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Microscale Tools for Targeted Protein Cytology

by

Elaine Jean Su

A dissertation submitted in partial satisfaction of the requirements for the
degree of

Joint Doctor of Philosophy
With University of California, San Francisco

in

Bioengineering

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Amy E. Herr, Chair
Professor Todd McDevitt
Professor Shaofan Li

Summer 2018

Microscale Tools for Targeted Protein Cytology

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Elaine Jean Su

Abstract

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Professor Amy E. Herr, Chair

Direct measurement of proteins in single cells is necessary in the study of human biology and disease. Protein signaling underpins a variety of cellular processes, such as stem cell differentiation, cell migration, and cancer metastasis. Extracellular factors such as paracrine signaling, biophysical properties of the substrate, and cell-cell interactions are known to regulate a variety of signaling processes. Yet, challenges in selectively measuring protein targets exist, and measuring signaling in adherent cells remains elusive as most conventional approaches to study single cell signaling involve cell detachment. To address these measurement gaps, we introduce methods to manipulate the cell-extracellular microenvironment and design new technologies for assessing protein signaling in adherent cells.

First, to determine how the biophysical environment influences cell behavior, we designed hydrogels of varying stiffness and evaluated how stiffness modulates reprogramming efficiency. We fabricated tunable hydrogels with dynamic stiffness modulation using DNA crosslinkers and demonstrated how stiffness modulation can enhance reprogramming of murine fibroblasts. These findings provide new insights into biophysical regulation of cellular reprogramming.

Next, we designed and characterized a suite of novel electrophoretic cytometry tools for highly selective analysis of proteins from single cells. We first introduced a microfluidic platform to perform isoelectric focusing of single adherent cells. By spatially separating proteins by isoelectric point, we conferred higher selectivity compared to conventional immunoassays. We characterized the separation performance of the assay and demonstrated detection of protein signaling events and surface receptors that are otherwise undetectable by conventional measurement techniques. Next, we leveraged the automation and facile handling of centrifugal microfluidics to design a microscale

device to capture rare cells. We demonstrated photopolymerization of polyacrylamide gels within single-cell capture arrays for enhanced cell capture and possible downstream electrophoresis. Finally, we discussed expandability of electrophoretic cytometry tools to study alternatively-spliced protein isoforms. We investigated fundamental limitations and tradeoffs in assay design parameters.

In addition to assay design, we developed a set of image processing scripts to analyze image data from non-arrayed protein separations. Building on existing algorithms for protein separation data, we expanded the capability of the analysis scripts to first segment protein peaks and then generate defined regions of interest for peak quantitation. Quality control checks were performed, guided by user interaction.

Finally, we proposed a model for understanding physical parameters that influence analytical sensitivity of electrophoretic cytometry. Sensitivity is a key metric in single-cell assays, as high concentrations must be maintained to ensure assays have applicable lower limits of detection. We characterized fluid films formed between hydrogels and used numerical simulation to characterize protein transport within these films. These findings provide insights into engineering design for enhanced sensitivity and has broader implications in drug delivery and tissue engineering strategies.

Overall, in this dissertation, we employed fundamental principles of mass transport and micro-scale electrokinetics to design, characterize, and validate new technologies for selective cell manipulation and protein measurement. Future directions may expand on improving separation metrics, sensitivity, multiplexing, and/or interfacing with complementary analysis tools such as mass spectrometry or nucleotide analysis (e.g., RNA-Seq).

To my family, friends, and the myriad
individuals in my support network

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TABLE OF CONTENTS

1 INTRODUCTION	1
1.1 CELL REPROGRAMMING	1
1.2 MOTIVATION FOR AND LIMITATIONS OF SINGLE-CELL PROTEIN MEASUREMENTS	1
1.3 DISSERTATION OVERVIEW	2
1.4 ELECTROKINETIC THEORY	3
REFERENCES	10
2 REGULATION OF CELL REPROGRAMMING VIA BIOPHYSICAL CUES	12
2.1 INTRODUCTION	12
2.2 MATERIALS AND METHODS	13
2.3 RESULTS AND DISCUSSION	13
2.4 CONCLUSIONS	22
REFERENCES	23
3 ELECTROPHORETIC CYTOMETRY OF ADHERENT CELLS	25
3.1 ABSTRACT	25
3.2 INTRODUCTION	25
3.3 MATERIALS AND METHODS	27
3.4 RESULTS AND DISCUSSION	30
3.5 CONCLUSION	47
REFERENCES	48
4 IMAGE PROCESSING ALGORITHMS FOR STREAMLINED ANALYSIS OF NON-ARRAYED OBJECTS	53
4.1 INTRODUCTION	53
4.2 RESULTS AND DISCUSSION	55
4.3 FUTURE DIRECTIONS AND IMPROVEMENTS	60
REFERENCES	60
ANALYSIS SCRIPT (MATLAB CODE)	61

5 SOLUBILIZATION OF ALTERNATIVELY-SPLICED ISOFORMS FOR SINGLE-CELL ISOELECTRIC FOCUSING	71
5.1 INTRODUCTION	71
5.2 MATERIALS AND METHODS	72
5.3 RESULTS AND DISCUSSION	73
5.4 CONCLUSIONS	82
REFERENCES	86
 6 ELECTROPHORETIC CYTOMETRY OF CENTRIFUGALLY- LOADED CELLS	 89
6.1 INTRODUCTION	89
6.2 MATERIALS AND METHODS	90
6.3 RESULTS AND DISCUSSION	92
6.4 CONCLUSIONS AND FUTURE DIRECTIONS	97
REFERENCES	98
 7 TRANSPORT OF PROTEINS IN HYDROGEL INTERFACIAL FLUID FILMS	 101
7.1 INTRODUCTION	101
7.2 MATERIALS AND METHODS	102
7.3 RESULTS AND DISCUSSION	104
7.4 CONCLUSIONS	112
REFERENCES	112
 8 CONCLUSIONS AND FUTURE DIRECTIONS	 114
REFERENCES	114

LIST OF FIGURES

1-1 THE ELECTRIC DOUBLE LAYER FORMS OVER A CHARGED SURFACE, IN WHICH AN ACCUMULATION OF COUNTERIONS IS FOUND NEAR THE SURFACE OF THE PLATE	4
1-2 SCHEMATIC OF ISOELECTRIC FOCUSING FOR ENRICHMENT OF PROTEINS	8
2-1 FLUORESCENCE MICROGRAPHS OF IMMUNOSTAINING OF COLONIES CULTURED ON SUBSTRATES OF VARYING STIFFNESS	14
2-2 BAR PLOT OF NUMBER OF COLONIES OBSERVED ACROSS THE SUBSTRATES OF VARYING STIFFNESS	15
2-3 BAR PLOT OF NUMBER OF COLONIES OBSERVED ACROSS THE SUBSTRATES OF VARYING STIFFNESS USING THREE-FACTOR (SKM) REPROGRAMMING	16
2-4 IMMUNOBLOTTING OF ESCS CULTURED ON GELS OF VARYING STIFFNESS IN THE ABSENCE OF LIF	17
2-5 IMMUNOBLOTTING OF ESCS CULTURED ON GELS OF VARYING STIFFNESS IN THE ABSENCE OF LIF AND PROBED FOR GERM LAYER MARKERS	18
2-6 SCHEMATIC OF DNA GEL	19
2-7 SCHEMATIC OF DNA GEL CROSSLINK REMOVAL	19
2-8 SCHEMATIC OF DNA GEL CHEMICAL STRUCTURE	20
2-9 IN REPROGRAMMING EFFICIENCY IS ENHANCED ON DYNAMIC (SOFTENED) HYDROGELS	20
3-1 <i>IN SITU</i> IEF OF CELLS (9000 CELLS/CM ² SEEDING DENSITY) USING THE PREVIOUSLY REPORTED DENATURING LYSIS BUFFER DEMONSTRATES LIMITED SOLUBILISATION	28
3-2 <i>IN SITU</i> IEF MEASURES PROTEIN PEAKS FROM SINGLE AND CLUSTERED CELLS ADHERED TO A SUBSTRATE GEL	31
3-3 SCHEMATIC OF MICROCONTACT PRINTING OF FIBRONECTIN-PATTERNED SUBSTRATE GELS	32
3-4 THE SUBSTRATE GEL CONTAINS A TUNEABLE, ECM-PATTERNED REGION ONTO WHICH CELLS ADHERE	33
3-5 <i>IN SITU</i> IEF OF CELLS SEEDED AT A DENSITY OF 500 CELLS/CM ² AND CULTURED FOR 4 DAYS, FOR A FINAL DENSITY OF ~2000 CELLS/CM ²	22

3-6 MEASUREMENT OF LAMIN A/C PI	34
3-7 <i>IN SITU</i> IEF SEPARATES AND DETECTS PROTEINS FROM SINGLE- AND CLUSTERED-CELLS	35
3-8 LAMIN A/C PEAKS ARE RESOLVED Laterally AT A LOW (500 CELLS/CM ²) CELL DENSITY	36
3-9 ANALYSIS OF TOTAL LAMIN A/C CONTENT OF CELLS INDICATE APPROXIMATELY PROPORTIONAL TOTAL PROTEIN CONTENT TO CELL DENSITY	37
3-10 <i>IN SITU</i> IEF OF CELLS SEEDED ON FIBRONECTIN-PATTERN ISLANDS	38
3-11 COMPARISON OF FIBRONECTIN FLUORESCENCE INTENSITY BEFORE AND AFTER <i>IN SITU</i> IEF	39
3-12 PROTEIN EXPRESSION DOES NOT CORRELATE WITH CELL MORPHOLOGY	40
3-13 COMPARISON OF β -TUBULIN PROTEIN CONTENT (ID), WHICH IS RETAINED ON THE FIBRONECTIN PATTERN, UNDER VARYING LYSIS AND EF DURATIONS	41
3-14 LAMIN A/C EXPRESSION DOES NOT CORRELATE WITH CELL MORPHOLOGY, AS MEASURED USING A CELL TRACKER DYE (CYTOPAINTER)	41
3-15 <i>IN SITU</i> IEF OF ADHERENT CELLS ASSAYS PROTEIN PROFILES THAT DIFFER FROM TRYPSIN-TREATED (DETACHED) CELLS	44
3-16 SEPARATION PERFORMANCE, AS MEASURED BY LAMIN A/C PEAK WIDTH, AND PROTEIN SOLUBILISATION ARE ENHANCED BY TUNING THE EF AND LYSIS DURATIONS, RESPECTIVELY	45
3-17 SCATTER PLOT OF LATERAL (PERPENDICULAR TO IEF AXIS) PEAK WIDTH AS A FUNCTION OF INCREASED EF TIME	46
3-17 SCATTER PLOT OF LATERAL (PERPENDICULAR TO IEF AXIS) PEAK WIDTH AS A FUNCTION OF INCREASED EF TIME	46
3-17 SCATTER PLOT OF LATERAL (PERPENDICULAR TO IEF AXIS) PEAK WIDTH AS A FUNCTION OF INCREASED EF TIME	46
4-1 WORKFLOW FOR IMAGE SEGMENTATION USING A MATLAB GUI	54
4-2 SCHEMATIC OF DATA STRUCTURE AND FIELDS (BRANCHED LINES)	55
4-3 LAMIN A/C IS PARTIALLY RETAINED ON THE FIBRONECTIN PATTERN	56

4-4 INPUT AND OUTPUT IMAGES OF THE ROI-GENERATION SCRIPT	57
4-5 GENERATION OF TEMPORARY ROIS FOR PEAK LOCATION ESTIMATION	58
4-6 UI QUALITY CONTROL ALLOWS USER INTERVENTION FOR “GOOD” AND “BAD” PEAKS	59
5-1 SCHEMATIC OF FIVE COMMON MODES OF ALTERNATIVE SPLICING	71
5-2 SEPARATION PERFORMANCE OF SCIEF	75
5-3 IPSCS AND NEURAL DIFFERENTIATED CELLS (NPCS AND MNS) INCREASINGLY EXPRESS THE AS ISOFORM OF EXCLUDED EXON 9 IN RNA-SEQ DATA	76
5-4 SEMI-BULK MICROFLUIDIC IEF OF U251-GFP LYSATES	77
5-5 INVERTED FLUORESCENCE MICROGRAPHS OF PKM IN NATIVE AND DENATURING CONDITIONS UNDER PREVIOUSLY REPORTED SCIEF CONDITIONS	77
5-6 NATIVE SCIEF OF U251- GFP CELLS DEMONSTRATES UNDETECTABLE LEVELS OF DYNC 1I2, DNMT1 AND TERF1	78
5-7 DENATURING SCIEF, DYNC 1I2, DNMT1, TERF1, AND ZCCHC10 (NOT SHOWN) ARE NOT DETECTABLE	79
5-8 BULK WESTERN BLOT INDICATES PRESENCE OF ZCCHC10 AND TWO ISOFORMS OF TERF1, BUT LITTLE DNMT AND DYNC 1I2 RELATIVE TO GAPDH	79
5-9 LYSIS AND EF DURATION CAN IMPROVE SOLUBILIZATION OF PKM	80
5-10 HIGHER DETERGENT AND CHAOTROPE CONCENTRATIONS DO NOT SIGNIFICANTLY IMPROVE PKM SOLUBILIZATION IN SINGLE CELLS	81
5-11 ADDITION OF REDUCING AGENTS RESULTS IN ONE RESOLVABLE PKM PROTEIN PEAK	82
6-1 PHOTOMASK DESIGN FOR PA-PDMS DEVICES	91
6-2 WORKFLOW OF CENTRIFUGAL ARRAYED CAPTURE DEVICE FOR ELECTROPHORETIC CYTOMETRY	93
6-3 NON-PLASMA BONDED DEVICES OPERATE WITHIN CENTRIFUGAL OPERATING RANGE WITHOUT DELAMINATION	94

6-4 CHO CELL LOADING IN PDMS DEVICES DEMONSTRATE LOW OCCUPANCY	95
6-5 SKBR3 LOADING IN PDMS DEVICES WITHOUT PA DEMONSTRATE LOW OCCUPANCY	95
6-6 PHOTOMASK USED FOR POLYMERIZING PA WITHIN THE PDMS CHANNELS	96
6-7 CHARACTERIZATION OF PA POLYMERIZATION WITHIN PDMS CHANNELS	97
6-8 CELL LOADING IN PA-PDMS DEVICES RESULTS IN HIGHER OCCUPANCY	97
7-1 CONCEPT SCHEMATIC OF DIFFUSIVE LOSSES AND THE EFFECT OF PARTITIONING ON MICROWELL PROTEIN CONCENTRATION	105
7-2 CONFOCAL XZ FLUORESCENCE MICROGRAPHS REVEAL THE PRESENCE OF A THIN FLUID FILM BETWEEN “LID” AND SUBSTRATE GELS	106
7-3 FLUID FILM THICKNESS BETWEEN LIDS AND BOTTOM GELS RESULTS IN DECREASED MICROWELL AND BOTTOM-GEL PROTEIN CONCENTRATION	108
7-4 <i>IN SILICO</i> SENSITIVITY ANALYSIS INDICATES IN-GEL PROTEIN CONCENTRATION IS DEPENDENT ON FLUID FILM THICKNESS AND PARTITION COEFFICIENT OF THE LID	109
7-5 EXPERIMENTAL VALIDATION OF SIMULATION USING PROTEIN-RELEASE MICROPARTICLES	111
7-6 PARTICLE VELOCIMETRY REVEALS ADVECTION INDUCED BY LID GEL PLACEMENT THAT IS TWO ORDERS OF MAGNITUDE LOWER THAN IN A POURED BUFFER SYSTEM FOR PA-PDMS DEVICES	111

Chapter 1.

Introduction

Cell Reprogramming

Induced pluripotent stem cell (iPSC) and induced neuron (iN) technology has shown significant progress towards the development of patient-specific therapies to treat a variety of conditions ranging from Alzheimer's to cardiac ischemia. iPSC technology is promising because a patient's own somatic cells, e.g., fibroblasts, can theoretically be reprogrammed into a pluripotent state using only four transcription factors: Oct4, Sox2, Klf4, and c-Myc.¹ In iN reprogramming, fibroblasts are directly converted to neurons using only three transcription factors: Brn2, Ascl1, and Myt1l.² However, challenges to clinical applicability of these technologies include low reprogramming efficiency, and the use of oncogenes c-Myc and Klf4, which have been shown to increase tumorigenicity in chimeric mice.³ Thus, approaches to reduce the number of exogenously expressed transgenes as well as improve reprogramming efficiency could make the use of reprogramming technologies more clinically feasible.

Several reports have demonstrated that the biophysical environment influences stem cell differentiation⁴ and cell reprogramming efficiency.⁵ These studies introduced hydrogels (polyacrylamide) or elastomeric polymers (PDMS) as substrates onto which cells adhere, altering internal signaling and epigenetic mechanisms. Materials modulation can thus be a promising avenue to increase the translation feasibility of cell reprogramming strategies from the bench side to the clinic.

Motivation for and limitations of single-cell protein measurements

To understand how signaling mechanisms change or are perturbed, such as in biophysical treatments of cells, direct measurement of protein-mediated signaling is required. Moreover, because cells are heterogeneous, single-cell measurements are crucial, as bulk, average measurements can mask the diversity between individual cells that drive events such as cancer metastasis⁶ or stem cell differentiation.⁷

Conventional single-cell approaches to study cellular proteins such as flow cytometry and immunocytochemistry are powerful but rely on antibody affinity-target specificity. Importantly, many antibodies do not exist for the many types of protein modifications, such as phosphorylation events that drive cancer metastasis,⁸ or alternatively-spliced isoforms. Moreover, fixation of cells presents artifacts that confound interpretation of results.⁹

To improve selectivity of immunoassays, electrophoretic separations such as Western blots and isoelectric focusing (IEF) introduce a size- or isoelectric point-based separation, respectively, prior to the immunoassay. The separation enables the identification of isoforms based on physical positioning of the protein analytes relative to

a standard. Using capillary IEF, several groups have reported detection of protein isoforms from as few as 25 cells pooled together.^{10,11} Recently, both Western blots and IEF were introduced at the single-cell level.^{12,13} These electrophoretic cytometry assays take advantage of micro-scale lengths to maintain relatively high local protein concentrations, making single-cell resolution possible.

To understand how substrates influence rapidly changing cell-signaling events at the individual cell level, there is a need for highly selective measurement tools that can assay these rapidly-changing, adhesion-dependent proteins at high concentrations.

Dissertation Overview

This dissertation reports on tools development that involves materials engineering, assay development for selective protein measurement in adherent cells, and fundamental understanding of diffusion-driven losses in electrophoretic cytometry.

In chapter 2, we present the design, measurement, and utilization of tunable-stiffness hydrogels for enhancement of cell reprogramming efficiency. We first report that static hydrogels of varying elasticity (stiffness) modulate murine fibroblast reprogramming to iPSCs and can potentially be used to replace exogenous factors. We then discuss the formulation and characterization of dual-crosslinked hydrogels, using a permanent bis-acrylamide crosslinker and a dynamic DNA-based crosslinker. Finally, we demonstrate how dynamic stiffness modulation can influence the reprogramming of murine fibroblasts directly to iNs.

Chapter 3 focuses on the development, demonstration, and characterization of an electrophoretic cytometry assay for adherent cells. Adherent cells express different protein signatures than cells that have been detached, either mechanically or biochemically, and thus we designed an assay to interrogate cells while still attached to a substrate. We combine microcontact printing to pattern cells to a defined region at single- and multiple- cell density, and we perform an *in situ* isoelectric focusing that lyses cells, separates the intracellular contents, and employs immunoprobing for target detection. We then correlate protein expression to morphological parameters and demonstrate detection of targets otherwise un-detectable in conventional single-cell assays. We also present further optimization considerations in the concluding section.

In chapter 4, we share the development and implementation of analysis algorithms for processing images of non-arrayed separations. Building on work previously described in the Herr lab,^{14,15} we expand the capability of the analysis scripts to segment protein peaks from electrophoretic separations that are non-arrayed. The script then generates regions of interests for analysis based on protein peak spacing. A user-interface for quality control is also implemented.

Approaches to improve the applicability of scIEF are discussed in chapter 5. In particular, we investigate protein solubility in non-ionic detergents and demonstrate a

wider-range pH gradient for the detection of alternatively spliced isoforms. We discuss tradeoffs in solubilization that occur from longer lysis and electrofocusing durations, which improve protein solubilization but concomitantly increase the amount of analyte diffusive losses. We also demonstrate that solubilization is protein-dependent by probing for various targets such as DYNC1I2, DNMT1, and TERF1. In the concluding remarks, we discuss improvement of separation resolution and important tradeoffs.

In chapter 6, we employ centrifugal microfluidics, combined with array-based cell capture and hydrogel photopatterning to improve cell capture and couple it with a downstream electrophoretic separation. We discuss varying design parameters (e.g., geometry, spacing) on capture efficiency of SKBR3 cells. We also demonstrate polyacrylamide photopolymerization that could ultimately enable downstream electrophoresis, photocapture, and antibody probing. Future directions are also discussed.

Chapter 7 proposes a model for understanding diffusion-driven losses in electrophoretic cytometry, specifically during cell lysis. We characterize thin fluid films between hydrogels in two configurations previously demonstrated to deliver lysis reagents^{16,17} and mitigate diffusive losses¹⁸. We employ the fluid film thickness as an added geometry into a numerical simulation and validate the model using Protein G-releasing microparticles. Finally, we employ the model to determine how varying assay parameters such as partition coefficient and fluid film thickness influence in-gel protein concentrations, which can inform engineering design for improved protein retention.

Electrokinetic Theory

In electrophoretic cytometry, separations are performed based on the molecular mass or charge (isoelectric point) of different protein species. In this section, electrokinetic theory will be described relevant to these separations.

Electric Double Layer (EDL)

Most surfaces will acquire charge when put into contact with an aqueous medium. This charged surface will influence the distribution of ions nearby in the solution such that counterions— or ions of opposite charge to the surface will be attracted to the surface (Figure 1). This attraction of counterions and repulsion of co-ions results in the formation of an electric double layer, which is a region close to the charged surface in which there is enrichment of counterions to neutralize the surface charge. Co-ions and counter-ions are distributed in a diffuse manner due to the thermal energy of the system. The thickness of EDL is characterized by the Debye length (λ_D), which is the length at which the potential energy is approximately equivalent to the thermal energy of the counterions. The zeta potential, ζ , is the potential at the distance of the double layer beyond which ion species can “slip” or move past the charged surface.

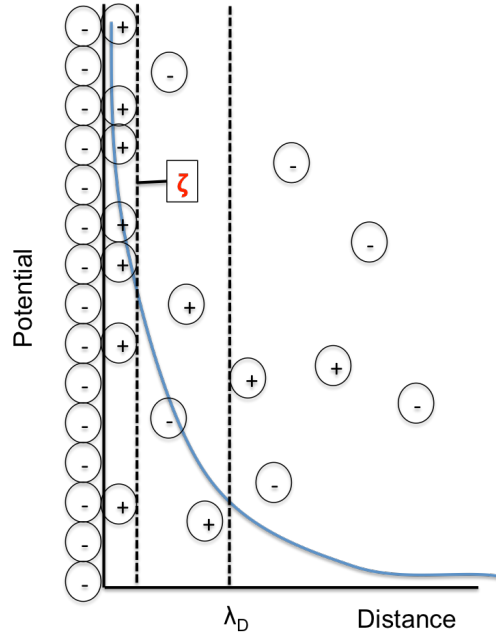


Figure 1. The electric double layer forms over a charged surface, in which an accumulation of counterions is found near the surface of the plate. The blue line represents to electric potential, which decreases exponentially as the distance from the charged surface increases. The zeta-potential is the potential where the distance of the double layer forms the slip plane.

Using Poisson's equation, we have:

$$\frac{\partial^2 \phi}{\partial x^2} = \frac{Fcz}{\epsilon} \quad (1)$$

where ϕ is the electric potential, F is Faraday's constant, c is the concentration of ions, z is the valency of the ions, and ϵ is the electrical permittivity.

Integrating, and applying boundary conditions, we obtain:

$$\phi = \frac{Fzc x^2}{2\epsilon} \quad (2)$$

The electric potential energy, W , can be calculated from the equation:

$$W = -Fz\phi \quad (3)$$

Substituting (2) into (3), we obtain the change in W across a plane layer of width x :

$$\Delta W = -\frac{F^2 z^2 c x^2}{2\epsilon} \quad (4)$$

At the Debye length, the absolute value of ΔW is equal to the thermal energy. Thus,

$$RT = \frac{F^2 z^2 c x^2}{2\epsilon} \quad (5)$$

And the Debye length can be defined in terms of the thermal energy:

$$\kappa \equiv \lambda_d = \left(\frac{2\varepsilon RT}{F^2 z^2 c} \right)^{1/2} \quad (6)$$

We can see from (6) that the Debye length increases as temperature increases and as valency or ion concentration decreases.

Now, implementing the Boltzmann distribution, we can write the concentration of species i as a function of the local potential ϕ as

$$c_i = c_0 e^{\left(\frac{-z_i F \phi}{RT} \right)} \quad (7)$$

where c_0 is the ion concentration far from the charged surface ($\phi \rightarrow 0$). The charge density can be defined in terms of the ion concentration.

$$\rho_E = \sum F z_i c_i \quad (8)$$

Substituting (7) into (8), we obtain

$$\rho_E = \sum F z_i c_0 e^{\left(\frac{-z_i F \phi}{RT} \right)} \quad (9)$$

which can be re-written as

$$\rho_E = -2Fz c_0 \sinh\left(\frac{zF\phi}{RT}\right) \quad (10)$$

Using (10) into the Poisson equation for uniform permittivity ($\frac{\partial^2 \phi}{\partial x^2} = -\frac{\rho_E}{\epsilon}$), we obtain:

$$\frac{\partial^2 \phi}{\partial x^2} = \frac{2Fz c_0}{\epsilon} \sinh\left(\frac{zF\phi}{RT}\right) \quad (11)$$

For small surface charges where $\frac{zF\phi}{RT} \ll 1$, we implement the Debye-Hückel approximation:

$$\frac{\partial^2 \phi}{\partial x^2} = \frac{\phi}{\lambda_d^2} \quad (12)$$

Applying boundary conditions that $\phi(0)=\zeta$, and $\phi=0$ when x approaches infinity

$$\phi = \zeta e^{\left(-\frac{x}{\lambda_d} \right)} \quad (13)$$

As can be seen from (13), the electric potential depends on the Debye length and the zeta potential.

Electrophoresis

If we consider a charged sphere with radius a in a an applied electric field, we know that to maintain electroneutrality in the bulk solution, the charge in the EDL must be qequal and opposite the charge of the sphere. We can calculate the charge by taking the volume integral of the charge density ρ_E of the EDL.

$$q = q_{particle} = q_{inside} = -q_{outside} = - \int_a^\infty \int_0^\pi \int_0^{2\pi} \rho_E \sin \vartheta r^2 d\theta d\vartheta dr \quad (14)$$

Once again using Poisson's equation, we obtain:

$$q = \int_a^\infty 4\pi\epsilon \frac{d}{dr} \frac{d\Phi}{dr} r^2 dr \quad (15)$$

$$q = 4\pi\epsilon a^2 \left(\frac{d\Phi}{dr} \right)_{r=a} \quad (16)$$

Using the Poisson-Boltzmann distribution and the characteristic length of the EDL, we obtain:

$$\frac{1}{r^2} \frac{d}{dr} \left(r^2 \frac{d\Phi}{dr} \right) = \frac{\Phi}{\lambda_d^2} \quad (17)$$

Integrating and applying the boundary conditions, we obtain:

$$\left(\frac{d\Phi}{dr} \right)_{r=a} = -\zeta \left(\frac{1}{a} + \frac{1}{\lambda_d} \right) \quad (18)$$

We can then substitute (18) into (16):

$$\zeta = \left(\frac{1}{4\pi\epsilon a} - \frac{q}{4\pi\epsilon a(a+\lambda_d)} \right) \quad (19)$$

If we assume that the EDL is much smaller than the radius of the particle, we can estimate the surface of the particle as planar and use the 1D Navier-Stokes equation for steady-state flow in a low-Reynolds number regime:

$$0 = \mu \nabla^2 \vec{u} + \rho_E \vec{E} \quad (20)$$

Where u is the velocity vector. Substituting Poisson's equation, we obtain:

$$\mu \nabla^2 \vec{u} - \epsilon \nabla^2 \Phi \vec{E} = 0 \quad (21)$$

Integration with boundary conditions that u_{EP} is zero in the bulk and that $\phi(a)=\zeta$:

$$u_{EP} = \frac{\epsilon\zeta}{\mu} \vec{E} = \mu_{EP} \vec{E} \quad (22)$$

where

$$\mu_{EP} = \frac{\epsilon\zeta}{\mu} \quad (23)$$

Polyacrylamide Gel Electrophoresis (PAGE)

In PAGE, molecules are separated differentially based on molecular mass as they are sieved through a porous polyacrylamide matrix. The electrophoretic mobility is related to the sieving matrix by the Ferguson relation:¹⁹

$$\log(\mu_{EP,gel}) = \log(\mu_0) - K_R T \quad (24)$$

where $\mu_{EP,gel}$ and μ_0 are the in-gel and free solution electrophoretic mobilities, respectively, and T is the concentration of polyacrylamide monomer in the gel. The retardation coefficient, K_R is empirically-derived and is a measure of how much the sieving matrix retards the electrophoretic mobility of the particle.

In SDS-PAGE, proteins are denatured and coated with sodium dodecyl sulfate (SDS), which stoichiometrically coats amino acids in proteins. The SDS confers a constant mass-to-charge ratio, and during PAGE, proteins are separated based on molecular mass.²⁰

Isoelectric Focusing (IEF)

In IEF, molecules are separated by isoelectric point (pI), the pH at which the molecule has no net charge, along an established pH gradient (Figure 2). In protein separations, proteins are typically embedded in a polyacrylamide gel. The pH gradient is established by applying an electric field; the gradient is established by a mixture of carrier ampholytes, which are amphoteric species that have good conductivity and buffer at their pI. Since proteins carry charge depending on the local pH of the environment, the proteins will electromigrate along the pH gradient until neutrally charged. At the pI, the protein has no net electrophoretic mobility and is at equilibrium, subject to diffusive band broadening, but subsequent electrophoretic restoration as the protein gains or loses charge.²¹

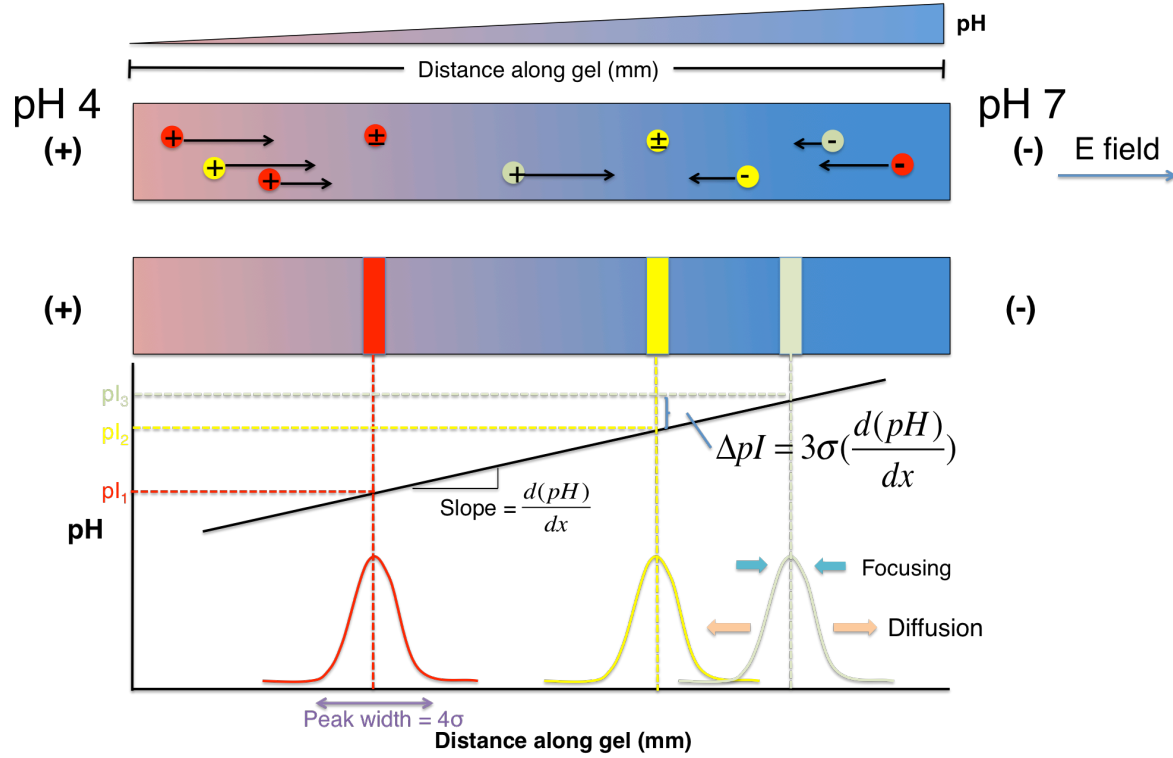


Figure 2. Schematic of isoelectric focusing for enrichment of proteins. A pH gradient is established along a gel or channel. Proteins will carry charge based on position along the pH gradient and will migrate to the isoelectric point, the pH at which the protein has no net charge. At the pI, the protein has no net electrophoretic mobility, and is at equilibrium between diffusional band broadening but subsequent electrophoretic restoration as the protein gains or loses charge (i.e., protein residues are protonated or deprotonated). The concentration distribution is Gaussian, and the peak width, along with the slope of the pH gradient can be used to determine the minimum resolvable pI difference.

The equation for equilibrium isoelectric focusing is:

$$\frac{d(C\mu_{EP}E)}{dx} = \frac{d}{dx}D \frac{dC}{dx} \quad (25)$$

where C is the protein concentration at a the isoelectric position x, μ_{EP} is the electrophoretic mobility of the protein, E is the electric field strength, and D is the diffusion coefficient. Integration of (25) yields:

$$C\mu_{EP}E = D \frac{dC}{dx} + c_1 \quad (26)$$

We assume c_1 is zero, and let a variable, p, represent the change in electrophoretic mobility with position:

$$p = -\frac{d\mu_{EP}}{dx} = -\frac{d\mu_{EP}}{dpH} * \frac{dpH}{dx} \quad (27)$$

We integrate equation (27) and apply the boundary condition that at the isoelectric point, $x = 0$, and there is no net electrophoretic mobility ($\mu_{EP} = 0$):

$$-\mu_{EP} = px \quad (28)$$

Substituting (28) into (26), we arrive at:

$$-CpxE = D \frac{dC}{dx} \quad (29)$$

Rearranging, we obtain:

$$\frac{dC}{C} = -\frac{pEx \cdot dx}{D} \quad (30)$$

We assume that E and D are constant, thus:

$$C = C_0 e^{-\frac{pEx^2}{2D}} \quad (31)$$

where C_0 is the local maximum concentration. We see from (31) that the focused zone has a Gaussian-distributed concentration where

$$\sigma = \left(\frac{D}{pE} \right)^{0.5} \quad (32)$$

If we substitute (27) into the (32), we obtain:

$$\sigma = \left(\frac{D}{E * \left(-\frac{d\mu_{EP}}{dpH} \right) * \left(\frac{dpH}{dx} \right)} \right)^{0.5} \quad (33)$$

We can see from (33) that the peak width is thus dependent on the applied electric field, the slope of the pH gradient (assay-dependent parameters), the electrophoretic mobility of the protein, and diffusion coefficient of the protein (protein-dependent parameters).

Separation Performance

To quantify separation performance, we can consider the separation resolution (R_s), which is a measurement of how well two species are resolved.

$$R_s = \frac{\Delta x}{2\sigma_1 + 2\sigma_2} \quad (34)$$

where Δx is the distance between the peak centers of each species, and σ_1 and σ_2 are the corresponding peak widths. Peak width is often defined as 4σ (Figure 2), which covers 99.99% of the peak.

Diffusion and Mass Transport in Hydrogels

When considering analytical sensitivity in electrophoretic cytometry, it is necessary to consider how diffusive transport occurs in the materials of interest, i.e., in free solution and in PA gels. In free solution, the diffusivity, D , of a protein can be calculated by the Stokes-Einstein equation:

$$D = \frac{kT}{6\pi\mu a} \quad (35)$$

where k is Boltzmann's constant, T is temperature, μ is the viscosity. If we assume unsteady 1-D diffusion with no reaction into a semi-infinite medium (i.e., the fluid reservoir is much larger than the molecule), we can solve Fick's laws to determine the characteristic length that the protein will travel before a large concentration gradient is reached:

$$\frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial x^2} \quad (36)$$

$$x \sim (Dt)^{0.5} \quad (37)$$

In hydrogels, diffusion of proteins can be estimated using the equation presented by Park et al²²:

$$\frac{D_{gel}}{D_{solution}} = e^{-3.03R_H^{0.59}T^{0.94}}$$

where R_H is the hydrodynamic radius of the protein, and T is the percentage of monomer concentration. Varying diffusion coefficients of proteins underpin diffusive losses during electrophoretic cytometry which limit analytical sensitivity; thus, understanding and measuring these parameters are important for designing methods to mitigate these losses.

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Chapter 2.

Regulation of Cell Reprogramming via Biophysical Cues

This work was performed in the laboratories of Prof. Song Li and Prof. Gerard Marriott.

2.1 Introduction

Neurological diseases such as Alzheimer's and Parkinson's affect over 30 million individuals,¹ and Alzheimer's is the sixth leading cause of the death in the United States. Current treatments are scarce and the pathology of neural degenerative diseases is poorly understood. Thus, it is critical to develop models that accurately recapitulate disease states in hand with high-throughput assays for screening drug candidates. In recent years, the advent of cellular reprogramming to induced pluripotent stem cells² (iPSCs) has granted unprecedented access to patient-specific neurons.³⁻⁷ Direct conversion of fibroblasts to neurons (iNs) has also been demonstrated using forced expression of three transcription factors: Brn2, Ascl1, Myt1l (collectively called BAM) in mice, and BAM plus one additional factor, NeuroD1, in humans.⁸ Yet, clinical therapeutics utilizing iPSC and iNs are stymied by low reprogramming efficiencies and a lack of understanding of the mechanisms regulating the conversion process.

Various biochemical effects have been reported to influence the reprogramming process and improve neural induction.^{9,10} However, fewer studies have investigated how the biophysical environment influences the reprogramming process. In 2013, Downing and colleagues reported that induced pluripotent stem cell (iPSC) reprogramming efficiency could be enhanced using topography through regulation of the cell epigenetics.¹¹ Specifically, topography downregulates histone deacetylase activity and increases H3 acetylation, thus opening up chromatin structure and enhancing iPS reprogramming. Similarly, neuron reprogramming efficiency and purity were shown to be enhanced on cells seeded on micro-grates, suggesting that underlying topography can influence the direct reprogramming of fibroblasts to iNs.¹²

Another biophysical cue, substrate elasticity, is involved in many signal transduction processes that have been shown to guide differentiation of stem cells to a specific lineage.¹³ Mesenchymal stem cells (MSCs) seeded on soft hydrogels (0.6 kPa) that match the physiologic stiffness of brain tissue tend to differentiate into neurons, while MSCs adhered on stiffer substrates (40 kPa) differentiate towards an osteogenic lineage. Furthermore, softer substrates have been shown to promote neuronal differentiation from neural progenitor cells¹⁴ and neuronal maturation during directed differentiation.¹⁵ Yet, few studies have shown whether substrate elasticity can influence cell reprogramming. Given the importance of stiffness on cell fate, we hypothesized that

modulating stiffness could also modulate reprogramming, which is a dynamic process.¹⁶ Here, we demonstrate that reprogramming of fibroblasts to iPSCs is independent of stiffness within physiologic ranges (0.6-40 kPa) but that maintenance of pluripotency is enhanced on intermediate stiffnesses (20 kPa). We also demonstrate reprogramming is possible with only three (of the four) canonical reprogramming factors. Finally, we design a dynamically-tunable DNA hydrogel for tuning substrate stiffness *in situ*. We modulate the stiffness from 20 kPa to 5 kPa and demonstrate improved iN reprogramming efficiency, which has clinical implications for therapeutics and disease modeling of neurological disorders.

2.2 Materials and methods

Cell culture

Mouse ear fibroblasts were harvested via primary culture from C57BL6 mice. Tissue was treated with liberase according to the manufacturer's instructions. Sample was then collected and resuspended on gelatin-coated dishes. Media was changed every 2 days. After 5 days, the cells were passaged onto gelatin-coated dishes and frozen on passage 2. All cells for reprogramming experiments were thawed and used directly from passage 2. Fibroblasts were cultured in DMEM media (Gibco) supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin/streptomycin. Nanog-GFP mouse embryonic stem cells (mESCs) were generously donated by the Qi lab. The Nanog was gene edited using CRISPR/Cas9 to replace the endogenous Nanog gene with a Nanog-GFP fusion construct. Fibroblasts reprogramming to iPSCs and mESCs were cultured in DMEM supplemented with 15% ES-grade FBS (ES-cult), 10% penicillin/streptomycin, 1% non essential amino acids (LifeTechnologies), 1% leukemia inhibitory factor (LIF, Millipore), and 0.8% beta-mercaptoethanol (Sigma). Fibroblasts reprogramming to iNs were cultured in N2B27 media (DMEM F12 + 1% N2 + 2% B27 + 1% P/S).

Cell reprogramming on hydrogels of varying stiffness

Viral synthesis and cell transduction were performed as previously described.^{8,11} After transduction, fibroblasts were re-seeded at a density of 3000 cells cm⁻² on substrate gels. Polyacrylamide gels of varying stiffness (0.6, 8, 20, and 40 kPa) were fabricated according to previously published protocols.¹⁷ Gels were incubated with 1 mg/mL sulfo-SANPAH (Pierce), activated under UV, washed and then coated with either 4mg/mL collagen (iPSC reprogramming) or laminin (iN reprogramming). Cells were allowed to reprogram for 14-20 days, then fixed and immunostained. For DNA hydrogels, Acrydite-modified DNA molecules were obtained from IDT technologies. Gel formulations for acrylamide and bis-acrylamide were modified from those previously described¹⁸. Sequences of DNA are described in the Results and Discussion section.

2.3 Results and discussion

Reprogramming of fibroblasts to iPSCs is independent of physiologically-relevant stiffness

To assess the effects of substrate stiffness on reprogramming of fibroblasts to iPSCs, we lentivirally transduced mouse ear fibroblasts with the iPSC cocktail of transcription factors Oct4, Sox2, Klf4, and c-Myc (OSKM) and plated them on collagen-conjugated hydrogels of varying stiffness (0.6, 8, 20, and 40 kPa). We then allowed cells to culture and grow on the substrates for 14 d, at which point we fixed the cells and immunostained for Nanog. Reprogramming efficiency was quantified by counting the number of Nanog+ colonies. Nuclear (DAPI) staining was performed to identify all colonies.

We observed colonies on all substrates of varying stiffness (Figure 1). Interestingly, we observed several colonies in all stiffness conditions that did not express Nanog (Figure 2). We hypothesize that these were premature, incompletely reprogrammed colonies, as reprogramming is a partially stochastic process¹⁹ and Nanog expression is a late-stage pluripotency marker.²⁰ Across all the stiffnesses that we assayed, we observed no significant difference in reprogramming efficiency (Figure 2). These data corroborate the results reported by Choi et al.,²¹ who demonstrated that reprogramming was improved on very soft substrates (0.1 kPa), but not those in physiologic range of stiffnesses (1 kPa and higher). Future studies could aim to expand the stiffness range, or combine with topography to further optimize reprogramming efficiency while minimizing exogenous factors.

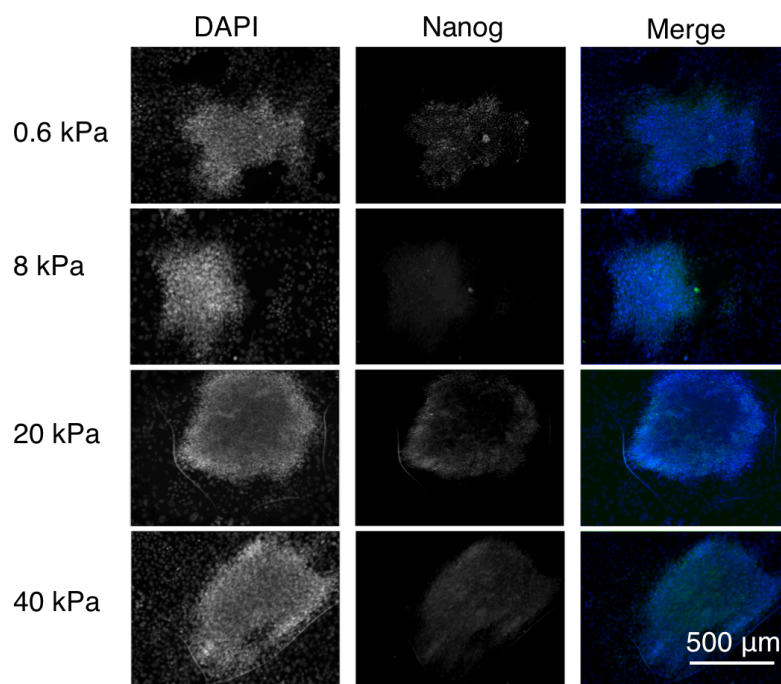


Figure 2-1. Fluorescence micrographs of immunostaining of colonies cultured on substrates of varying stiffness. We observed pluripotent cell colonies on all stiffnesses tested, as evidenced by Nanog expression.

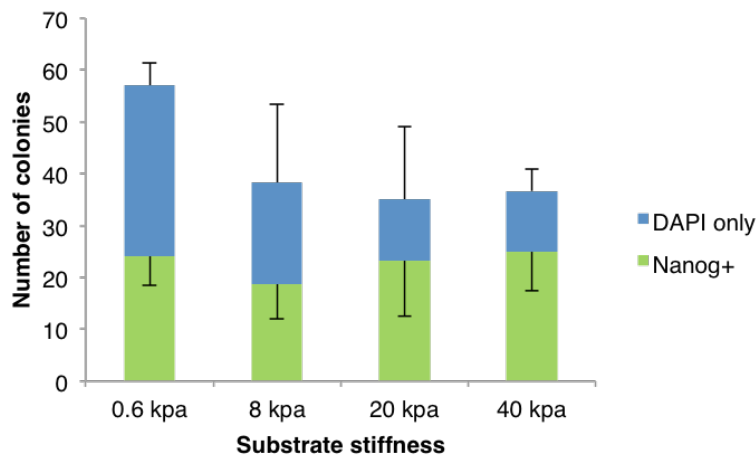


Figure 2-2. Bar plot of number of colonies observed across the substrates of varying stiffness. We observed no statistical significant groups across the range of stiffness tested.

Reprogramming using three-factors (SKM) is possible by using substrate stiffness

Current efforts in reprogramming seek to reduce the number of exogenously expressed transgenes during reprogramming, since the use of oncogenes c-Myc and Klf4 increase tumorigenicity.²² Redmer and colleagues demonstrated that E-cadherin, a membrane protein involved in cell-cell junctions, could be exogenously expressed to replace Oct4, which was considered to be the most difficult factor to replace due to its importance in the primary pluripotency genetic circuitry.²³ This study demonstrated that reprogramming was not solely a nuclear phenomenon, and that transmembrane environmental cues are important in the reprogramming process. More recently, Hou and colleagues demonstrated that Oct4 could be replaced in mouse cells by the small molecule forskolin, a cAMP agonist that is also involved with microenvironmental sensors.²⁴ Given the importance of substrate stiffness stem cell differentiation¹³ and in inducing mesenchymal-to-epithelial transition and upregulation of E-cadherin on soft substrates,²¹ we hypothesized that modulating the mechanical environment could replace Oct4 during reprogramming and reduce the number of exogenously expressed genes necessary for pluripotency induction.

To test this hypothesis, we transduced mouse fibroblasts with a polycistronic lentiviral vector containing three of the four reprogramming factors, excluding Oct4 (SKM). We then seeded cells on substrates of varying stiffness and allowed the cells to reprogram for 14 d, then fixed and stained for Nanog. We observed colonies (high-density clusters of cells) on all substrates. Interestingly, Nanog+ colonies were present on stiffer substrates (20 kPa and higher), with the highest number of colonies on the stiffest hydrogel (40 kPa). Since E-cadherin adhesion mechanisms change depending on stiffness²⁵ (i.e., from 30 kPa to 60 kPa), we hypothesize that at 40 kPa, a switch of E-cadherin interaction with the cytoskeleton occurs and may be responsible for partial

replacement of the reprogramming factor Oct4. Future studies thus aim to elucidate E-cadherin mechanisms of reprogramming, such as determining whether upstream cAMP regulators are influenced by loss and gain of function of stiffness-mediated E-cadherin expression.

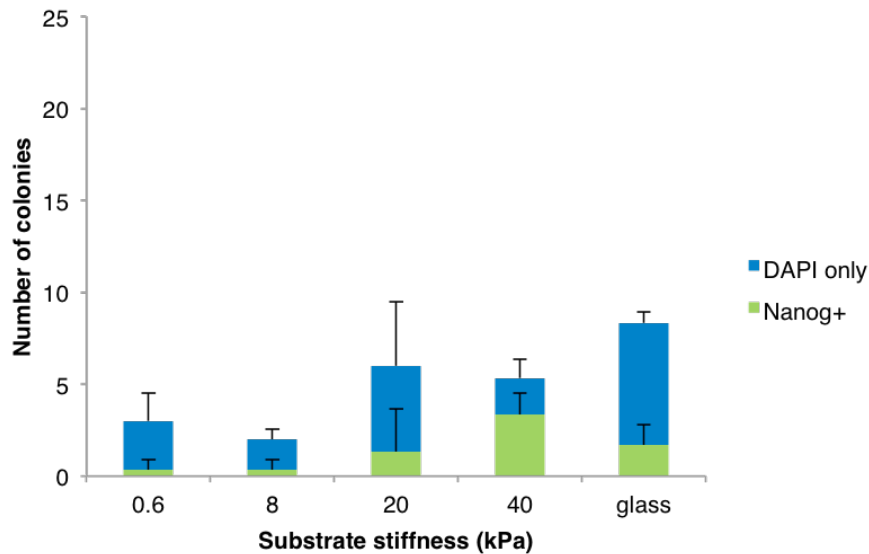


Figure 2-3. Bar plot of number of colonies observed across the substrates of varying stiffness using three-factor (SKM) reprogramming. We observed Nanog+ colonies on stiffer gels (20 and 40 kPa), with the highest number of colonies on the stiffest 40 kPa gels.

Pluripotency is maintained optimally on intermediate stiffness substrates

Given our results of stiffer substrates (40 kPa) enhancing three-factor reprogramming efficiency and how very soft (0.1 kPa) substrates enhance four-factor reprogramming,²¹ we wanted to investigate whether substrate stiffness could also be optimized for maintenance of pluripotency of iPSCs and ESCs. We thus cultured Nanog-GFP mESCs on substrates of varying stiffness (0.6, 8, 20, and 40 kPa) in the absence of LIF for 5 d. After 5 d, we trypsinized the cells and analyzed them for GFP presence via flow cytometry. Interestingly, we observed the greatest percentage of cells that were GFP+ on the 20 kPa substrate (n = 2 trials), suggesting that pluripotency maintenance is optimal on intermediate substrate stiffnesses. We validated this measurement by performing immunoblotting (Western Blot) of ESCs cultured on the different stiffnesses for 5 d, also in the absence of LIF, and comparing to a positive control of cells cultured on a soft substrate (0.6 kPa) with LIF (Figure 4a). The culturing of ESCs on soft substrates with LIF was chosen as a positive control as softer substrates have previously been demonstrated to optimally maintain pluripotency when a range of 0.6-8 kPa was tested.²⁶ We again noted the highest levels of Nanog in ESCs cultured on the 20 kPa substrate, similar to levels of the ESCs cultured in LIF (Figure 4b). This biphasic trend suggests that optimal biomechanical environments can aid in reducing the number of exogenous reagents introduced to ESCs to maintain pluripotency.

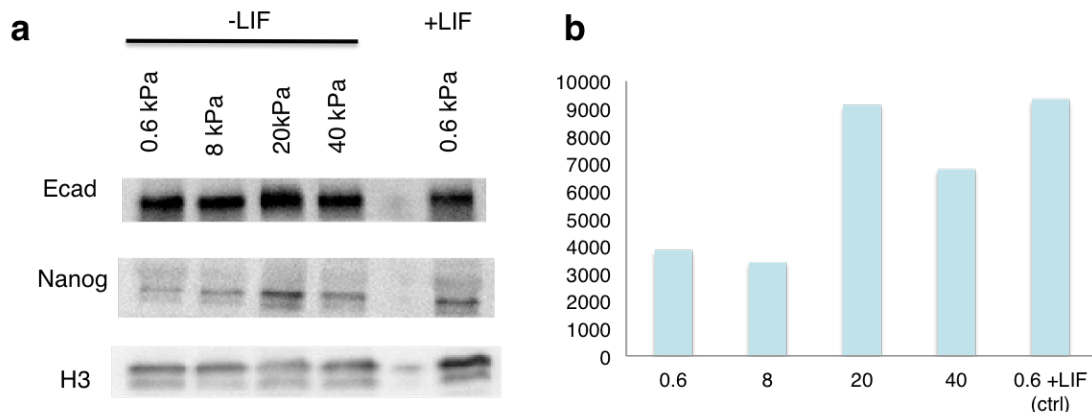


Figure 2-4. Immunoblotting of ESCs cultured on gels of varying stiffness in the absence of LIF.

We observed the highest Nanog levels (normalized to H3 fluorescence) in ESCs cultured on the 20 kPa substrate. Cells cultured on soft (0.6 kPa) gels with LIF were used as a positive control.

Next, we aimed to elucidate into which germ layers (endoderm, mesoderm, or ectoderm) the non-LIF treated ESCs preferentially differentiated. Thus, we repeated the experiment and probed for Nestin (ectoderm), FGF2 (mesoderm), and Gata4 (endoderm) and normalized each intensity profile to GAPDH (Figure 5). We noted that both Gata4 expression and Nestin appeared inversely biphasic. Gata4 expression was lowest for cells cultured on the 20 kPa gels, whereas Nestin was highest on the 20 kPa of the gels and also on glass. Interestingly, we observed no detectable FGF2 in any of the samples. These results suggest that 20 kPa is optimal for stem cell pluripotency maintenance, with some cells expressing ectoderm markers. These results corroborate what others have noted, i.e., Nanog is able to maintain pluripotency in the absence of LIF, although some primitive ectoderm characteristics arise.²⁷ Gata4, an endoderm marker, was visibly lower in the 20 kPa gels ($n = 3$ trials). Higher stiffness has previously been demonstrated to decrease BMP signaling;²⁸ since BMP regulates Gata4, we hypothesize that an optimal stiffness may promote pluripotency by down-regulating BMP pathways that induce endoderm differentiation. Further loss and gain of function studies thus aim to elucidate biophysically-regulated signaling mechanisms of germ layer differentiation.

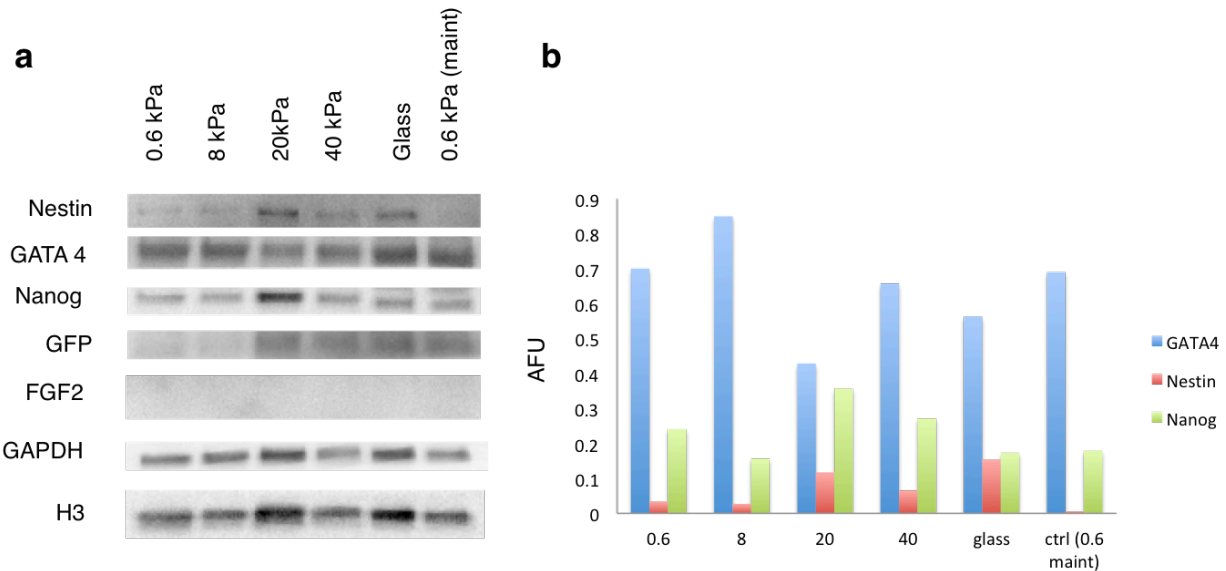


Figure 2-5. Immunoblotting of ESCs cultured on gels of varying stiffness in the absence of LIF and probed for germ layer markers. We observed the highest Nanog levels in ESCs cultured on the 20 kPa substrate and opposing trends for Nestin and Gata4.

Dynamic modulation of stiffness via DNA-crosslinked hydrogels

In addition to reprogramming fibroblasts to iPSCs, we characterized whether dynamic stiffness modulation could influence the direct reprogramming of fibroblasts to neurons (iNs). Previous work in the Li lab demonstrated that fibroblasts preferentially reprogrammed to iNs, with up to 5-fold increase in the number of iNs generated, on an intermediate stiffness of 20 kPa (unpublished data). These fibroblasts were cultured on PA gels of a set stiffness during the entire reprogramming duration. However, neuronal differentiation from neural progenitor cells is enhanced on soft (0.6 kPa) substrates,¹⁵ Yeung and colleagues reported that fibroblasts spread optimally at higher stiffnesses (10 kPa) compared to neurons.²⁹ Given that reprogramming is a dynamic process, we hypothesized that dynamic modulation of stiffness could further enhance reprogramming efficiency. Specifically, we hypothesized that an optimal starting stiffness enhances the initiation of reprogramming by establishing the necessary epigenetic conditions for the binding of pioneering reprogramming factor Ascl1,³⁰ and that subsequent softening of the cell substrate during reprogramming would improve maturation of iNs.

To test whether dynamic modulation of stiffness was achievable within our desired range (i.e., 0.6 to 20 kPa), we fabricated a hybrid bis-acrylamide, DNA-crosslinked hydrogel (DNAgels). We chose DNA as our tunable stiffness parameter as other strategies for dynamic stiffness modulation involve a change of pH³¹, temperature³², electric field³³, or UV irradiation³⁴, which limit their applicability in live-cell systems. In contrast, DNA hydrogels are cell-compatible³⁵. DNA crosslinks are controllably broken (dehybridized) or re-formed (hybridized) by the addition of reversal or crosslinking strands, respectively. However, the maximum demonstrated stiffness of this system was

10 kPa. Thus, we re-formulated the gels with a hybrid of bis-acrylamide and DNA-crosslinked hydrogel (Figure 6). The two types of cross-linking exist to provide a wider and more physiologically-relevant stiffness range for the gels. Specifically, N,N'-methylene-bis(acrylamide) is used as a permanent, irreversible crosslinker that serves to establish the basic structural integrity of the material, as well as to give a basal level of stiffness. Altering the ratios of acrylamide and bis-acrylamide determines the baseline stiffness of the gels, as previously described¹⁸. The other crosslinking mechanism utilizes the concepts of DNA-powered molecular machine³⁶ and is comprised of a three-oligonucleotide system that allows for reversible, dynamic modulation of stiffness³⁵ (Figure 7).

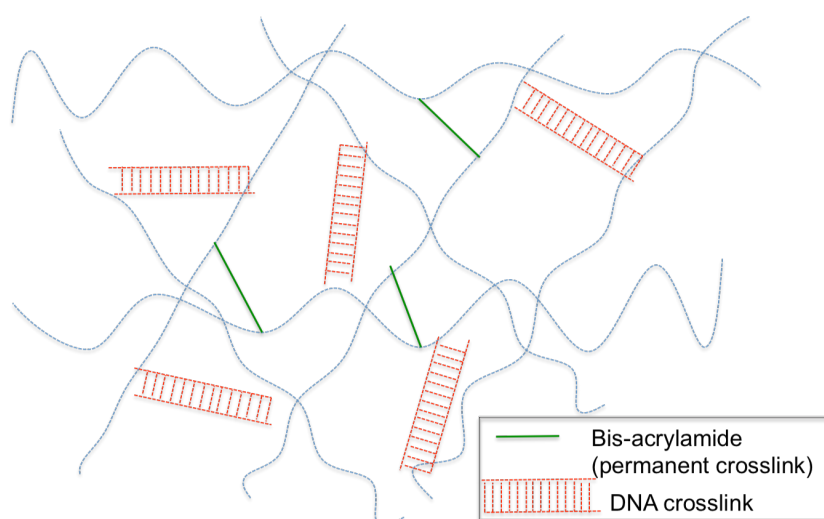


Figure 2-6. Schematic of DNA gel. Bis-acrylamide crosslinks are irreversible, but DNA crosslinks can be removed by addition of a removal crosslinking strand.

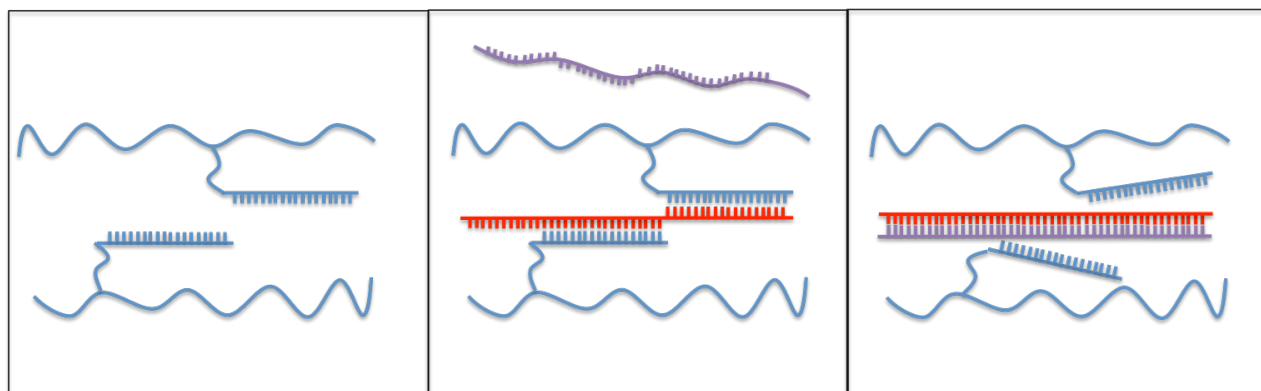


Figure 2-7. Schematic of DNA gel crosslink removal. Addition of a crosslinking strand (red, called L2) forms the DNA-gel crosslink. Addition of a removal strand (purple, called CL2) binds complementarily to the crosslinking strand and removes the DNA crosslink.

Fabrication and characterization of stiffness of DNA hydrogels.

We fabricated DNAgels by altering the ratios of acrylamide and bis-acrylamide, which determine the baseline stiffness, and including 10—base pair DNA molecules (SA1 and SA2) that were modified at the 5' end with Acrydite™. The acrydite allows for incorporation of the pendant DNA chains into the polyacrylamide gel. Next, sections complementary to each of the strands were incorporated into a 30-base pair crosslinker strand (L2, Figure 8). The ten remaining base pairs that do not bind SA1 and SA2 form the toehold. A removal strand (CL2) spans 30-base pairs in length and is complementary to L2; addition of CL2 to the gel dehybridizes the DNA. Stoichiometric alteration of L2 and CL2 determine the level of crosslinking of DNA that is achieved. The sequence of the four oligonucleotides are listed in Table 1.

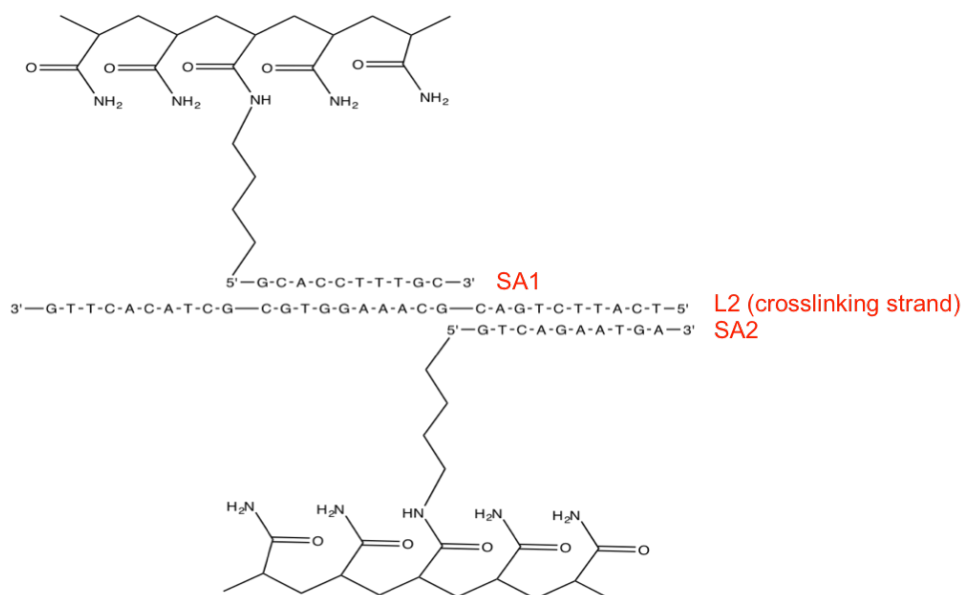


Figure 2-8. Schematic of DNA gels chemical structure. A polyacrylamide backbone and DNA oligonucleotides that form the DNA crosslink. Here, addition of a crosslinking strand (L2) forms the DNA-gel crosslink. Addition of a removal strand (CL2) binds complementarily to the crosslinking strand and removes the DNA crosslink.

Table 2-1. Sequences of the oligonucleotides used in DNAgels.

Name	No. Bases	Sequence
SA1	10	5'Acryd-GCA CCT TTG C-3'
SA2	10	5'Acryd-GTC AGA ATG A-3'
L2	30	5'-TCA TTC TGA CGC AAA GGT GCG CTA CAC TTG-3'

CL2	30	5'-CAA GTG TAG CGC ACC TTT GCG TCA GAA TGA-3'
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To characterize the stiffness of the mechanical properties of the gels, we fabricated various formulations based on modifying stoichiometric ratios of bis-acrylamide, acrylamide, and DNA crosslinker and measured the elastic moduli of the gels using atomic force microscopy. Using a composition of 30% v/v Acrydite DNA (15% SA1 and 15% SA2), 8% w/v acrylamide, 0.132% w/v bis-acrylamide and the remaining volume in dl water, we obtained hydrogels that were tunable from 23 kPa ($n = 3$ regions of a single gel) when fully crosslinked, that lowered to a stiffness of 5 kPa ($n = 3$ regions of a single gel) when fully-decrosslinked by adding equal stoichiometry of CL2 DNA. Although we were not able to achieve de-crosslinking down to the desired 0.6 kPa, further optimization of gel compositions is likely possible by altering ratios of acrylamide, bis-acrylamide, and using co-polymer systems, such as acid-labile decrosslinking gels³⁷.

Changing stiffness during reprogramming improves iN conversion efficiency

We next tested whether stiffness modulation would alter iN reprogramming efficiency. We transduced mouse fibroblasts with a lentiviral vector containing BAM transcription factors. 24 h after transduction, the cells were seeded onto laminin-coated DNAgels and cultured in neurogenic (N2B27) media. We softened the DNA hydrogels by addition of the CL2 strand at day 5 of reprogramming. After 14 d of reprogramming, the cells were fixed and analyzed for iN reprogramming efficiency. The iN cells were identified by immunostaining for beta-III-tubulin (Tuj1) and by morphology, i.e., if the prosthesis was at least three times the size of the neuron body. Reprogramming efficiency was calculated by dividing the number of iNs by the plating efficiency of the substrate (i.e., how many fibroblasts adhered to each substrate).

Reprogramming efficiency indeed improved on substrates that were softened part-way through reprogramming (“dynamic” gel, Figure 9). We observed 8.7% iN efficiency in the softened gels, compared to 4.4% in the 20 kPa static gel and 4.3% in the 5 kPa static gel (all samples $n = 3$ gels, one experiment). As a control, we also included cells seeded on rigid (glass, ~ 1 gPa) substrates, and on glass with DNA added, to ascertain that addition of the DNA alone did not alter iN reprogramming efficiency. Altogether, these results suggest that reprogramming is indeed a dynamic process which can benefit from finely tuned biophysical parameters. Future studies aim to elucidate the mechanism of biophysical regulation of cell reprogramming. For example, one could probe for substrate-induced differences in histone markers such as H3K4me1, H3K27ac, and H3K9me3, which are characteristic of the “trivalent” chromatin state that allows the pioneering factor Ascl1 to bind and initiate reprogramming of fibroblasts to neurons³⁰. These studies could be followed by ChIP-qPCR against the histone modifications and then analyzed for neuronal genes, such as MAP2 and NeuN, as well as Zfp38, a

downstream target of Ascl1, or alternatively, ChIP-Seq. Since the cell is initially in a fibroblast state, it is likely that H3K9me3 enrichment for neuron genes will be high, indicating that these neuronal genes are repressed, and that H3K4me1/2/3 and AchH3 for fibroblast genes will be high, since these genes are activated. However, once Ascl1 has bound to the closed chromatin, “opened” up the neuronal genes, and begun the rest of the reprogramming cascade, the subsequent maturation of the induced neurons can then be regulated by further culture on a softer substrate. At this time point, fibroblast genes will likely be repressed and marked with the condensed chromatin marker H3K9me3 and/or H3K27me3.

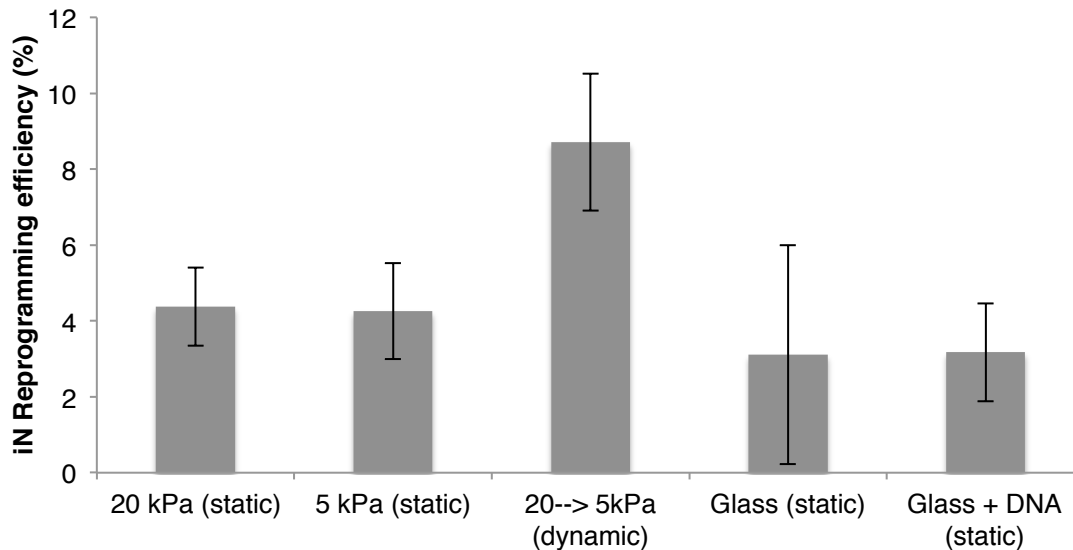


Figure 2-9. iN reprogramming efficiency is enhanced on dynamic (softened) hydrogels. iN reprogramming efficiency was quantified as the number of neurons divided by the plating efficiency. A glass negative control was included to ensure that addition of the CL2 DNA strand had no contribution to the observed reprogramming efficiency increase.

Conclusions

Substrate rigidity plays a key role in cell function and behavior. Here, we have demonstrated that substrate stiffness is important for cell reprogramming of fibroblasts to both iPSCs and iNs and for maintenance of pluripotency. Moreover, since reprogramming of fibroblasts is a dynamic process, dynamic modulation of stiffness also improves reprogramming efficiency.

Understanding cell interaction with the mechanical environment is necessary to evaluate reprogramming mechanisms and better comprehend how the complex network of biochemical and biophysical factors influence cell behavior. Thus, more mechanistic insights into how the biophysical environment regulates cell reprogramming are necessary. Overall, these insights can lead to improvements in direct reprogramming efficiency, making iNs more clinically relevant for more accurate disease modeling and drug screening.

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Chapter 3.

Electrophoretic Cytometry of Adherent Cells

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3.1 Abstract

Cell-matrix and cell-cell interactions influence intracellular signalling and play an important role in physiologic and pathologic processes. Detachment of cells from the surrounding microenvironment alters intracellular signalling. Here, we demonstrate and characterise an integrated microfluidic device to culture single and clustered cells in tuneable microenvironments and then directly analyse the lysate of each cell *in situ*, thereby eliminating the need to detach cells prior to analysis. First, we utilise microcontact printing to pattern cells in confined geometries. We then utilise a microscale isoelectric focusing (IEF) module to separate, detect, and analyse lamin A/C from substrate-adhered cells seeded and cultured at varying (500, 2000, and 9000 cells/cm²) densities. We report separation performance (minimum resolvable pI difference of 0.11) that is on par with capillary IEF and independent of cell density. Moreover, we map lamin A/C and β -tubulin protein expression to morphometric information (cell area, circumference, eccentricity, form factor, and cell area factor) of single cells and observe poor correlation with each of these parameters. By eliminating the need for cell detachment from substrates, we enhance detection of cell receptor proteins (CD44 and β -integrin) and dynamic phosphorylation events (pMLC^{S19}) that are rendered undetectable or disrupted by enzymatic treatments. Finally, we optimise protein solubilisation and separation performance by tuning lysis and electrofocusing (EF) durations. We observe enhanced separation performance (decreased peak width) with longer EF durations by 25.1% and improved protein solubilisation with longer lysis durations. Overall, the combination of morphometric analyses of substrate-adhered cells, with minimised handling, will yield important insights into our understanding of adhesion-mediated signalling processes.

3.2 Introduction

Studying and understanding human biology and disease has benefited from systems biology, including efforts to unify proteomics, transcriptomics, and genomics. High-resolution subcellular maps, such as the Human Protein Atlas, have identified single-cell, intracellular variations that drive disease.¹ Extracellularly, cells interact dynamically with the surrounding microenvironment, and these cell-cell and cell-matrix interactions regulate key biological processes such as cell survival,² stem cell differentiation,³ cell

migration,^{4,5} and cancer metastasis.⁶ For example, cancer cells differ in adhesive properties,⁷ with cell adhesion molecules such as integrins and selectins known to play a significant role in cancer progression and metastasis.^{8–10} Other cell receptor proteins, such as CD44, possess multiple alternatively spliced variants, of which certain isoforms promote resistance to apoptosis and mediate the migratory behaviour of cancer cells.^{11–13} These cell receptor proteins influence the adhesive strength of the cell, which correlates with the metastatic capability.^{14–16} Various other adhesive proteins, such as N-cadherin and β -integrin, have important roles in matrix mechanosensing, highlighting the importance of microenvironmental cues on cell behaviour.^{5,10,17–21} Other biophysical features, such as geometry,²² topography,²³ and substrate stiffness,⁴ also influence cell signalling. Thus, understanding the mechanisms underlying adhesion and downstream protein-mediated signalling is necessary for understanding disease progression and for identifying therapeutic targets.

It is unclear whether contemporary protein analyses accurately recapitulate the cellular environment. Flow cytometry offers high throughput analysis of multiple targets, but adherent cells are removed from the culture environment, often using enzymatic digestion, which proteolytically cleaves important cell-matrix receptors.^{24,25} Moreover, the long handling times may influence protein expression and modification, especially for rapid dynamic events such as phosphorylation.²⁶

Immunocytochemistry (ICC) is the workhorse immunoassay that detects cell-matrix interactions based on antibody specificity. Recent work has demonstrated multiplexed detection of intracellular and secreted cytokines in microfluidic systems.^{27,28} However, relying solely on antibody specificity for protein detection can be misleading, as nonspecific antibody binding to other cellular constituents can occur.²⁹ Moreover, chemical fixation of the cells has been demonstrated to cause artefacts in the dynamic detection of transcription factors.³⁰ Live-cell imaging can visualise the movement of select proteins dynamically, but is limited to transgenic cell lines, which limits multiplexing (number of targets measured concurrently).

Assays such as the western blot³¹ offer multiplexing capability and high selectivity by inclusion of an electrophoretic size separation step. However, conventional western blots require large numbers of cells and also entail disruption of the cell-matrix interactions by proteolytic and/or harsh mechanical conditions. Efforts to append an electrophoretic (capillary) separation to adherent cell lysis have shown promise;³² however, serial cell analysis limits throughput. Moreover, in all the aforementioned assays, protein isoforms that have small molecular mass differences are not separable.

In isoelectric focusing (IEF), proteins are resolved electrophoretically by isoelectric point (pI), the pH at which a protein is net neutrally charged. Since proteoforms of similar mass often differ in pI, IEF confers extraordinary selectivity. During IEF, a linear pH gradient is established along a gel or capillary. Proteins carry charge depending on the local pH of the environment, and as such, electromigrate along the pH gradient until

neutrally charged.³³ In capillary IEF, protein isoforms were identified from the lysates of < 25 cells and from tumour aspirates.^{34,35} Our recently reported work has demonstrated that proteins with just a single charge difference are resolvable in single-cell lysates.³⁶ However, all of these techniques require cell dissociation from the microenvironment, which can alter protein signalling^{7,11,24,25,37} and limit the ability to monitor spatial, morphometric, and dynamic signalling information.

Given the high selectivity of IEF and the importance of cell environment on signalling processes, we designed a tool that analyses proteins from cells *in situ*. We combine the selectivity of IEF with controlled micropatterning of cells. We measure proteins from single and clustered cells and correlate areas of individual cells with protein expression. Importantly, we identify protein targets from intact cells that are otherwise undetectable in detached cells. Finally, we discuss and optimise assay parameters to improve protein solubilisation in our system.

3.3 Materials and methods

Microcontact printing of fibronectin templates

PDMS stamps for microcontact printing were made by casting PDMS (Dow-Corning) at a 10:1 ratio onto a silicon wafer with SU-8 3050 (Microchem) features 40 µm in height. The wafer moulds were created using standard SU-8 soft lithography; photomasks with 30 - 400 µm features were created in AutoCAD and printed onto Mylar positive masks (CAD/Art services). After PDMS casting and polymerisation, the stamps were peeled off, and incubated with 10 mg/mL rhodamine-fibronectin (Cytoskeleton Inc.) at room temperature for 30 min. The fibronectin was then rinsed away with deionised water, and the mould dried under an air stream. PDMS stamps were inverted onto ethanol-cleaned glass slides (VWR International) and pressed down with forceps until contact was made for ~ 1 min. Stamps were then peeled off, leaving printed features on the glass.

Device fabrication

Substrate gel for cell patterning: 6%T, 3.3%C polyacrylamide gels containing 5 mM N-[3-[(3-benzoylphenyl) formamido]propyl] methacrylamide (BPMAC, Pharm-Agra Laboratories) were fabricated by pipetting the precursor solution between an acrylate-silanized microscope slide and the fibronectin-patterned glass slide described above. 0.08% (w/v) APS (Sigma-Aldrich) and 0.08% (v/v) TEMED (Sigma-Aldrich) were used as the initiator and catalyst. Prior to peeling the fibronectin-patterned slide, the gels were incubated in distilled water for 30 min at room temperature. The process transferred the fibronectin pattern from the imprinted glass to the surface of the gel, as has been previously described (Figure 1A).^{22,38} Prior to cell seeding, gels were sterilised in 70% ethanol for 10 min in a biosafety cabinet, then washed in PBS.

Lid gel: The lid gel is chemically-imprinted, using the protocol described previously.³⁶ Briefly, we fabricated 500- μm thick polyacrylamide gels with three adjacent compartments. The outermost regions define the pH boundary conditions (pH 4 and pH 10) for the IEF assay. The centre focusing gel serves two functions: (i) delivery of lysis reagents to attached cells and (ii) delivery of carrier ampholytes required to establish the pH gradient between the pH boundary gel sections. We used different carrier ampholytes (ZOOM®, 1% (v/v), Thermo Fischer Scientific), as the previously described carrier ampholytes³⁶ were discontinued. We also increased the denaturing and lysis reagents—81 mM CHAPS (Amresco), 2.5% (v/v) Triton X-100 (Sigma-Aldrich), 7 M urea (Sigma-Aldrich) and 2 M thiourea (Sigma-Aldrich) to enhance solubilisation (Figure 1).

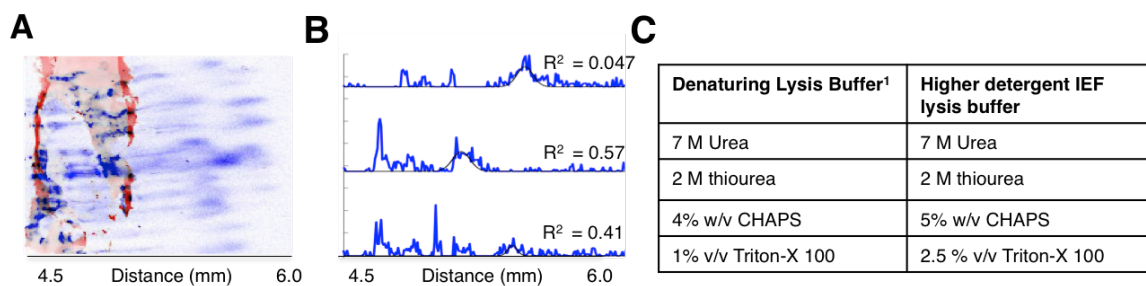


Figure 3-1. *In situ* IEF of cells (9000 cells/ cm^2 seeding density) using the previously reported¹ denaturing lysis buffer demonstrates limited solubilisation. (A) False-colour inverted fluorescence micrographs of lamin A/C resulting from *in situ* IEF using the previously reported denaturing lysis buffer. (B) Example fluorescence intensity profiles of probed lamin A/C indicate poor Gaussian fit and unanalysable data. (C) Table of reformulation of the lysis buffer to include a higher concentration of detergents CHAPS and Triton-X 100. This reformulation was used for all data presented herein.

Cell culture and cell patterning

Human BJ fibroblasts were provided by the Dillin laboratory (UC Berkeley) and validated by short tandem repeat identification (UC Berkeley Cell Culture Facility). These cells were mycoplasma-negative and cultured using DMEM+GlutaMAX media (ThermoFisher Scientific) supplemented with 1% Penicillin/Streptomycin (Life Technologies), 1x MEM non-essential amino acids (Life Technologies), and 10% foetal bovine serum (Gemini Bio-products). Cells were cultured at 37 °C with 5% CO_2 in a humidified incubator. All cells were between passage number 8 and 13.

For cell patterning, cells were detached using 0.25% Trypsin-EDTA (Gemini Bio-products), re-suspended, and applied to the fibronectin-patterned gel slide and incubated at 37 °C for 1 h to allow for cell attachment. The gels were then rinsed with PBS to remove unadhered cells and cultured in culturing medium overnight (to allow for recovery of receptor proteins) or up to four days (to obtain 2000 cell/ cm^2 density). For studies comparing GFP expression, BJ fibroblasts were first transduced with GFP using

a BacMam GFP Transduction kit (Thermo Fischer Scientific) at a 5% (v/v) concentration. Cells were then used 24 h after transduction.

For fluorescence-labelling of cells, BJ fibroblasts were stained using Cytopainter Cell Tracking Staining Kit (abcam) according to the manufacturer's instructions.

***In situ* and microwell IEF**

For *in situ* IEF, cell-patterned substrate gels were incubated in PBS for 10 min, then transferred to a buffer solution of 1% carrier ampholytes in Milli-Q water for ~ 30 s prior to lysis. Substrate gels were then placed in a custom-built electrophoresis chamber. To initiate lysis and IEF, the chemically-imprinted lid gel (centre focusing gel region) was interfaced with the substrate gel for 30 s, or, for solubilisation studies, for 45 or 70 s. A glass slide was placed on top of the lid gel to minimise evaporation. Next, an electric potential of 600 V was applied using a PowerPac high-voltage power supply (Bio-Rad) for 6 or 8 min. Following IEF, the proteins were immobilised into the substrate gel upon UV light exposure (Hamamatsu LC5) for 45 s, as previously described.^{39,40} The slide was then washed with 1x TBS + Tween 20 (TBST, Santa Cruz Biotechnology) for 30 min prior to antibody probing.

For microwell IEF, the same procedure as above was performed, except the substrate gel was replaced with a bottom gel layer stippled with a single row of microwells, as previously described.³⁶ Cells were trypsinized and seeded into wells via gravity sedimentation for 10 min. Excess cells were then washed with PBS and quickly rinsed with carrier ampholytes diluted 1:100 in MilliQ water prior to lysis. IEF was then performed immediately after washing.

Antibodies

Primary and secondary antibodies were diluted in 2% (w/v) BSA in 1x TBST. Mouse anti-CD44 antibody (Pierce, MA5-15462), rabbit anti-pMLCS19 (Cell Signaling, 3671T), goat anti-GFP (abcam, ab6673), mouse anti- β -integrin (abcam, ab30394), and rabbit anti- β -tubulin (abcam, ab6046) antibodies were diluted at 1:10, and mouse anti-lamin A/C (Pierce, mab636) antibody was diluted at 1:5 concentration. Secondary antibodies, AlexaFluor 488-, 594-, and 647-conjugated donkey anti-mouse, anti-goat, and anti-rabbit antibodies (Life Technologies), were diluted 1:50. Immunoprobings were conducted as previously described.⁴¹

Image acquisition and analysis

Brightfield images were obtained on an Olympus IX-71 inverted microscope with an Olympus UPlanFi 4X (Numerical Aperture (NA) 0.13) or 10X (NA 0.3) objectives and an EMCCD Camera iXon2 (Andor). Fluorescence images for ICC were obtained with the 10X objective and an X-Cite Exacte mercury arc lamp illumination source.

Immunoprobed gels were imaged on a microarray scanner (Genepix, Molecular Devices) under the 488, 535, and 635 excitation wavelength channels. Images were

then analysed using an in-house MATLAB (R2017a) script. The custom MATLAB algorithm segments peaks by first applying a threshold to identify protein peaks and centroids of each peak. A Gaussian is then fit laterally to each peak to generate a custom region of interest “lane” containing the entire peak (8σ). Intensity profiles of each peak were obtained by summing the intensities of each pixel along the separation axis and dividing by the number of pixels. The intensity profiles were fit to Gaussian curves with a minimum signal-to-noise ratio (SNR) of 3 and R^2 threshold of 0.7. Protein mass of focused protein peaks was calculated by summing the area under the curve (AUC) of the fit Gaussian curve. Protein mass of protein retained on the fibronectin pattern was calculated by summing the background-subtracted intensities along the fibronectin pattern (Integrated Density, ID).

Immunocytochemistry (ICC)

For ICC, BJ fibroblasts were seeded at a concentration of 5E3 cells/mL onto fibronectin-patterned gels and allowed to adhere overnight. Slides were then washed in PBS, fixed in 4% PFA (Electron Microscopy Sciences) for 10 min, then washed in PBS three times for 5 min. Cells were permeabilized in 0.25% Triton-X 100 for 10 min, washed in PBS, and blocked with 1% (w/v) BSA in PBST for 30 min. Primary antibodies were diluted at 1:100 (anti-lamin A/C) or 1:200 (anti- β -tubulin) in PBST and incubated overnight at 4°C on a shaker. Cells were then washed in PBS and incubated with fluorescent secondary antibody at 1:500 dilution in 1% (w/v) BSA in PBST for 1 h in the dark. Cells were then washed and imaged as described above.

Statistical analyses

Statistical analyses were performed in MATLAB. To determine significance between protein masses, we first tested for normality (QQ plot) and homoscedasticity (Levene’s test). For normal distributions with equivalent variance (Levene’s test, $p > 0.05$), comparison of two-sample groups was conducted using a Student’s t-test. For non-normal or non-homoscedastic samples, we performed a Mann-Whitney U-test for comparison of two groups, or a Kruskal-Wallis test, followed by Dunn’s post-hoc test for comparison of multiple groups. To detect correlation in protein expression with cell area, a Spearman’s rank correlation was performed. The correlation of protein expression with cell area was expected to be monotonic. Correlations with $p < 0.05$ were considered significant.

3.4 Results and discussion

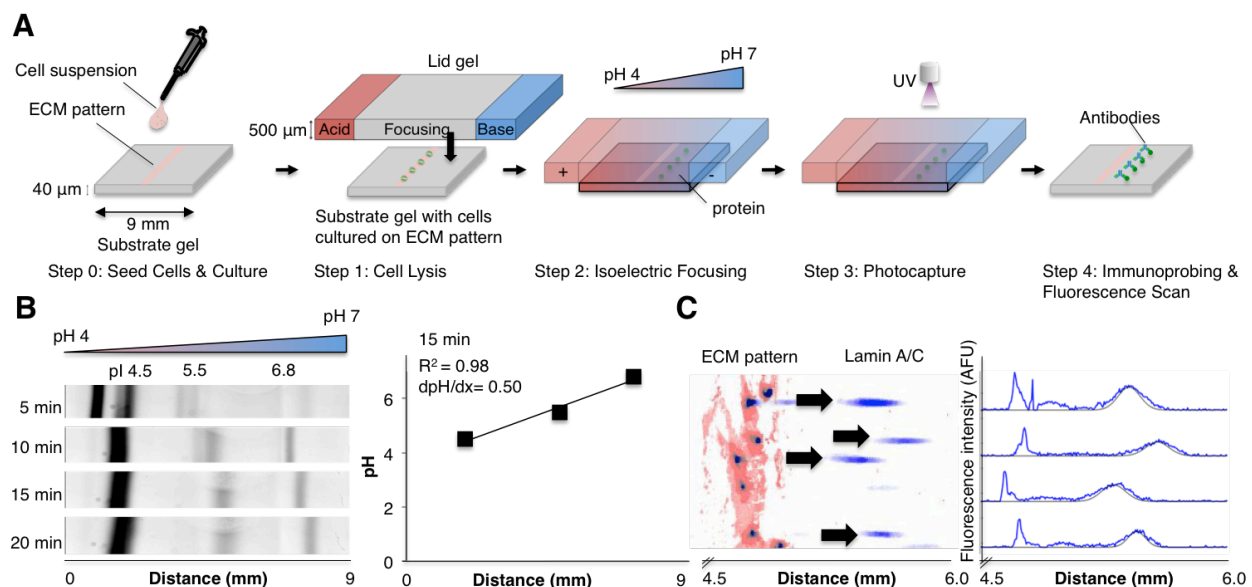
***In situ* IEF performs rapid protein analysis of single and clustered cells**

We designed an assay that measures protein signalling from cultured, adherent cells. The *in situ* IEF assay couples a substrate gel with cells cultured on an extracellular matrix (ECM) pattern and a microfluidic, lid-gel delivery system (Figure 2A). The assay is designed for (i) control of cell patterning and attachment to a defined ECM

environment, (ii) rapid lysis of attached cells and (iii) separation of proteins via IEF. IEF retains high protein concentrations due to electrophoretic restoring forces that limit diffusion to two dimensions.^{33,36} Microfluidic integration is key to minimising the time for diffusion-driven dilution and losses of the lysate from each cell.

The assay consists of two gels that are interfaced together (Figure 2A). The substrate gel, which also comprises the analytical unit, houses cells that are cultured on pre-determined, fibronectin-patterned regions (Figure 2A, Step 0). For the lid gel, we employ our previously reported single-cell IEF technology (Figure 2A, Step 1).³⁶ The lid gel consists of three regions— a central focusing region that contains denaturing, lysis reagents (Triton-X 100, thiourea, CHAPS, and urea) and carrier ampholytes, flanked by acidic and basic regions, which establish the pH boundary conditions (Figure 2A). The lid gel is denser than the substrate gel to reduce diffusive losses of cell lysate proteins into the lid. To maximise separation resolution, we chose a shallow pH gradient of 4-7.

Figure 3-2. *In situ* IEF measures protein peaks from single and clustered cells adhered to a substrate gel. (A) Concept schematic of *in situ* IEF (not-to-scale). Step 0: Cells are cultured on pre-determined regions by seeding cells on the ECM-patterned substrate gel. Step 1: A chemically-imprinted lid gel, which contains the lysis reagents and carrier ampholytes, is interfaced with the substrate gel, which contains the cells attached to the ECM-patterned region, for 30 s. Step 2: An electric potential (600 V) is then applied for 6 min, establishing a stable, linear pH gradient. Step 3: After focusing, proteins are covalently immobilised in the substrate gel via UV activation of a benzophenone moiety. Step 4: Gels are then washed and immunoprobed for targets of interest. Individual protein peaks are then quantified. (B) Inverted fluorescence kymograph of pI markers incorporated into the substrate gel and focused. The established pH gradient is linear and stable for > 20 min. (C) *Left*: False-colour inverted fluorescence micrograph of micropatterned rhodamine-fibronectin, onto which cells were cultured, lysed, separated via IEF, photocaptured, and immunoprobed for lamin A/C. *Right*: fluorescence intensity profile of lamin A/C protein peaks that have passed the SNR > 3 and Gaussian fit $R^2 > 0.7$ threshold. Black line indicates Gaussian fit. IEF, isoelectric focusing. pI, isoelectric point. ECM, extracellular matrix. AFU, arbitrary fluorescence units.



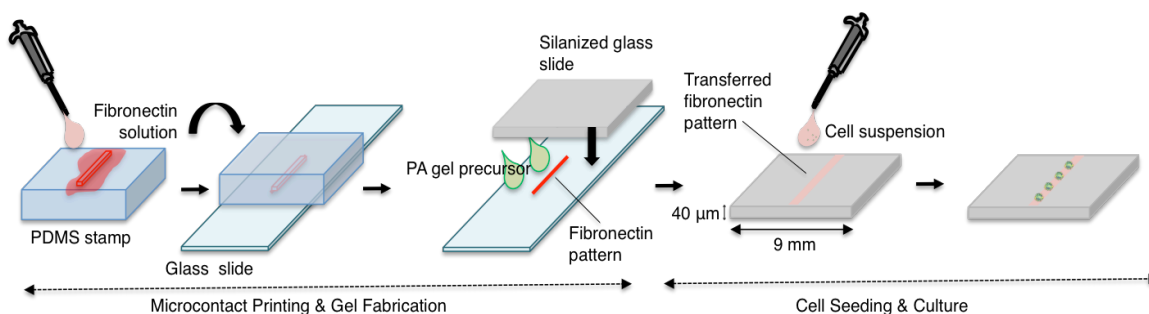


Figure 3-3. Schematic of microcontact printing of fibronectin-patterned substrate gels. The protocol for creating the substrates is described in the Materials and Methods section. Fibronectin solution is pipetted onto a PDMS stamp and inverted onto a standard microscope slide. Polyacrylamide gel is then polymerised between the patterned slide and a silanized glass slide. The fibronectin pattern transfers to the polyacrylamide gel. After, cells are seeded on top of the fibronectin-patterned substrate gel, and non-adhered cells, i.e., in the unpatterned regions, are washed away.

IEF is conducted *in situ*, i.e., at the site of the cells, by contacting the lid gel to the cell-patterned substrate gel (Figure 2A, Step 1) for 30 s. During this time, the carrier ampholytes and lysis reagents diffuse into the substrate gel layer. Upon application of an electric potential (600 V), the pH gradient is established, and the proteins migrate along the pH gradient until neutrally charged (at the pI, Figure 2A, Step 2). As previously utilised carrier ampholytes³⁶ have been discontinued, we prepared and characterised the formation of the pH gradient over time (Figure 2B). The pH gradient was linear and stable for at least 20 min; thus, we achieved similar pH gradient characteristics. After focusing for 6 min, the electric field is removed and the focused proteins are covalently immobilised to the gel via UV excitation of a benzophenone moiety⁴¹ in the gel (Figure 1A, Step 3). After washout, the gels are immunoprobed to measure proteins of interest (Figure 2A, Step 4 and Figure 2C).

The substrate gel, onto which cells are adhered and cultured, is fabricated by patterning a 400 μm -wide line of fibronectin along the centre of the substrate gel, as described above (Figure 3). Cells are seeded onto the patterns, and non-adhered cells, i.e., in the unpatterned regions, are washed away. We utilised 400 μm -wide ECM features for facile fabrication and increased cell occupancy; however, varying geometries and size of patterns can be achieved by tuning photomask design. For example, we patterned a 200 μm -wide line, as well as circular features as small as 30 μm , approximately the diameter of a suspended cell (Figure 4A). The 30-mm fibronectin pattern had a SNR of 55, which implies that patterning of even smaller features is possible (Figure 4A). Indeed, various groups have previously demonstrated features smaller than 200 nm using a similar nanocontact printing approach.^{42–45}

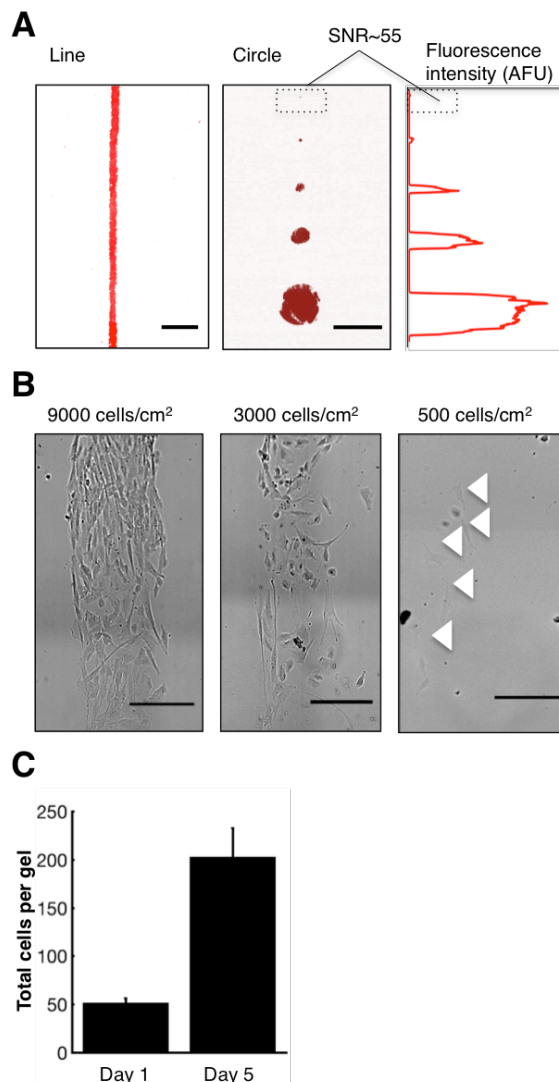
***In situ* IEF analysis of intracellular proteins of single and clustered cells**

We first sought to tune cell-to-cell spacing and verify cell proliferation on our patterned gels. We seeded human BJ fibroblasts at varying cell densities (500, 3000, or 9000 cells/ cm^2) to control the spacing between the cells (4B). Notably, even for the highest seeding density, the cells remained constrained to the pattern. We also verified cell culture on fibronectin-patterned substrate gels by seeding BJ fibroblasts at a low density (500 cells/ cm^2) and monitoring cell growth over four days. The total number of cells per device quadrupled

over this time period ($n = 3$ devices, Figure 4C), a growth rate that is similar to those previously reported for fibroblasts seeded on fibronectin substrates.⁴⁶

Next, we characterised separation performance of *in situ* IEF in comparison to the gold-standard technique of capillary IEF. First, we assessed whether higher cell densities would negatively impact separation performance. We patterned cells at two densities (500 and 9000 cells/cm²) and cultured the cells for 18 h prior to analysis. In addition, we also assessed the effects of culture duration on assay performance by seeding cells at a density of 500 cells/cm² and culturing for four days on the fibronectin pattern (final density ~ 2000 cells/cm², Figure 5). We then performed *in situ* IEF, followed by

Figure 3-4. The substrate gel contains a tuneable, ECM-patterned region onto which cells adhere. (A) False-colour inverted micrographs of gels patterned with varying geometries and pattern sizes of rhodamine-fibronectin. The SNR of the smallest circular feature ($d = 30 \mu\text{m}$) was 55. (B) Brightfield images of cells seeded at varying densities. The density of cells controls the cell-to-cell spacing. (C) Cells cultured at a starting concentration of 500 cells/cm² on the fibronectin-patterned gels proliferate over a period of four days, quadrupling in cell number. Error bars represent the standard deviation. Scalebars, 500 μm . SNR, signal-to-noise ratio.



photocapture and immunoprobings for lamin A/C, a nuclear protein (Figure 6). We quantified separation performance by measuring peak width (4σ , where σ is the standard deviation of the Gaussian fit to the fluorescence intensity profile) and ΔpI , the minimum resolvable pI difference.

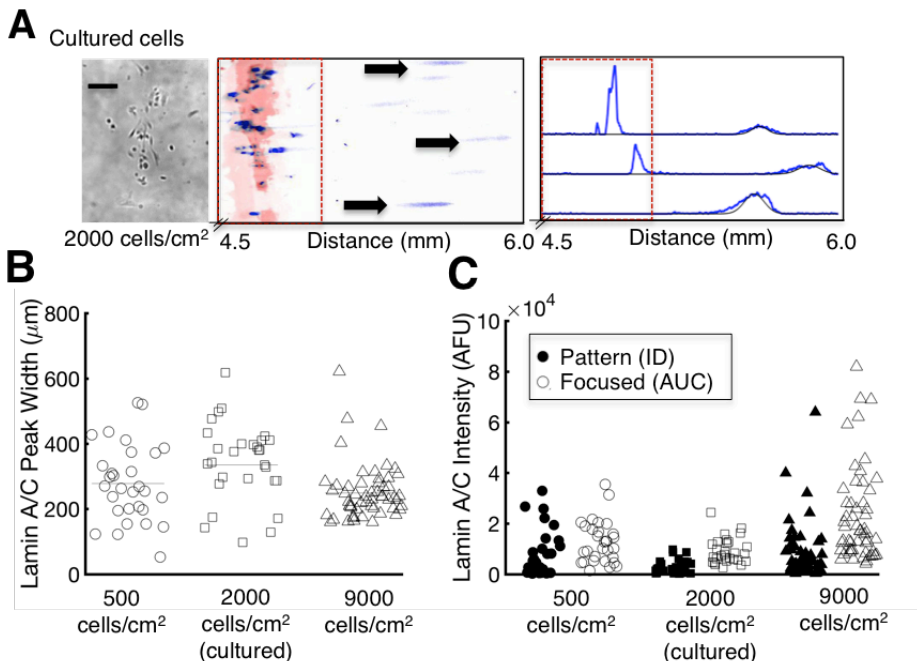


Figure 3-5. *In situ* IEF of cells seeded at a density of 500 cells/cm² and cultured for 4 days, for a final density of ~2000 cells/cm². (A) Brightfield images of cultured cells (left), and false-colour inverted fluorescence micrographs of lamin A/C that is retained on the fibronectin pattern and focused into a Gaussian peak (middle) and Gaussian fit of intensity profiles (right). (B) Separation performance, as measured by lamin A/C peak width, does not significantly change with culture duration. (C) Protein mass measurements of lamin A/C that is retained on the fibronectin pattern (ID) and focused (AUC). The data from the 500 and 9000 cells/cm² are the same as in the main text and are presented here for comparison purposes. Bars represent the mean. Scalebars, 200 µm. AFU, Arbitrary fluorescence units. ID, Integrated density. AUC, Area under curve. pI, isoelectric point.

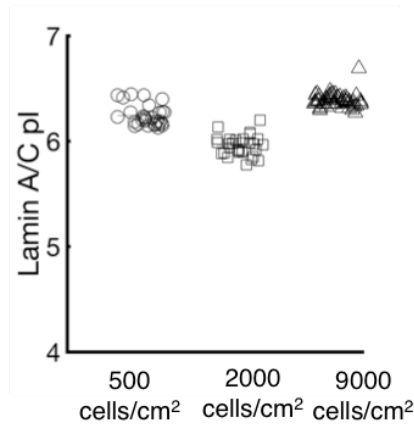


Figure 3-6. Measurement of lamin A/C pI. The pI was estimated for lamin A/C by using the peak location. The measured pI was 6.4 ± 0.06 (mean $\pm$ s.d., $n = 112$ peaks). Across devices and densities, the estimated pI of lamin A/C ranged from 6.0-6.4, which is close to those reported previously² and the predicted pI of 6.51 from proteomics software ExPASy.

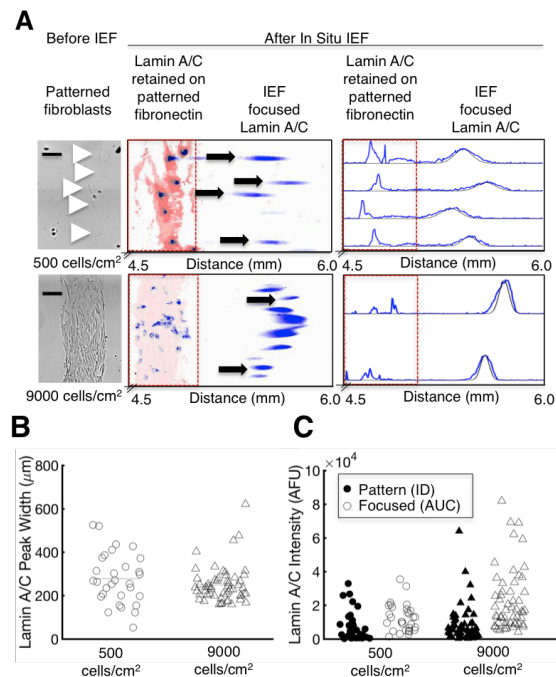


Figure 3-7. *In situ* IEF separates and detects proteins from single- and clustered-cells. (A) Brightfield images of single cells and a ~80% confluent pattern of cells prior to *in situ* IEF (left), and false-colour inverted fluorescence micrographs of lamin A/C that is retained on the fibronectin pattern and focused into a Gaussian peak (middle). The protein peaks are segmented and fluorescence intensity profiles are generated for each peak (right). Black line indicates Gaussian fit. (B) Separation performance, as measured by lamin A/C peak width, does not significantly change with higher seeding density, indicating that protein is not overloaded. (C) Protein mass measurements of lamin A/C that is retained on the fibronectin pattern (ID) and focused (AUC). Bars represent the mean. Scalebars, 200 μ m. AFU, Arbitrary fluorescence units. ID, integrated density. AUC, Area under curve. pI, isoelectric

Increasing the cell density from single cells (500 cells/cm²) to an ~ 80% confluent “line” (9000 cells/cm²) did not increase the median peak width (Figure 7, Mann-Whitney, $p > 0.05$, $n_{500} = 30$ cells, $n_{9000} = 54$ peaks). The equivalent peak widths suggest that even for a highly confluent “line” of cells, protein was not overloaded, which would saturate buffering capacity of the carrier ampholytes, and thus increase the peak width.^{47,48} We further estimate that the total local protein concentration of the highest density condition (9000 cells/cm²) is $\sim 1.6\text{E-}5$ mg/cm, which is 5 orders of magnitude lower than the predicted buffering capacity of 1-10 mg/cm;⁴⁹ thus, our system can assay millions of cells without reducing separation performance. Moreover, we calculated a ΔpI value of 0.11 pH units (ΔpI = the pH gradient slope $\times$ peak width), which is similar to the separation performance of capillary IEF³⁵ and on the order of a single phosphorylation event.⁵⁰

In addition to characterising separation performance as a function of cell density, we also measured the pI and protein mass (AUC) of each lamin A/C peak (Figure 7C and 5). We only analysed peaks that were minimally resolved laterally, (i.e., perpendicular to the IEF axis), to ensure that each peak had minimal ($< 4\%$) crosstalk with other

peaks. For the 500 cells/cm² density, ~70% of lamin A/C peaks were minimally resolved and could thus be indexed back to a single, adherent cell (Figure 7A and 8). Importantly, for the high (9000 cells/cm² density condition, only ~56% of peaks were resolvable laterally and passed our minimum Gaussian fit threshold ($R^2 > 0.7$). Thus, peaks that contained the highest protein content were excluded from analysis. The lamin A/C protein mass of the excluded peaks can be found in Figure 9.



Figure 3-8. Lamin A/C peaks are resolved laterally at a low (500 cells/cm²) cell density. To pattern single cells that were resolved laterally (perpendicular to the IEF axis), we used the characteristic diffusion equation ($\tau \sim X^2/D$) to determine the cell-to-cell spacing for minimally resolved lamin A/C peaks. We used already reported lamin A/C diffusion coefficients,³ and factored in in-gel diffusivity of proteins in a 6%T, 3.3%C gel.⁴ Given our 390 s assay time, we estimated cells needed to be spaced 43-187 μm (depending on which diffusivity used) apart. For the 500 cells/cm² seeding density, the peaks were spaced $74 \pm 71 \mu\text{m}$ (mean $\pm$ s.d., $n = 38$ cells) apart. Thus, most (~70%) focused IEF protein peaks are minimally resolved and thus can be indexed back to a single adherent cell.

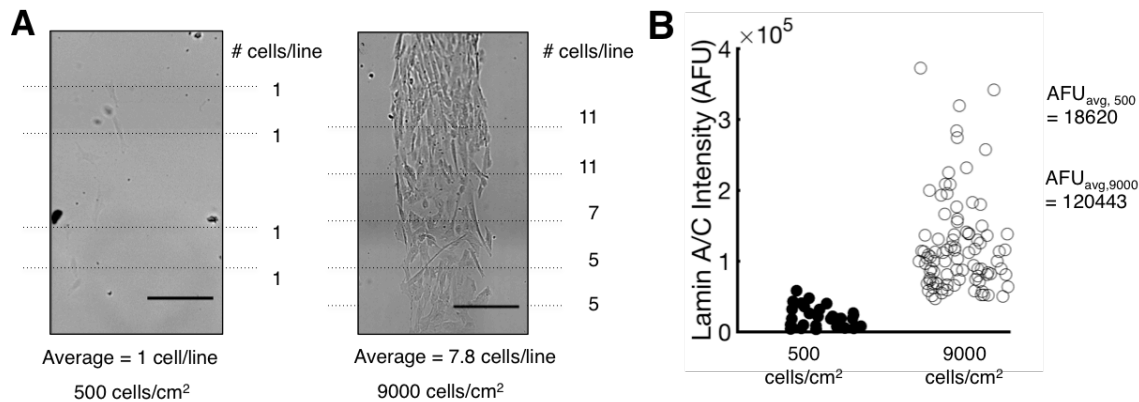


Figure 3-9. Analysis of total Lamin A/C content of cells indicate approximately proportional total protein content to cell density. A) Brightfield images of cells seeded at 500 and 9000 cell/cm² density. The total number of cells per peak were counted manually and indicated that ~7.8x the density of cells were adhered using the 9000 cell/cm² density, as compared to that of the 500 cell/cm² condition. B) Total AUC content of lamin A/C peaks that were identified. These peaks include those that were *not* minimally resolved laterally or had a Gaussian-fit $R^2 < 0.7$. In the main text, only peaks that were minimally resolved laterally with Gaussian-fit $R^2 > 0.7$ were included in the analyses. Thus, many (44%) of the peaks, which contained the highest amount of protein, were excluded.

As expected, the highest cell density (9000 cells/cm²) yielded the highest quantity of focused protein, as each peak corresponds to the greatest number of cells (Figure 7C, Levene's test, $p = 1.2E-7$, Kruskal-Wallis and Dunn's test, $p = 1.4E-7$). The measured lamin A/C content was independent of starting cell-cluster geometry (Figure 10). Interestingly, the focused protein mass was lowest for the medium (2000 cells/cm²) density, the only cells that were cultured for multiple (four) days prior to analysis (Figure 5). Furthermore, we observed that lamin A/C was partially retained on the fibronectin pattern (which did not electromigrate) after cell lysis, regardless of the cell density (Figure 7A and 11). We hypothesize that longer culture times of adherent cells result in a more difficult-to-lyse phenotype. Adhered fibroblasts produce and secrete ECM proteins, such as fibronectin, and become more strongly adhered to the substrate with time.^{51,52} These secreted ECM proteins limit biomolecular diffusion extracellularly,⁵³ whereas the network of transmembrane proteins and the cytoskeleton act as a "fence" that limits diffusion of intracellular contents.^{54,55} Altogether, we hypothesize that adhered cells possess a tethered network of ECM-transmembrane-cytoskeletal proteins that act as a protein "barrier" that diminishes cell lysis and protein solubilisation. We present improvements to protein solubilisation below.

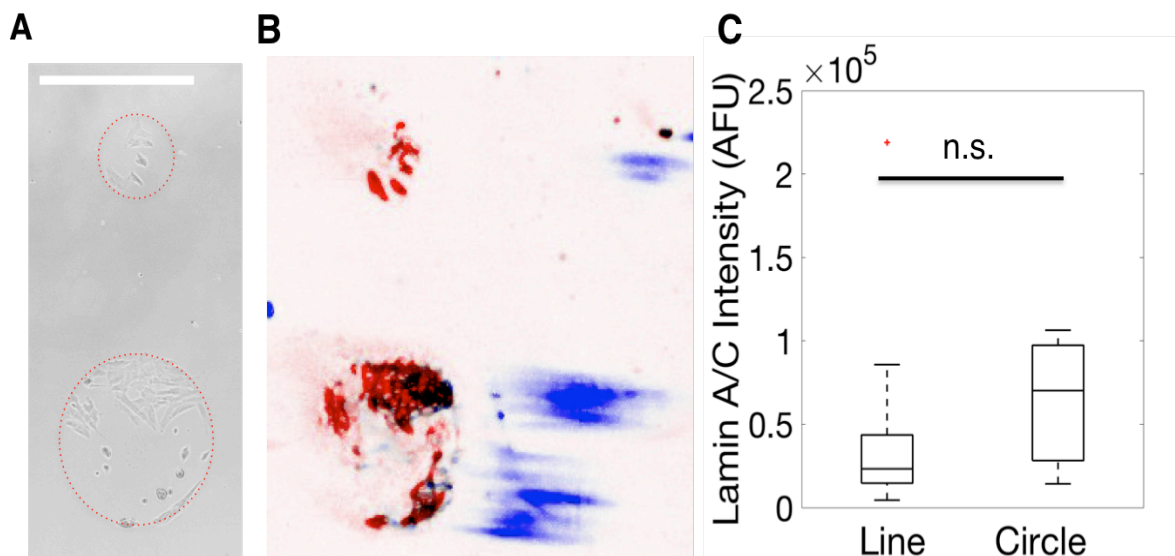


Figure 3-10. *In Situ* IEF of cells seeded on fibronectin-pattern islands. (A) Brightfield image of cells seeded at 9000 cells/cm² on islands of 200 and 500 µm diameter. (B) False-colour inverted fluorescence micrographs of lamin A/C separated via *in situ* IEF. (C) Total measured lamin A/C content between the 400-µm line and circle was not significantly different (n = 3).

Since the protein retained on the fibronectin pattern was not electrophoretically separated and thus not Gaussian, we chose to analyse the protein content similarly to a Reverse Phase Protein Assay (RPPA).⁵⁶ We thus measured the ID of the immunoprobed signal retained in the fibronectin-patterned area (Figure 12). The ratio of focused protein (AUC) to total protein (AUC + ID) was similar for all densities ($n_{500} = 30$ cells; $n_{2000} = 40$ peaks; $n_{9000} = 54$ peaks, non-normal distribution from QQ plot, not shown, Kruskal-Wallis $p > 0.05$), further suggesting that IEF performance in our system is independent of the starting density of cells.

Cell morphology and protein expression

Using *in situ* IEF, we next correlated individual protein peak measurements back to single-cell morphometric information. We hypothesized that protein expression would increase linearly with cell area, as previous reports have suggested.^{57–59} Most of these studies, however, were conducted in bulk populations of yeast cells or for multiple cell lines, in which the cell areas were averaged.⁶⁰ Similar studies using transcriptomics have also linked RNA content with cell size.⁶¹

We first seeded BJ fibroblasts at single-cell density (500 cells/cm²) overnight. We then imaged BJ fibroblasts under brightfield microscopy, then performed the *in situ* IEF and probed for lamin A/C and β -tubulin (Figure 12A). We quantified the total lamin A/C and β -tubulin content by summing the protein focused into a peak (AUC) and that retained on the pattern (ID). Interestingly, our measured lamin A/C and β -tubulin protein mass

did not correlate well with cell area (Figure 12B, Spearman's correlation test, $p = 0.05$ for lamin A/C and $p = 0.21$ for β -tubulin, $n = 16$ cells). Since image segmentation and accurate identification of cell borders is challenging using brightfield microscopy, we further assessed cell area using fluorescently-labelled cells, which also yielded poor correlation between cell area and lamin A/C protein mass (Figure 13, Spearman's test, $\rho = 0.33$, $p = 0.08$, $n = 28$ cells).

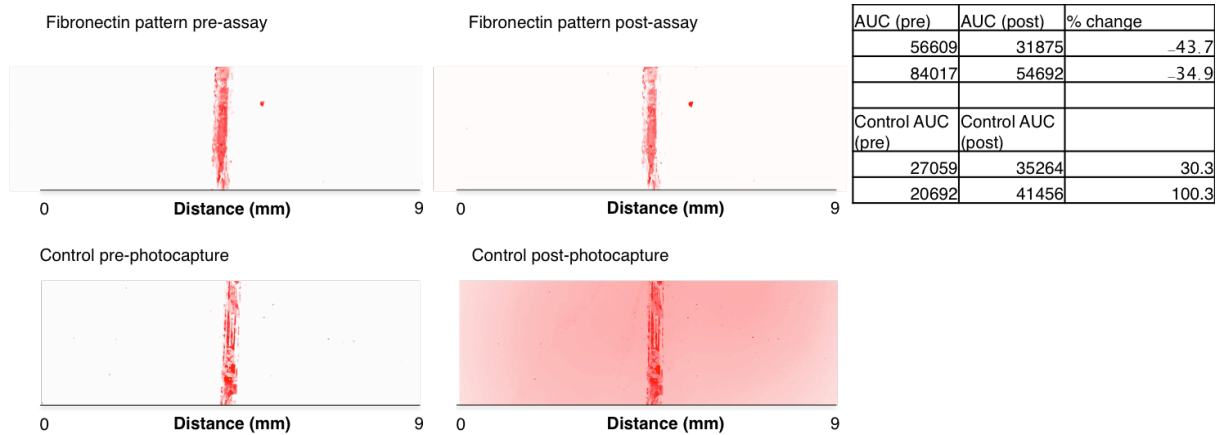
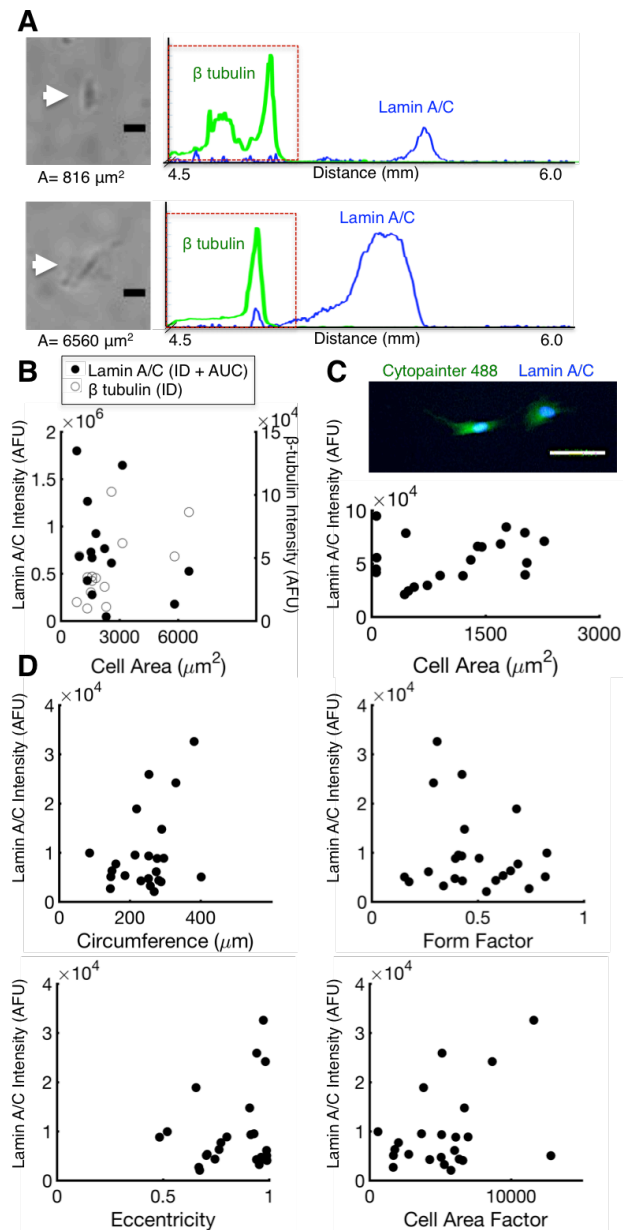


Figure 3-11. Comparison of fibronectin fluorescence intensity before and after *in situ* IEF. *Left:* False colored inverted fluorescence micrographs of the printed fibronectin pattern prior to seeding cells, lysis, IEF, photocapture, and immunoprobing. The fibronectin pattern did not electromigrate during the assay. *Bottom:* False colored inverted fluorescence micrographs of a printed fibronectin pattern prior to and after photocapture only. Photocapture alone increased the background intensity and the measured (background-subtracted) measured area under the curve (AUC). *Right:* Table of AUC values pre- and post-assay, and the percent change for two devices and two control devices (exposed to UV only with no other steps). Notably, the fluorescence intensity of the fibronectin pattern decreased after running the assay; however, we attribute these signal losses not to photobleaching (45 s exposure to UV for photocapture), but to other steps in the assay, such as gel sterilization, cell lysis, and multiple wash steps, as the control gel exposed only to UV actually increased in fluorescence intensity. Fibronectin is a large (~440 kDa) protein and was patterned on the substrate gel at $pI \sim 5.5$, which is close to the pI of fibronectin ($pI \sim 5.5-5.8$). The large size and proximity to pI will limit electrophoretic mobility of the protein⁵. The increase in fluorescence intensity of the fibronectin pattern and the background signal after UV exposure is possibly attributable to benzophenone phosphorescence⁶, which is currently under further examination.

Figure 3-12. Protein expression does not correlate with cell morphology. (A) Brightfield micrographs of cells of varying area and corresponding fluorescence intensity profiles of lamin A/C and β -tubulin. (B) Comparison of lamin A/C and β -tubulin protein content (AUC and ID), which correlate poorly with cell area, as measured via brightfield. (C) ICC fluorescence micrograph of fluorescently-labelled (Cytopainter) cells seeded on patterned gels. Lamin A/C protein expression does not correlate with cell area. (D) Scatter plots of lamin A/C protein mass as a function of cell circumference, form factor, eccentricity, and cell area factor (cell area/form factor). Lamin A/C protein mass did not correlate with these morphometric parameters. Scalebars, 100 μm . ICC, Immunocytochemistry. AFU, Arbitrary fluorescence units. ID, Integrated density. AUC, Area under curve.



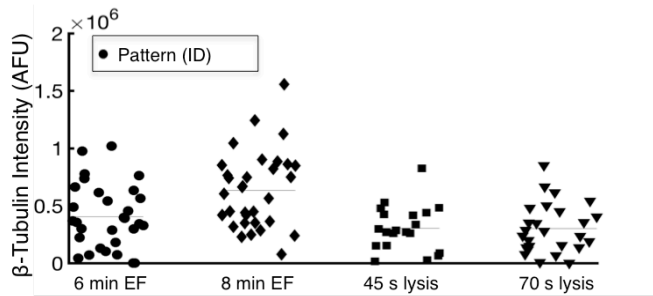


Figure 3-13. Comparison of β -tubulin protein content (ID), which is retained on the fibronectin pattern, under varying lysis and EF durations. For the higher EF duration, the mean β -tubulin protein content was higher (normal distribution from QQ plot, data not shown, and Student's two-sample t-test, $p = 0.0049$). The higher β -tubulin content may arise from the longer assay duration, which increases duration for protein solubilisation and denaturation. Across the different lysis durations, there was no significant difference in the measured protein content (Kruskal-Wallis, $p > 0.05$) of β -tubulin, thus suggesting that lysis times must be tuned to optimise solubilisation for different protein types. We hypothesize that transmembrane and cytoskeletal proteins, which are linked together and involved in cell anchoring, require more stringent solubilisation conditions.

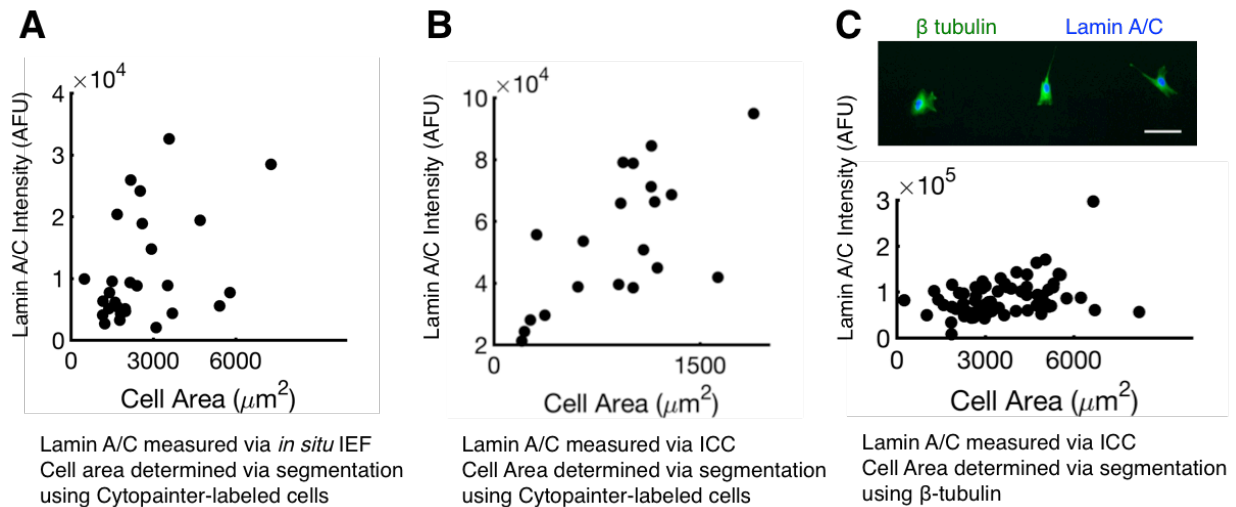


Figure 3-14. Lamin A/C expression does not correlate with cell morphology, as measured using a cell tracker dye (Cytopainter). (A) Scatter plot of lamin A/C intensity, as measured via *in situ* IEF (AUC + ID), which does not correlate well with cell area, as measured by segmentation using Cytopainter. (B) ICC corroborates the poor correlation of lamin A/C expression (ID) with cell area, as measured by segmentation using Cytopainter. (C) Lamin A/C intensity does correlate with area when cells are segmented using β -tubulin fluorescence. Scalebar, 100 μm . ICC, immunocytochemistry. AUC, area under the curve. ID, integrated density.

To validate protein mass and cell area measurements, we performed conventional ICC of fluorescently labelled BJ fibroblasts seeded on the fibronectin-patterned gels (Figure 12C and 13). The calculated lamin A/C and β -tubulin protein mass (ID) demonstrated similar variability ($CV_{\text{laminA/C}} = 41\%$, $CV_{\beta\text{-tubulin}} = 60\%$, $n = 20$ cells) as in situ IEF ($CV_{\text{laminA/C}} = 60\%$, $CV_{\beta\text{-tubulin}} = 67\%$, $n = 16$ cells). Moreover, we again observed poor correlation between cell area and lamin A/C protein mass (Figure 12C, Spearman's correlation test, $p = 0.22$, $n = 20$ cells). Interestingly, we observed a positive correlation between lamin A/C expression and cell area when β -tubulin was used for segmentation (Figure 14, Spearman's test, $p = 0.36$, $p = 0.0027$, $n = 68$ cells). The correlation of lamin A/C to β -tubulin-segmented area, but not to cell area itself, is possibly attributable to the association of lamin proteins with the cellular cytoskeletal network, which in turn regulates cytoskeletal reorganisation.⁶²

We next hypothesized that cell area could be an indicator of cell division stage, and that normalising the protein expression values to the pre-measured cell area would thus account for different cell cycle stages and decrease the variation. We chose to examine endogenous proteins, without the use of chemicals or serum starvation, which may alter cell biology.⁶³ Both proteins demonstrated similar levels of variability ($CV_{\text{laminA/C}} = 60\%$, $CV_{\beta\text{-tubulin}} = 67\%$, $n = 16$ cells). After normalisation to cell area, lamin A/C yielded a $CV = 56\%$; in comparison, the CV for most proteins in a human cell, when normalised *in silico* to cell cycle, is 12 - 28%.⁶⁴ Since the variation of our area-normalised protein expression is not representative of the CV of protein normalised to cell cycle, we conclude that cell area alone is not a proxy for cell division. The higher variance of protein expression in our system is possibly attributed to the protein types we assayed. For example, proteins involved in ECM interactions exhibit higher cell-to-cell variability.⁶⁵ Lamin A/C and β -tubulin are both mechanosensitive,^{66,67} thus, some of the variation may arise from the cells' differential interaction with the substrate.

In addition to scrutinising the relationship between the area of each cell and protein mass of lamin A/C expressed in each same cell, we assessed the relationship between lamin A/C expression and a set of morphometric parameters, including: cell circumference (perimeter), form factor (circularity), eccentricity, and cell area factor (cell area normalised to form factor). We observed no appreciable dependence of lamin A/C protein mass (AUC + ID) on any of the morphometric parameters investigated (Figure 12D). Interestingly, a predictive model of Src and MAPK1/2 expression using cell membrane eccentricity shows a dependence on Src and MAPK1/2 expression on eccentricity.⁶⁸ However, these approaches were modelled in 3-D and excluded consideration of cytoskeletal-mediated membrane signalling events. Thus, integration with flow cytometry or 3-D imaging modalities may yield complementary data to further elucidate how 3-D morphology influences or is predictive of intracellular protein expression.

***In situ* IEF detects distinct, adherent-cell biomarkers**

Since signalling can be quickly disrupted by detachment of cells (minute timescales),^{11,12,26} we compared the protein expression of single BJ fibroblasts that were detached and seeded into individual microwells (single-cell IEF)³⁶ with that of BJ fibroblasts patterned at single-cell density on a substrate gel and cultured overnight (*in situ* IEF). In both cases, we used the same lid-based IEF system. We compared expression of three markers: β -integrin, mono-phosphorylated myosin light chain (pMLC^{S19}), and CD44 (Figure 15A). β -integrin mediates cell attachment to fibronectin; pMLC^{S19} is involved in the adhesion-dependent Rho GTPase pathway and is diminished upon detachment,⁶⁹ and CD44 is a cell surface receptor protein subject to proteolytic cleavage.¹¹ The BJ fibroblasts were transduced with GFP to serve as a loading control. Characterisation of focused GFP protein content indicated no significant difference in the median measured amount of protein in the microwell (detached) cells, as compared to the fibronectin-patterned (attached) cells (Figure 15B, Mann-Whitney $p = 0.16$, $n = 6$ cells in microwells, $n = 3$ cells on fibronectin-patterned substrate gels), indicating that detection sensitivity of both systems are comparable. For β -integrin, pMLC^{S19}, and CD44, all of the detectable protein ($\text{SNR} > 3$) remained on the fibronectin pattern (Figure 15A). We hypothesize that the amount of protein that is retained on the fibronectin pattern may be a proxy for cell attachment, i.e., proteins that tether to the cytoskeleton and focal adhesion complexes are more highly retained on the fibronectin pattern. Accordingly, β -tubulin and pMLC^{S19}, cytoskeletal proteins, remained on the fibronectin pattern, as did transmembrane proteins CD44 and β -integrin (Figure 15A). In contrast, lamin A/C and GFP, a nuclear membrane and cytosolic protein, respectively, focused into protein peaks (Figure 15A).

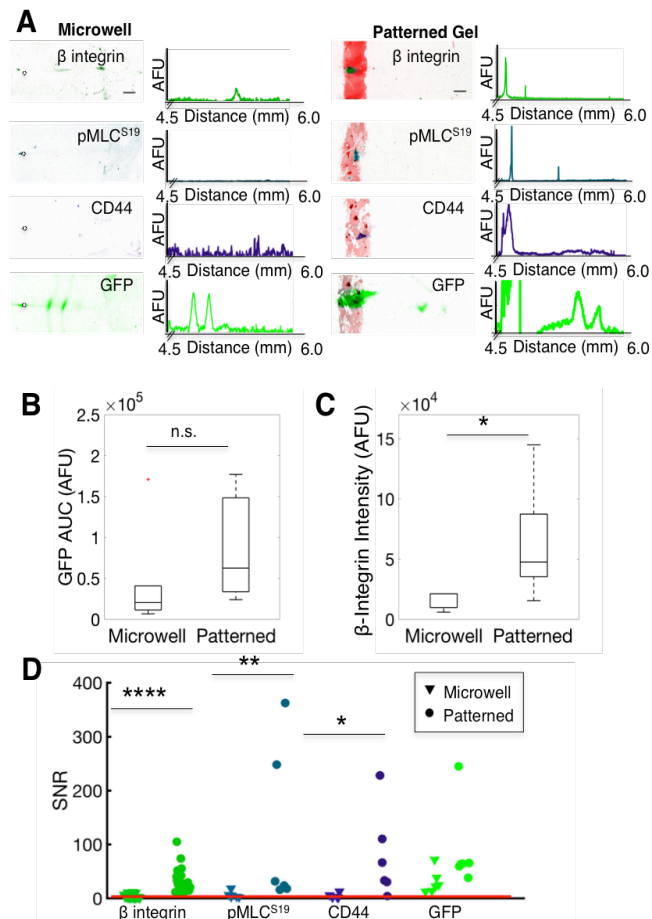
To measure the differences in protein expression of attached versus detached cells, we measured AUC of focused peaks and ID of protein retained on the fibronectin pattern. For β -integrin, we observed a significant difference in the protein expression of the focused peaks (AUC) from the microwells as compared to the protein retained on the fibronectin pattern (ID, Figure 15C, Mann-Whitney, $p = 0.01$, $n_{\text{microwell}} = 3$ cells and $n_{\text{patterned}} = 29$ cells). We attribute the lower expression and detection of β -integrin to trypsin cleavage of the receptor protein.⁷⁰ Similarly, for both pMLC^{S19} and CD44, no protein peaks were detectable ($\text{SNR} < 3$, or Gaussian fit $R^2 < 0.7$) in the microwells, but detectable signal was measured for the attached cells (Figure 15D, $n_{\text{microwell}} = 6$ cells and $n_{\text{patterned}} = 6$ cells). These results corroborate previous research demonstrating that proteolytic cleavage and/or cell detachment alters detection of surface proteins and downstream signalling.^{11,69} Altogether, our results implicate that minimised handling, obviation of enzymatic treatments, and direct *in situ* analysis of cell lysates analyses proteins that are otherwise undetectable or disrupted by the sample preparation of conventional single-cell studies, such as flow cytometry. Using our *in situ* IEF assay, we detect protein expression from substrate-adhered cells and thus assay different protein signatures compared to detached cells. This unique capability is important in assaying rapidly changing events (e.g., phosphorylations) or various adhesion-mediated

signalling pathways. Overall, our results highlight the critical importance on consideration of sample preparation parameters for single-cell analysis.

Tuning assay parameters for improved separation performance and protein solubilisation

Given the notable intracellular protein adhesion to the fibronectin pattern ($31 \pm 20\%$ lamin A/C retained on fibronectin pattern, $n = 30$ cells) that was observed under the single-cell condition, and variation of the peak width ($CV_{\text{laminAC}} = 39.8\%$, $n = 30$ cells), we sought to improve protein solubilisation and separation performance by

Figure 3-15. *In situ* IEF of adherent cells assays protein profiles that differ from trypsin-treated (detached) cells. (A) False-colour inverted fluorescence micrographs and intensity profiles of IEF of single cells detached using trypsin and seated into microwells (left) or analysed via *in situ* IEF of fibronectin-patterned (attached) cells (right). (B) Box plot of GFP control protein content (AUC) of focused peaks indicates comparable expression between microwells and fibronectin-patterned cells. Line in the box represents the median and box ends represent the 25th and 75th percentiles. Red dots indicate outliers. (C) Box plot of β -integrin indicates higher expression in patterned cells. (D) Comparison of measured SNR of various targets. Compared to microwell IEF, *in situ* (adhered cells on fibronectin pattern) IEF results in higher SNR of β -integrin, pMLC^{S19}, and CD44, targets that are adversely affected by trypsin treatment. The red line indicates SNR threshold of 3. Scalebars, 200 μm . AUC, Area under curve. SNR, signal-to-noise ratio. AFU, arbitrary fluorescence units. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$



systematically increasing the lysis or electrofocusing (EF) duration (Figure 16). We hypothesized that the high variation in peak width arose from proteins continuing to focus, i.e., equilibrium had not been reached within the 6 min. Increasing the assay duration presents trade-offs, as longer durations may improve separation performance but concomitantly increase diffusive losses of the cell lysate.^{41,71} We thus increased the

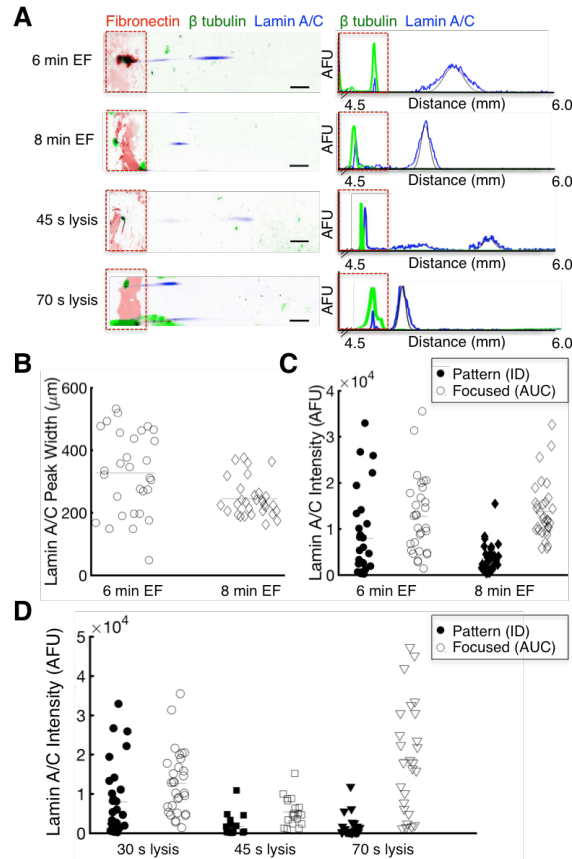


Figure 3-16. Separation performance, as measured by lamin A/C peak width, and protein solubilisation are enhanced by tuning the EF and lysis durations, respectively. (A) False-colour, inverted fluorescence micrographs and intensity profiles of single cells lysed for 30 s and electrofocused for 6 min, compared to either a longer EF duration of 8 min, or longer lysis durations of 45 s or 70 s. Contrast adjusted for improved visualisation. Black line indicates Gaussian fit. (B) Analysis of peak width for longer EF duration indicates improved separation performance (lower peak width). Bars represent the mean. (C) Comparison of lamin A/C protein mass retained on the fibronectin pattern or focused to lamin A/C pl as a function of EF duration. (D) Comparison of lamin A/C protein mass retained on the fibronectin pattern or focused to lamin A/C pl as a function of lysis duration. The longest lysis duration (70 s) improved lamin A/C solubilisation, as evidenced by higher focused protein content. Data from 30 s lysis duration are the same as the 6 min EF duration. Scalebars, 200 μ m. AFU, Arbitrary fluorescence units. ID, Integrated density. AUC, Area under curve. EF, electrofocusing.

EF duration modestly, from 6 min to 8 min, and immunoprobed for lamin A/C and β -tubulin (Figure 16A and 17).

The longer EF duration improved separation performance without decreasing lateral resolution (Figure 16B and 17). The average peak width of lamin A/C decreased by 25.1%, from $327 \pm 130 \mu\text{m}$ (mean $\pm$ s.d., $n_{6\text{min}} = 30$ cells) to $245 \pm 60 \mu\text{m}$ ($n_{8\text{min}} = 31$ cells), with lower variation (Figure 16B, $\text{CV}_{6\text{min}} = 39.8\%$, $\text{CV}_{8\text{min}} = 24.3\%$, Levene's test, $p = 4.2\text{E-}5$). These results imply that equilibrium is not yet reached for *in situ* IEF within 6 min, and that longer EF durations enhance separation performance.

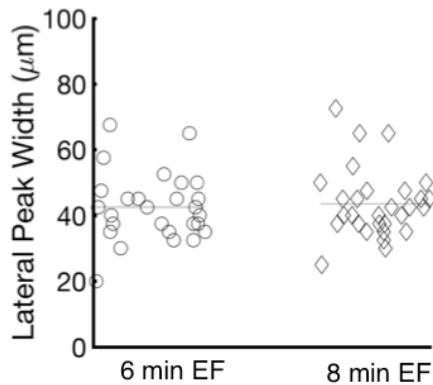


Figure 3-17. Scatter plot of lateral (perpendicular to IEF axis) peak width as a function of increased EF time. Lateral peak width did not change significantly. We estimated that for a 40% in gel diffusivity⁴ for a 3.33% C gel and a diffusivity of lamin A/C⁷⁹ to range from 0.58-5.2, we would expect lamin A/C peak width to increase 6.1-26.7 μm from 2 additional min of electrophoresis. However, since the concentration of protein decreases exponentially away from the peak center, the lateral peak width likely did not increase measurably, as the lamin A/C falls below the limit of detection.

Next, to assess how EF duration influences solubilisation, we assessed the protein content both remaining on the fibronectin pattern (ID) and focused into a peak (AUC) for the longer EF durations (Figure 16C). The longer EF duration increased the detected amount of β -tubulin, likely due to increased denaturation and thus photocapture efficiency (Figure 17).⁷² For lamin A/C, the median protein retained on the fibronectin pattern (ID) did not significantly change for a longer EF duration (Mann-Whitney, $p > 0.05$); however, the distribution was narrower ($\sigma^2_{6\text{min}} = 9090$, $n_{6\text{min}} = 30$ cells; $\sigma^2_{8\text{min}} = 3115$, $n_{8\text{min}} = 32$ cells, Levene's test, $p = 2.9\text{E-}5$), suggesting that more protein was solubilised (and migrated to the pl) with the longer EF duration. However, for the focused protein peak, there was no significant difference in the mean protein content, (normal distribution from QQ plot, not shown, Levene's test, $p > 0.05$, two sample t-test, $p = 0.69$). Although longer focusing duration may have increased protein solubilisation, the longer assay duration concomitantly increased diffusive losses of the lamin A/C.

Thus, EF duration improves separation performance, but with the trade-off of higher diffusive losses.

We next analysed whether longer lysis durations could improve protein solubilisation (Figure 16D). We assayed modest increases in lysis duration, from 30 s to 45 s or 70 s, and maintained a 6 min focusing duration. For β -tubulin, we observed no difference in the protein content from the longer lysis (Figure 17). For lamin A/C, we observed a narrower distribution of protein content retained on the fibronectin pattern for longer lysis durations (Figure 16D, $\sigma^2_{30s} = 9090$, $n_{30s} = 30$; $\sigma^2_{45s} = 2673$, $n_{45s} = 19$, $\sigma^2_{70s} = 2629$, $n_{70s} = 26$ cells, Levene's test, $p = 8.5E-7$). Longer lysis durations also decreased the mean amount of protein retained on the fibronectin pattern (non-normal distribution from QQ plot, not shown, Kruskal-Wallis and Dunn's test, $p = 2.5E-5$), suggesting increased protein solubilisation of lamin A/C with lysis duration. Interestingly, for the focused lamin A/C peak, we noticed a biphasic trend (Levene's test, $p = 7.9E-6$; Kruskal-Wallis and Dunn's test, $p = 4.2E-6$). It is possible that the intermediate lysis duration (45 s) was insufficient for protein solubilisation, and thus the increased duration only increased diffusive losses of the cell lysate, whereas the longest lysis duration (70 s) increased solubilisation sufficiently, thus resulting in higher protein content in the focused peak. Altogether, these results suggest that optimising EF and lysis duration can significantly improve separation performance and protein solubilisation. Further improvements of solubilisation may be achievable via chemical modulation, as has previously been reported in conventional preparatory IEF,^{73,74} or perhaps by modulating the substrate to allow for laser-based lift off of adherent cells⁷⁵ prior to lysis and IEF.

3.5 Conclusion

The cell microenvironment plays an important role in modulating cell behaviour. Here, we report on a microfluidic device that is designed for direct lysis of cells seeded on a tuneable microenvironment and subsequent analysis of the protein contents *in situ*, thereby interrogating cell-matrix mediated signalling. We utilise a tuneable cell-patterning technique and demonstrate the separation, detection, and quantitation of protein targets of single and clustered cells. We also assess separation performance of the assay in terms of peak width, measured pl, and Δ pl. Furthermore, we correlate morphometric information with protein expression levels of two commonly used housekeeping genes, demonstrating high variation that is independent of cell area. Finally, we analyse protein expression of cells that are attached to a fibronectin-patterned substrate and compare to those of detached cells, and demonstrate detection of targets that are otherwise masked or cleaved in conventional single-cell analysis approaches.

In future device work, we aim to expand the applications of the assay. Currently, the cell type determines the seeding specificity; seeding U251 cells, a glioblastoma cell line, did not result in specific cell adhesion as was observed for the BJ fibroblasts (results not

shown). Since different cell types vary in their adhesive strength and response to extracellular matrix environment,¹⁸ special attention to lysis conditions will be necessary to ensure sufficient solubilisation of the intracellular proteins. Moreover, for higher densities of cells, we cannot decouple protein signal from individual cells as of now; nonetheless, we can envision approaches using fluorescently tagged proteins, such as in co-culture systems, to determine the origin of each protein to a specified cell type.

While select microenvironmental cues are discussed herein, the value of the direct lysis IEF technology extends beyond studying individual cell-cell or cell-matrix interactions. Indeed, the platform could be combined with combinatorial soluble factor gradients and ECM microarrays/niches, such as those previously described elsewhere,^{76–78} to address the role of complex environments on stem cell behaviour or cancer metastasis. Since the analytical module (substrate gel) is grafted onto a conventional microscope slide, our device is particularly amenable to microscopy, which opens possibilities to further corroborate morphometric and spatial information with signalling and also enabling longitudinal measurements. Another important capability of this platform is to study cell dynamics. Minimal handling requirements and direct study of adherent cells lends a unique opportunity to explore rapidly changing events, such as protein phosphorylation, or other applications wherein detachment of the cells would drastically alter signalling. Moreover, IEF is uniquely suited to study protein-signalling events such as phosphorylations because of the ability to physically separate the phospho-forms. Ultimately, our findings move us one step closer to better recapitulating the *in vivo* environment and has applications to study stem cell and tissue engineering.

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Chapter 4.

Image Processing Algorithms for Streamlined Analysis of Non-Arrayed Objects

This work was performed in collaboration with Dr. Kevin A. Yamauchi and Julea Vlassakis.

4.1 Introduction

Separation science has existed since the mid-19th century, beginning with chromatographic separations that separated compounds based on their differential adsorption (i.e., interactions) with a substrate material. Today, separations remain an important method for selective measurement of proteins. In assays such as Western Blots or in isoelectric focusing, separations confer higher selectivity by physically separating multiple species. In immunoassays, in which antibody specificity to a target is exploited for target detection, separations are particularly important, as antibodies may not exist for protein isoforms or, as mentioned in Chapter 3, may bind to off-target cellular constituents.

In order to analyze separations, analysis tools are necessary that distinguish the compound of interest (e.g., immunoprobed protein peaks) from background signal. Advancements in microfluidic technologies have miniaturized and increased throughput, such that many separations can be performed in parallel^{1–4}. As such, analysis methods must also complement the increase in throughput. In addition, automation of analysis is ideal, as this increases robustness and improves user interface (UI).

In electrophoretic cytometry, tens to thousands of single cells are analyzed within a given microfluidic chip^{3,5}. Separations of subcellular constituents have been demonstrated⁶, and are advantageous because spatial separation of intracellular constituents eliminates the need for cell segmentation, which can be semi-subjective. Typically, the data generated from these assays are images of arrayed protein peaks (immunoprobed targets of interest), and the analysis--- which includes ROI generation and peak quantitation---of such arrays have been previously described⁷. For arrayed data, generation of ROIs is relatively simplistic, as only the dimensions and spacing of the array need to be input to quantify thousands of single cells. However, as described in Chapter 3, separation data can also arise in non-arrayed formats, which present challenges in peak identification and thus quantitation.

One major challenge with identifying protein peaks and generating ROIs around non-arrayed protein peaks lies in the initial image segmentation and generation of a

separation “lane” that encompasses the entire peak. As mentioned earlier, attention to image segmentation approaches is crucial to avoid semi-subjective quantitation (e.g., if a peak has been truncated from over-segmentation from noisy data⁸). Here, we describe a technique to analyze protein peaks resulting from IEF separations of cells patterned onto microcontact-printed ECM regions in non-arrayed formats. We utilize an initial segmentation step to identify protein peaks, generate ROIs that encompass the entire protein peak from the original (non-segmented) image, and include a UI-guided selection process to remove artefacts such as dust, overlapping peaks, etc. from the generated ROIs. The full annotated analysis script (MATLAB code) is included at the end of this chapter.

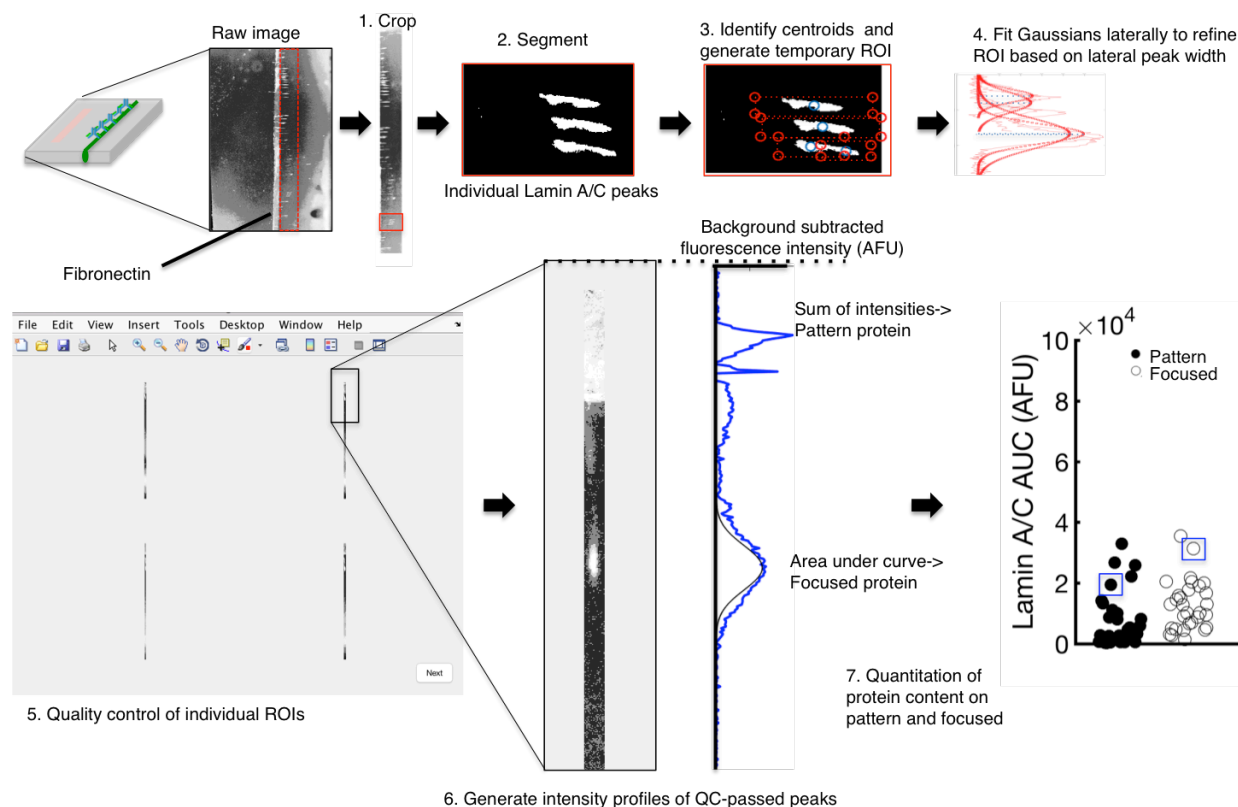


Figure 4-1. Workflow for image segmentation using a MATLAB GUI. The raw fluorescence image is first cropped based on user-based inputs on the bounds of the extracellular matrix protein pattern and end of separation lane. In the second step, the peaks are segmented via thresholding, converting to binary, and identifying centroids of each object (peak). Based on locations of the centroids, a temporary ROI is generated based on the values of the major and minor axis of the ellipse fitted to the identified peaks. Next, in step four, a Gaussian is fit *laterally* across each ROI from the cropped original (non-binary) image to identify the peak mean and width. From there, a new ROI is generated, which encompasses the entire peak ($\text{mean} \pm 4\sigma$). A quality control (QC) step is performed in Step 5 by the user to remove overlapping peaks or other artefacts caused by dust, debris, etc. Following QC, background-subtracted intensity profiles are extracted from each ROI. The background is subtracted on a per-pixel basis. For quantitation, the sum of the intensities of the background-subtracted profile (ID) are obtained for the pattern protein content, and the area-under-the-curve (AUC) of the Gaussian fit to the focused peak for the focused protein content.

4.2 Results and discussion

Design Approach

Building on work developed previously in our group⁹, we sought to update a series of scripts to automate image processing, extending the analysis schemes to non-arrayed data. The final script suite entails five distinct and sequential scripts designed to 1. Generate a user-defined region-of-interest (ROI), which designates the analysis areas (e.g., ECM-patterned region and separation axis), 2. Generate individual ROIs for each non-arrayed protein peak, 3. Display intensity profiles for each peak, 4. Fit Gaussian profiles to each intensity profile, 5. Perform quality control. Integration with the previously described analysis scripts⁹ to improve user friendliness was also an important design criterion. Thus, only steps 1-2 (image segmentation) were altered, whereas steps 3-5 were minimally modified.

Continuation of Data structure setup

Our previously reported analysis script uses a structure, which allows for assignment of multiple data types. The data structure was chosen because single-cell data come in multiple forms ranging from images to strings to vector/arrays. By including all these data types (fields) into a structure, one structure can store all the output data (Figure 2). For example, fluorescence intensity profiles for each cell is stored in a 3D matrix, but the final index of the lanes to analyze are stored a 2D matrix. Each field is called using a string, i.e., the name of the data set. Overall, use of a data structure minimizes output locations and is designed to improve user-interface experience.

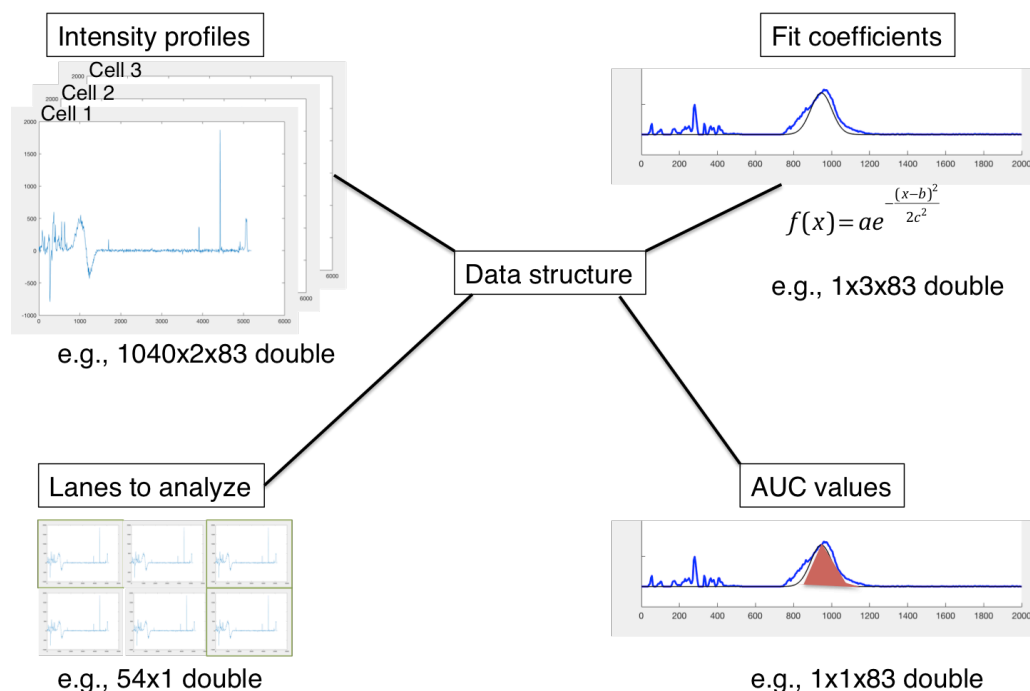


Figure 4-2. Schematic of data structure and fields (branched lines). The fields can be a variety of data types such as strings, vectors, arrays, etc. For example, in intensity profiles, a sample field could comprise a 1040x2x83 double, which is a 3D array comprising the fluorescence intensity measurement as a function of length (first two dimensions) for each cell (third dimension). Fit coefficients, which lanes to analyze, and the area-under-curve measurements for each of these cells are included in the

User-defined ROIs

The first step in the analysis is to define an ROI that contains all of the protein content, while eliminating regions that are irrelevant or contain artifacts such as dust, debris, etc. As we had observed protein retained on the patterned region from individual and clusters of cells (Figure 3, and Chapter 3), we designed the ROI generation to also characterize the amount of protein retained on the pattern, in addition to the focused protein peaks. To achieve this designation, the user inputs the region where the cells are patterned and the end of the separation axis. Since a linear geometry was used for ECM patterning, the user designates four click-points: the top (two) corners of the ECM pattern, followed by the end of the ECM pattern (i.e., the thickness of the line) and the end of the separation axis (Figure 1, Step 1). In MATLAB, the function *ginput* will convert the point that is clicked into x- and y-coordinates, which are then stored in the structure.

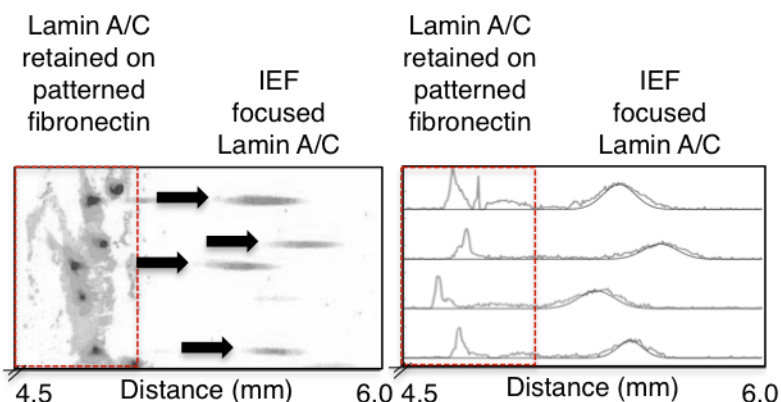


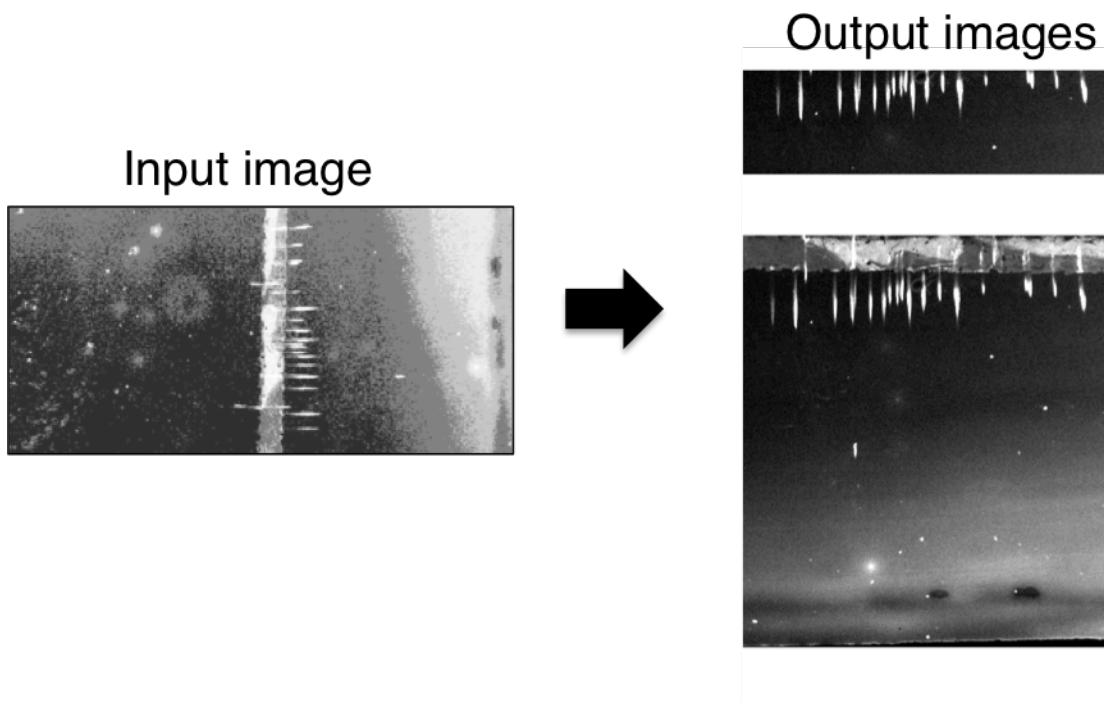
Figure 4-3. Lamin A/C is partially retained on the fibronectin pattern. To quantify the amount of lamin A/C retained on the pattern and focused into a peak, we generate intensity profiles that extend to the region where lamin A/C is patterned.

After the user has input the points of interest, the image is rotated, cropped, and two images are saved to the data structure. The first image is a cropped, rotated image that excludes the ECM-patterned region and only contains the focused protein peaks. The second image is identical to the first, except the ECM-patterned region is included (Figure 4). The first image (excluded ECM) is used in the subsequent step to define the boundaries of the individual lanes for each peak; the second generated image (included ECM) is used in the analysis, thereby allowing quantification of the protein retained on the pattern.

Image segmentation and generation of individual ROIs

Following image cropping, individual ROIs are generated around each focused protein peak that extend to the ECM pattern to quantify protein both within the focused peak and the protein retained on the pattern. Since the protein peaks are not arrayed, i.e., spaced equally apart, we chose to use a two-step approach to increase the fidelity of the ROI by ensuring that at least 95% of the lateral width of the peak is included in the generated ROI. To do so, we first generate a temporary ROI based on the peak center and relative size based on an ellipse fitted to the peak. This temporary ROI gives an estimate of the coordinates of the peak and estimate of the lateral (perpendicular to the separation axis) peak width. In the second step of ROI generation, a final (analytical)

Figure 4-4. Input and output images of the ROI-generation script. The input image is the raw image. The image is rotated 90 degrees and the user selects the top two corners of the ECM pattern, followed by the end of the ECM pattern and the end of the separation axis. After the user designates the corners of the ECM pattern, the coordinates of each designated click-point is saved, and the resultant output images are generated. The output image with no ECM pattern is used for peak identification to generate individual lanes, and the output image with ECM pattern is used for the intensity profile generation, so protein retained on the pattern could be quantified.



ROI is generated that includes the entire focused peak region, based on Gaussian fitting of the temporary ROI, explained below.

First, to identify the protein peaks, a threshold is applied to the image and the image is converted to binary (Figure 5 and Figure 1, step 2). The user can adjust the applied threshold, but for consistency, it is important to use the same threshold across images

run and imaged under the same conditions. After thresholding, the function *imfill* in MATLAB is used to fill in holes, which prevents over-segmentation. Next, the centroids of each peak are identified (Figure 5, blue circles). An ellipse was chosen as the structural element to fit to each peak, but various geometries are available for fitting. We selected an ellipse as this approximates the shape of separated protein peaks. A size threshold is imposed to prevent artefacts, such as punctate antibody trapped in-gel, from being identified as protein peaks.

Following ellipse fitting, a temporary ROI is generated by calculating a rectangle that encompasses each peak. To build a rectangle surrounding an elliptical feature, the major and minor axes of the ellipse must be known. These properties (centroid, major and minor axis), along with many others, are available in the built-in function in MATLAB called *regionprops*. From the major and minor axes, a temporary ROI is generated—i.e., a rectangle that encompasses the peak, based on the coordinates of the centroid, and with the length and width of the rectangle identified by the fitted ellipse major and minor axis lengths. The x and y coordinates of the rectangle are obtained by taking the centroid coordinates and adding and subtracting the major and minor axis lengths. Because subtraction of major or minor axis lengths may result in negative or zero coordinate values that will result in error (images cannot have negative spatial coordinates), any negative values must first be set equal to a positive integer (i.e., 1).

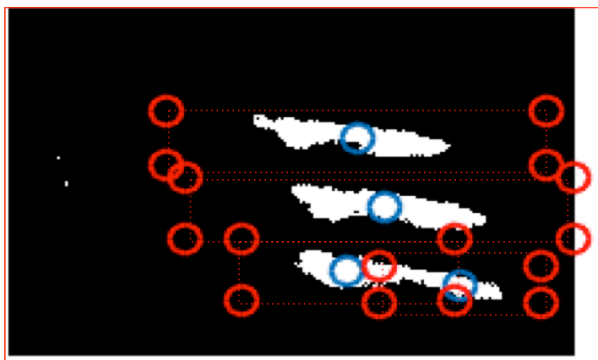


Figure 4-5. Generation of temporary ROIs for peak location estimation. A fluorescence intensity micrograph, after cropping, is thresholded and converted to binary. The centroids of each peak is identified and an elliptical structural element is fitted to each peak. The minor and major axis lengths are obtained and used to generate a temporary “box” ROI around each peak. From the temporary ROI, a permanent ROI is generated that contains > 99% of the entire peak via Gaussian fitting to the lateral (perpendicular to the separation axis).

While the temporary ROIs can also be used for peak quantitation, we wanted to generate a robust method to ensure that the ROIs encompassed > 95 % of the peak laterally (perpendicular to the separation axis) for all peaks. Since separations result in Gaussian concentration profiles, we used a Gaussian fit parameter to determine the bounds of the analytical ROI to ensure > 95% of the peak was included. To do so, we apply the temporary ROI to our cropped *non-threshold* (non-segmented) image (as thresholded images have sharp cutoffs and are not Gaussian). We then take an intensity profile (average intensity across the column) along the lateral axis of each

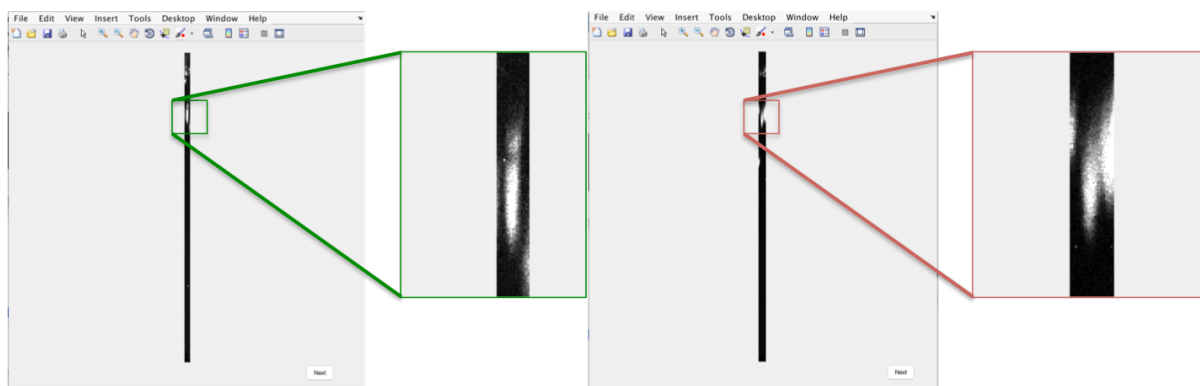
temporary ROI. The background is subtracted, which is crucial for Gaussian fitting, and then Gaussian peaks are fitted to the cropped image along the lateral axis (in MATLAB, using the function *gauss1*). Of the Gaussian fit parameters, the most important ones for our ROI generation are standard deviation (σ), which will give the lateral peak width, and the peak center, which, like the centroid, determines the coordinates of the peak for subsequent ROI generation.

After these parameters (Gaussian fit standard deviation and peak center) are obtained, a new ROI is finally generated on the non-threshold, ECM-*included* image. To do so, we begin by using the peak center of the Gaussian fit and generate an ROI with a (lateral) width of $8 \times \sigma$. Since the coordinates obtained from the Gaussian fit are isolated from the image, we use the coordinates of the centroids as a baseline of coordinates for the analytical ROIs. The choice of $8 \times \sigma$ was to ensure inclusion of the entire peak, and the choice to use the ECM-included image is so that the ROI “lane” can extend to the ECM pattern, thus analyzing the protein retained on the pattern.

Quality control of individual ROIs

As mentioned earlier, quality control should be performed on the image to ensure that artifacts such as dust/debris, overlapping peaks, etc. are not included and counted as “peaks” (although these will typically not pass the downstream Gaussian threshold). The final step of the ROI generation script thus involves a UI quality-control step (Figure 6). The UI populates a window into which the ROI “lanes” appear. The user can then select lanes that are “good” and discard those that contain other debris/non-protein peaks. All the ROIs are stored in the structure, and the “good” devices are indexed for further analysis.

Figure 4-6. UI quality control allows user intervention for “good” and “bad” peaks. The GUI populates each of the analytical ROIs, at which point the user can determine whether a peak is “good” (green) or not (red).



Continuation of image analysis

For the remainder of the MATLAB scripts, we refer the reader to our previously published work⁷, as the steps remain largely the same. Briefly, background-subtracted intensity profiles are generated across each ROI and saved to the data structure. Then Gaussian curves are fitted to the intensity profiles, this time along the *separation* axis. SNR and Gaussian fit thresholds are applied to select for “good” peaks, which are added to the data structure. In the final step of the analysis script suite, a quality control is performed on the intensity profiles to ensure any dust/debris/bright particles that passed the Gaussian fit threshold are removed.

4.3 Future directions and improvements

Although the analysis script suite is functional and automates many of the analysis steps, further improvements can achieve even higher automation and quality control steps. For example, during the first UI-quality control step (selection of peaks), one way to prevent repetition of protein peaks (i.e., if there are two protein isoforms that are identified and generate separate “lanes” despite arising from the same cell) is to include a nearest-neighbor algorithm. Peaks that fall within a designated range can automatically be concatenated/combined with the existing lane. This further prevents the same peak from being analyzed more than once (although various quality control steps already restrict this).

Moreover, the script is designed for analysis of protein peaks in one fluorescent channel. The ROI is easily applied to subsequent images of different channels; however, this step is performed manually. For increased automation, an input that takes two images of the same region in separate channels would further decrease analysis time and possible human error.

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Analysis script (MATLAB code)

```
function [struct] = ROI_ECM_final(filename,vertspacing,struct)
%This function rotates/aligns the raw image and segments the image into ROIs
% Outputs:
%Struct [structure]: A data structure containing objects (matrix
% with coordinates of rotated image)
% Inputs:
%filename [string]: A string containing the name of the fluorescence image
% to be processed
%vertspacing [num]: Well-to-well spacing (vertical)
%struct [structure]: input structure for just the Fibronectin pattern
%
%% versions
% 0.1-April 11, 2017
%updated on June 3, 2017 to include a ROI that encompasses the whole image
%updated on 6/4/17 to include an output that tells what the ECM length is;
%this value is to set the input for figuring out the residual protein

%% Check input arguments
switch nargin
    %If the user only provides the image
    case 1
        transform=0;
    case 2
        transform=1;

    tf = isstruct(struct);
    if tf==0
        error('Input argument "struct" is not a structure.');
```



```
    return

    end

%retrieve previously determined angle for transformation of image
angle=struct.angle;

%retrieve previously determined array boundaries
array_bounds=struct.array_bounds;
x_upperleft=array_bounds(1,1);
y_upperleft=array_bounds(1,2);
x_upperright=array_bounds(2,1);
y_upperright=array_bounds(2,2);
```

```

otherwise

    error('Invalid number of input arguments');

    return

end
%%

%Load the image file in Matlab
img=imread(filename);

if transform==0
    %Display more contrasted img in window
    contrasted_img=imadjust(img);
    imshow(contrasted_img);

    title('Take a look at the image and determine if the ECM pattern is oriented left or right of the protein
peaks. Then press any key');
    pause()

    % Construct a questdlg to ask the user how the image is currently oriented
    % for coarse rotation
    choice = questdlg('Is ECM pattern left of the bands or right of the bands?', ...
'Current array orientation', ...
'ECM is left of bands','ECM is right of bands','ECM is right of bands');

    % Handle response
    switch choice
        case 'ECM is left of bands';
            disp([choice 'Okay, the image will be rotated to the right!'])
            rotate = -90;
        case 'ECM is right of bands';
            disp([choice 'Okay, the image will be rotated to the left!'])
            rotate = 90;
        end
    struct.rotate=rotate;%save the rotation angle into the struct
    else
        rotate=struct.rotate;
    end
    imgrotated=imrotate(img,rotate);
    contrasted_img_r=imadjust(imgrotated);
    imshow(contrasted_img_r);

%Orient the image so that the ECM pattern is aligned
test=1;
while test==1
    %Prompt user to select the upper right edge of the ECM pattern.
    title('Please zoom in on the the middle of the upper left corner of pattern and press any key.');
```



```

zoom on; % use mouse button to zoom in or out
pause()
zoom off;

%preallocate array bounds matrix
array_bounds=zeros(4,2);

title('Please click on the middle of the upper left corner of the pattern.');
```

[x_click,y_click]=ginput(1);%xclick and yclick are the coordinates given from ginput

```

x_upperleft=x_click;
y_upperleft=y_click;
zoom out;

array_bounds(1,:)= [x_upperleft,y_upperleft];

%Prompt user to select the upper right well
title('Please zoom in on the middle of the upper right corner and press any key.')
```

zoom on; % use mouse button to zoom in or out

```

pause()
zoom off;
title('Please click on the middle of the upper right corner of the pattern.');
```

[x_click,y_click]=ginput(1);

```

x_upperright=x_click;
y_upperright=y_click;
zoom out;
array_bounds(2,:)= [x_upperright,y_upperright];

%Prompt user to select the lower left well
title('Please zoom in on the middle of the upper right corner and press any key.')
```

zoom on; % use mouse button to zoom in or out

```

pause()
zoom off;
title('Please click on the middle of the lower left corner of the pattern.');
```

[x_click,y_click]=ginput(1);

```

x_lowerleft=x_click;
y_lowerleft=y_click;
zoom out;

array_bounds(3,:)= [x_lowerleft,y_lowerleft];

%Prompt user to select the end of the separation lane; this is to
%constrain the subsequent peak identification step so that random
%cells are not incorporated into the analysis
title('Please zoom in on the end of the separation lane and press any key.')
```

zoom on; % use mouse button to zoom in or out

```

pause()
zoom off;
title('Please click on the middle of the lower right corner of the pattern.');
```

[x_click,y_click]=ginput(1);

```

x_bottomleft=x_click;
y_bottomleft=y_click;
zoom out;
```

```

array_bounds(4,:)= [x_bottomleft,y_bottomleft];

% Construct a questdlg to ask the user if they are happy with their
% selection
choice = questdlg('Are you happy with your selections?', ...
'Well selections for array boundaries', ...
'Yes','No','Yes');

% Handle response
switch choice
case 'Yes';
    disp([choice 'Great, let"s keep going then!'])
    test = 0;
case 'No';
    disp([choice 'That"s okay, try again!'])
    test = 1;
end

if (x_upperright<x_upperleft)
    test=1;
    title('Oh no! We detected you selected the wells in the wrong order. Please try again. Press any key
to continue')
    pause()
else
    test=0;
end
end

%store the array bounds in the struct
struct.array_bounds=array_bounds;
%store the coordinates of the direction vector that extends from the upper left well to the right most
point of the array
dir_vector1=[x_upperright,y_upperleft]-[x_upperleft,y_upperleft];
%store the coordinates of the direction vector that extends from the upper left well to the upper right well
dir_vector2=[x_upperright,y_upperright]-[x_upperleft,y_upperleft];
%Find angle between the two direction vectors [angle in degrees]
cosangle=dot(dir_vector1,dir_vector2)/(norm(dir_vector1)*norm(dir_vector2));
angle=acosd(cosangle);
if (y_upperright<y_upperleft)
    angle=-angle;
end
struct.angle=angle;
transform=1;

%Rotate the image so the pattern is aligned
b=imrotate(imgrotated,angle,'nearest','crop');
b_contrasted=imadjust(b);
imshow(b_contrasted);
hold on
sz=size(b)/2;

```

```

rotation_matrix=[cosd(-angle),-sind(-angle);sind(-angle),cosd(-angle)];
new_upper_left=rotation_matrix*[(x_upperleft-sz(2));(y_upperleft-sz(1))];
new_upper_right=rotation_matrix*[(x_upperright-sz(2));(y_upperright-sz(1))];

x_new_upper_left=new_upper_left(1)+sz(2);
y_new_upper_left=new_upper_left(2)+sz(1);
x_new_upper_right=new_upper_right(1)+sz(2);
y_new_upper_right=new_upper_right(2)+sz(1);

%also get the coordinates of the image without the ECM pattern (only the y
%coordinates change); round it to the nearest integer
y_new_upper_left_noECM=round(y_new_upper_left+(y_lowerleft-y_upperleft));

%Crop the image (anything above the ECM pattern)
%round the bounds first to obtain an integer
y_up_left=round(y_new_upper_left);
y_bottom_left=y_bottomleft;
y_end_image=round(size(b));
x_up_left=round(x_new_upper_left);
x_up_right=round(x_new_upper_right);
ROI_cropped=b(y_up_left:y_end_image,x_up_left:x_up_right);

%obtain the ROI of the same cropped image, with the ECM pattern removed,
%and with the input end of the separation lane
ROI_cropped_noECM=b(y_new_upper_left_noECM:y_bottom_left,x_up_left:x_up_right);

%create an ROI that encompasses the entire separation lane (above and below
%the ECM pattern, for pls that are above the pattern)
ROI_entirelane=b(1:y_end_image,x_up_left:x_up_right);

%show the contrasted image
figure
ROI_contrasted=imadjust(ROI_cropped);
imshow(ROI_contrasted)
%also create and save the ROI that excludes the ECM strip

figure
ROI_contrasted_noECM=imadjust(ROI_cropped_noECM);
imshow(ROI_contrasted_noECM);

struct.rois=ROI_cropped;
struct.rois_noECM=ROI_cropped_noECM;
struct.rois_whole=ROI_entirelane;
struct.ECMlength=round(y_lowerleft-y_upperleft);

function [struct] = findPeaks(struct)
%This function takes a rotated/cropped image and returns ROIs around each

```

```

%protein peak that extend to the fibronectin pattern
% Outputs:
%Struct [structure]: A data structure containing objects (3D matrix
% with each ROI contained in a different z, and coordinates of the ROIs)
% Inputs:
%
%struct [structure]: input structure from ROI_ECM script

%updated 5/27/17 to include QC steps
%updated 6/3/17 to include coordinates (column start and end) of each ROI

%open the image

img=struct.rois_noECM;
I=struct.rois;
contrasted_I=imadjust(img);
imshow(contrasted_I)
%threshold
level=0.7
Ithresh=im2bw(contrasted_I,level);
imshow(Ithresh)
Ifilled=imfill(Ithresh,'holes');

figure
imshow(Ifilled)
se=strel('disk',1);
Ifit=imopen(Ifilled,se);

%filter by size
Ifit=bwareafilt(Ifit, [20 100000]);

figure
imshow(Ifit);

%% Extract data

Iregion=regionprops(Ifit, 'Centroid');
%concatenate centroid values into a single matrix
centroids=cat(1,Iregion.Centroid);
Majoraxis=regionprops(Ifit, 'MajorAxisLength');
Majoraxis=cat(1,Majoraxis.MajorAxisLength);
Minoraxis=regionprops(Ifit, 'MinorAxisLength');
Minoraxis=cat(1,Minoraxis.MinorAxisLength);

%Obtain the coordinates for a rectangle
%that houses the centroid and has dimensions of 2*minor axis x 2*major axis
xbounds=[centroids(:,1)-Minoraxis, centroids(:,1)+Minoraxis];
xbounds=round(xbounds); %first column is lower bound and second is upper
ybounds=[centroids(:,2)-Majoraxis, centroids(:,2)+Majoraxis];
ybounds=round(ybounds); %first column is lower bound and second is upper
s=size(xbounds,1);

```

```

%if the bounds are negative set them equal to 1
xbounds(xbounds<1)=1;
ybounds(ybounds<1)=1;

xlowerbound=xbounds(:,1);
xupperbound=xbounds(:,2);
ylowerbound=ybounds(:,1);
yupperbound=ybounds(:,2);

%if the bounds are greater than the size of the image, set them equal to
%the outermost bound of the image
xupperbound(xupperbound>size(img,2))=size(img,2);
yupperbound(yupperbound>size(img,1))=size(img,1);

%%define rectangular ROIs around the centroids from the original image
%first, initialize the cell array that will ultimately contain the ROIs
ROI_new=cell([s, 1]); %this creates s number of matrices
figure
for i=1:s
    ROI=img(ylowerbound(i):yupperbound(i), xlowerbound(i):xupperbound(i));
    X=1:size(ROI,2); %specifies a vector of the length (in pixels) of the lateral (perpendicular to
    % separation axis). These will be the xvals that are plotted for the intensity profile

    %take intensity plot along the lateral axis with background subtraction
    %NOTE: background subtraction is necessary to allow for proper Gaussian
    %fitting
    Int_prof=mean(ROI,1); % intensity across the columns
    backgroundwidth=2;
    top_backgroundregion=ROI((1:backgroundwidth),:);
    bottom_backgroundregion=ROI((((end+1)-backgroundwidth):end),:);
    top_background_int=(sum(top_backgroundregion,1))/backgroundwidth;
    bottom_background_int=sum(bottom_backgroundregion,1)/backgroundwidth;
    mean_background=(top_background_int+bottom_background_int)/2;
    bsub_int=Int_prof-mean_background;
    % lane_profile=[X',bsub_int];
    % int_profiles(:,i)=lane_profile;

    plot(X',bsub_int,'r');
    hold on

    % fit gaussian to plots
    ft=fitype('gauss1');
    opts=fityptions('Method','NonlinearLeastSquares','Lower',[0 0 0]);
    [f,g]=fit(X.', bsub_int.',ft,opts);%perform gaussian fit
    FitCoeffs=coeffvalues(f);
    A1(i)=FitCoeffs(1);
    B1(i)=FitCoeffs(2);
    C1(i)=FitCoeffs(3);
    sigma(i)=C1(i)./sqrt(2);

```

```

    %plot the Gaussian

    %plots Gaussian as a function of sigma-- this is to ensure good fit.
    f1 = @(x) A1(i).*exp(-((x-B1(i)).^2/(2*(sigma(i)).^2))); %use for troubleshooting
    %use for troubleshooting
    plot(X.',f1(X.'),'rs'); %plot all the gaussian fits

    %NOTE: THE GAUSSIAN FITS ARE TO THE NEW INT PLOT COORDINATES--NEED TO
    %DRAW BACK TO ORIGINAL ROI

    %From these b values, draw a new ROI across the image that contains the
    %coordinates of B +/- 3*sigma in the y direction (perpendicular to the
    %separation axis) and that extend along the length of the original image
    B=B1';
    sigma_col=sigma';
    col_start(i)=round(xlowerbound(i)+B(i)-4*sigma_col(i));
    %if negative, then set to 1
    col_start(col_start<1)=1;
    col_end(i)=round(xlowerbound(i)+B(i)+4*sigma_col(i));
    %if exceeds dimension of image, then set to edge of the image
    ysize=size(struct.rois,2);
    col_end(col_end>ysize)=ysize;
    row_start=1;
    row_end=size(struct.rois,1);

    %create a cell array to store each of the ROIs; take the ROIs around
    %the original image, not the one with ECM cropped

    mat=l(row_start:row_end,col_start(i):col_end(i));
    size(mat);

    %populate the cell array with each of the ROIS
    ROI_new{i}=mat;

end

%%INCLUDE A QC STEP TO REMOVE PEAKS THAT OVERLAP

    %set number of rows/columns of subplots to display in each figure window
    n=1;
    num_subplots=n*n;
    plots_display=size(ROI_new,1);
    good_lanes=ones(size(ROI_new,1)); %initializing the vector that contains all the lanes
    number_subplots=ceil(plots_display/(n*n));%number of subplots, given 5x5 ROIs displayed per subplot
    z_dim=size(ROI_new,1);
    dev_to_analyze=zeros(z_dim,1);%number of devices to analyze

    good_subplots = ones(plots_display,1);

```

```

disp(number_subplots);

%show images of lanes

% for loop to generate subplots for user inspection of the intensity profiles
for i=1:number_subplots %ex: if you have 120 lanes, then number_subplots is 120/25= 5
    disp(i);
    figure
    if i==1
        devices_subplot=(1:(n*n));
    elseif i*n*n<=plots_display
        devices_subplot=((i*n*n)-(n*n)+1):((i*n*n));
    else
        devices_subplot=((i*n*n)-(n*n)):(plots_display);
    end
    next=0;
    for j=1:length(devices_subplot) %for each subplot
        dev_number=devices_subplot(j);
        device=ROI_new(dev_number);
        subplot(n, n, j);
        %plot(xval,yval,'LineWidth',2,'Tag', sprintf('%d', dev_number), 'buttondownfcn',...
        % @clickTest);
        h=imshow(imadjust(device));
        hold on
        scatter(centroids(dev_number,1),centroids(dev_number,2))
        % h.Color='black';
        set(h,'Tag',sprintf('%d',dev_number),'buttondownfcn',...
            @clickTest);

    end
    next=0;
    btn = uicontrol('Style', 'pushbutton', 'String', 'Next',...
        'Position', [500 15 50 30],...
        'Callback',@continueButton2);
    while next==0
        pause(0.01);
    end

    close(gcf)
end

data_struct.dev_to_analyze = good_subplots;
good_subplot_ind=find(good_subplots==1)
%data_struct.ROI_dev_to_analyze=good_r2(good_subplot_ind);
struct.fittedROIs=ROI_new(good_subplot_ind);
struct.col_start=col_start(good_subplot_ind);
struct.col_end=col_end(good_subplot_ind);

function [next]=continueButton2(qstring,title,str1,str2,default)
%UNTITLED5 Summary of this function goes here
qstring='Are you done selecting devices to throw out?';
title='Device Quality Control';

```

```

str1='Yes';
str2='No';
default='Yes';
choice = questdlg(qstring,title,str1,str2,default);
    % Handle response
    switch choice
        case 'Yes';
            disp([choice 'Great, let"s keep going then!'])
            next=1;
        case 'No';
            disp([choice 'Okay, please finish selecting devices to throw out'])
            next=0;
    end

assignin('caller', 'next', next); %in the caller -- i.e. in the Main workspace, assign next (i.e. 0 or 1) to the
variable "next"

end

function clickTest(edge_handle, event)
good_subplots = evalin('caller', 'good_subplots');
current_tag = get(edge_handle, 'Tag');

subplot_num = str2num(current_tag);
%subplot_state = str2num(split_tag{1, 2});
subplot_state = good_subplots(subplot_num);

disp(sprintf('%d, %d', subplot_num, subplot_state));

% Toggle the selection based on the last character in the tag
% (0 = off, 1 = on)

if (subplot_state)
    set(edge_handle, 'CData', [1, 0, 0]); %changes the image color data
    good_subplots(subplot_num) = 0;

else

    set(edge_handle, 'CData', [0, 0, 1]);

    good_subplots(subplot_num) = 1;

end
assignin('caller', 'good_subplots', good_subplots);

end
    %select lanes
    %confirm selections
    %save index of good lanes

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Chapter 5.

Solubilization of Alternatively-Spliced Isoforms for Single-Cell Isoelectric Focusing

This work was performed in collaboration with Dr. Olga Botvinnik, Dr. Yan Song, and Prof. Gene Yeo.

5.1 Introduction

Alternative splicing (AS) is a process by which multiple mRNA variants are produced from a single gene,^{1,2} and this process underpins isoform diversity, cellular identity, and homeostasis within eukaryotic cells. During AS, particular exons of a gene are included or excluded within a final, translated mRNA. Multiple modes of AS are common³ (Figure 1), and it is estimated that AS occurs in over 95% of mammalian genes.^{4,5} Moreover, an estimated 15-50% of human disease mutations affect AS splice selection.⁶

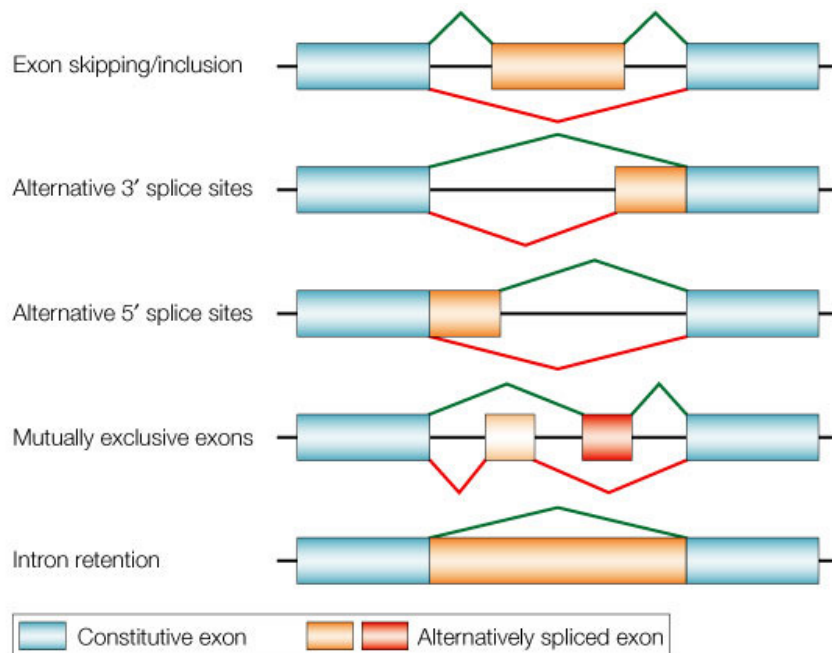


Figure 5-1. Schematic of five common modes of alternative splicing. Modified from Ref. 3. The green and red lines indicate one AS path. For intron retention, the AS pathway corresponds to no splicing.

In induced pluripotent stem cells (iPSCs), AS is a crucial requirement for stem cell fate and differentiation into a specific lineage. Recent advances in RNA-Seq have enabled detection of AS in single cells, thus enabling discovery of novel splice junctions and elucidating the complexity of AS in single cells.⁷⁻⁹ Importantly, differences in AS events

occur amongst seemingly homogeneous populations; for example, up to 20% of iPSCs undergoing neuronal differentiation exhibit bimodal AS,⁹ thus highlighting the importance single-cell analyses.

Yet, little is known how these AS events translate on the protein level. Multiple studies have indicated that mRNA levels do not correlate with protein levels.^{10–13} Unsurprisingly, proteins have longer half-times, partially explaining the lack of correlation between mRNA and protein.¹⁴ Since proteins are the drivers of cell function, the detection of these AS events on the protein level is necessary in order to interrogate key biologic and pathologic processes.

Western blot and ICC are the workhorse immunoassays for protein detection. However, ICC suffers from low selectivity resulting from off-target binding, and Western blot, while offering higher selectivity, cannot resolve protein isoforms with low molecular weight differences. In contrast, in isoelectric focusing (IEF), higher selectivity is conferred by separation of proteins by isoelectric point. Nonetheless, tradeoffs in IEF exist, particularly in protein solubilization, arising from the required utilization of non-ionic detergents.¹⁵

Here, we report on a scIEF device for separation of alternatively spliced isoforms. Using U251-GFP cells as a model cell line, we demonstrate a wide-range pH gradient with high separation performance for resolution of protein isoforms. Next, we discuss solubilization improvements—first by varying lysis and electrofocusing (EF) durations, and then by modifying chemical constituents in the cell lysis buffer. Overall, we believe improving separation performance via sample preparation are important for high performance single-cell IEF.

5.2 Materials and methods

Device fabrication

Microwell substrate gel: 6%T, 3.3%C polyacrylamide gels containing 5 mM N-[3-[(3-benzoylphenyl) formamido]propyl] methacrylamide (BPMAC, Pharm-Agra Laboratories) were fabricated by pipetting the precursor solution between an acrylate-silanized microscope slide and casting onto a SU-8 wafer stippled with microposts of 32 μm diameter and 40 μm height, as previously described.^{16,17} 0.08% (w/v) APS (Sigma-Aldrich) and 0.08% (v/v) TEMED (Sigma-Aldrich) were used as the initiator and catalyst.

Lid gel: The lid gel is chemically-imprinted, as previously described.¹⁷ Briefly, we fabricated 500- μm thick polyacrylamide gels with three adjacent compartments. The outermost regions define the pH boundary conditions (pH 4 and pH 10) for the IEF assay. The center focusing gel consisted of a mixture of carrier ampholytes (ZOOM®, 1% (v/v), Thermo Fischer Scientific, pH 4-7 and 6-9). The lid gel in denaturing conditions also contains 81 mM CHAPS (Amresco), 2.5% (v/v) Triton X-100 (Sigma-

Aldrich), 7 M urea (Sigma-Aldrich) and 2 M thiourea (Sigma-Aldrich). In native conditions, only the CHAPS and Triton are added as detergents.

PI Marker for Determining Separation Performance

Fluorescent pI markers of known isoelectric point were obtained from Sigma. The markers were incorporated into the microwell substrate gel precursor solution at a 1:100 ratio. For determining separation performance, the lid gel was applied for ~30 s, then a 600 V electric potential was applied. The gel was imaged using a UV-longpass filter on a 4X objective, as described below and using the scan slide feature of Metamorph imaging software. Images were taken every 5 minutes. Gaussian peak-fitting was performed using a custom, in-house MATLAB script. The linearity of the pH gradient was obtained by plotting the peak mean (distance along the gel) as a function of the known pI and using a linear regression to determine the slope and R-squared value.

Single-cell IEF (scIEF)

For scIEF, a suspension of 1E6 cells/mL was pipetted onto the microwell-stippled gel and gravity-sedimented for 10 minutes. Excess cells were removed with PBS and rinsed in carrier ampholytes diluted 1:100 in MilliQ water prior to lysis. IEF was then performed by applying a potential of 600 V for 6 or 8 min. Following IEF, the proteins were immobilized into the substrate gel upon UV light exposure (Hamamatsu LC5) for 45 s, as previously described.^{18,19} The slide was then washed with 1x TBS + Tween 20 (TBST, Santa Cruz Biotechnology) for 30 min prior to antibody probing.

Antibodies

Primary and secondary antibodies were diluted in 2% (w/v) BSA in 1x TBST. Rabbit PKM 1/2 (Bethyl laboratories, A303-660A), Rabbit ZCCHC10 (abcam, ab104531), Mouse TERF-1, Rabbit DYNC 112 (abcam, ab167399), Goat DNMT1 (abcam, ab109981), goat anti-GFP (abcam, ab6673), and rabbit anti- β -tubulin (abcam, ab6046) antibodies were diluted at 1:10 or 1:50 (tGFP). Secondary antibodies, AlexaFluor 488-, 594-, and 647-conjugated donkey anti-mouse, anti-goat, and anti-rabbit antibodies (Life Technologies), were diluted 1:50. Immunoprobings were conducted as previously described.¹⁶

Image acquisition and analysis

Brightfield images were obtained on an Olympus IX-71 inverted microscope with an Olympus UPlanFi 4X (Numerical Aperture (NA) 0.13) or 10X (NA 0.3) objectives and an EMCCD Camera iXon2 (Andor). Fluorescence images for pI marker experiments were obtained with the 4X objective and an X-Cite Exacte mercury arc lamp illumination source. Immunoprobed gels were imaged on a microarray scanner (Genepix, Molecular Devices) under the 488, 535, and 635 excitation wavelength channels. Images were then analyzed using an in-house MATLAB (R2016 and R2017a) script.

5.3 Results and discussion

Alternative splicing regulates iPSC differentiation into motor neurons

Using a computational approach, Song et al demonstrated that iPSC differentiation into motor neurons was driven by several alternatively-spliced events of varying genes⁹. While demonstrated on the mRNA level, protein levels of these AS events have not been documented in single cells. To corroborate whether these key alternatively spliced transcripts also resulted in translation of the corresponding protein isoforms, we designed an immunoassay to separate and detect alternatively spliced protein isoforms via isoelectric focusing (IEF). The list of these targets, with predicted isoelectric points (pI), are presented in Table 1.

Table 5-1. List of proteins that influence neuronal differentiation of iPSCs and the predicted pIs of the AS isoforms.

pH 4-7	pH 7-8	pH 8-9	pH 9-10	pH >10
DYNC1/2 (5.16 and 5.10)	PKM (7.61 and 7.96)	RBPJ (8.12 and 8.43)	ZNF207 (9.21 and 9.20)	RPS24 (10.79 and 10.89)
SMARCE1 (4.76 and 4.84)		VDAC3 (8.84 and 8.84)	EWSR1 (9.37 and 9.33)	
SNAP25 (4.74 and 4.66)			BOLA3 (9.94 and 9.66)	
			MRPL33 (9.10 and 10.81)	
			THYN1 (9.38 and 9.30)	

Design of an expanded, high-resolution pH gradient for separation of protein isoforms

Expanding on our previously reported single-cell isoelectric focusing (scIEF) technology,^{17,20} we utilized a high-resolution mixture of carrier ampholytes to maximize separation performance while maintaining a broad pH (4-9) range to assay multiple proteins of interest. The workflow of the assay is designed for single-cell targeted protein analysis. Briefly, individual cells are settled by gravity sedimentation into microwells, followed by lysis, electrofocusing, and covalent immobilization of proteins into the gel scaffold. Subsequent diffusion of antibodies into the gel scaffold is used for fluorescent readout of protein content. Microfluidic integration is key for rapid assay times and minimization of diffusion-driven losses of proteins within cell lysates.

In our modified design, we utilized a mixture of commercially available carrier ampholytes. Since utilizing different carrier ampholytes will affect separation performance²¹, we characterized the linearity of the pH gradient and the separation resolution (ΔpI , the minimum resolvable pI difference). We incorporated fluorescent markers of known pI (4.5, 6.6 and 8.6) and imaged the gradient formation within a 9 mm gel. The gradient was linear, with a slope of 0.733 pH units/mm (Figure 2A).

Next, to characterize the separation performance of the assay, we used the Rilbe criterion²¹ for determining ΔpI , the minimum resolvable pI difference using the equation $\Delta pI = 4\sigma$ (dpH/dx), where 4σ is the peak width of the separated species of interest, and dpH/dx is the slope of the pH gradient. For our species of interest, we used tGFP from U251 cells to characterize our peak width (Figure 2B). Single cells were seated into individual microwells, lysed, and focused for 6 minutes, prior to photocapture and immunoprobing, as previously described¹⁷. We measured the peak width of GFP as $97.3 \pm 43.4 \mu m$ (Figure 2C). Our ΔpI thus is 0.07, meaning that within our system, we resolve proteins with a pI difference as small as 0.07 pH units. Since GFP has a molecular weight of ~ 27 kDa and thus has a larger diffusivity than the median-sized protein, we expect our calculation of ΔpI to be a conservative estimate of our separation performance.

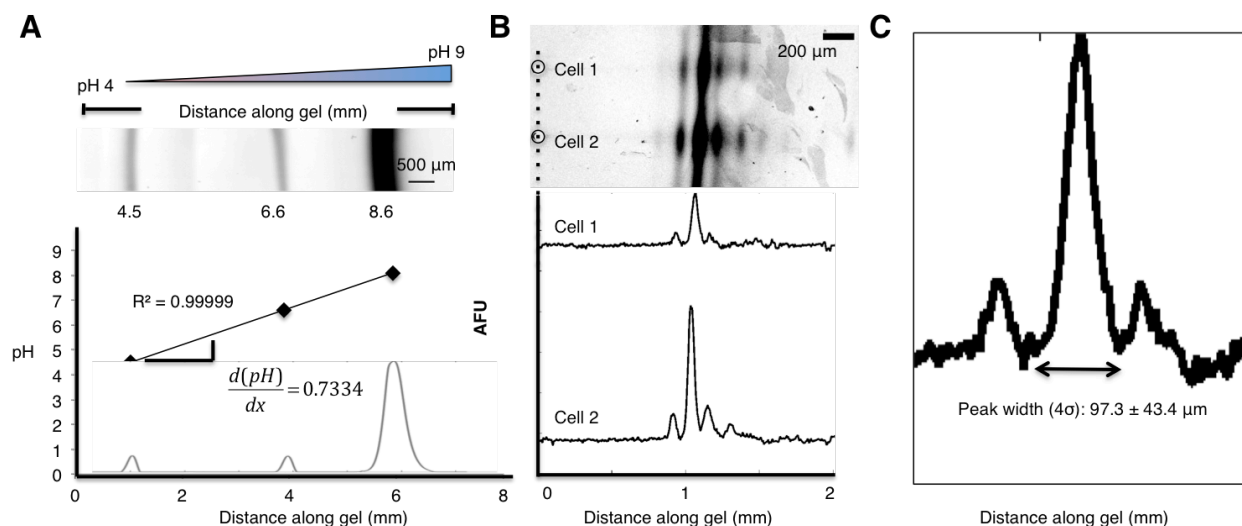


Figure 5-2. Separation performance of scIEF. A) inverted fluorescence micrograph of pI markers incorporated into the gel and focused for 6 minutes. The gradient was linear with a slope of $0.7334 \text{ pH units mm}^{-1}$. B) scIEF of U251-tGFP and immunoprobing of tGFP demonstrates separation of tGFP isoforms. C) The peak width of the widest GFP peak was ~ 97.3 microns, which yields a ΔpI (minimum resolvable pI difference) of 0.07 pH units.

Sensitivity of scIEF assay for targets of interest

Given our designated pH 4-9 gradient and separation resolution of 0.07 pH units, we next sought to ensure we had the necessary sensitivity to detect the targets of interest. Again, we used U251-GFP cells as a model cell line and probed for pyruvate kinase (PKM) 1/2, as this target has a relatively high ΔpI between the isoforms. In RNA-Seq of single cells, PKM is present almost exclusively in the excluded form in iPSCs, but is alternatively spliced during differentiation into NPCs and is more skewed toward inclusion in fully-differentiated motor neurons (Figure 3A). We hypothesized that these changes would also manifest on the protein level, and we would visualize single cells with only one isoform in iPSCs, but a distribution of NPCs would have only one or both isoforms (Figure 3B).

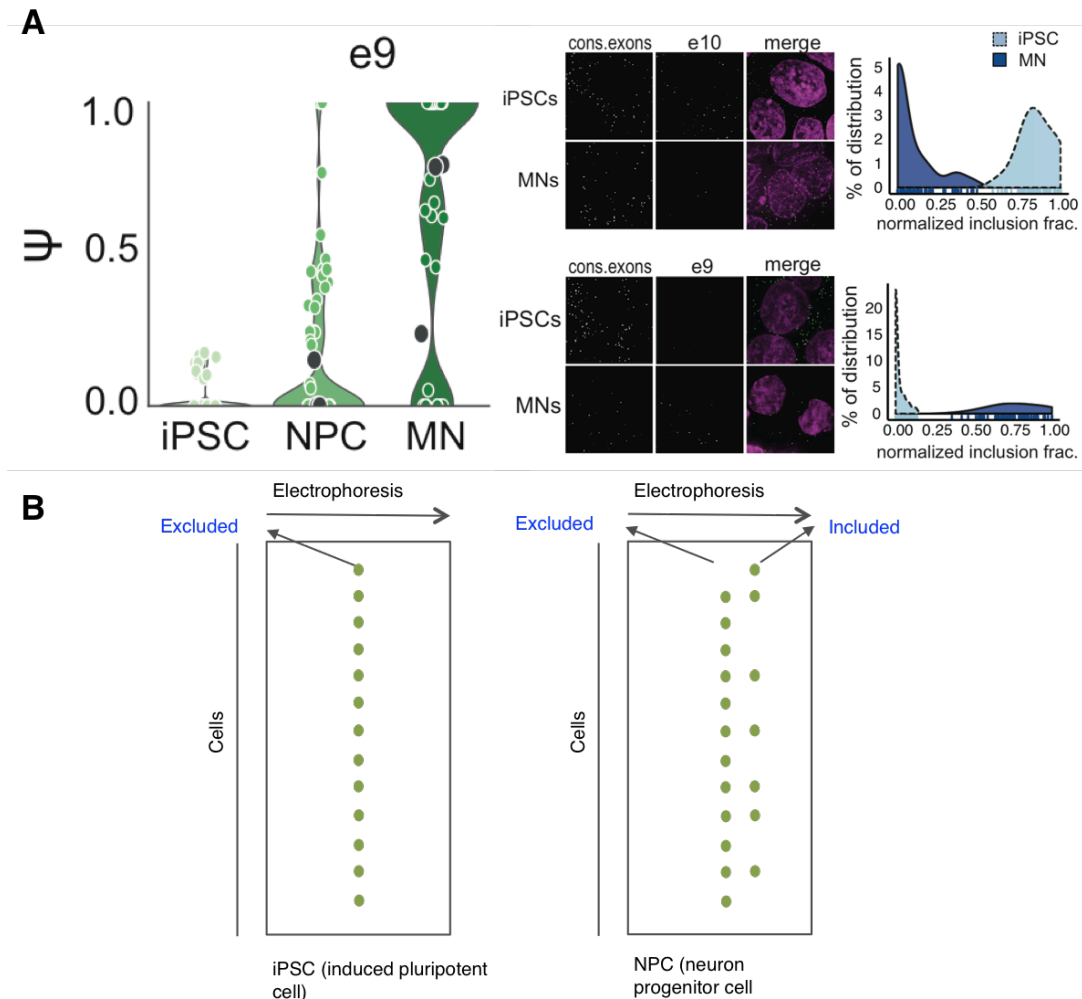


Figure 5-3. iPSCs and neural differentiated cells (NPCs and MNs) increasingly express the AS isoform of excluded exon 9 in RNA-Seq data A) Violin plots of distribution of cells in single-cell RNA Seq that express the exon 9 isoform, and ICC of iPSCs and MNs demonstrate higher expression of exon 9 (included isoform) in MNs. B) Schematic of expected protein isoforms during scIEF. NPCs would possess populations of cells that possess both isoforms. Adapted from EJS, OB, YS, GY, and AEH, *unpublished*.

To test this hypothesis, we conducted a semi-bulk microfluidic IEF using lysates from U251-GFP cells, which are easily culturable. We detected one isoform of PKM1/2, which is likely the PKM 2 isoform present in glioblastoma cells²² (Figure 4). However, when assaying single cells in both native and denaturing IEF conditions, PKM demonstrated low solubilization, remaining largely in the microwells, with considerable injection dispersion (Figure 5).

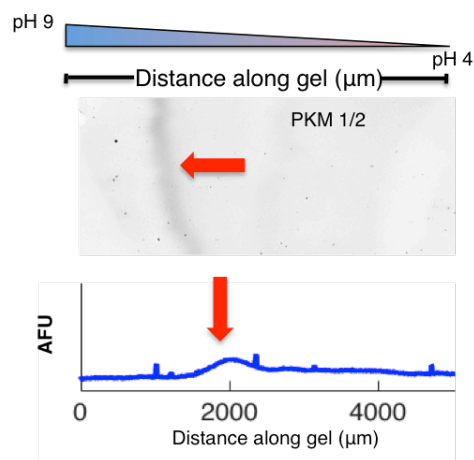


Figure 5-4. Semi-bulk microfluidic IEF of U251-GFP lysates. Cells were lysed in the same concentration of detergents and concentrations as would be applied in a lid gel, i.e., The lid gel also contains 81 mM CHAPS (Amresco), 2.5% (v/v) Triton X-100 (Sigma-Aldrich), 7 M urea (Sigma-Aldrich) and 2 M thiourea (Sigma-Aldrich). One PKM isoform was detectable from U251-GFP cells.

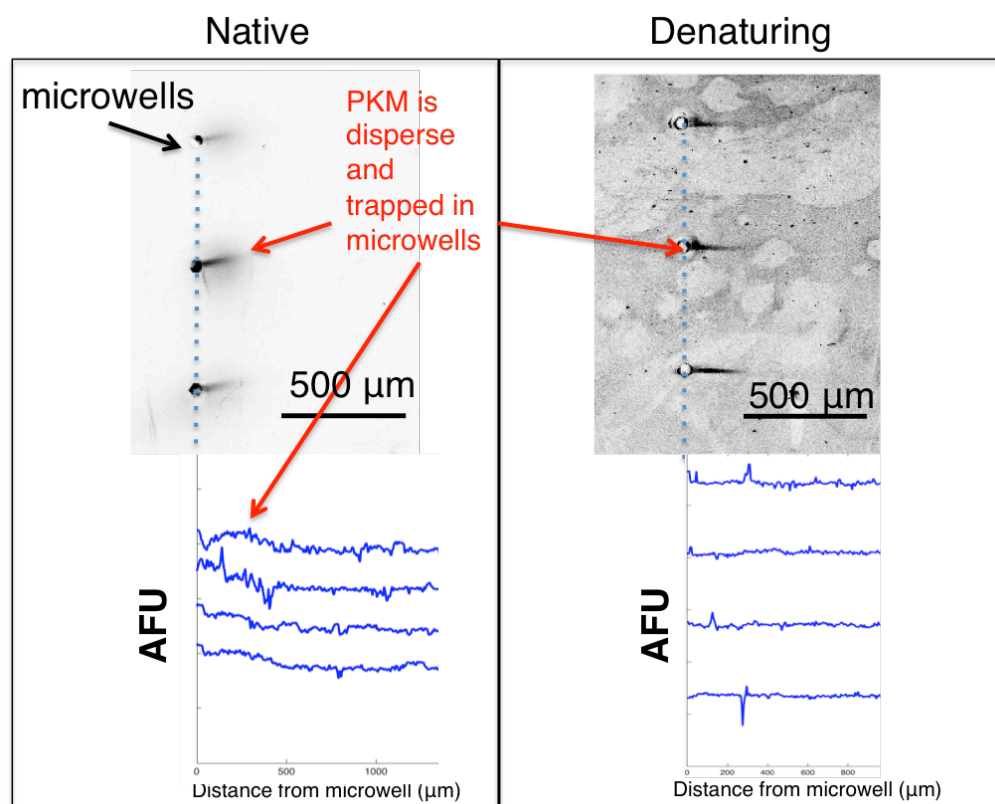


Figure 5-5. Inverted fluorescence micrographs of PKM in native and denaturing conditions under previously reported scIEF conditions. PKM is disperse and trapped in microwells, which leads to poor Gaussian fitting and inability for quantitation of protein content from each cell.

In addition to PKM, we assessed isoform presence in four other targets using our U251-GFP cell line: DNMT1, DYNC 1I2, TERF1, and ZCCHC10. We selected these targets based on their expected $\Delta pI > 0.07$ for each isoform, expected high abundance,^{23,24} and availability of commercially-available, specific antibodies (i.e., did not possess nonspecific or non-identifiable bands in the manufacturer's information and Western Blot validation). In native IEF, we observed one isoform of GFP (positive control), but undetectable signal of DYNC 1I2, DNMT1, and ZCCHC10. (Figure 6, data not shown for ZCCHC10). Notably, we detected two peaks of TERF1, but the SNR < 3 and only occurred in 50% of samples (n=4). We also conducted scIEF in denaturing conditions and probed for the same targets of interest (Figure 7). Again, we did not observe any detectable signal of DYNC 1I2, DNMT 1, ZCCHC10, or TERF1. To investigate whether the lack of signal was due to the limitations of the analytical sensitivity of our assay, we assayed these targets in a bulk Western Blot (Figure 8). TERF1 and ZCCHC10 were both abundant, but DNMT1 and DYNC 1I2 were undetectable. Altogether, these results suggest that the limit of detection of scIEF may be higher than the suggested 42,000 molecules.¹⁷

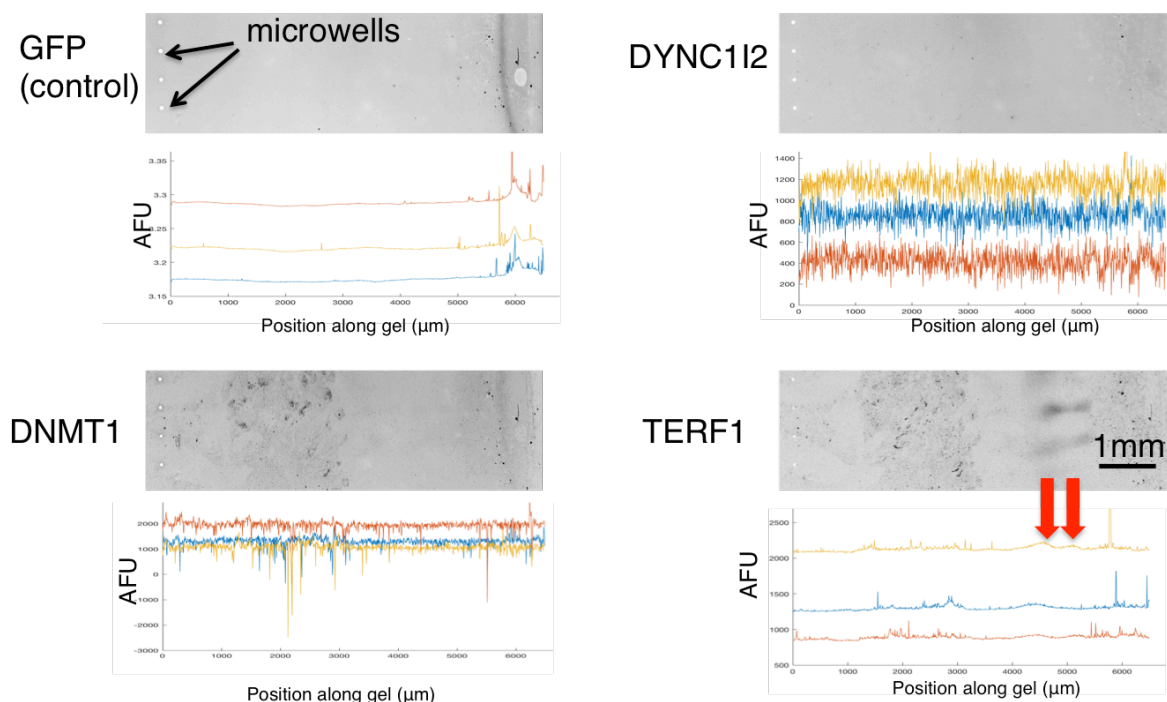


Figure 5-6. Native scIEF of U251- GFP cells demonstrates undetectable levels of DYNC 1I2, DNMT1 and TERF1. TERF1 possessed two visual isoforms but with unquantifiable signal (SNR < 3).

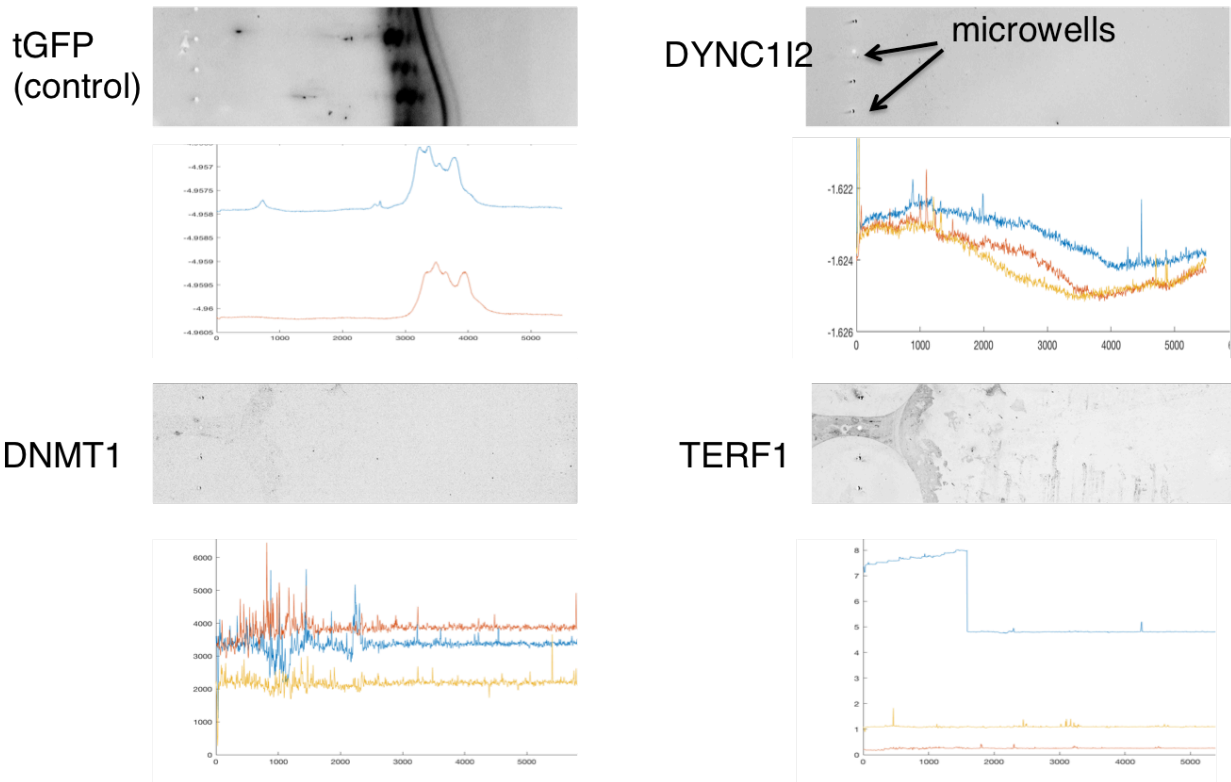


Figure 5-7. Denaturing scIEF, DYNC 112, DNMT1, TERF1, and ZCCHC10 (not shown) are not detectable. Denaturation leads to the characteristic isoforms of tGFP, but no signal for all other targets of interest.

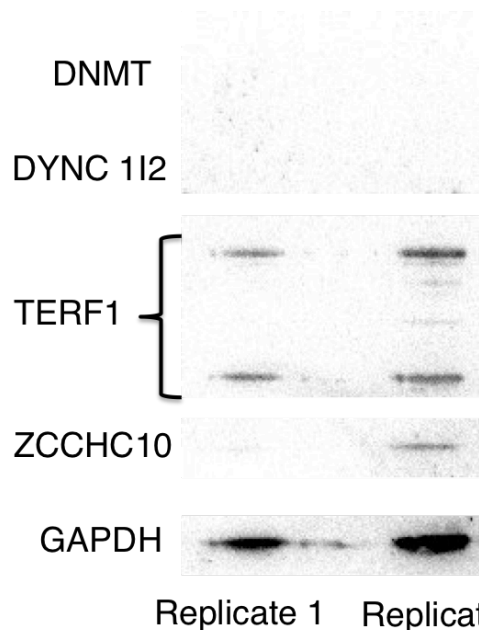


Figure 5-8. Bulk Western Blot indicates presence of ZCCHC10 and two isoforms of TERF1, but little DNMT and DYNC 112 relative to GAPDH. Presence of TERF1 and ZCCHC10 in bulk assays but not in single-cell IEF suggest that the sensitivity of the assay is insufficient for these targets.

Effect of longer lysis and EF durations on PKM solubilization

Previous work has demonstrated that longer lysis and electrophoresis durations present tradeoffs, resulting in improved protein solubilization but concomitant increased diffusive losses.^{20,25} Longer lysis durations aid in solubilizing and unfolding larger, hydrophobic proteins and prevent kinetic selection of fast-solubilizing proteins.²⁶ However, milder, non-ionic detergents such as Triton X-100 have a reduced tendency to denature proteins and dissociate protein complexes and thus may require even longer solubilization times.²⁷ To determine if lysis and electrofocusing (EF) durations would improve PKM solubilization, we increased the lysis duration to 50 s and separately increased EF duration to 8 min. Interestingly, longer lysis durations did not improve solubilization of PKM, but longer EF times did (Figure 9A, 9B). The median signal-to-noise ratio (SNR) of PKM increased with the longer 8 min focusing duration, regardless of lysis duration. In addition, the longer lysis and EF durations (50 s lysis, 8 min EF) increased the R-squared value of the Gaussian fit of the protein (Figure 9C), indicating improved solubilization.

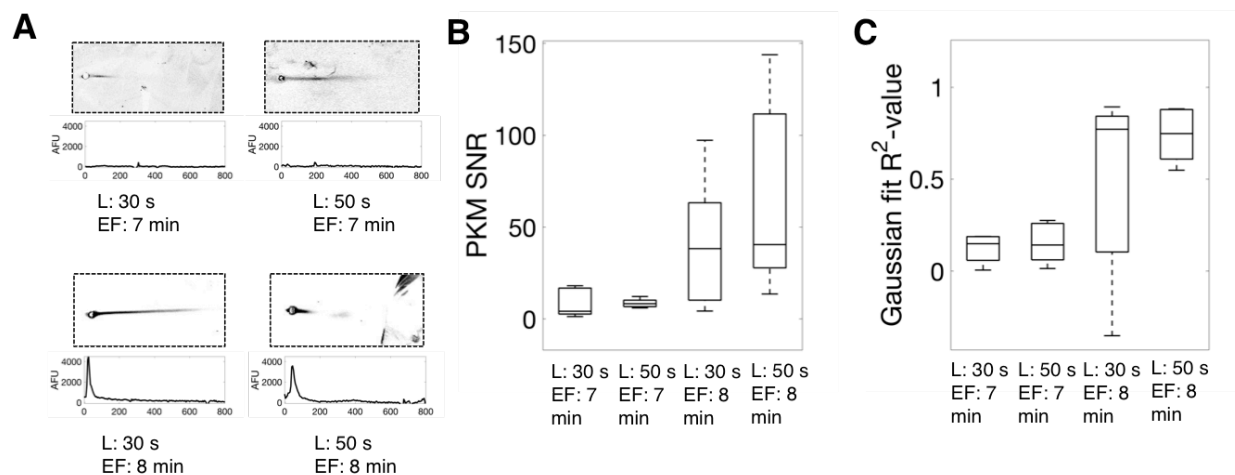


Figure 5-9. Lysis and EF duration can improve solubilization of PKM. A) inverted fluorescence micrographs and corresponding fluorescence intensity profiles of single cells lysed and focused for varying durations. B) Box plot of PKM SNR as a function of varying lysis and EF times indicates that longer lysis and EF duration improve solubilization. C) Box plot of Gaussian fit R^2 -value for varying lysis and EF times. The 50 s lysis and 8 min EF resulted in the best Gaussian fit, suggesting improved solubilization and injection into the PA gel.

PKM solubilization is independent of assayed detergent concentrations

In addition to increasing lysis and EF time, we sought to further enhance solubilization via chemical modification to our lysis and EF buffers. We hypothesized that use of higher detergent and chaotrope concentrations could maintain shorter assay durations and thereby mitigate diffusion-driven losses of cell lysate. Thiourea disrupts lipid association and helps maintain protein solubility during IEF,²⁸ whereas the mechanism of urea is disputed and either 1) disrupts water structures around proteins, weakening hydrophobic interactions or 2) interacts with the protein and strengthens electrostatic interactions or preferential van der Waals attractions.²⁹

We increased the concentration of detergents and chaotropes to 8M urea, 2.5 M thiourea, 4% w/v CHAPS and performed the assay with a 30 s lysis, 6 min EF duration, or for 50 s lysis and 8 min EF. Interestingly, the high detergent concentration did not remarkably improve solubilization of PKM 1/2, and disperse bands were visible (Figure 10A). Moreover, the Gaussian fit R-squared value was lower for the higher detergent concentration compared to the lower concentration group, with greater variation (Figure 10B). The wider variation likely arises from high injection dispersion, which occurs when cell lysis conditions are sub-optimal and influences the peak width.¹⁶ The overall measured PKM quantity (area-under-curve, AUC) was equivalent in both the low and higher detergent cases, suggesting that increasing chaotrope and detergent concentration alone does not improve solubilization of all proteins (Figure 10C). Sufficient protein solubilization requires combinations of chaotropes, surfactants, ampholytes and complete reduction of alkylation of cysteine bonds, followed by the removal of all salts and conductive species.¹⁵ PKM is active as a homotetramer and contains multiple cysteine residues, which may contribute to difficulty in solubilization using non-ionic detergents.

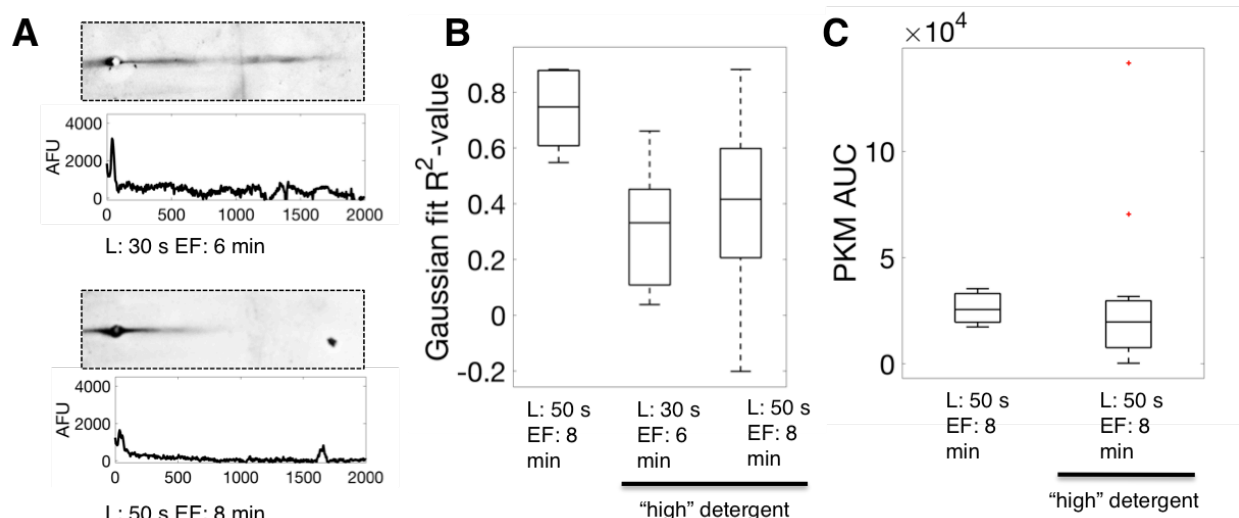


Figure 5-10. Higher detergent and chaotrope concentrations do not significantly improve PKM solubilization in single cells. A) Inverted fluorescence micrographs of single cells lysed using a higher detergent and chaotrope concentration. B) Box plot indicates that Gaussian fit has greater variation with the high detergent concentration. C) Amount of PKM detected is not improved with the higher detergent and chaotrope concentration.

Drawing from principles from preparatory IEF, we also tested the effects of adding reducing agents (dithiothreitol, DTT) and Tris base to our detergent and chaotrope formulation. Reducing agents disrupt disulfide bridges within proteins. Since PKM forms tetramers³⁰, we hypothesized that adding reducing agents and elevating the pH would effectively solubilize these protein-protein interactions. Interestingly, incorporation of 50 mM DTT and 40 mM Tris base into the formulation resulted in one resolvable PKM peak from scIEF (Figure 11). However, PKM was still trapped in the microwell, indicating incomplete solubilization. Nonetheless, these results suggest that careful sample

preparation and formulations of lysis and solubilization reagents are necessary, depending on the protein of interest. Future studies could aim to improve solubility of proteins, e.g., by using different detergent formulations²⁶, including combinations of reducing and/or alkylating agents such as tributyl phosphine³¹ or unpolymerized acrylamide¹⁰, adding nucleases to reduce viscosity, or adding other reagents for solubilization such as amidosulfobetaines^{28,32}. Altogether, sample preparation is crucial for quantitative readout of protein expression.

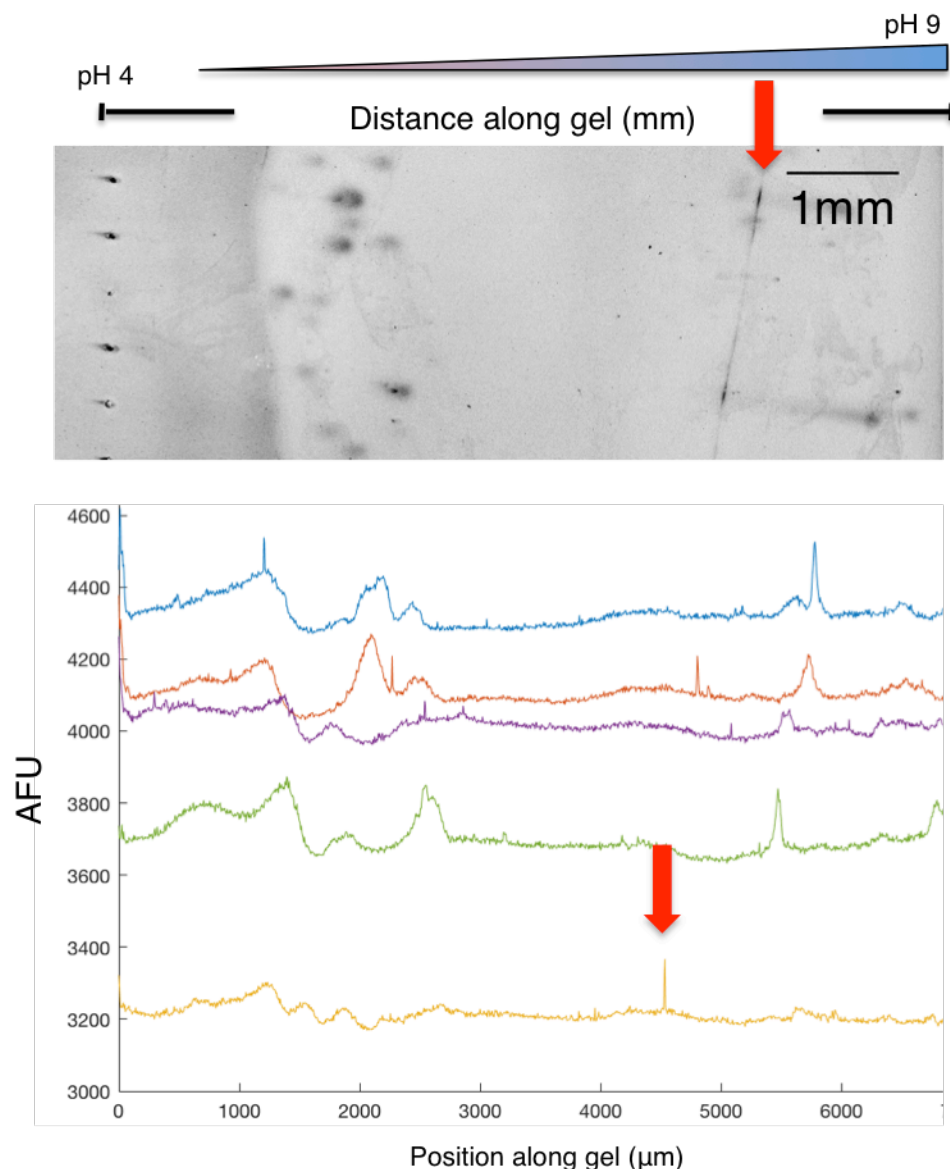


Figure 5-11. Addition of reducing agents results in one resolvable PKM protein peak. However, substantial amount of protein remained in the microwells.

5.4 Conclusions

Single-cell IEF is a powerful tool that provides protein expression data to complement gene expression data, such as that obtained from single-cell RNA-seq. While providing various advantages over SDS-PAGE, such as high selectivity for proteins with low molecular mass differences, scIEF is hindered by the sample preparation step; since short assay durations are required to preserve high local concentrations of proteins, solubilization poses a major challenge, even if higher concentrations of detergents, chaotropes, and reducing agents are incorporated. Future demonstrations of scIEF to decouple lysis from EF would be advantageous to increase the solubilization durations and/or chemistries.

One caveat of immunoassays is the dependence of the assay on available antibodies. Of the targets listed in Table 1, over 50% of the commercially available antibodies produced non-specific bands in conventional Western Blot, based off the manufacturer's characterization. While IEF confers higher selectivity based on spatial separation of proteins, the lack of specific antibodies hinder robust validation of target measurement and thus hinder accurate quantitation. A potential complementary technique to bypass this limitation is to use non-targeted protein tools, such as mass spectrometry, such as those demonstrated in small numbers of cells.³³

Future Directions: Improving separation resolution

While we reported a separation resolution that was on par with microfluidic and capillary IEF, some targets possessed isoforms with predicted pI differences as small as 0.01 pH units (Table 1). A minimum pI difference of 0.01 is below our current limit of separation ($\Delta pI \sim 0.07$), but has been demonstrated in IEF systems utilizing immobilized pH gradients.³⁴ However, in carrier ampholyte (CA)-only systems, this resolution has not yet been achieved, due to the limited availability of amphoteric species that buffer at pIs at such fine resolution, and also due to limitations of joule-heating that decrease separation resolution.^{21,35} We posit here that with careful tuning of IEF parameters and consideration of the interplay of parameters, it may be theoretically possible to obtain the desired minimum pI difference ($\Delta pI = 0.01$) within our platform, as detailed below.

As outlined in Chapter 1, we may consider the minimum resolvable pI difference of two proteins as the separation resolution of our system, given a linear pH gradient that scales with distance along the gel. Separation resolution is given by $SR = \frac{\Delta X}{2\sigma_1 + 2\sigma_2}$, where SR is the separation resolution, ΔX is the distance between the peak centers, and 2σ denotes half the peak width. If we average the peak widths or assume they are equivalent, then we obtain the equation $SR = \frac{\Delta X}{4\sigma}$. For a separation resolution of unity, which is often the criterion for minimally resolved peaks, we obtain the equation $\Delta X = 4\sigma$. Since we are interested in finding the minimum pI difference, we substitute in the equation $\Delta pH = \Delta X \left(\frac{dpH}{dx} \right)$ to obtain $\Delta pH = 4\sigma \left(\frac{dpH}{dx} \right)$. Thus, the minimum resolvable pI difference (in pH units) is equivalent to the peak width times the slope of the pH gradient. In practice, the ΔpI can be measured by measuring the peak width of a protein of interest.

In theory, the ΔpI is dependent on several parameters, as given by the equation $\Delta pI = 4 \sqrt{D \left(\frac{dpH}{dx} \right) / (E * -\frac{du}{dpH})}$ where D is the diffusion coefficient of the protein, dpH/dx is the slope of the pH gradient, E is the applied electric field, and $-du/dpH$ is the electrophoretic mobility of the protein.³⁶ We can examine each of these parameters individually to determine the range in which $\Delta pI \sim 0.01$ would be possible, if at all.

Let us begin with E , the applied electric field. To determine what electric field is needed to obtain a theoretical $\Delta pI \sim 0.01$ within our system, we need to first determine what is the effective electric field through the polyacrylamide gel. The electric field is simply the applied voltage over the distance across which the potential is applied, $E = V/L$. From Ohm's law, $V = IR$, where I is the current and R is the resistance. The resistance of the material, R is measurable by taking the inverse of conductivity times the cross sectional length, i.e., $R = L/A * 1/C$, where C is the conductivity of the medium, L is the length, and A is the area. The total applied voltage (600 V) is equal to the sum of the voltage across each area of the gel. $V_{total} = V_{acid} + V_{focusing} + V_{base}$. The total current through the circuit is equivalent across each gel, i.e., $I_{total} = I_{acid} = I_{focusing} = I_{base}$. Since we can calculate R , and we know the applied voltage (600 V), we determine I_{total} and substitute I_{total} into Ohm's law to determine V_{acid} , $V_{focusing}$, and V_{base} . Within the focusing region, $E_{focusing} \sim 333.2$ V/cm.

$$\text{Since } \frac{\Delta pI_{final}}{\Delta pI_{initial}} = \frac{4 \sqrt{D_i \left(\frac{dpH}{dx} \right)_i / (E_i * -\frac{du}{dpH}_i)}}{4 \sqrt{D_f \left(\frac{dpH}{dx} \right)_f / (E_f * -\frac{du}{dpH}_f)}}. \text{ Keeping all other variables constant, } \frac{\Delta pI_{final}}{\Delta pI_{initial}} =$$

$\sqrt{E_i/E_f}$. Given our current resolution of $\Delta pI = 0.07$, we would need $E_f = 29400$ V/cm to obtain a $\Delta pI \sim 0.01$. While this seems extraordinarily high, we can also take advantage of geometry to increase applied electric field.³⁷ Recall that $E = V/L = IR/L = I/(WHC)$, where w and h are the width and height of the gel, respectively, and C is the conductivity. We could thus tune the geometry, e.g., decrease the width, of the gel to obtain much higher electric fields. Of course, decreasing the width presents tradeoffs as throughput will be severely compromised if width is decreased. Another caveat is that joule heating will decrease separation performance.^{37,38} For example, increasing the applied electric field by 5.5 times would correspondingly increase the heat flux by 30.25 times. Since $D = \frac{k_b T}{6\pi\eta r}$, increasing the heat flux would dramatically increase D and thus decrease ΔpI . Thus, tuning electric field alone would be insufficient to obtain the required separation performance.

Next, let us consider the next parameter in the calculation of ΔpI . Similar to E , we can tune dpH/dx and determine which value is necessary for $\Delta pI \sim 0.01$. Keeping E (333.2 V/cm), D , and $-du/dpH$ remain constant, and assuming our current assay setup of 4 pH units over a 9 mm focusing region, we would need $dpH/dx = 0.0000113$ pH/ μm , which could be obtained in a setup e.g., 1 pH unit across 88.2 mm separation axis.

In addition to altering the applied electric field and decreasing dpH/dx , which are considered the assay-dependent parameters, we can also tune the system to alter the other terms of the equation. D , the diffusion coefficient of the protein, is often considered independent of the tunable parameters of the assay. However, D can be tuned by modifying the sieving matrix material. For example, Park and colleagues modeled change in D as an exponential function of the hydrodynamic radius and %T (monomer concentration) in the gel³⁹ using the equation $\frac{D}{D_0} = \exp\left(-13.5\left(\frac{R_h}{\xi}\right)^{0.59} C^{0.56}\right)$, where R_h is the hydrodynamic radius of the protein, ξ is the mesh size of the gel, and C is the PA concentration. Using GFP as a model protein and using the hydrodynamic radius of 2.4 nm estimated by Bhunia et al,⁴⁰ we would expect the diffusivity in a 8%T versus a 6%T gel to be 0.64.

One other consideration in obtaining $\Delta pI \sim 0.01$ is tuning the stringency of our requirement for minimally resolved peaks. Recall that we used a criterion of 1 as the minimal separation resolution. However, a separation resolution > 0.5 is considered acceptable.⁴¹

Overall, improving separation resolution within gels for detection of AS isoforms of iPSCs and iPSC-derived neurons requires orchestration of assay design principles. Importantly, to maximize separation resolution, E should be optimized for maximal separation efficiency while minimizing joule heating, dpH/dx can be adjusted by changing device geometries, D can be tuned using gel density, and the criterion itself for minimally resolved peaks can be altered. One example of how a $\Delta pI \sim 0.02$ may be put together is if we assumed a separation resolution of 0.75, used a 8% T gel (resulting in diffusion coefficient change from 31 to 19.84), a pH gradient of 2 pH units over a 9mm separation axis, and an applied electric field of 400 V/cm. Obtaining $\Delta pI \sim 0.01$ would require further tuning and optimization of parameters. Note that in the described case, we assume a stable IEF system based on utilization of carrier ampholytes. Use of immobilized pH gradients has already obtained ΔpI values of 0.001 and could be a useful future direction for tuning the assay.²¹

Future Directions: Improving analytical sensitivity

In addition to improving separation resolution, another improvement to utility of the scIEF assay could be to enhance the analytical sensitivity. As mentioned above, various alternatively spliced targets were not detectable in scIEF, possibly due to low expression that falls below our reported lower limit of detection. Similar to separation resolution, the analytical sensitivity of the assay can be improved by tuning various assay parameters. For example, in chapter 7, we discuss how the partition coefficient of the lid gel used for lysis reagent delivery can be altered to decrease protein partitioning into the lid gel, which would retain higher concentrations in the substrate gel that is used for immunoprobng. By both slowing the diffusivity of proteins in the lid gel and acting as an unfavorable thermodynamic partitioning barrier, high % T lid gels can be used to

mitigate protein losses in the lid gel during electrofocusing and thus increase in-gel concentrations and analytical sensitivity.

Moreover, similar to how the criterion of separation resolution can be made less stringent, we can change the way we process our outputs, i.e., alter our signal processing techniques, to improve analytical sensitivity. As described in chapter 4, our analysis algorithms perform a quality-control method that apply thresholds of Gaussian fit r-squared values of 0.7 and SNR > 3. These criteria can reduce the number of detected protein peaks. Ongoing research in the group is elucidating the contribution of r-squared values to true signal and AUC values and suggest that r-squared values are not the only useful criteria for assessing the quality of a peak.

Two other assay parameters that are tunable that may affect assay sensitivity are temperature and gel porosity/density. Joule-heating induced by high applied electric fields will increase dispersion and protein diffusivity, thus increasing losses and decreasing analytical sensitivity.³⁸ Thus, methods to reduce heating, such as cooled chambers, or introducing cooling channels, may be a feasible approach to decrease diffusive losses and improve sensitivity. The other parameter that can be tuned is the gel density to balance between photocapture efficiency and probing efficiency. Ongoing research had indicated that increasing gel density (%T) can decrease the pore size in gels and improve photocapture efficiency; however, increasing gel density concomitantly decreases probing efficiencies due to size exclusion of antibodies in gels with smaller pores and higher partition coefficients.^{41,42} Thus, a delicate tradeoff between pore size and immunoprobng exists that remains to be optimized.

Overall, careful combinations of assay parameters and considerations on the definition of analytical sensitivity and separation resolution can improve the utilization of electrophoretic cytometry.

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Chapter 6.

Electrophoretic Cytometry of Centrifugally-Loaded Cells

This work was performed in collaboration with Dr. Damien King, Dr. Rohit Mishra, and Prof. Jens Duccrée in the laboratory of Prof. Duccrée at Dublin City University, Ireland. Primary financial support for this work was provided through a Graduate Research Opportunities Worldwide (GROW) fellowship through NSF.

6.1 Introduction

Sample preparation, beginning with the isolation of rare cells, is critical to analyzing and understanding biological and pathological processes such as stem cell differentiation or cancer metastasis. In cancer biology, both the collection of rare circulating tumor cells (CTCs) from peripheral blood,¹⁻⁴ as well as sensitive, multiplexed target detection are necessary to elucidate mechanisms of cancer progression. Moreover, expression of variant isoforms have been linked to enhanced tumor cell migration and metastatic ability^{5,6}; thus, distinguishing and detecting protein isoforms in rare cells is crucial.

Yet, technical challenges in the selection, capture, and analysis of rare cells stymie our ability to understand the intricate, networked signaling pathways of individual cells. To address the capture and isolation of rare cells, various groups have developed microfluidic technologies for rare cell isolation and capture based on properties such as surface marker expression, cell size, and deformability.⁷⁻⁹ Further enhancements of purity have been obtained by appending an optically-induced dielectrophoresis step for individual cell manipulation.¹⁰ However, these isolation approaches often require a large starting quantity of cells (on the order of 1E6 cells), and labeling methods may alter intracellular signaling.^{11,12} Furthermore, for downstream analysis, these techniques rely on single-stage immunoassays, such as immunocytochemistry, which are subject to analytical artifacts from non-specific antibody binding¹³ and are limited in protein-isoform detection based on availability of isoform-specific immunoreagents.

In electrophoretic cytometry, additional selectivity is conferred via a separation stage, e.g., by molecular mass¹⁴⁻¹⁶ or isoelectric point.^{17,18} Rare cells can be analyzed via transfer of individual cells into microwells, followed by cell lysis, electrophoresis, and immunodetection of proteins within the cell lysate. Although this technique offers the advantages of high selectivity and multiplexing, the individual cell transfer process is laborious. In addition, protein solubilization, a critical step for immunodetection, is constrained by the short lysis durations required to prevent diffusion-driven losses of proteins within the cell lysate.^{16,19}

In the past two decades, centrifugal microfluidics has emerged as a promising technology in automated sample preparation. Ducrée, Zengerle, and colleagues have demonstrated lab-on-a-disc (LoaD) devices that meter and split volumes,²⁰ mix reagents on-chip,^{21–23} generate droplets,²⁴ route fluids precisely,²⁵ and couple with other optical detection systems,²⁶ amongst a multitude of functionalities. More recently, LoaD devices have been used for rapid isolation of cells with high capture efficiencies. Kim et al demonstrated a centrifugally loaded LoaD device prepended to an electrophoretic separation that obtained 75% cell settling efficiencies and 70% microwell occupancy.²⁷ Capture efficiency and occupancy can be further enhanced using arrays of physical traps, such as v-cups,^{28,29} which have demonstrated near 100% capture efficiency and occupancy under a given set of conditions. Given the high capture capabilities, facile operation, and minimal handling requirements, centrifugal microfluidics is a particularly attractive method to capture rare cells.

Here, we design and characterize a centrifugally operated microfluidic device that utilizes array-based cell capture combined with a polyacrylamide (PA) photopatterning approach for downstream single-cell lysis, electrophoresis and immunodetection of proteins. We first demonstrate fabrication of a reversibly bound PDMS-glass microchip that operates within centrifugal gravity-settling regimes, modified from previously reported v-cup arrays.³⁰ Next, we characterize geometry-based cell capture using three configurations. Finally, we demonstrate array-based photo-polymerization of individual PA gel islands within the PDMS channels for downstream electrophoretic cytometry. We discuss implications and hypotheses of exploiting low protein permeability properties of PDMS for reduction of diffusion-driven protein losses during electrophoresis. Future work will aim to implement on-chip electrodes for facile electrophoresis of captured and lysed rare cells.

6.2 Materials and methods

Chemicals/Reagents

30% T, 3.3% C acrylamide/bis-acrylamide (29:1) (A3574), benzophenone (B9300), methanol (34860), 30 μ m polystyrene beads (84135), fluorescein sodium salt (F6377), and acetone (000000001000121000) were purchased from Sigma-Aldrich. VA-086 (2,2'-Azobis[2-methyl-*N*-(2-hydroxyethyl)propionamide]) was purchased from Wako Chemicals. Tris-glycine 10x native electrophoresis buffer (1610734) was purchased from Bio-Rad laboratories. Methacryloxyethyl thiocarbamoyl rhodamine B (23591) was purchased from Polysciences, Inc. Alexa-Fluor labeled OVA (O34781) and BSA (A34786) were purchased from Invitrogen (Bio-sciences, Ireland).

Device Fabrication and Operation

The v-cup array device was fabricated using standard soft lithography techniques and PDMS casting on to SU-8 silicon molds. Features were 28 μ m in height using SU-8 3025 (Microchem). Three feature dimensions were designed with either 10 or 20 μ m

channel widths, and 32 or 40 μm capture heights (Figure 1). The diameter of each trap was 32 μm . Devices were designed in AutoCAD (Autodesk) and printed on chrome masks on silica glass (JD Photo, United Kingdom). Feature heights on each wafer were verified using an optical profilometer (Bruker). PDMS (Sylgard, Dow Corning) was mixed at a 10:1 w/w ratio, cast onto the silicon SU-8 mold, and degassed under strong vacuum for 1 hour before curing at 70° C for 2 hours. PDMS devices were then gently peeled off, cut, and two inlets were punched using a biopsy puncher. The second inlet facilitates introduction of solution into the device. PDMS devices were then laminated (adhered) onto laser-cut glass-PMMA-PSA cartridges. To fabricate the glass-PMMA-PSA cartridges, PSA (Adhesives Research) was adhered onto PMMA (Radionics). The PSA-PMMA was then laser cut to the cartridge geometry. Finally, 24 mm x 60 mm glass coverslips (Menzel-Gläser) were laminated onto the adhesive side of the PSA. Prior to use, the devices were degassed for 1 hour, primed with 8 microliters of 1% Bovine Serum Albumin (Sigma-Aldrich) in PBS, then loaded with either cells or microbeads.

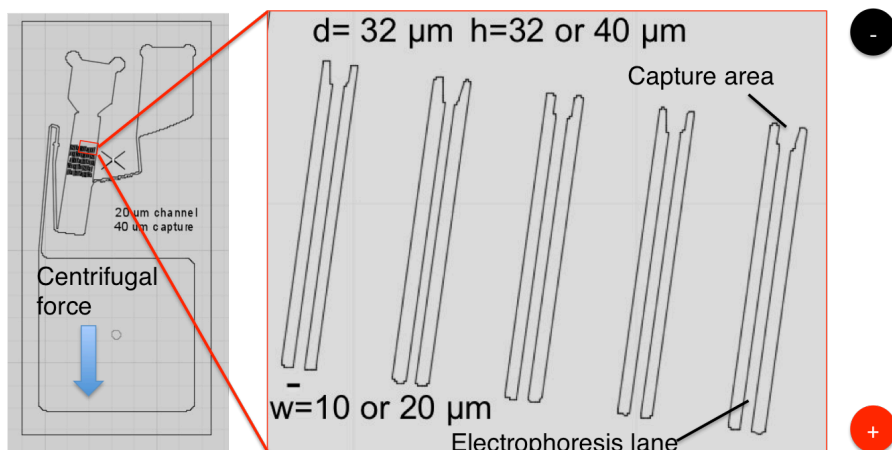


Figure 6-3. Photomask design for PA-PDMS devices. The device contains 70 arrayed traps. The trap capture diameter is 32 μm in diameter and 32 or 40 μm in height. The gap between the PDMS, into which PA is polymerized, is 10 or 20 μm in width. Loading of cells occurs via application of centrifugal force, which drives the cells into the capture area.

For devices with PA, PDMS was first treated by pipetting 20 μL of 10% w/v benzophenone in acetone onto an open PDMS device and allowed to adsorb for 10 minutes. Devices were then aspirated and gently rinsed with deionized water and dried with a nitrogen stream. Next, the devices were gently laminated onto glass coverslips and degassed for 1 hour. The PA precursor solution, comprising of 15% T PA, 0.02% VA-086, and 0.005% Rhodamine-polyacrylamide, was degassed for 10 minutes and introduced into the closed PDMS-glass devices via degas-driven flow. A photomask was then overlayed over the glass and aligned using an optical mask aligner (OAI). The devices were then exposed to UV for 4 minutes. Following polymerization, the devices were gently removed from the glass, placed under a nitrogen stream for ~5 seconds, and then re-laminated on a new glass coverslip. The PA-PDMS-glass devices were then placed under a strong vacuum for 1 hour before use.

For cell or bead loading, devices were primed with 20 μ L of 1% solution BSA in PBS, then loaded with 8 μ L of cell or bead solution. The devices placed in a custom, 3D-printed rotary adaptor and centrifuged at 10 or 20 Hz for one hour. Devices were then imaged using a brightfield and epifluorescence microscope (Hamamatsu).

Cell Culture

SKBR3 and CHO cells were generously provided by the National Institute for Cellular Biotechnology at DCU. For most studies, cells were fixed in 4% PFA prior to loading.

6.3 Results and discussion

Centrifugally-loaded single-cell electrophoresis with minimized protein loss

We designed a microfluidic device that combines the high capture efficiency of array-based, centrifugally-driven cell loading with the selective measurement of protein targets in individual cells afforded by an electrophoretic separation (Figure 2). The assay is designed for (i) centrifugal loading of single cells into an array of 70 physical v-cup traps (Figure 2A) (ii) rapid introduction of lysis buffer into the device, (iii) separation of proteins via electrophoresis, with minimized losses of cell lysate through the PDMS channel walls, and (iv) immunoprobing through passive diffusion via a removable glass lid (coverslip). Microscale features are key to minimizing assay durations and thus diffusion-driven dilution and losses of proteins within the cell lysate during electrophoresis.

The device is comprised of a PDMS- PA hybrid body, which is adhered onto a glass coverslip (Figure 2A and 2B). Several alterations to the previously described v-cup array²⁸ have been implemented to enable downstream electrophoretic cytometry. First, the capture geometry of the trap is heightened with a capture height-to-diameter ratio of 4:3, as this configuration has been reported to be optimal for single cell capture of U251 cells in microwell systems.¹⁵ The heightened walls of the capture area also serve to reduce diffusive losses of cell lysate after introduction of lysis buffer, as PDMS is not permeable to proteins (Figure 2A). Each PDMS feature is also elongated and bisected by a polyacrylamide channel of 10 or 20 μ m width and 500 μ m length. The selection of 500 μ m length for the PA-PDMS channel was designed based on dimensions of separation lanes within an arrayed single-cell Western Blot.¹⁴ The PDMS-PA device is adhered onto a glass coverslip attached to a laser-cut PMMA cartridge. Importantly, the device is not covalently attached to the glass (i.e., plasma bonded) to allow for downstream delamination (toggle “open”) and introduction of antibodies for targeted protein analysis. The glass is also used to introduce polyacrylamide and align a photomask for polymerization within the channels, which is described further in the Material and Methods section (Figure 2C). Finally, although not implemented in the design reported here, the toggle open-closed system (i.e., removable glass coverslip) design enables facile introduction of electrodes for high-voltage on-chip electrophoresis.

For device operation, cells are first loaded into the traps via centrifugal-driven sedimentation (Figure 2D). After cell lysis and upon application of an electrical potential, the PA acts as the sieving matrix during electrophoresis, and the PDMS “rails” limit lateral diffusion of the protein bands. Protein is then photocaptured into the gel, using a benzophenone chemistry, as previously described,^{14,31} and the glass coverslip is then removed for passive introduction of antibodies (Figure 2D). The toggle open-closed system, i.e., the removable glass coverslip, facilitates device fabrication (gel polymerization) and diffusive immunoprobings.

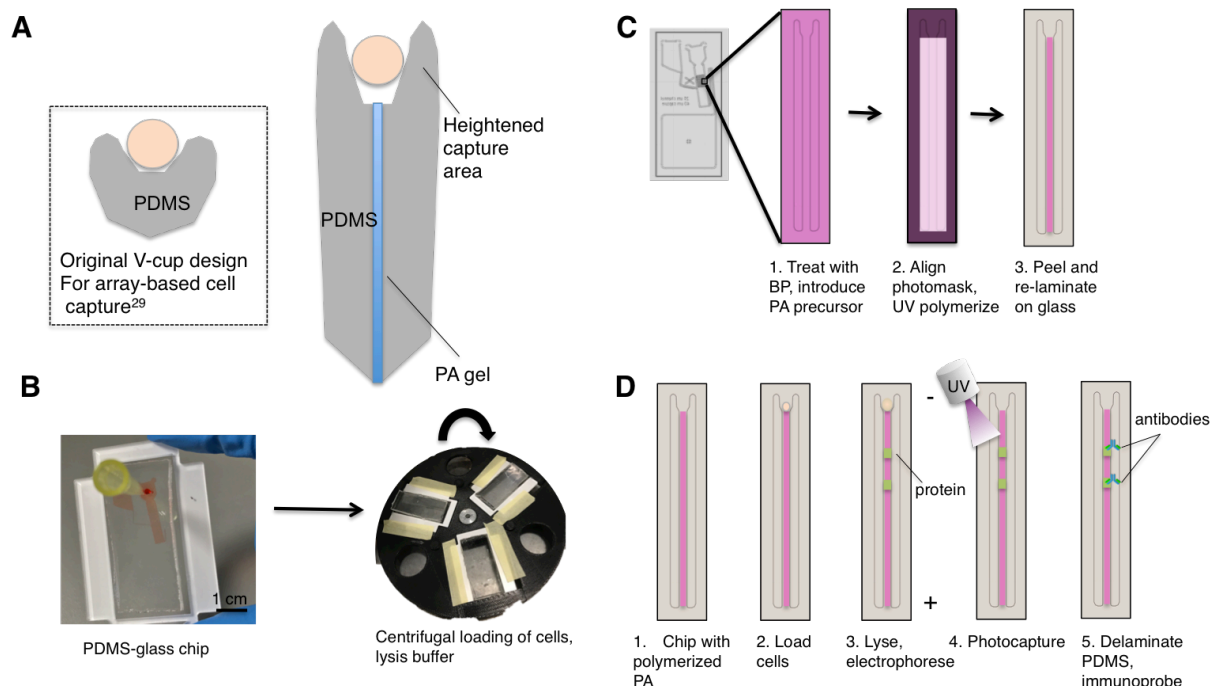


Figure 6-2. Workflow of centrifugal arrayed capture device for electrophoretic cytometry. A) Schematic of the modifications made to the v-cup array previously reported,²⁹ which featured hundreds of single-cell capture traps. The geometry reported here is reconfigured with a heightened capture area and a polyacrylamide channel for downstream single-cell lysis and electrophoresis. B) Photograph of PDMS-PA-glass chip loaded with red food dye. For cell and bead loading, cells and beads are loaded into the inlet of the chips and inserted into a 3D-printed centrifugal adaptor. Chips are then centrifuged at 10-20 Hz. C) Schematic of workflow of PA polymerization within the PDMS channels. PDMS devices are first imbibed with BP, then PA is introduced into the device and photopolymerized. Following polymerization, the PA-PDMS is peeled from the glass and re-laminated on a new coverslip. D) Schematic of workflow of proposed downstream cell lysis and electrophoretic separation. After loading of cells, the cells are lysed and the cell lysate is electrophoresed and photocaptured. After, the device is delaminated and immunoprobed via passive diffusion. BP, benzophenone. PA, polyacrylamide.

Centrifugal cell loading and capture

The v-cup array serves as a physical trap for the cell, as well as the chamber for cell lysis. As mentioned before, two key modifications to a previous capture cup design^{29,32} make it suitable for the appended electrophoretic separation. First, the capture area contains heightened walls constructed from PDMS. The wall heights are designed to be 1 and 1.33 times the diameter (and capture diameter) of a 30 μm cell for optimal capturing of single cells while preventing diffusive losses¹⁵. Second, the geometry of the

v-cup is modified to house individual 500 μm -long PA gel lanes (either 10 or 20 μm in width) within each individual capture cup. Thus, immediately after capture, the contents of each cell can be interrogated via lysis and electrophoresis of the cellular lysate into the channel. The PDMS walls and glass lid are designed to reduce diffusion of proteins within cell lysate to just one dimension (along the separation axis).

Although PDMS is typically bonded to glass via plasma treatment, we opted for a non-bonding fabrication approach to allow for downstream introduction of antibodies into the PA-PDMS device via passive diffusion. To test the feasibility of using the device under centrifugal operating conditions while toggling the “open” and “closed” features of the device, we tested the failure mode of the different non-bonded configurations under normal centrifugal operating conditions (10 or 20 Hz). Operation of an “open-able” system, i.e., removal of the glass lid layer, provides several key advantages, such as diffusive immunoprobng and readout³³. We adhered our PDMS devices onto glass coverslips adhered via PSA attachment to PMMA cartridges, and centrifuged the devices at 10-20 Hz, increasing the spin rate over time ($n = 3$ devices, Figure 3). Within the typical centrifugal operating range of 10-20 Hz, all of the devices remained laminated. Increasing the spin rate to 29 Hz resulted in device failure (PDMS delamination from glass). Although increasing spin rate will increase the speed of cell loading,³⁰ beads can be effectively loaded at close to 100% efficiency at lower spin rates. These results indicate the adhesion of PDMS onto glass is sufficiently strong to resist delamination under typical centrifugal operating conditions.

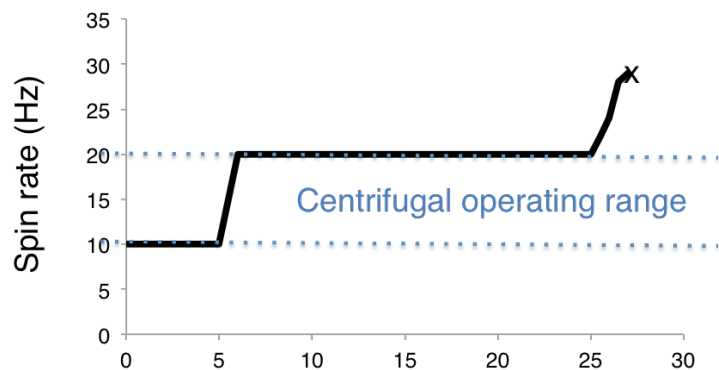


Figure 6-3. Non-plasma bonded devices operate within centrifugal operating range without delamination. Time course plot of PDMS-glass devices operated within varying centrifugal spin rates. Within the 10-20 Hz operating range, the PDMS remained laminated to the glass. The devices failed when centrifuged at 29 Hz.

Next, we tested the capture efficiency and occupancy of bead and cell loading in our v-cup array, when PA was not polymerized in the channels. We designed and characterized three different geometries: (i) a 40 μm height trap, 10 μm channel width (ii) a 40 μm height trap, 20 μm channel width (iii) a 32 μm height trap, 20 μm channel width. We first loaded fixed CHO cells ($d \sim 10\text{-}20 \mu\text{m}$) into empty, i.e., non-PA loaded, PDMS channels. For all configurations of the non-PA PDMS devices, cell loading into the traps was poor ($< 30\%$ of traps were occupied, Figure 4), with many cells flowing

through the array and ending near the outlet at the end of the channel. We hypothesized that the non-PA channels were too wide and that the cells flowed through the gaps between the PDMS rails during loading. Next, we loaded the devices with larger SKBR3 cells ($d \sim 20 \mu\text{m}$), which resulted in slightly increased v-cup occupancy. Of all the configurations, the occupancy was highest in the $10 \mu\text{m}$ channel width, suggesting that the $20 \mu\text{m}$ channels were too wide when unfilled, even for the larger cell type (Figure 6). Overall, these results indicate the importance of careful optimization of geometry for enhanced capture of single cells.

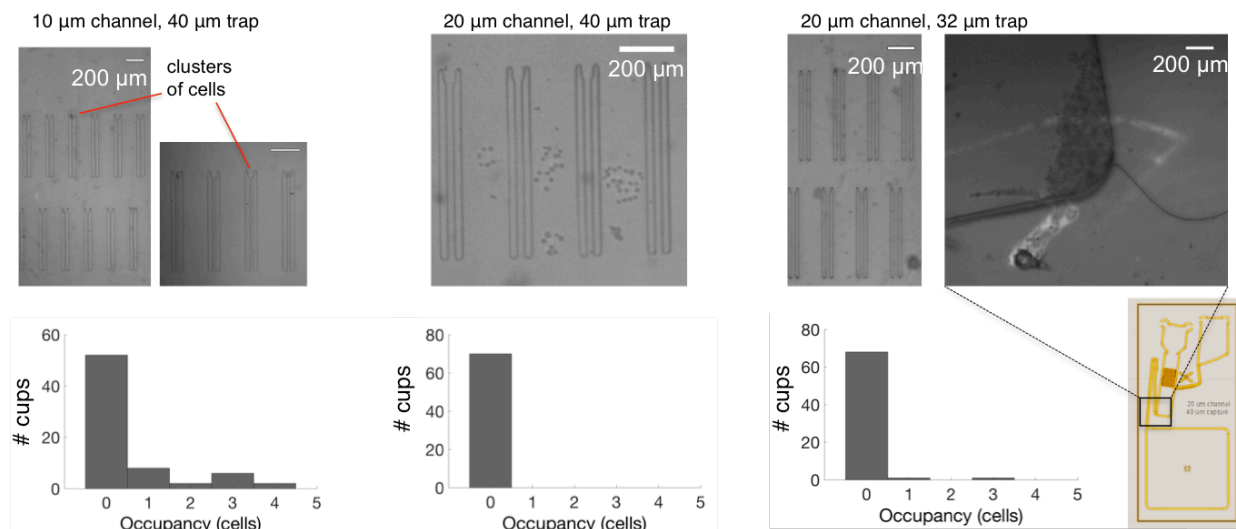


Figure 6-4. CHO cell loading in PDMS devices demonstrate low occupancy. In all $20 \mu\text{m}$ channels, the occupancy of the v-cups was $< 5\%$

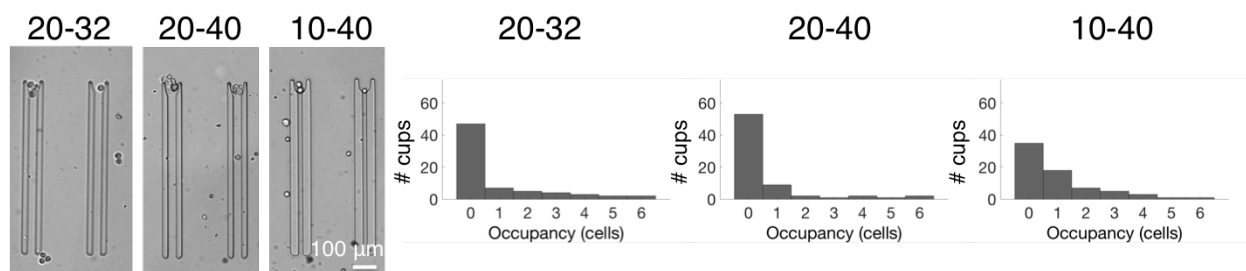


Figure 6-5. SKBR3 loading in PDMS devices without PA demonstrate low occupancy. Left: brightfield micrographs of different device configurations and captured SKBR3 cells. Right: histogram of occupancy of v-cups demonstrate low v-cup occupancy, despite using a larger cell.

Polymerization of individual PA channels in PDMS

Given the low occupancy in the v-cup arrays without filled channels, and to allow for downstream electrophoretic separations, we polymerized Rhodamine-conjugated PA into the PDMS channels, i.e., between the “rails”. PA can be introduced and chemically polymerized in oxygen-permeable PDMS via imbibition of PDMS with benzophenone^{33,34}. We used a photopatterning approach to polymerize 15% T (3.3% C)

PA only within the PDMS channels and not elsewhere in the device (Figure 2B). For facile fabrication, we designed the mask with fiducial markers for manual alignment (Figure 6) under a UV mask aligner.

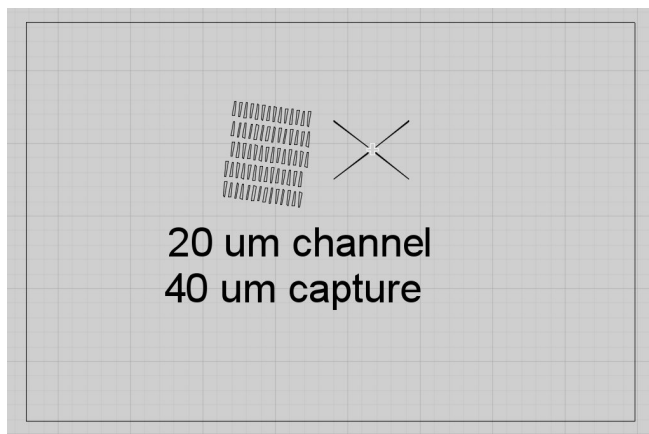


Figure 6-6. Photomask used for polymerizing PA within the PDMS channels. Rhodamine PA is introduced into the device. The “X” with cross in the center is used for alignment of the mask with the PDMS device, and the device is exposed to UV for 4 minutes, which polymerizes the PA within the channels, but not elsewhere in the device.

Next, we characterized the accuracy of the manual alignment of the features by calculating the x- and y- offsets of the polymerized gel from the designated area (Figure 7). Since cell capture and subsequent electrophoresis is conducted in the x-direction, any x-offset (i.e., to the left or right of the capture area) will either influence cell capture (e.g., if the PA is offset to the left and the capture chamber is obstructed by PA), or result in multiple cells captured (if the chamber is too far right and cells are captured within the channels). We defined the tolerance of this offset as the diameter of a cell ($\sim 20\ \mu\text{m}$), to prevent capture of multiple cells. For the y-offset, an offset of $30\ \mu\text{m}$ (the width of the “rails”) would result in a gel that is polymerized outside the channel region; thus, $30\ \mu\text{m}$ was the tolerance set for the y-offset. Of our devices, the mean x-offset was $13.1 \pm 3.1\ \mu\text{m}$ and the mean y-offset was $23.7 \pm 12.5\ \mu\text{m}$ (Figure 7B, $n = 3$ devices). These PA polymerization within these devices was thus within our tolerance range.

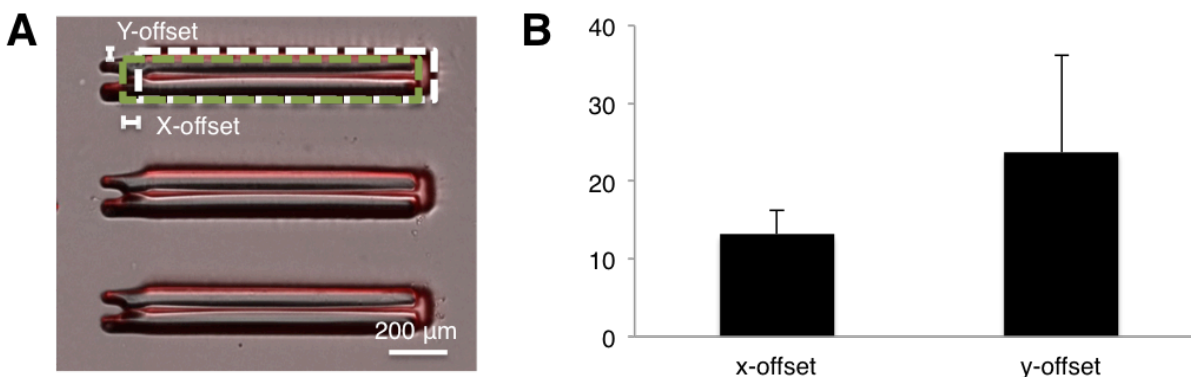


Figure 6-7. Characterization of PA polymerization within PDMS channels. A) Overlay micrograph of brightfield and rhodamine fluorescence. Rhodamine-PA was polymerized within the PDMS channels using a photomask. B) Bar graph of X- and Y- offset, which characterizes the displacement of the polymerized PA based on manual alignment of photomask with the device ($n = 3$ devices).

Cell loading in PA-PDMS devices

To test whether cell loading would be improved in the PA-PDMS hybrid devices, we centrifugally loaded SKBR3 cells into devices with the 40 μm height trap, 20 μm channel configuration. We calculated the occupancy of the v-cup array and the distribution of the number of cells captured and observed that for our 30 μm -diameter capture area, multiple cells were captured per device (Figure 8A). Overall occupancy of the v-cup array was higher, with over 85% of cups occupied (Figure 8B). Most of the cups, however were occupied with > 1 cell (Figure 8C). These results suggest that the PA polymerization in the chips is necessary for obstruction of cells from flowing through the channels. Careful tuning of geometry is necessary to optimize cell capture and occupancy.

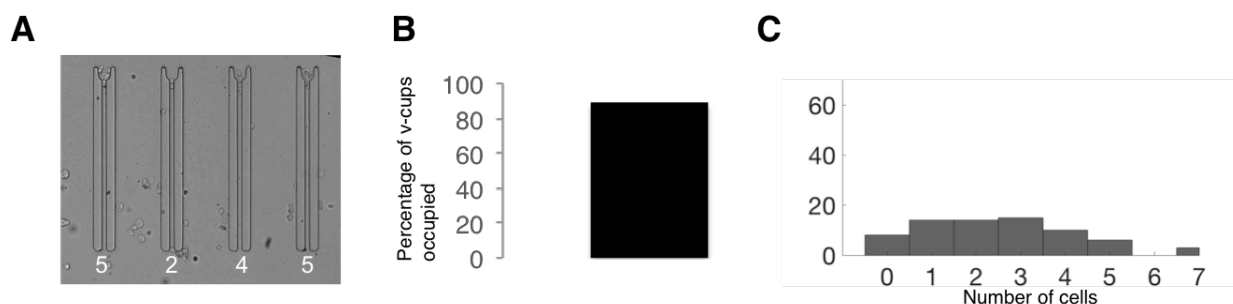


Figure 6-8. Cell loading in PA-PDMS devices results in higher occupancy. A) Brightfield micrographs of SKBR3 cells loaded into a v-cup array demonstrate multiple cells loaded per trap. B) Bar graph of cell occupancy in PA-PDMS devices. Over 80% of traps are filled with cells, albeit mostly with more than one single cell (C).

6.4 Conclusions and future directions

Capture and analysis of rare cells that underpin key pathologic processes such as cancer is necessary for understanding, modeling, and designing treatments for disease.

We report herein on a device that integrates centrifugal, array-based capture of cells for downstream PAGE while minimizing out-of-plane diffusion of proteins from cell lysate. We fabricated various device geometries and tested non-covalent adhesion of PDMS to glass within centrifugal operating parameters. Next, we loaded cells centrifugally into PA-polymerized PDMS with high occupancy (> 85%). Further improvements to capture efficiency can be designed by decreasing the pitch,²⁹ optimizing capture geometry, and wall height.

For downstream electrophoresis, on-chip electrodes can be fabricated using a variety of techniques ranging from gold patterning to integration of interdigitated electrodes, which can generate high local electric fields.^{35–39} Combined with high cell capture efficiency and improved sensitivity afforded by a material (PDMS), we view our technology as advancing electrophoretic cytometry and targeted proteomics to further understand pathology.

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Chapter 7.

Transport of Proteins in Hydrogel Interfacial Fluid Films

This work was performed in collaboration with Shaheen Jeeawoody.

7.1 Introduction

Molecular transport in hydrogels has important functions for tissue engineering, drug delivery through tissues, and in immunoassays. Determining protein diffusion through hydrogels is necessary for protein delivery to ensure viability of cell encapsulation,^{1,2} and efforts to better understand kinetics of protein release by determining in-gel diffusion coefficients have facilitated development of novel and facile characterization techniques.³ In immunoassays, protein transport through and inhibited by different media dictate analytical sensitivity. For example, secreted proteins from single cells in microwells diffuse through free solution to reach immobilized antibodies along the walls or lid of the microwell,^{4–7} and the spatial positioning of the cell relative to the measurement tool (e.g., antibodies) determines signal strength.⁸ Furthermore, partitioning and permeability of the material properties also inform engineering design to ensure protein analytes do not escape the detection area.⁵ Thus, understanding how time-dependent mass transfer of proteins depends on operational parameters such as partition coefficient, geometries, and diffusion coefficient provide a physical framework for determining design of bioanalytical tools with high sensitivity.

In electrophoretic cytometry (EPC), understanding of micro-scale transport of proteins is crucial for determining critical timescales that prevent dilution and diffusive losses of analytes of interest, which fundamentally limit analytical sensitivity. In size-based separation of single-cell lysates, 40% of protein losses were estimated during cell lysis, partially due to a pouring method of lysis buffer that caused advection.⁹ To mitigate these losses, Kang et al. demonstrated that using a glass slide conjugated with a polyacrylamide (PA) gel would improve protein retention.¹⁰ Similarly, for isoelectric point-based separations, Tentori et al. modeled and experimentally determined that delivery of lysis reagents using a higher density PA lid gel mitigated losses compared to an “open” free solution-only system.¹¹ However, this result did not corroborate experimental in lid-gel delivery of lysis reagents for subcellular fractionation, i.e., use of a high-density gel lid did not significantly mitigate losses compared to a pouring method when a different detergent composition was utilized.¹²

To understand where and when dilution/diffusive losses that limit analytical sensitivity arise, it is necessary to decouple lysis and denaturation of proteins that occur during

lysis from actual diffusive losses. Here, we use numerical simulation to determine diffusive transport of proteins in hydrogels. We first characterize fluid film thickness in two setups of EPC- one with a glass lid conjugated to PA, and one with a high-density PA gel lid. Fluid film thicknesses were then input into a numerical model to predict how microwell protein concentrations are impacted by fluid film thickness as well as partition coefficient of the lid. Finally, we validate the model using protein-releasing microparticles. Understanding diffusion-driven transport of proteins in hydrogels and free solution ultimately aids in engineering design for improvements of hydrogel properties to retain higher concentrations of protein for a variety of applications from drug delivery to high-sensitivity diagnostics.

7.2 Materials and Methods

Reagents

Acrylamide/bis-acrylamide 30% (w/w) solution, ammonium persulfate (APS), N,N,N',N'-tetramethylethylenediamine (TEMED), imidazole, sodium phosphate, sodium chloride, sodium hydroxide, sodium deoxycholate, sodium dodecyl sulfate, and Triton X-100 were obtained from Sigma-Aldrich. 10x Tris-glycine was obtained from Bio-Rad. Tris-HCl, pH 6.8 buffer was obtained from Teknova. PureProteome nickel magnetic microparticles, 10 μm , were obtained from Millipore-Sigma. TBST was obtained from Cell Signaling Technologies. Methacryloxyethyl thiocarbamoyl rhodamine B was obtained from Polysciences. VA-086 was obtained from Wako. N-[3-[(3-benzoylphenyl)formamido]propyl] methacrylamide (BPMA), was obtained from Pharm-Agra Laboratories. Silicon wafers were obtained from WaferNet. SU-8 developer and photoresist (SU-8 3050) were obtained from Microchem. Recombinant protein G with His Tag were obtained from abcam and labeled in-house with Alexa Fluor 647 NHS ester (Life Technologies). 0.5 μm rhodamine-microbeads were obtained from Life Technologies.

Fabrication of rhodamine-labeled polyacrylamide gels

6% T, 3.3% C polyacrylamide gels containing 5mM BPMA and 0.005% (w/v) methacryloxyethyl thiocarbamoyl rhodamine B were fabricated by degassing and pipetting the precursor solution between an acrylate-silanized microscope slide and a silicon wafer patterned with 40 μm SU-8 features. PA gels were crosslinked using 0.08% (w/v) APS as the initiator and 0.08% (v/v) TEMED as the catalyst. After 20 min of polymerization, the gels were peeled off the wafer, rinsed in deionized water, and gently dried under a nitrogen stream or stored in 1x TBST solution. 15% T, 3.3%C lid gels containing 0.005% (w/v) methacryloxyethyl thiocarbamoyl rhodamine B were fabricated using photopolymerization, as previously described.¹¹ The 500 μm thickness was obtained by patterning the gel between two glass plates separated by a 500 μm thick spacer (CBS Scientific).

Imaging

Confocal imaging experiments were conducted on an inverted Zeiss LSM 710

AxioObserver (Zeiss, Oberkochen, Germany). Images were acquired at room temperature using a 40x water objective (LD C-Apochromat 40x/ 1.1 NA W Corr M27, Zeiss) with the correction collar set for a No. 1 coverslip.

Methacryloxyethyl thiocarbamoyl rhodamine B was imaged using a DPSS-561 laser at 0.25% power, using the MBS488/561/633 beam splitter and the Zen 2010 software (Zeiss). AlexaFluor 647 IgG was imaged using a HeNe-633 laser at 4% power. Z-stack images were acquired with 0.42 μm step size with line scanning at $x=y=z=0.42 \mu\text{m}$ pixel size.

Widefield epifluorescence images for microparticle imaging were obtained on an Olympus IX-71 inverted microscope with an Olympus LCPlanFI 40X/ 0.6 NA objectives and an EMCCD Camera iXon2 (Andor). Fluorescence images for ICC were obtained with the 10X objective and an X-Cite Exacte mercury arc lamp illumination source.

Numerical simulation

Mass transport of proteins during cell lysis were simulated in COMSOL Multiphysics 5.3. Input parameters were obtained from literature or were experimentally determined. Partition coefficients for GFP were 0.51, 0.24, and 0.10 for 6%T PA gel to free solution, 15% T PA gel to free solution, and 6%T PA gel to 15%T PA gel, respectively.¹¹ The gel lid was 500 μm in height, the bottom gel was 30 μm in height, and the microwell was 30 μm in height and width. These geometries were input into a 2D axisymmetric model. The maximum and minimum mesh element sizes were 30 and 0.3 μm , respectively. Initial conditions were modeled in the Transport of Dilute Species module as a uniform starting concentration of GFP in a 28- μm diameter cell. The diffusivity of GFP was $4.2\text{E}-12 \text{ m}^2 \text{ s}^{-1}$, $3.13\text{E}-11 \text{ m}^2 \text{ s}^{-1}$, and $1.691\text{E}-10 \text{ m}^2 \text{ s}^{-1}$ in 15%T gel, 6%T gel, and free solution, respectively. The time steps for lysis was 1 s. C_t/C_0 was estimated by taking a volume integral of the bottom gel and microwell.

For MP simulations, the same parameters as above were used. However, the bottom gel was adjusted to 90 μm in height, as this was the measured height of the 6%T gels when allowed to swell to equilibrium in TBST. The 500 μm , 15%T PA lid gel did not change substantially from incubating in TBST. To simulate what would be measured via widefield microscopy, we included a 30- μm wide rectangle directly above the microwell to include the fluid film and lid gel. For the MP, we modeled the species of protein G released from the bead and free to diffuse. We assumed that fluorescence intensity was equivalent to the number of fluorescent particles. The fluorescence of the bead fit the function $y(t)=1135.8*\exp(-0.037*t)$. This fluorescence as a function of time was assumed to be the amount of protein “bound” to the MP. For the free species, the fluorescence was calculated as the difference between C_0 and C_t . The flux, which represents the amount of free protein leaving the bead over the surface area per unit time, of the bead was thus calculated using the expression $N_{\text{free}}(t)= d(y(0)-y(t))/dt * 1/(4*\pi*r^2)$. The final concentration was determined by taking a volume integral of the free species over the surface of the microwell, including the column directly above the microwell encompassing the fluid layer and lid gel, and summing to the measured

fluorescence ($y(t)$) of the MP. The summed values were then normalized to the initial summed value.

Particle tracking

Particle tracking was performed as previously described.^{9,13} 0.5 μ m fluorescent beads were placed on hydrated bottom gels (6%T PA gels). A lid gel (15%T PA gel) was then interfaced to the bottom gel while imaging at 500 ms exposure. The length of the streak lines was quantified to determine the migration velocity.

Image processing and analysis

Fluid film thicknesses were determined from confocal images in a method described by Kuypers et al.¹⁴ Briefly, the fluorescence intensity of the XZ profiles were first background subtracted. The thickness of the fluid film was obtained by determining the “half shoulder” points, which were determined by finding the local minima and maxima and determining the halfway point. The thickness was calculated as the difference in the half shoulder points.

Microparticle fluorescence quantification

Microparticle fluorescence was quantified using an in-house Matlab script for segmentation of the bead. We used a Canny edge detection approach for segmentation.

7.3 Results and Discussion

Characterization of fluid films in electrophoretic cytometry

First, we sought to characterize the thickness of fluid films between hydrogels used in EPC (Figure 1a and 1b). In EPC, single cells are settled into a bottom gel consisting of a 30-90 μ m thick PA gel stippled with microwells. This PA gel microarray is conjugated onto a microscope slide and the monomer concentration (%T) is tunable depending on the desired separation characteristics.¹⁵ Lysis reagents are then delivered to the cells via pouring of lysis buffer or through delivery of a pre-incubated lid gel or gel-glass lid. The lid (Figure 1b) is constructed either of a high density (15% monomer concentration, %T) PA gel or an inverted “bottom gel”, i.e., a microscope slide conjugated with 6%T PA gel (Figure 1a). As the cell lyses, the intracellular contents (starting at a concentration C_0) are released into the microwell and the surrounding gel, and the concentration of protein diminishes due to dilution and diffusion-driven losses as a function of time (concentration C_t).

In previous numerical simulation of diffusion-driven transport of proteins in EPC, the system was modeled as two hydrogels of different density (%T) that were perfectly interfaced with no fluid film.¹¹ However, in hydrogels, interfacial films exist and vary in size according to the topography and morphology of the gel, interfacial tension, interface potential, adsorption, partitioning, and chemistry of the gel,¹⁶ and fluid films between hydrogels and human tissues, such as the cornea, have demonstrated fluid films

ranging from nanometers to tens of microns.^{17–21} Thus, we anticipated non-negligible fluid films to exist between hydrogels and that previous work may have underestimated the diffusive losses of proteins from microwells. In addition, we expected that changing the properties of the lid gel, such as the equilibrium partition coefficient (K_{eq}) would also impact the amount of protein retained in the microwell (Figure 1c). Specifically, we anticipated to improve protein retention in the microwell with decreasing partition coefficient, as fewer protein molecules would diffuse into the lid gel.

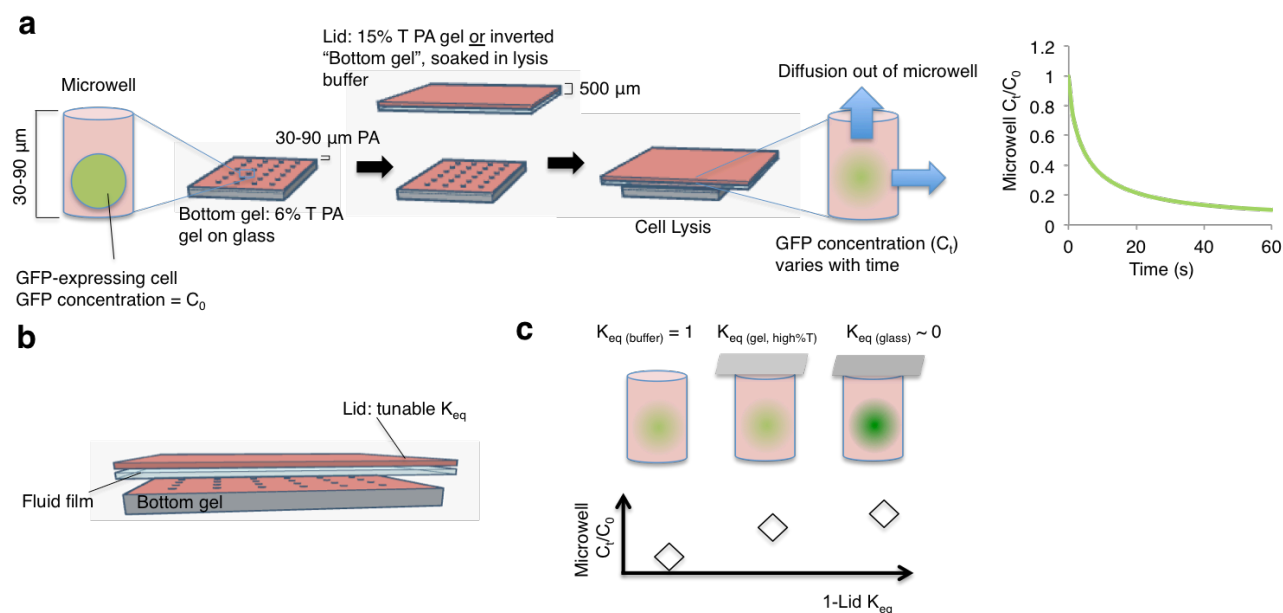


Figure 7-1. Concept schematic of diffusive losses and the effect of partitioning on microwell protein concentration. A) In microwell-stippled bottom gels, which consist of a 6% T PA gel conjugated to a glass slide, individual cells are seated in microwells and contain a concentration, C_0 , of GFP. A lid consisting of either a 15% T PA gel, or an inverted “bottom gel” (6%T PA gel conjugated to a glass slide), is soaked in lysis buffer and then interfaced with the bottom gel to initiate cell lysis but also prevent diffusive losses (e.g., of GFP) out of the microwell. The microwell concentration decreases with time. B) Schematic of a fluid film forming between gels. A lid with tunable K_{eq} is interfaced with the bottom gel. At the interface of the gels, a fluid film forms. C) Schematic of how the microwell concentration is influenced by the K_{eq} of the lid. PA, polyacrylamide; K_{eq} , equilibrium partition coefficient.

To measure the fluid film thickness, we incorporated rhodamine methacrylamide into the PA gels, incubated them in a buffer solution (TBST) overnight, and then imaged the gels and interfaces using confocal microscopy. We measured the thickness of the void between a bottom gel and a 15%T lid gel (Figure 2a), as well as that between a bottom gel and an inverted bottom gel (Figure 2b) in a similar method employed by Kuypers et al.¹⁴ For the bottom gel-15%T PA lid gel interface, we noticed a relatively small range of fluid thickness (Figure 2c). However, for the bottom gel-inverted bottom gel setup, we noticed a much larger range of observable fluid films (Figure 2d and 2f). In addition, 8 of 17 total samples from the bottom gel-15%T PA lid gel setup had non-quantifiable (i.e.,

no resolvable drop in fluorescence in the void) fluid film thickness, whereas only 3 of 15 total were non-quantifiable in the bottom gel-inverted bottom gel setup. The larger range of the fluid film heights from the inverted bottom gel, compared to inverted bottom gel was 1.7-28.8 μm , with a median of 3.7 μm ($n = 12$), whereas the range for the 15% T lid gel was 1.3-1.9 μm , with a median of 1.7 μm ($n = 9$). The variation of the fluid film in the PA-conjugated glass lid system was higher than that of the 15% T lid gel (Levene's Test, $p = 0.007$). For the inverted bottom gel case, we would larger film thickness values, since lower %T gels have increased permeability and contain more voids that permit quick escape of fluid from the gels.²² We also hypothesize that the higher variation is caused by the lower %T gel conjugated to the glass; establishing fluid film is time-dependent and previous reports have demonstrated that more variations occur depending on the permeability of the hydrogel.²³

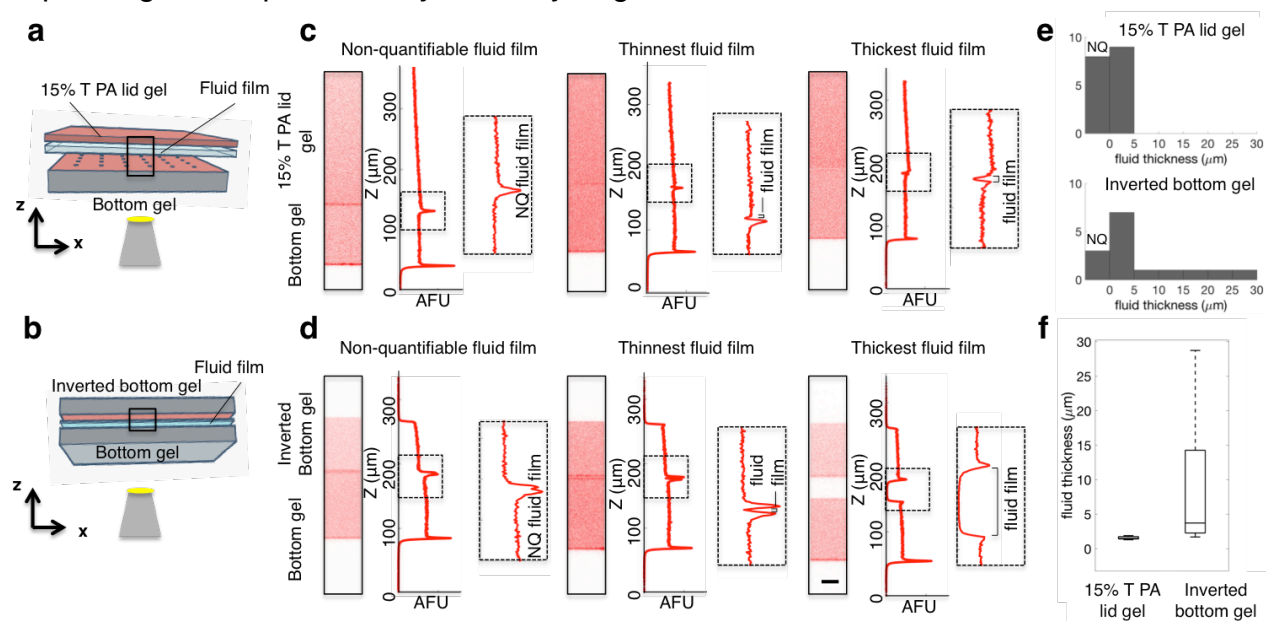


Figure 7-2. Confocal XZ fluorescence micrographs reveal the presence of a thin fluid film between “lid” and substrate gels. A) Schematic of imaging setup of a bottom gel with a 15% T PA lid gel. B) Schematic of imaging setup of a bottom gel covered with an inverted bottom gel. Both gels are 6% T gels conjugated to glass. C) Inverted fluorescence intensity micrographs of the bottom gel interfaced with a 15% T PA lid gel, obtained via confocal microscopy. The two micrographs demonstrate the range of fluid films encountered. D) Fluorescence micrographs of the inverted bottom gel interfaced with the bottom gel and corresponding fluorescence profiles. Two micrographs depict the range of fluid films encountered in this setup, with a zoomed in view on the right. E) Histogram of fluid film thickness measured in the setups from A and B. F) Box plot indicating that the measured fluid film was more variable in the bottom gel-inverted bottom gel interface, compared to the bottom gel-15% T lid gel setup. Scalebar is 25 μm .

Model of diffusive losses of proteins in fluid films

We next sought to characterize how the fluid film thickness and partition coefficient would affect in-gel protein concentrations during lysis. Losses occur during lysis as proteins diffuse and partition between the different media (i.e., gel, free solution). We performed numerical simulation, using GFP as our model protein and previously

reported partition coefficient measurements.¹¹ Using GFP as a model serves as a conservative estimate of intracellular protein losses, since the relatively small (26 kDa) protein has a higher diffusion coefficient compared to the median-sized mammalian protein, which is ~53 kDa.²⁴ The lid was modeled as a 500 μm -thick 15% T lid gel (Figure 3a and 3b) or an inverted bottom gel (30 μm -thick 6%T PA-conjugated glass slide, Figure 3c and 3d). For simplicity, we assumed the partition of coefficient into glass was 0.

Using the simulation, we assessed the concentration of protein retained in the microwell and bottom gel during lysis. To estimate the losses, we determined the concentration of GFP in the microwell and bottom gel as a function of time (C_t) and normalizing to the starting concentration (C_0). In both lid systems, we compared to a perfectly sealed system (i.e., 0 μm fluid film thickness) and also a completely open system (i.e., 500 μm fluid film). A thickness of 500 μm was chosen to represent a completely open system, as this simplification was previously estimated to represent a free solution bath, given the time scales of tGFP diffusion.¹¹ Our model corroborates this estimate, as placing a glass lid 500 μm away from the substrate gel did not significantly alter the concentration profile compared to the 500 μm free solution bath alone (Figure 3a and 3c).

From the concentration profiles, we noted that even in a perfectly sealed system (i.e., fluid film height of 0 μm), using an inverted bottom gel as a lid resulted in substantial losses of lysate in the microwell and bottom gel (C_t/C_0 , Figure 3d). Within 15 s, C_t/C_0 in the gel dropped to 0.61, compared to 0.96 in the 15% T lid gel case. The lower concentration of the in-gel concentrations of the glass-PA lid gel are expected, due to i) the increased diffusivity of GFP in a lower %T gel and ii) a lower partition coefficient of the conjugated gel.

Next, we determined how the median measured fluid film thickness would influence the amount of protein retained in the substrate gel during lysis. Since proteins have a higher diffusion coefficient in free solution compared to in gel, we anticipated lower C_t/C_0 when accounting for the fluid film. With a 15% T lid gel, we noted a substantial drop in C_t/C_0 for a 2 μm fluid film thickness. After 30 s, C_t/C_0 decreases to 0.77, compared to 0.95 in the sealed system. For the inverted bottom gel, we also observed substantial losses from a 4 μm fluid film, with a C_t/C_0 of 0.45 after 30 s. Overall, the inverted bottom gel resulted in lower substrate-gel protein concentrations for the measured fluid heights, compared to the 15% T lid gel case. Given that simulated protein losses were lower in the 15% T lid gel system, we used this system for further analysis on how the interfacial fluid film thickness would influence observed microwell and bottom-gel concentrations.

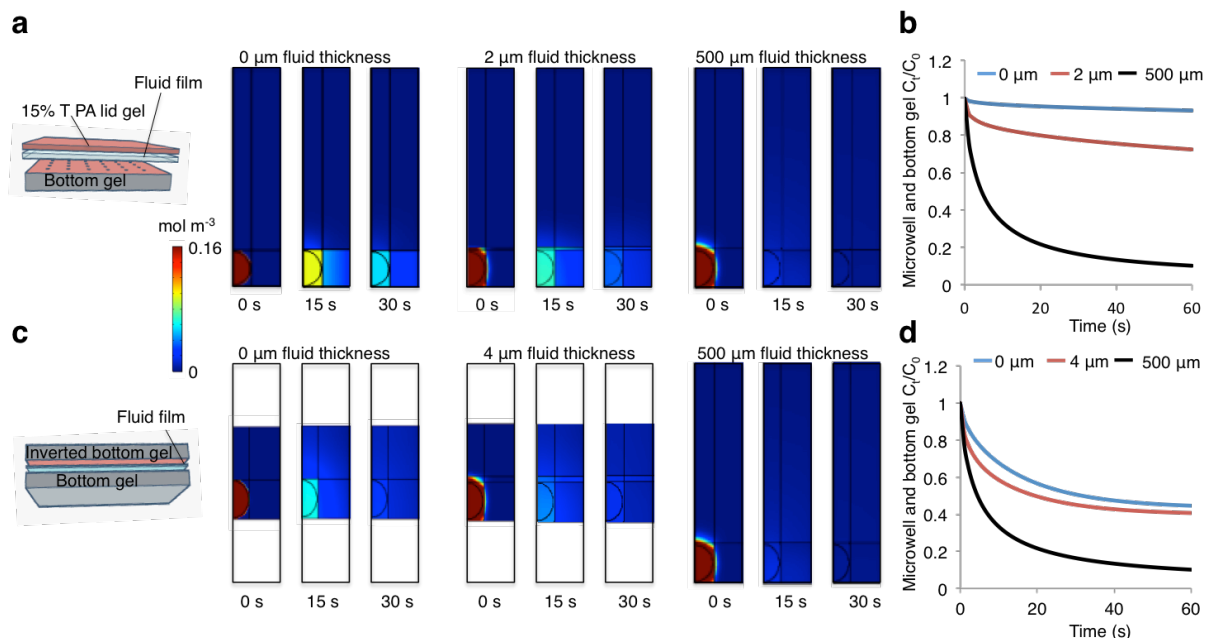


Figure 7-3. Fluid film thickness between lids and bottom gels results in decreased microwell and bottom-gel protein concentration. A) Schematic of COMSOL model setup and simulation results. A 40 μm bottom gel (6%T) is interfaced with a 15%T lid gel, generating a median fluid film thickness of 2 μm . The COMSOL heatmaps depict GFP concentration in a theoretical sealed (0 μm) system, a 2 μm -thick fluid film, and a 500 μm -thick “open” or free-solution only system (no lid). B) Kymograph of the summed microwell and bottom gel GFP concentration, normalized to initial concentration for fluid film thicknesses of 0 μm , 2 μm , and 500 μm . C-D) same as above, but for the bottom gel-inverted bottom gel setup and a median fluid film thickness of 4 μm .

Sensitivity analysis of in-gel concentration to fluid film thickness

To understand how the thickness of the fluid film influences microwell and bottom gel-concentration when using a 15% T lid gel, we performed a sensitivity analysis using the COMSOL model. We varied the fluid film thickness to 5, 10, 20, and 50 μm , and observed the microwell and bottom gel C_i/C_0 at $t = 15$ s, which is halfway through the duration of lysis of a single cell (Figure 4a).⁹ C_i/C_0 drops rapidly with the height of the fluid film, from 0.95 for a 0 μm fluid film thickness, to 0.69, 0.57, 0.42, and 0.28, for 5, 10, 20, and 50 μm fluid film thicknesses, respectively. At 20 μm , the change in C_i/C_0 as a function of fluid film thickness (i.e., the slope of the curve) decreased below 2%. These results indicate that at 20 μm fluid film thickness, the free solution volume begins to behave as a semi-infinite medium. Overall, the fluid film thickness plays a major role in estimated substrate-gel concentrations and design strategies to minimize this fluid film thickness are critical.

Next, we sought to test the sensitivity of the microwell and bottom-gel concentration to partition coefficient (K_{eq}) of the lid, given our median-observed, 2 μm fluid film thickness. We set K_{eq} to 0 to simulate non-partitioning of GFP in free solution to glass, compared to 0.24 in free solute ion to a 15%T lid gel (Figure 4b). From our simulations, we observed the microwell and bottom gel C_i/C_0 ($t=15$ s) to be 0.81 with a 15% T PA lid gel

compared to a 0.91 if the bottom gel were paired with a glass lid. Similarly, with thicker fluid films, C_t/C_0 depends on the partition coefficient of the lid up to the 20 μm thickness. For a 10 μm fluid film, C_t/C_0 at $t=15\text{s}$ is 0.58 for a 15%T PA lid gel compared to 0.67 for a glass lid, whereas for a 20 μm fluid film thickness, C_t/C_0 at $t=15\text{s}$ is 0.42 and 0.44 for a 15%T PA lid gel and glass lid, respectively. These *in silico* results are self-consistent and imply that 20 μm fluid film thickness is a critical distance after which the fluid film behaves as a semi-infinite medium.

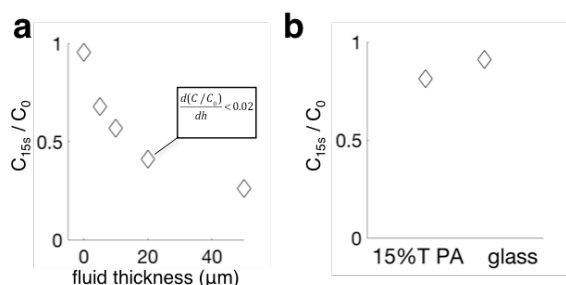


Figure 7-4. *In silico* sensitivity analysis indicates in-gel protein concentration is dependent on fluid film thickness and partition coefficient of the lid. A) The summed microwell and bottom-gel GFP concentration at 15s was obtained and normalized to the initial concentration (C_{15s}/C_0). The C_{15s}/C_0 drops rapidly as a function of fluid film thickness, stabilizing to <2% change at a 20- μm fluid film thickness. A thicker fluid film allows a greater amount of protein to be lost to the fluid film, where the diffusivity of GFP is high. B) For a 2- μm fluid film thickness, the partition coefficient also influences the substrate-gel concentration.

Experimental validation of model

We next sought to experimentally validate our model of microwell and bottom gel losses of protein. In our model of cell lysis, we assumed an initial uniform concentration of GFP within the cell. However, this simplification means that we do not account for solubilization times of the plasma membrane,⁹ which takes on the order of $\sim 10\text{ s}$ and can vary from cell to cell,²⁵ or of denaturation of fluorescent proteins that occurs. Thus, instead of modeling cell lysis, we utilized and modeled a microparticle (MP)-based chemistry for rapid release of proteins (Figure 5a). Using MPs, his-tagged proteins are loaded onto a 10 μm MP and are released on the order of seconds upon delivery of imidazole. The release profiles are used as protein standards in electrophoretic cytometry (J. Kim, et al. *unpublished data*), and the MP approach is advantageous over alternative approaches for protein release such as from protein-PA conjugations^{26–28} photolabile polymers^{29–31}, caged particles³², photoactivatable probes³³, drug-releasing nanogels³⁴, photo-assembling and photo-cleavable microcapsules³⁵, photocontrolled release micelles³⁶, protein-protein conjugations, and click chemistry, which have long “switching” periods (order of minutes to hours), low efficiency, or poor spatial control.

To input the release kinetics of the MPs into our model, we first characterized the release of Alexa Fluor 647-labeled protein-G from MPs placed in a microwell and interfaced with a 15%T PA gel lid. We selected protein G, as this protein is commercially available and has the same molecular mass as GFP. These MPs were loaded into

individual microwells, as previously described (J. Kim, et al. *unpublished data*), then interfaced a 15%T PA lid gel that was pre-incubated overnight in imidazole. The imidazole diffuses into the well within 1 s (data not shown). We measured the fluorescence intensity of the MP as a function of time (Figure 5b), normalized to the initial fluorescence intensity (Figure 5b). The fluorescence decay was exponential and matched our previously reported model of imidazole delivery using a pour system ($y = 1.2789 \cdot \exp(-0.035 \cdot x)$, $R^2 = 0.98$, compared to $y = 1.18 \cdot \exp(-0.04 \cdot x)$ reported in J. Kim, et al. *unpublished data*).

From the fluorescence profiles of the MPs, we input a flux boundary condition in our COMSOL model. First, we assumed the raw fluorescence intensity after background subtraction was equivalent to the number of fluorescent molecules/proteins. Since we normalize the final number of fluorescent molecules to the initial, the absolute number of fluorescent molecules is not important. Next, we defined a flux boundary condition for the protein that is released from the MP into the microwell and surrounding fluid films and gels. The flux ($N(t)$) was defined as:

$$N(t) = \frac{d(\text{Number molecules}(0) - \text{Number molecules}(t))}{dt} * \frac{1}{4\pi r^2 * s}$$

where r is the radius of the MP ($5E-6$ m). We used the same partition coefficients for a 6% T and 15% T PA lid gels and diffusion coefficients for GFP and input a 2 μ m-thick fluid film (Figure 5c). Advection from the lid gel placement was assumed to be negligible, as the Péclet number was two orders of magnitude lower than in a pour system (Figure 6).

Since we measured fluorescence of the microwell as a function of time, which represents a 2D projection of a 3D volume, we aimed to also simulate the amount of protein that would be visible via epifluorescence microscopy. Thus, we obtained a volume integral of the protein molecules in the surface arising from the 30 μ m-wide microwell, including the 30 μ m width of the (2- μ m thick) fluid film and 15% T PA lid gel (Figure 5c). To obtain the microwell fluorescence, we summed the integral at each time point with the measured fluorescence of the MP at each time point. The summed value was then normalized to the initial summed value ($t=0$) to obtain the normalized microwell fluorescence value. Results from our model correlated reasonably well with our experimental results (Figure 5c and 5d). The discrepancy in the model likely arises from self-quenching of fluorophores in the experimental condition; at higher concentrations, cyanine dyes, e.g., Cy5 with similar spectra as Alexa Fluor 647, aggregate, causing quenching of up to 90% of fluorescence signal until the dye molecules are spaced sufficiently far apart.³⁷ Nonetheless, the similar slopes of the curves indicate that our model can predict diffusion of proteins between hydrogel layers and fluid films, and that our estimates of losses during cell lysis can accurately reflect protein diffusion, decoupled from the effects of lysis and protein denaturation.

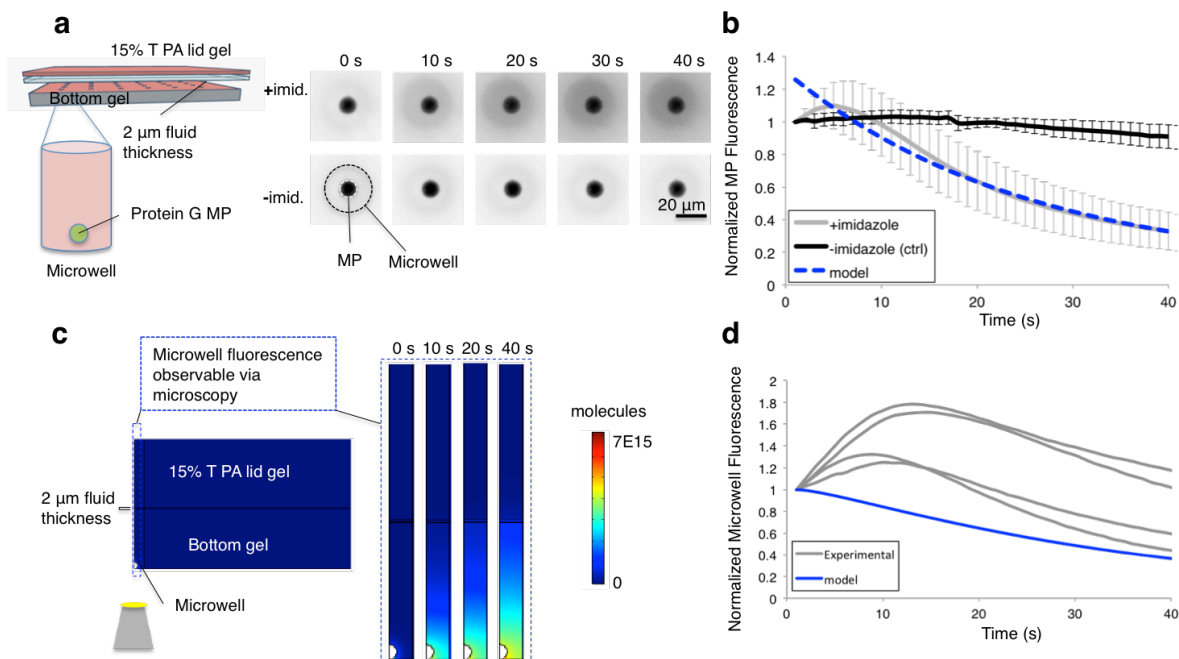


Figure 7-5. Experimental validation of simulation using protein-release microparticles. A) Schematic of experimental setup and inverted fluorescence micrographs of release of protein G (26 kDa) from MPs at varying time points. A bottom gel was loaded with protein G-MPs. At $t = 0$ s, a 15% T PA lid gel soaked in imidazole buffer was interfaced with the bottom gel to initiate protein release. B) The fluorescence intensity of the bead at each time point, normalized to the initial concentration, decreases exponentially. C) An exponential function for the fluorescence decrease was input as a concentration boundary condition of a $10 \mu\text{m}$ sphere in an axisymmetric model in COMSOL. The flux of the bead, simulating the amount of GFP released per unit area, was calculated as the number of molecules released per unit time, divided by the surface area of the sphere. The heatmaps depict the expected concentration of GFP from a volume that contains the microwell, as well as the fluid film and 15%T PA lid gel directly above the microwell. This volume was selected to represent what would be observed via epi-fluorescence microscopy. D) Predicted normalized microwell fluorescence of the microwell from the model match reasonably with experimental replicates.

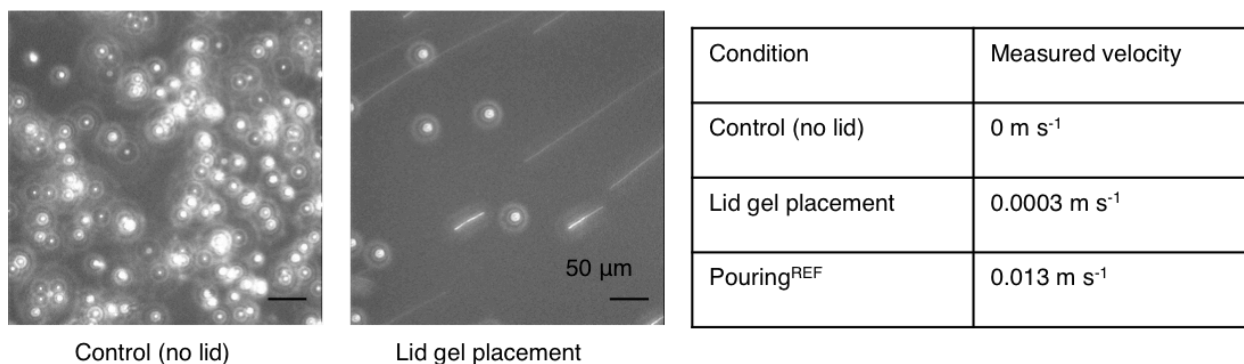


Figure 7-6. Particle Velocimetry reveals advection induced by lid gel placement that is two orders of magnitude lower than in a poured buffer system.

7.4 Conclusions

Understanding protein transport in hydrogels has applications in drug delivery, tissue engineering strategies, and immunoassays. Here, we characterized fluid film thicknesses that occur between interfaces of hydrogels, to better understand where losses occur during electrophoretic cytometry. We next input these film thicknesses into numerical simulations, and validated our model results using protein-releasing microparticles. Overall, these results allow for accurate estimation of losses due to diffusion during cell lysis. The ability to accurately recapitulate hydrogel systems and determine the losses from microwells lend insights into design criteria for higher protein retention. For example, incorporating lid gels that possess higher % T lid gel; e.g., 20% T lid gel conjugated to glass can aid to reduce diffusive losses that occur during cell lysis. Ultimately, these insights have implications in engineering design for a variety of applications where protein transport through hydrogels needs to be precisely tuned for success of the device or measurement tool.

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Chapter 8.

Conclusions and Future Directions

In this dissertation, we identified measurement gaps in single-cell protein analysis and sought to address them by elucidating the underlying physics that drive losses in analytical sensitivity and designing novel microfluidic tools for assaying proteins from single, adherent or rare cells. The culmination of sample preparation ranging from materials engineering to precise patterning of single cells, along with *in situ* separation and detection in single microfluidic tools, enable sensitive and highly-selective protein measurements.

While we have explored protein measurements from single cells, a future area of interest is integration with other complementary protein measurement techniques, such as mass spectrometry (MS). Exciting progress has been made on single-cell MS measurements,¹ but immunoassays can provide complementary information on target isoforms and relative quantitation. Moreover, while proteins are the signal transducers of the cell, there is also considerable interest in other macromolecules, such as nucleotides (RNA, DNA), lipids, and carbohydrates. An integration of protein assays with a suite of different –omics (e.g., transcriptomics, secretomics, metabolomics) would provide a comprehensive picture of the cell and the many intricacies and subtleties afforded by biology.

Finally, while we have discussed some of the design tradeoffs of electrophoretic cytometry, some of these limitations are inherent to our limited understanding of systems biology. For example, while there is no “magic bullet” for protein solubility, a better understanding of protein structure and folding could enhance rational design for microfluidic assays. We anticipate expanding protein databases and predictive *in silico* models of protein folding will usher in new insights into targeted and discovery proteomics approaches.

Overall, novel tools to better understand single cell biology will in turn lead to more effective targeted strategies for advancing and engineering biology.

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