Using Design of Experiments to Optimize Microfluidic Transfection Platform for Cas9 RNP and mRNA Delivery in Various Primary Cell Types

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Introduction

We have developed a microfluidic consumable designed to deliver gene modifying payloads into human primary cells using mechanoporation. However, due to the large microchannel parameter space, thorough optimization for a specific application, or cell types, can require lengthy development times. We use a statistical approach to multivariate optimization using design of experiments (DoE) and response surface methodology (RSM) to assess the effects of several parameters and simultaneously optimize for maximal transfection efficiency and cell viability. Using this approach, we can decrease the number of conditions necessary to find an optimal device parameter operating window. To test this approach, we delivered enhanced green fluorescence protein (EGFP) messenger RNA (mRNA) in primary hematopoietic stem/progenitor cells (HSPCs) as a proof of concept. In addition, we expanded this approach to the transfection of either mRNA or Cas9 ribonucleoprotein (RNP) complex into fresh primary peripheral blood mononuclear cells (PBMCs) and CD3+ T-Cells subpopulations, respectively, due to the clinical needs for T-cell engineering for cell therapies.

Objectives

- To discover a working set of instrument and microchannel parameters that simultaneously optimize for maximum mRNA and RNP transfection efficiencies and cell viability.
- To evaluate the effects of the design and test variables on the outcome cell metrics.
- To show the accuracy of the reduced response surface models created using DOE methods.

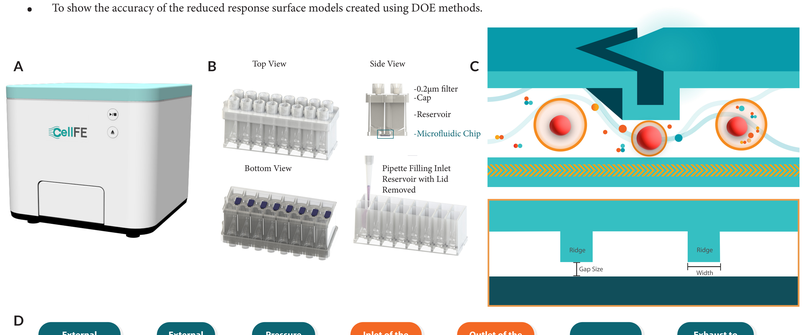

Figure 1| The CellFE Zephyr and Microfluidic Device for Intracellular Delivery via Mechanoporation.
A) The CellFE Zephyr (approximately 11.5 x 11.5 x 11 in) is a Research Use Only instrument that can process cell samples using pressure-driven flow. B) Images of the CellFE microfluidic device (approximately 3 x 1.25 x 1.5 in) from different viewpoints, which has the capacity to process 8 individual cell samples with sterile inlet and outlet reservoirs. C) The top image shows a cross section of the microfluidic chip (Figure1. B Side View) that the cells and payload pass through that creates the transfection event. The bottom image defines the parameters of gap size, ridge width, and number of ridges explored in this study. D) A simplified flow chart showing the inner mechanisms of the CellFE Zephyr instrument that allows it to process the samples in the consumable. Teal shows instrument components, while orange shows components of the microfluidic device.

Experimental Design

For all applications in this study, cell health was characterized using standard AO/DAPI staining (solution 13; ChemoMetec, DE) with NC-3000 automated cell counter (ChemoMetec) to assess cell viability and cell count. All the antibodies that were used for flow cytometry in this study were manufactured by Miltenyi Biotec. The design of the experiment conducted was generated using JMP software (JMP International, NC) to create a D-Optimal design for a response surface model (RSM), which includes all main effects, two-way interactions, and quadratic terms for the parameters tested (see Table 1 for parameters explored in this study, exceptions are noted in the respective applications experimental design section). Analysis of the resulting data was also completed using JMP to fit the data to the RSM and perform the subsequent backward stepwise model reduction was conducted by minimizing the Akaike Information Criterion (AIC). A residual maximum likelihood model (REML) was constructed for each response of interest, mRNA and CRIPSR RNP transfection/editing efficiency and viability, to assess fixed parameters, such as microchannel design, instrument parameters, and the relevant random parameters.

Factor	Design Role	Levels		
Device	Blocking (8 runs) & Random	1	2	3
Donor	Categorical [Random]	A	B	
Gap Size	Continuous	-1	0	1
Supply Pressure	Continuous	-1	0	1
Ridge Number	Discrete Numeric	-1	0	1
Ridge Width	Continuous	-1	0	1

Table 1| Factors of Interest for Design of Experiments
A list of factors, or parameters, explored in this study along with the role that it played in the experimental design and analysis. The level of device shown indicates run order for each block of 8 samples per device consumable. Exact measurements of levels tested are coded as -1, 0, 1 for the minimum, midpoint, and maximum values tested, respectively.

Hematopoietic Stem / Progenitor Cells (HSPCs)

Frozen, human CD34+ primary cells (AllCells, CA) were obtained from two different cell donors and were resuspended in X-Vivo 10 media (Lonza, MD) with 50µg/mL of eGFP mRNA (TriLink Biotechnologies, CA) and processed with the CellFE Zephyr (CellFE Biotech, CA). Phenotypes were analyzed with flow cytometry (CytoFlex; Beckman Coulter, CA), after 1 day, primarily eGFP to verify mRNA delivery, CD34 & CD38 expression to verify stemness, and and SYTOX AADvanced (viability stain). By treating the factors of device and donor as random effects, we can make inference on the population of device consumables and potential donors that could be tested. Total sample size for this experiment is N_{HSPC} = 24, n = 12 per cell donor. A follow up study was conducted to confirm the model predictions where 2 proposed optimized conditions were tested and compared to verify values.

Peripheral Blood Mononuclear Cells (PBMCs)

Freshly sourced human peripheral blood leukopak (StemCell Technologies, Vancouver, Canada) from 2 cell donors was obtained, isolated into its PBMC subpopulation. The resulting PBMCs were immediately resuspended to a density of 3.5x107 cells/mL in serum-free CTS OpTimizer T-cell expansion media (ThermoFisher, MA) containing 100 mM of D-Mannitol with 70µg/mL of eGFP mRNA (TriLink Biotechnologies, CA) and processed with the CellFE Zephyr (CellFE Biotech, CA). Health and phenotypes of the cells were characterized using methods stated earlier. Flow cytometry was done to observe eGFP, CD4, CD8, and SYTOX AADvanced (viability stain) expression. The random factor of device was not explored in this study, a random block was added to explore the effect, if any, of performing the experiment on a different day. The original D-optimal design, which contained 15 runs, was augmented and 15 new runs were added to explore the confounded effect of experiment day and cell donor. The total sample size for this experiment, after augmenting the design, is N_{PBMC} = 30, n_{Block} = 15.

T Cells (CD3+)

Freshly sourced human peripheral blood leukopak (StemCell Technologies, Vancouver, Canada), from 3 cell donors was obtained, isolated into its CD3+ subpopulation, activated with a 1:100 part per volume ratio of amount of TransACT (Miltenyi Biotec, DE). After culturing for 48hr, the cells were resuspended to a density of 4.0x107 cells/mL and combined with 25µg/mL of pre-complexed CRISPR/Cas9, sgRNA:Cas9 molar ratio of 3:1, and processed with the CellFE Zephyr (CellFE Biotech, CA). Viability and phenotypes of the cells were characterized using methods stated earlier. Flow cytometry was done to observe TCR-a/b, CD4, CD8, and SYTOX AADvanced (viability stain) expression. This experiment was designed to have 4 random replicates built into the design to assess variance. The original D-optimal design, which contained 15 runs, was augmented twice and 15 new runs were added each time to explore the confounded effect of experiment day and cell donor. The total sample size for this experiment, after augmenting the design, is N_T = 45, n_{Block} = 15. A follow up study was conducted to verify the model predictions where 2 proposed optimized conditions were tested and compared to expected values.

Results

mRNA Delivery in Hematopoietic Stem / Progenitor cells (HSPCs)

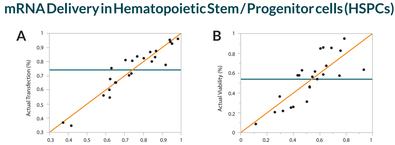

Figure 2| Actual vs Predicted Plots for Transfection Efficiency and Viability.
Orange diagonal line indicates line of fit from reduced model. Teal horizontal line indicates response average. A) A comparison of actual mRNA transfection efficiency measures and model predictions indicate a good fit (RSq=0.94, RMSE =0.0542, p<0.0001, N=24). B) A comparison of actual viability measures and model predictions indicates a good fit (RSq=0.79, RMSE =0.1241, p=0.0006, N=24).

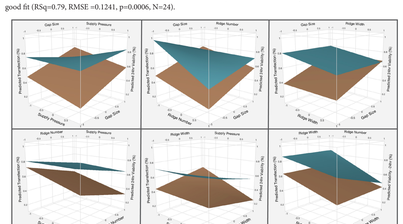

Figure 3| Surface Profilers for Transfection Efficiency and Viability.
Response surface plots showing the predicted mRNA transfection (shown in blue) and viability (shown in orange) values by a combination of two factors of interest. This allows for visualization of model effects in 3-dimension. A curvature in the response surface indicates a significant interaction between the two plotted factors that remained in the model. Example: top left image shows an interaction between gap size and pressure that is significant in modeling transfection efficiency.

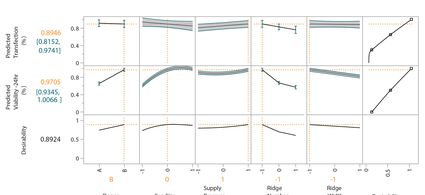

Figure 4| Prediction Profiler to Maximize Desirability for Transfection Efficiency and Viability.
A snapshot of the prediction profiler that is used to obtain an optimal set of factor levels to achieve a simultaneously maximized mRNA transfection efficiency and viability. Curves for each response and factor combination are generated by holding the level of each factor level constant indicated by the orange vertical dashed lines. Shaded area around the curve shows 95% prediction interval and teal text on the y-axes display those values at the constant factor level.

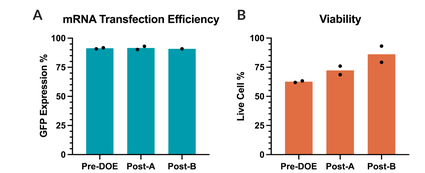

Figure 5| Confirmation of Predictions
Results of a confirmations study testing 2 potential optimized conditions suggested by DOE predictions. Each data point presented for each condition denotes a unique cell donor (n=2). A) High mRNA transfection efficiencies were maintained after device optimization. Transfection efficiencies shown confirm model predictions for the device and instrument settings tested (Condition A Predicted = 87.05% +/- 6.63%; Condition B Predicted = 99.69% +/- 7.50%). B) Cell health was able to be improved by more than 20%, as measured on Day 0 after mechanoporation. Model predictions were accurate and within prediction intervals for the optimized parameters. Viabilities shown confirm model predictions for the device and instrument settings tested (Condition A Predicted = 68.87% +/- 23.29%; Condition B Predicted = 82.71% +/- 22.08%).

mRNA Delivery in Peripheral Blood Mononuclear Cells (PBMCs)

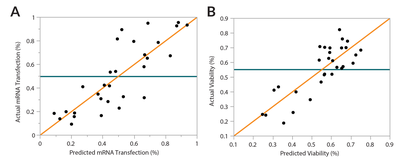

Figure 6| PBMC mRNA Delivery Actual vs Predicted Plots for Efficiency and Viability.
Orange diagonal line indicates line of fit from reduced model. Teal horizontal line indicates response average. A) A comparison of actual mRNA transfection efficiency measures and model predictions indicate a good fit (RSq=0.67, RMSE =17.893, p<0.0001, N=30). B) A comparison of actual viability measures and model predictions indicates a good fit (RSq=0.67, RMSE =10.529, p=0.0006, N=30).

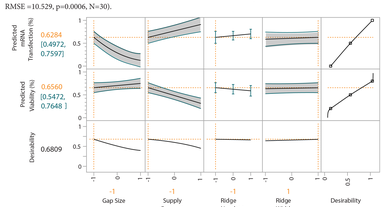

Figure 7| Prediction Profiler to Maximize Desirability for Transfection Efficiency and Viability.
A snapshot of the prediction profiler is used to obtain an optimal set of factor levels to achieve a simultaneously maximized mRNA transfection efficiency and viability. Curves for each response and factor combination are generated by holding the level of each factor level constant indicated by the orange vertical dashed lines. Shaded area around the curve shows 95% prediction interval and teal text on the y-axes display those values at the constant factor level.

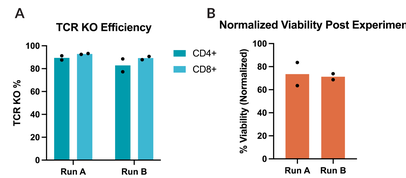

Figure 8| Results of Optimized Conditions
Results of DOE testing with 2 distinct PBMC donors transfected with eGFP mRNA. No pre-DOE reference condition was available due to this being the initial test performed in this application. A) Several channel designs and processing conditions also led to high mRNA transfection efficiency (80 – 95%). While the top results show similar outcome metrics the nature of the design and processing conditions is very different bringing functional benefits to the mechanoporation process including decreasing processing time and increasing cell throughput. B) Cell health was maintained with several conditions evaluated in the DOE, as measured 1-day after mechanoporation.

T Cells (CD3+) Knock-Out using CRISPR RNP

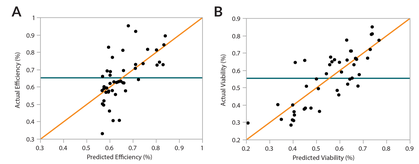

Figure 9| Actual vs Predicted Plots for CRISPR RNP Knock-Out Efficiency and Viability.
Orange diagonal line indicates line of fit from reduced model. Teal horizontal line indicates response average. A) A comparison of actual TCR Knock Out efficiency measures and model predictions indicate a good fit (RSq=0.32, RMSE =0.1142, p=0.0023, N=45). B) A comparison of actual viability measures and model predictions indicate a good fit (R Sq=0.79, RMSE =0.084, p<0.0001, N=45).

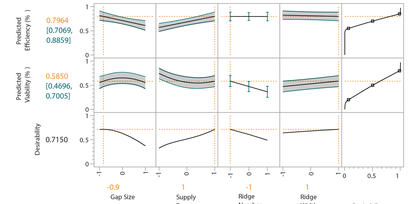

Figure 10| Prediction Profiler to Maximize Desirability for Transfection Efficiency and Viability.
A snapshot of the prediction profiler used to obtain an optimal set of factor levels to achieve a simultaneously maximal TCR Knock Out efficiency via CRISPR RNP and viability. Curves for each response and factor combination are generated by holding the level of each factor level constant indicated by the orange vertical dashed lines. Shaded area around the curve shows 95% prediction interval and teal text on the y-axes display those values at the constant factor level.

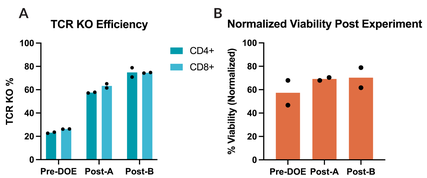

Figure 11| Confirmation of Model Predictions
Results of a confirmation study testing 2 more optimal conditions, relative to initial design/processing parameters as suggested by DOE predictions. The increase shown from an independent experiment is as expected from model predictions, which leads us to believe its accuracy. A) The new channel design and processing conditions led to a consistent increase in Knock Out efficiency of more than 35% of the TCR-a/b in both the CD4+ and CD8+ expressing populations of T-cells compared to pre-DoE reference condition. Each dot represents a distinct CD3+ T-cell donor. Viabilities shown confirm model predictions for the device and instrument settings tested (Condition A Predicted = 57.46% +/- 8.81%; Condition B Predicted = 64.13% +/- 15.72%). B) Cell health was maintained with the change in parameters, as measured on 1-day after mechanoporation. Viabilities shown confirm model predictions for the device and instrument settings tested (Condition A Predicted = 64.13% +/- 11.10%; Condition B Predicted = 59.35% +/- 10.94%).

Conclusion

In this set of experiments, we aimed to quickly assess the importance of device and instrument parameters for maximizing transfection and viability in three different primary cell types (HSCs, PBMCs, and CD3+ T-cells) with two different payloads (mRNA and CRISPR RNP). By using DOE methodologies, we were able to quickly assess the importance of these parameters and compare trends between the various cell types and payload. DOE has allowed for less iterations of experimentation, each with fewer runs necessary to acquire quality data while assessing for statistical significance. In future studies, we plan to extend the use of these methods to a further variety of cell types and payloads, further optimizing our technology.

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