](image)

Figure 5-13 Schematic of a NEP-transfected cell increasing the secretion of EVs endogenously loaded with intact and functional overexpressed RNAs via a heat-shock-protein-involved chaperone mediated autophagy triggered by a transient yet drastic heat shock during the NEP process.
Indeed, we found that NEP could substantially increase the expression of several HSPs. When HSP inhibitors were added in cell culture medium after electroporation, EV secretion was suppressed. As shown in Figure 5.14, 50%, 40% and 70% decrease of EV secretion of NEP transfected MEF cells in terms of EV number with treatment of HSP 70 inhibitor (VER 155008), HSP90 inhibitor (NVP-HSP990), and their mixture respectively at their EC-50 concentrations was seen. Medium was collected at 24 h post-transfection and EV numbers were detected by dynamic light scattering (DLS) goniometer.
Effect of heat shock protein 70 (HSP70) and heat shock protein 90 (HSP90) inhibitors on EV secretion from NEP transfected MEF cells. After NEP transfection, the cell culture was replaced with fresh medium containing HSP70 inhibitor (VER 155008), HSP90 inhibitor (NVP-HSP990), or their mixture. Medium was collected at 24 h post-transfection, and EV numbers were detected by dynamic light scattering (DLS) goniometry.

Figure 5-14 EV production by NEP is heat-shock-mediated.
Physically, during the NEP process, the electric voltage pulse applied would create an intense electric field in the nanochannel, which facilitates electrophoresis-based cargo delivery and localized poration on the cell membrane, as discussed before. Meanwhile, a sharp temperature gradient was also formed in and around the NEP-transfected cell near the nanochannel, resulting in a local “heat source” with a power density of ~1 x 10^{14} W/m^3. Numerical simulation results showed that a 200 V and 10 ms pulse could create a localized “hot spot” in the nanochannel outlet with a peak temperature up to 55 °C from ambient temperature, which could lead to a strong ‘heat shock’ stimulation to the transfected cells (Figure 5.15). However, once the pulse ended, the temperature field would vanish rapidly (<2ms) to ambient temperature is reached, due to the extremely small volume of the heated fluid inside the nanochannel (V_{nanochannel} \approx 1 \times 10^{-18} m^3) compared to the bulk solution outside the nanochannel (V_{bulk} \approx 1 \times 10^{-7} m^3). Therefore, the transfected cells would not be permanently damaged due to any pro-longed exposure to a unphysiologically overheated cell buffer.

Since BEP does not have the nanoscale spatial confinement like NEP, it could not contribute to the generation of any transient and yet drastic temperature field. Consequently, BEP only slightly increased EV secretion despite a much higher voltage applied (>1,000 V).

Together, these results imply that the NEP-induced large EV secretion from the transfected cells could be partially attributed to the heat-shock-protein-mediated cellular responses triggered by a transient yet drastic heat shock during the NEP process.
(a) A snapshot of the temperature gradient at 10.4 ms, indicating a high temperature up to 55.5°C in the nanochannel region (red color). (b-c) The temperature at different locations from top (point 1) to bottom (point 5): z = 5, 0.5, 0 (nanochannel interface), -2 and -5 µm as a function of time. While peak temperature inside the nanochannel reached > 60 °C, the temperature gradient vanished rapidly (<2 ms) once the pulse ended.
5.2.8 Comparison EV Loading Methods: Endogenous RNA Loading by NEP Transfection of Donor Cells with Plasmids vs. Exogenous RNA Loading into Collected Blank EVs by BEP Post-insertion

Here, we compared the efficacy of producing therapeutic EVs using our NEP based cell transfection and the BEP post-insertion approach used by several researchers (Alvarez-Erviti et al., 2011; Kamerkar et al., 2017). For the former, the miR-128 plasmid was co-transfected with CD63-GFP plasmid to MEF cells by NEP to generate EVs containing miR-128 according to aforementioned procedures. For the latter, blank EVs were first harvested from MEF cells transfected with CD63-GFP plasmids at 24 h after NEP. In parallel, miR-128 was collected from MEF cells transfected with miR-128 plasmids at 24 h post-transfection by NEP. The collected miR-128 (1 µg) was mixed with blank EVs (1.0 x 10^6) and electroporated by BEP (1250 V, 30 ms) according to conditions used by other researchers (Alvarez-Erviti et al., 2011; Kamerkar et al., 2017). EVs from the two approaches were tested using a tethered lipoplex nanoparticle (TLN) biochip on a total internal reflection fluorescence (TIRF) microscope. Figure 5.16A shows the TLN-TIRF assay schematic (Lee et al., 2016)(Wu et al. 2013). Briefly, a molecular beacon (MB) for the RNA target was designed and encapsulated in cationic liposomal nanoparticles. These cationic lipoplex nanoparticles were tethered on a glass slide, which are able to capture negatively charged EVs by electrical static interactions to form a larger nanoscale complex. This lipoplex-EV fusion led to mixing of RNAs and MBs within the nanoscale confinement near the biochip interface. TIRF microscopy was capable of detecting a single biomolecule
and it measured fluorescence signals <300 nm near the interface, which was where the tethered liposomal nanoparticles locate.

Figure 5-16 Comparison of secreted EVs containing miR-128 by NEP transfection of DNA plasmid to MEF cells vs. existing EVs loaded with pre-collected miR-128 by BEP post-insertion.
Figure 16B shows the representative TLN-TIRF images of the captured EVs. The green fluorescence was from EVs containing CD63-GFP, while the red fluorescence was from the hybridization of miR-128 molecules and the Cy5-miR128 MBs in the captured EVs. It is clear that our NEP approach was able to produce more EVs containing higher copies of miR-128 than the BEP post-insertion approach. Figures 16C-E show a quantitative comparison of those two approaches. Although both approaches were able to produce EVs containing miR-128 (~80% of total captured EVs), the EV miR-128 concentration in EVs (~3 times MB fluorescence intensity) was much higher in NEP based direct cell transfection than in BEP based microRNA post-insertion. Furthermore, BEP post-insertion tended to break nearly half of the blank EVs leading to a very low yield of therapeutic EVs.

A similar comparison was also carried out for a much larger RNA, Brn2 mRNA (6272 bases for Brn2 mRNA vs. 21 bases for miR-128) using the same approach as for miR-128. Figure 17 shows that our NEP approach could produce >70% EVs containing Brn2 mRNA, while only very few existing EVs could be loaded with the same mRNA by the BEP post-insertion approach. The concentration of Brn2 mRNA in NEP produced EVs was high, while that in BEP post-insertion was very poor.
Figure 5-17 Comparison of secreted EVs containing Brn2 mRNA by NEP transfection of DNA plasmid to MEF cells vs. existing EVs loaded with pre-collected Brn2 mRNA by BEP post-insertion.
5.2.9 Improvement of Multiple mRNAs Co-localized in the Same Secreted EVs by Sequential NEP Transfection of DNA Plasmids to MEF Cells

Figure 18 shows the EV secretion and content profiles as a function of time after NEP transfection with Ascl1, Brn2 and Myt1l DNA plasmids. The Ascl1 plasmid is the smallest one (7k bp) among the three, while the Myt1l plasmid is the largest (9k bp) with the Brn2 plasmid in between (8k bp). EVs in the cell culture medium was collected at the indicated time points, and the culture medium was replaced with fresh medium. The EV numbers were detected by the DLS goniometer, while the EV mRNA expressions were detected by qRT-PCR as described before. The results showed a quick increase of EV secretion within 4 h post-transfection, and peaked at 8 h with continuous EV secretion for more than 24 h. EVs containing Ascl1 and Brn2 mRNAs also appeared within 4 h post-transfection with profiles matching well with that of EV secretion. EVs containing Myt1l mRNA appeared at a later time, but still within 24 h. This data implies that different mRNA targets could be transcribed at different times and rates in the transfected cells, even though multiple DNA plasmids were delivered to the cells at the same time, due to the size difference of plasmids or other reasons. This may lead to individual EVs containing only one or few mRNA targets. For better therapeutic efficacy, it would be valuable if more or all mRNA targets can be encapsulated in the same secreted EVs. By sequentially deliver each DNA plasmid into MEF cells using NEP based on its transcription time, Figure 19 shows that we could substantially increase the secreted EVs containing all three mRNAs, Ascl1, Brn2 and Myt1l (>50% vs. <25%), needed for iN reprogramming. For NEP transfection, Ascl1, Brn2 and Myt1l plasmids were transfected
at the same time as described before. For sequential-NEP, the Myt1l plasmid was transfected first, Brn2 plasmid was transfected 4 h later, while Ascl1 plasmid was transfected 4 h after Brn2 transfection. At 24 h post Myt1l transfection, culture medium was collected for TLN assay. Equal amount of FAM-Ascl1, Cy3-Brn2, and Cy5-Myt1l MBs were encapsulated in tethered lipoplex nanoparticles for EV-mRNA detection. In the figure, the yellow arrow means EVs containing all 3 mRNAs, the blue arrow means EVs containing 2 mRNAs, while the pink arrow means EVs containing only 1 mRNA.
MEF cells were transfected with DNA plasmids by NEP. The cell culture medium was collected at indicated time points, and replaced with fresh medium. The EV numbers were detected by DLS goniometer. The mRNA expressions were detected by qRT-PCR.

Figure 5-18 EV-mRNAs secretion profiles from NEP transfected MEF cells.
For NEP transfection, Ascl1, Brn2 and Myt1l plasmids were transfected at the same time as described before. For sequential-NEP, the Myt1l plasmid was transfected first, Brn2 plasmid was transfected 4 h later, while Ascl1 plasmid was transfected 4 h after Brn2 transfection. At 24 h post Myt1l transfection, culture medium was collected for TLN assay. Equal amount of FAM-Ascl1, Cy3-Brn2, and Cy5-Myt1l MBs were encapsulated in tethered lipoplex nanoparticles for EV-mRNA detection. Yellow arrow: EVs containing 3 mRNAs; Blue arrow: EVs containing 2 mRNAs; and Pink arrow: EVs containing 1 mRNA.

Figure 5-19 Increased mRNA co-localization in the same EV by sequential-NEP
5.3 Materials and Methods

5.3.1 Cell Culture

Mouse embryonic fibroblasts (MEFs) were purchased from Millipore. MEFs were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Thermo Fisher Scientific) containing 10% Heat-Inactivated Fetal Bovine Serum (FBS) (Catalog number: 10438034 Thermo Fisher Scientific) and 1% Non-Essential Amino acid (NEAA) (Catalog number: 11140050, Thermo Fisher Scientific).

5.3.2 Cell Transfection

For NEP-based transfection, a single layer of donor MEF cells attached and spread on the 3D NEP silicon chip surface after overnight cell incubation. DNA plasmids pre-loaded in PBS buffer were injected into individual donor cells via nanochannels using a 220 volts electric field across the nanochannels. Various electroporation conditions such as voltage level, pulse number and pulse length can be chosen.

BEP (Neon Transfection System, Thermo Fisher Scientific) and lipofectamine transfection were conducted according to manufacturer’s instruction.

Ascl1/Brn2/Myt1l plasmids at a weight ratio of 2/1/1, according to the protocol in literature (Gallego-Perez, Otero, et al., 2016), were pre-mixed for transfection. A mixture of those DNA plasmids is known to reprogram donor cells into induced neurons (iNs).

5.3.3 Collection of EVs Secreted by MEF Cells

MEF cells were cultured in cell culture medium containing serum until they were transfected. The cell culture medium containing serum was removed. The cells were washed with PBS three times and cultured in serum-free cell culture medium.
For RNA content measurement experiments including qRT-PCR, NanoDrop™ and *in vitro* translation, EVs were collected from cell culture supernatants using ExoQuick™ precipitation by centrifugation at 1500 g for 10 mins.

For EV particle measurement experiments including DSL and NanoSight™, EVs were collected from cell culture supernatants by a series of centrifugation and ultracentrifugation steps as described in literature. (Thery, Amigorena, Raposo, & Clayton, 2006)

5.3.4 *in vitro* Protein Translation

A same amount of total RNA (1 µg) from each transfection method was applied for *in vitro* protein translation using Rabbit Reticulocyte Lysate System (Promega) according to manufacturer’s instruction. After the translation procedure was accomplished, samples were separated by SDS-PAGE and the proteins were detected with various antibodies as shown in the Western blotting plot.

5.3.5 Isolation of Exosome and Microvesicles from Total EVs

For the collected total EVs, the larger microvesicles were sorted by ultracentrifugation at 10,000 g for 30 min. The supernatant was further centrifuged at 100,000 g for 2 h to collect the smaller exosomes, following the detailed protocol described in the literature. (Thery et al., 2006).

5.3.6 Whole-cell Patch Clamp for Induced Neuron (iN) Characterization

The whole-cell patch clamp recording was used to measure excitability. Cell were continuously superfused with an extracellular bath solution containing 115 mM NaCl, 2 mM KCl, 1.5 mM MgCl₂, 3 mM CaCl₂, 10 mM HEPES, and 10 mM Glucose (pH 7.4). Glass electrodes (3-4 MΩ) were filled with a pipette solution containing 115 mM K-
gluconate, 10mM N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid (HEPES), 4 mM NaCl, 0.5 mM ethylene glycol tetraacetic acid (EGTA), 1.5 mM MgCl2, (pH 7.3). Cells had a patch resistance of >100 MOhm after whole-cell access was gained, and series resistance was compensated 40-50%. Data were collected using an Axopatch 200B amplifier, Digidata 1322A digitizer, and Clampex 9 software (Molecular Devices, Sunnyvale, CA). For analysis of voltage-gated currents, the basal holding potential was -70 mV and cells were stepped for 400 ms in 10 mV increments from -120 mV to 80 mV. Transient inward currents, due to activity of voltage-gated sodium channels, were isolated from measuring the peak amplitude. Sustained plateau currents, reflective of voltage-gated potassium currents, were measured as the average of the last 50ms of the voltage step in the plateau phase of the current. Action potential induction was measured using current clamp. Current was held at 0 pA and then stepped in 20 pA intervals for 1 sec.

5.3.7 FEM Heat Transfer Simulation of the NEP Process

The temperature field near a nanochannel was simulated using COMSOL® Multiphysics 5.0. (COMSOL Inc.) “heat transfer in fluids” module, by solving the governing equation

\[
\rho c_p \frac{\partial T}{\partial t} + \rho c_p \mathbf{u} \cdot \nabla T = k \nabla^2 T + Q,
\]

where \( \rho \): density \( c_p \): heat capacity \( \mathbf{u} \): flow rate \( k \): thermal conductivity

Initial temperature = 295.15K (22°C, ambient temperature)

The nanochannel is regarded as a pulsed heat source with a power density \( Q_{nc} = P/V \approx 1 \times 10^{14} \text{ W/m}^3 \).

Simulated data was exported to MATLAB (MathWorks, Inc.) for analysis and plotting.

5.3.8 Dynamic Light Scattering (DLS) Goniometer
Size distributions of cell-secreted EVs were determined using dynamic light scattering goniometer (BI-200SM Goniometer, Brookhaven Instruments Corporation., Holtsville, New York, USA). Absolute numbers of exosomes and larger microvesicles secreted per cell were quantified by NanoSight™.

5.3.9 qRT-PCR Quantification of EV-containing RNA Target Expression Levels

The expression of Ascl1, Brn2, Myt1l mRNAs and miR-128 in EVs was measured using qRT-PCR following the manufacturer recommended protocol.

5.3.10 Total Internal Reflection Fluorescence (TIRF) Imaging

TIRF microscopy (Nikon Eclipse Ti Inverted Microscope System) was used to detect the fluorescence signals from the samples.

5.3.11 TLN assays

EVs were tested using a tethered lipoplex nanoparticle (TLN) biochip on a total internal reflection fluorescence (TIRF) microscope, following the same procedures as described in details elsewhere (Lee et al., 2016); (Wu et al. 2013). Briefly, a molecular beacon (MB) for the RNA target was designed and encapsulated in cationic liposomal nanoparticles. These cationic lipoplex nanoparticles were tethered on a glass slide, which captured negatively charged EVs by electrical static interactions to form a larger nanoscale complex. This lipoplex-EV fusion led to mixing of RNAs and MBs within the nanoscale confinement near the biochip interface. TIRF microscopy was used to measure the fluorescence signals within 300 nm range of focal plane interface, which is where the tethered liposomal nanoparticles locate.
5.4 Conclusion

In this chapter, a new concept of NEP-based exosomal RNA transfer among cells was introduced. For the first time, we reported that NEP can serve as a unique platform for engineering therapeutic exosomes containing specified RNAs via endogenous loading within cell-based bioreactors, by orchestrating a series of transient (within only milliseconds) and yet drastic (~2 x 10^7 V/m electric field strength) physical stimulations of the NEP-transfected cells, including non-endocytic plasmid transfection and a Joule heating induced temperature shock triggering cell secretion of EVs due to chaperone mediated autophagy.

NEP-stimulated cells could secret 10~100 folds more EVs compared to other transfection methods. Loaded with overexpressed RNAs from exogenous DNA plasmids, NEP-transfected cells produced EVs containing high copies of intact mRNA and miR targets up to thousands of folds more than those in EVs secreted from the non-transfected donor cells, not achievable by any exiting methods.

As a novel bio-inspired non-viral method, EV-based intracellular delivery, though still at early stage, is very attractive because of its inherent advantages including superior delivery efficiency, low cell toxicity, versatility to encapsulate various RNAs and the ability to travel across major biological barriers such as the blood–brain barrier (BBB). Thus, therapeutic EVs produced by NEP-based transfection could have an impact on a diversity of biomedical applications including regenerative medicine and targeting of oncogenes in cancer.
Chapter 6 Conclusions and Recommendations

6.1 Conclusions

In this PhD work, three micro/nanofabrication-based nanochannel electroporation (NEP) biochip platforms are presented for high-precision in vitro living cell manipulation, transfection and/or interrogation at the individual cell level. They include a living cell interrogation platform with cell migration monitoring, intracellular marker probing, and drug resistance functions (Chapter 3), a microfluidics based 3D NEP platform with high throughput cell capture, transfection and release functions, and an integrated 3D NEP - tethered lipoplex nanoparticle (TLN) platform that can produce a large amount of therapeutic extracellular vesicles (EVs) and in situ characterize the EV RNA content at the individual EV level. Unlike conventional cell transfection and cell/EV characterization methods in which cell and EV lysis or fixing is inevitable, our three biochip platforms are designed to allow temporal monitoring of cellular/subcellular activities in and out living cells.

Our unique NEP technology is implemented as the key component in all three platforms to form a powerful physical cell transfection and manipulation tool capable of deterministic and uniform individual cell characterization and transfection. A variety of biomolecules ranging from molecular beacon probes, oligo microRNAs (anti-miRs), to DNA plasmids
encoding mRNAs or miRs can be benignly introduced into target cells in a controllable and high-throughput manner.

The 3D NEP - migration biochip shown in Chapter 3, with engineered micro-features on the chip surface which mimics the in vivo microstructures with parallel tracks, allows real-time monitoring of guided single-cell migration behaviors. Coupled with NEP’s unique functions, we, for the first time, achieved the screening of intracellular markers to identify either the relatively benign proneural (PN) phenotype or the much more aggressive mesenchymal-like (MES) phenotype, and monitored the guided single-cell motility in one single chip. Migration behaviors of single cells can be linked to cell’s gene signatures screened by NEP-based transfection of molecular beacons (MBs). This biochip platform can be utilized to follow the dynamics of intracellular marker expression levels and in vitro cell motility during induced phenotypic shift of cancer stem cells such as EMT or PMT and thus has the potential to shed a new perspective for future solid tumor research. Furthermore, it is feasible for in situ quantitative probing of the decrease/increase of gene expression and detection of the variation of cell migration speed of aggressive mesenchymal stem cells caused by NEP-based transfection of novel therapeutic gene under different NEP conditions for medical treatment evaluation.

In Chapter 4, a novel microfluidic-based passive hydrodynamic weir-like cell trap approach enabling rapid, benign and efficient parallel cell manipulation with single-cell resolution was integrated with a high throughput NEP platform, which has been demonstrated with high transfection efficiency, improved transfection dosage uniformity, and enhanced controllable exogenous gene expression, not achievable by any of the
existing transfection tools. A simple micro-/nano-fabrication protocol was developed to engineer massive nanochannel arrays and a dense cell trap array. Using this unique device, both adherent cells (i.e., mouse embryonic fibroblasts (MEFs)) and suspension cells (i.e., natural killer (NK) cells,) can be massively trapped in the micro-cap array with a capture efficiency of ~90% in a few minutes. Various cargoes from large plasmids to small oligos have been uniformly NEP-delivered into trapped cell array in a controllable and uniform manner, thus showing the versatility of this 3D microfluidic-NEP platform. Such innovative nanotechnology-enabled cell transfection platform capable of precise, rapid and benign introduction of biomolecules into a large population of cells at single cell resolution has great potential to resolve the cell transfection bottleneck in many potentially promising cell therapy concepts such as CAR-T/ CAR-NK cancer immunotherapy.

In Chapter 5, a new concept of NEP-based exosomal RNA transfer among cells was introduced for the first time. We reported that NEP enabled the engineering therapeutic exosomes containing specified RNAs via endogenous loading within cell-based bioreactors, by orchestrating a series of transient and yet drastic physical stimulations of the NEP-transfected cells, including the non-endocytic plasmid transfection and a Joule heating induced temperature shock to trigger cell secretion of EVs due to chaperone mediated autophagy. NEP-stimulated cells could secret 10~100 folds more EVs compared to other transfection methods. Loaded with overexpressed RNAs from exogenous DNA plasmids, EVs produced from NEP-transfected cells contained high copies of intact mRNA and miR targets up to thousands of folds more than those in EVs secreted from the non-transfected donor cells, not achievable by any exiting methods.
This novel and bio-inspired non-viral method, alternative to physical membrane-disrupted method like bulk electroporation, for EV-based intracellular delivery may become very attractive because of its inherent advantages including superior delivery efficiency, low cell toxicity, versatility to encapsulate various endogenous RNAs, and the ability to travel across major biological barriers such as the blood–brain barrier (BBB). Thus, therapeutic EVs produced by NEP-based transfection could have an impact on a diversity of biomedical applications including regenerative medicine and cancer therapy.

6.2 Recommendations

6.2.1 Large-scale drug screening based on multi-well 3D NEP platform

We have demonstrated the large-scale intracellular screening capability of our 3D NEP device in Chapter 3. Such platform can be easily scaled up by dividing the NEP chip into multiple wells to achieve high-throughput drug screening, with each well containing a certain type and/or concentration of a new drug or drug mixture (Figure 6.1). Conventional chemo drug can be simply applied in the cell culture medium in different wells with a concentration gradient. Novel regulator RNA interfering gene therapy (such as miR or anti-miR) can be delivered via NEP-based cell transfection. Controllable dosage delivery of nucleic acid cargos can be achieved by programmed pulse duration and numbers of pulses, without affecting cell viability.

Intracellular biomarker measurements can be done by NEP-based MB delivery. It enables fast and efficient readout of intracellular dynamics in response to various drug treatment conditions at the single-cell level.
Such platform could be useful to find out the optimal drug treatment conditions among a large number of drug candidates.

Figure 6-1 Schematic of multi-well 3D NEP platform for large-scale drug screening
6.2.2 On-chip migration assay after cell trapping

It is possible to develop an integrated NEP - migration biochip for on-chip multi-functional living cell interrogation at single clone level. A design concept is given in Figure 6.2. Here, the 3D NEP chip, with the ‘U-shaped’ microstructures facing up, is vertically dipped in a monodispersed cell mixture for a few minutes, and then is lifted-up stably and slowly (with an angle of $60^\circ$ – $90^\circ$ to the perpendicular direction). Microfluidic cell trapping described in Chapter 4 can be used as an alternative method. In this procedure, individual cells can be hydrodynamically trapped in the micro-caps (step (ii)). This automated cell trapping method is much faster and efficient, and can easily handle thousands of cells. After cell positioning in the cell trap array, cell migration is immediately conducted as each captured cell will travel along a microgroove track towards a 20 µm diameter well located 300 µm away (step (iii)). A chemical gradient driven by fetal bovine serum can be established from the nanochannels to guide unidirectional migration to the transfection location. Cell motility can be monitored via time lapse microscopy for up to many hours or days. Nanochannels are located at the bottom of each well for NEP delivery of MBs using a pulsed electrical stimulation (e.g. 250 V, 10 ms pulses, 10 pulses) to detect the expression of intracellular biomarkers such as MLK4, NF-kB and CD109 for gliomas stem cells (GSCs) after fast-migrating cells have reached their corresponding microwells. For comparison, one may also detect CD133 and CD44 expression. On-chip sorting of high/medium motility GSCs can be done by washing away the slower migrating cells still on the tracks (step (iv)) after a pre-specified migration time, and then releasing the fast-migrating cells trapped inside the
microwells with trypsin. Fluorescence-activated cell sorting (FACS) can be used for sorting based on high/low expression of MLK4/CD109. Scrambled MBs can be used for control purposes. Beacon hybridization dynamics post NEP can be monitored via fluorescence microscopy, and GSC clones can be categorized into subtypes of high/low expression of CD109, MLK4 and/or NF-kB based on a normalized fluorescence intensity scale. The ability of siRNA (e.g. MLK4) to downregulate a specific gene in GSC subtypes can be subsequently evaluated via NEP-based siRNA delivery. MBs targeting the same targets can then be NEP-delivered to monitor changes in target factors following siRNA delivery. One may use fluorescent probes at different wavelengths so that one can distinguish hybridization events from the first MB delivery vs. the second one. For example, these experiments will allow us to correlate MES GSCs with high/low expression of CD109, MLK4 and/or NF-kB to their motility capabilities. These experiments will allow us to establish a correlation between the expression levels of these key factors and the cell’s ability to resist siRNA therapy. This biochip platform is capable of high-throughput intracellular screening and migratory behavior monitoring of cancer cells on a single chip, which has not been achieved by any existing on-chip assay. On-chip in situ living cell interrogation can be demonstrated by the detection and characterization of proneural–to-mesenchymal (PN-MES) transformation in patient-derived glioma stem cell (GSCs). This novel 3D NEP-migration platform can not only accomplish single-cell migration assay, but also correlate individual cell’s quantitative migratory behaviors with their distinct intracellular
signatures, which could potentially be used for targeted therapy development as well as better understanding of tumor cell biology.
(a) NEP device patterned with cell trap array for cell positioning

(b) Cells can be trapped in the cell trap array via simple de-wetting or microfluidic cell trapping, as described in Chapter 4.

(c) NEP-transfected cells can migrate along the biomimetic micro grooves.

(d) High-motility cells can be enriched by removing the slow-moving cells from the chip.
6.2.3 Integrated NEP - TLN assay for time lapse studies of EV secretion at the single EV level in response to NEP-based stimulations

We may develop a novel chip platform that combines the advantages of the NEP and TLN technologies into a single system (Figure 6.3).

6.2.3.1 Study of EV release from heterogeneous cancer cells under therapy treatment

This platform is suitable for understanding the inherent tumor heterogeneity by comparing the number of cell-derived EVs containing certain oncogene targets (e.g. oncomiR miR-21) quantified by TIRF-measured fluorescence intensity for individual cells with distinct phenotypes examined by NEP-based MB hybridization. It is reasonable to hypothesize that MES CSCs will secret more EVs containing oncogenes due to its much higher tumor-initiating ability compared to PN CSCs. It is also interesting to investigate the cellular response to external stimulations such as irradiation therapy, and NEP-based anti-miR injection, in terms of EV secretion. Our hypothesis is that apoptotic cells induced by treatment will secret less number of EVs (excluding apoptotic bodies) encapsulating oncogene; however, drug-resistant cells may secret more EVs that are likely to play an important role in devastating tumor recurrence.

6.2.3.2 Characterization of therapeutic EVs

Such setup will also be used to controllably transfect autologous dendritic cells, which are known to produce high amounts of EVs(Hall et al., 2016), with plasmids encoding for specific proteins (targeting CD109) that can then be packed with MLK4 siRNA by post-insertion and used for therapeutic purposes. The TLN component can be used to study the dynamics of EV release as well as the EV content.
Studies in Chapter 5 have shown that NEP stimulation can tremendously enhance the release of EVs compared to conventional non-viral cell transfection methods. We propose to integrate NEP and TLN into a unique nanotechnology platform where specific plasmids or their combinations can be systematically delivered into donor cells by 3D NEP to secret EVs with targeting molecules and define optimum conditions (via TLN-based monitoring) to produce high-quality therapeutic EVs.
Figure 6-3 Schematic of NEP-TLN biochip system for *in situ* detection and characterization of EVs secreted from NEP-stimulated living cells.
6.2.4 Polymer-based soft 3D NEP devices

Currently, our 3D NEP platform is based on the silicon substrate with the limitations of high cost, brittleness, non-transparency, and labor-demanding fabrication protocol. While Si-based NEP chip is suitable for proof-of-concept research purposes, a low-cost and mass producible polymer version of the 3D NEP devices would be beneficial for NEP-related assays ranging from large-scale NEP cell transfection, \textit{in vivo} NEP transfection, to fundamental single cell analysis of EV secretion after NEP stimulation.

Here, a nanoimprinting fabrication method is proposed to make polymer-based NEP devices. (Figure 6.4) A silicon master mold with a nano-pillar array is fabricated as a template (Figure 6.5-6) and nanopores can be created in the polymer by nanoimprinting. A water soluble sacrificial layer can be used to detach the polymer NEP thin layer from the substrate and also to protect the tips of nano-pillars on the silicon template from being broken.
Figure 6-4 Fabrication of soft NEP patch by nanoimprinting
Figure 6-5 Design of a silicon master mold with a nano-pillar array

Figure 6-6 Fabrication of nano-pillars using different deep etching methods

(a) Cryo-Process (image courtesy of NIL Technology ApS)

(b) Bosch process
References


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Appendix A: Multi-gene detection by NEP-delivered MBs

High-throughput NEP-based intracellular multi-gene screening via \textit{in situ} molecular beacon (MB) hybridization was demonstrated in Glioma Stem Cells (GSCs).

Case 1 Regulation of AXL by PDGFRa

Here we studied the effect of downregulation of PDGFRa via shPDGFRa on the AXL expression. To quantify mRNA expressions of 2 genes (i.e. AXL and PDGFRa) in individual GSC clones, high-throughput NEP-based injection of molecular beacon probes for large-scale intracellular screening of both non-treated (NT) GBM 157 samples and shPDGFRa infected GBM157 samples were conducted. The single-clonal fluorescence intensity distributions quantifying biomarker expressions in two groups are displayed in Figure A-1. Experimental results of \textit{in situ} molecular beacon (MB) hybridization showed both AXL and PDGFRa MBs signal distributions shifted to the left and shPDGFRa viral infection. This result indicates a connection between these two genes in which downregulation of PDGFRa would lead to the less expression level of AXL at mRNA level.
Case 2 Multi-gene and oligo RNA detection experiments showed increased expression of MLK4.

The results of quantification of miR363 and MLK4 expression before and after anti-miR363 delivery are shown in Figure A-3. The levels of miR363 in the surviving cell population decreased as expected. The levels of MLK4, on the other hand, increased, which further suggests that the surviving cell population has a more mesenchymal-like phenotype (Kim et al., 2016). Fluorescence micrographs showing multi-gene and oligo RNA detection capabilities in the surviving cell population, which has low/negligible expression of proneural marker CD133, and high expression of mesenchymal markers CD44 and MLK4.
Figure A - 1 NEP/MB-based detection of AXL and PDGFRa in shPDGFRa-treated GBM157 population.
Figure A-2 Fluorescence images showing MB signals in shPDGFRa-treated GBM157 population, in comparison to control.
Figure A-3 NEP/MB-based detection of multi-gene-expression and oligo RNA
Appendix B: Induced Endothelium by in vivo NEP transfection for Ectopic Osteogenesis post bone scaffold (BMP-2/MBG) Subcutaneous implantation

This is a collaborative *in vivo* NEP study with East China University of Science and Technology (ECUST). We conducted the animal experiments for the pilot study of *in vivo* NEP-based induced endothelium (iE) for ectopic osteogenesis post bone scaffold (BMP-2/MBG) subcutaneous implantation. The *in vivo* NEP transfection device set-up is shown in Figure B-1. The qRT-PCR assays and confocal fluorescence observation 24 hours after the *in vivo* transfection were used to determine the transfecting efficiency of Etv2/Foxc2 plasmids and BMP-2 plasmid. (Figure B2-3). The results indicated successful transfection of both plasmids.

According to ectopic bone formation protocol, we took samples for histological section 2 weeks after implantation and transfection. By observation with naked eye while taking samples, we could see that vascularization at the ectopic bone site of EFF transfection groups (both skin and muscle) seemed better than the un-transfected control group; however, the ectopic bone formation of BMP-2 plasmid transfection group seemed inferior to the BMP-2 protein group.

Experiments to further characterize the vascularization at the implantation/transfection site will be conducted.
Figure B-1 *in vivo* NEP transfection system set-up
Verify *in vivo* NEP co-transfection of induced endothelia (iE) factors: ETV2&FOXC2 without reporter genes at two transfection locations, i.e., skin and muscle.

Figure B-2 ETV2 mRNA expression determined by qrt-PCR 24h post NEP.
Figure B-3 Confocal images of NEP-transfected skin and muscle tissues.

(a-b) – Epidermis NEP-transfection of BMP2-GFP plasmids compared to control (PBS). (c-d) Muscle NEP-transfection of BMP2-GFP plasmids compared to control (PBS)
Appendix C: Therapeutic effect study for EV-mediated drug delivery to AML MV4

Our 3D NEP-TLN biochip platform can be used to controllably deliver plasmids encoding for specific surface markers to produce functionalized EVs to specifically target cancer cell populations of interest, and characterize the EV release profile in real time using the TLN component. Proof of concept experiments demonstrated that NEP-based delivery of CEPβα to 293T cells can lead to secretion of a tremendous number of EVs rich in miR-181a. (Figure C-1).

Such EVs have been found to be cytotoxic against Raji leukemia cells. (Figure C-2) EVs collected from NEP-PBS transfected cells were used as control.
Figure C-1 NEP-based transfection of CEBPα plasmids induces upregulation of miR-181a in transfected cells and cell-secreted EVs

A. Fluorescence micrograph of 293T cells showed high miR-181a MB signal. Image taken 24h post NEP-based transfection of CEBPα plasmids.

B. TIRF images of miR-181a-containing EVs detected by TLN chips. CEBPα plasmids transfection group showed significantly higher Cy3 miR-181a MB signal compared to PBS transfection group. Scale bar: 10 μm
Figure C-2 EVs rich in miR-181a can kill Raji cells in 24 h. (a) Representative images of LIVE/DEAD Assay. (b) Cell viability percentage after treatment of miR-181a-containing EVs secreted from CEPBA transfected cells.