Optimizing Transfection Efficiency with High-throughput Live Cell Imaging

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Abstract

Many bioengineering applications require optimized DNA transfection efficiency in the biological system of interest. But developing a transfection protocol involves manipulating multiple variables, followed by several lengthy rounds of iterations to achieve the optimal transgene transfection efficiency. The main experimental variables that usually affect lipofection-mediated transfection efficiency are cell seeding density, transfection reagent concentration and DNA amount. With high-throughput live cell imaging and analysis, these variables are quickly assessed in a controlled and optimal environment. In this application note, the CELLCYTE X was used to monitor in real time the expression of the fluorescent reporter mCherry protein to achieve optimal transfection efficiency of a pcDNA3.1 vector in HEK 293T/17 cells. In addition, transfection efficiency was further evaluated by examining the penetrance (proportion of cells being transfected) and expressivity (the relative degree of expression of a transgene in a single cell).

Introduction

The introduction and expression of foreign genetic material into mammalian cells is a major scientific advancement whose application has enabled the emergence of genetic engineering. The procedure is known as transfection and involves inserting DNA vectors into cultivated animal cells. Transfection is a very valuable tool with broad applicability, from exploratory research to clinical therapies, but wider adoption is hindered by numerous technical challenges. The first challenge is that plasma membranes of animal cells are physiologically impermeable to DNA vectors. To overcome this impermeable barrier, different biological, mechanical and chemical processes have been devised to increase the permeability of cells selectively and temporarily to nucleic acids. Each methodology comes with unique advantages and disadvantages that must be weighed against the scientific question (Kim, 2010).

The many benefits of live cell imaging include enabling the real-time quantitative assessment of transfection efficiency in biological systems. The ideal transfection conditions are the ones that ensure maximal transgene expression, minimize impact to cellular physiology while requiring less intervention. Therefore, imaging cells in standard humidified incubators at 37°C and 5% CO2, while keeping culture plates undisturbed for days or weeks, allows the user to obtain more physiological and quantitative data, essential to determining robust transfection conditions.

Cellular confluency as an indicator of cellular health can be tracked with the CELLCYTE X from images acquired with the Enhanced Contour imaging feature, a form of high-contrast transmission light-based microscopy. In parallel, the uptake and expression of transgenic materials can be assessed in real time from the same experiment with fluorescent microscopy when the genetic product is fluorescent. The CELLCYTE X allows the user to monitor the expression of fluorescent proteins of interest in the blue, green and red portions of the visible spectrum, either individually or all at the same time.

This application note demonstrates how the CELLCYTE X can be used to optimize the transfection of a pcDNA3.1 vector in HEK 293T/17 cells, using the red fluorescent protein mCherry and Lipofectamine 2000 as a chemical-based transfection agent. An example of the high-throughput image analysis that the CELLCYTE X makes possible in 96- or 384-well plate formats is showcased.

Materials and methods

Cell culture

HEK 293T/17 cells (ATCC, CRL-11268) were cultured in DMEM High Glucose 4.5 g/L media (Gibco, 31053-028) supplemented with 10% fetal bovine serum (Gibco, 10270-106), 1 mM sodium pyruvate (Gibco, 11360-070), 2 mM GlutaMAX supplement (Gibco, 35050-038), and 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco, 15140-122). The day before the transfection experiment, cells at a confluency of about 80% to 90% in T75 flasks were detached by incubation with 3 mL of TrypLE Select Enzyme 1x (Gibco, 12563029) for 5 minutes at 37°C. Viability was assessed with Trypan Blue exclusion and only cells displaying values above 85% were deemed healthy enough for transfection. hese healthy cells were appropriately diluted in complete media and seeded in 100 µL per well on top of 90 µL of complete media predistributed in a 96-well plate. To achieve

optimal seeding homogeneity across each well's surface, the plates were left undisturbed at room temperature for about 30 minutes before being transferred to an incubator at 37°C and 5% CO_2.

Lipofectamine-mediated DNA transfection

Sixteen hours after seeding, cells were transfected via lipofection. DNA vectors (CYTENA) and Lipofectamine 2000 (Thermo Fisher, 11668027) were each diluted separately in 5 µL of Opti-MEM I Reduced Serum medium (Gibco, 31985-070) per reaction well. Loaded lipoparticles were obtained by combining equal volumes of the two solutions and were subsequently incubated for 5 minutes at room temperature. Then, 10 µL of the mixture was added in each well on top of 190 µL of complete DMEM media.

This study evaluated a matrix of conditions of lipoparticles made of 0.2 µL to 0.5 µL of Lipofectamine 2000, loaded with a combination of 20, 50 or 100 ng per well of pcDNA3.1 vector, either with or without the transgene codifying for the mCherry-B-catenin protein (Ehyai, 2018), at three different cell seeding densities of 10,000, 20,000 or 40,000 cells per well. Each combination was assessed in technical triplicates, and images were acquired with the CELLCYTE X every 4 hours using the Enhanced Contour mode and the red channel (400 milliseconds exposure, 4 dB of gain) with four fields of view (FOVs) per well.

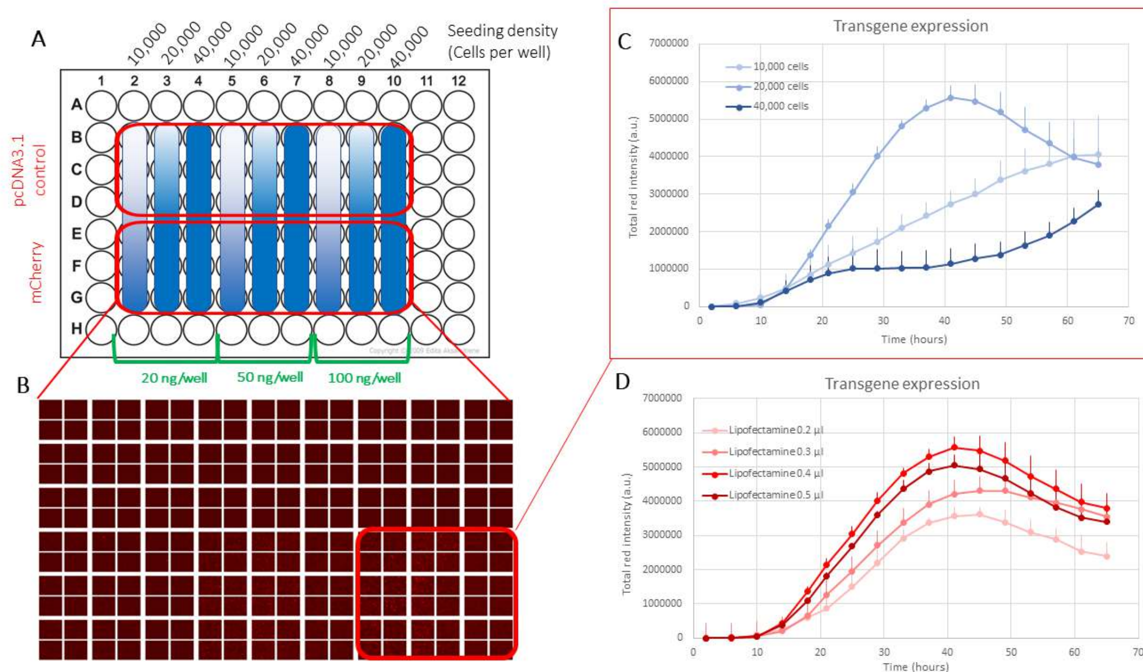

Figure 1. A) *Plate map schematic of a 96-well plate treated with different amounts of DNA vector. B) CELLCYTE's plate map view after 48 hours in the red channel with 4 FOVs per well. C) Total fluorescent intensity signal of different cell numbers transfected with 100 ng of mCherry DNA vector and 0.4 µL of Lipofectamine 2000. D) Total red fluorescence signal intensity in 20,000 HEK 293T/17 cells transfected with 100 ng of DNA and the indicated amount of Lipofectamine 2000. Time points are shown as averages of the signal, and error bars indicate the standard error of 3 wells.*

Results and discussion

Optimizing transfection efficiency protocols using live cell imaging

High-throughput culture plates, such as 96- or 384-well plates, allowed the study in parallel of a vast library of experimental conditions with minimal amounts of consumables and time. Technical variability was reduced by averaging data from

multiple replicate wells and acquiring multiple FOVs per well. This study evaluated three main factors dictating optimal transfection efficiency, namely cell concentration, Lipofectamine 2000 volume and the amount of DNA to be transfected. In a single 96-well plate, in triplicate wells, the use of different numbers of seeding cells and different amounts of mCherry expressing vector were evaluated, controlling for the respective empty DNA vector control **(Figure 1A)**. Forty-eight hours after transfection, images displayed in CELLCYTE Studio **(Figure 1B)** confirmed that the maximum mCherry expression could be achieved by delivering higher amounts of DNA in 20,000 cells per well. The quantitative measurement of total red fluorescence intensity over the length of the study offered by the CELLCYTE Studio confirmed this finding **(Figure 1C)**. With these two parameters, the ideal volume of Lipofectamine 2000 was then pinpointed; among the tested volumes, 0.4 µL per well offered the highest expression of the transgene **(Figure 1D)**. The third most important experimental variable dictating transfection efficiency is the amount of DNA being transfected. In particular, we saw a dose-dependent relationship, whereby the highest amounts of DNA were resulting in the highest expression of mCherry **(Figure 2A)**. The total red intensity metric only included the total fluorescent output of cells and not the intensity of the background signal. This was obtained by extrapolating intensity data only from high-fidelity masks of the cells of interest **(Figure 2B)**. This highly sensitive, and specific fluorescence signal displayed a peak 2 days after transfection. Parallel analysis of cell proliferation at the different cell densities showed that 20,000 cells reached full confluency at this specific time point **(Figure 2C)**, indicating how an exponential growth is needed for a continuous expression of mCherry in HEK 293T/17 cells. High amounts of DNA and Lipofectamine 2000 can also affect cell health. Nonetheless, even with the highest amounts of DNA and optimal amounts of Lipofectamine 2000, we saw no impact on cellular growth **(Figure 2D)**, making these conditions ideal for this cell line.

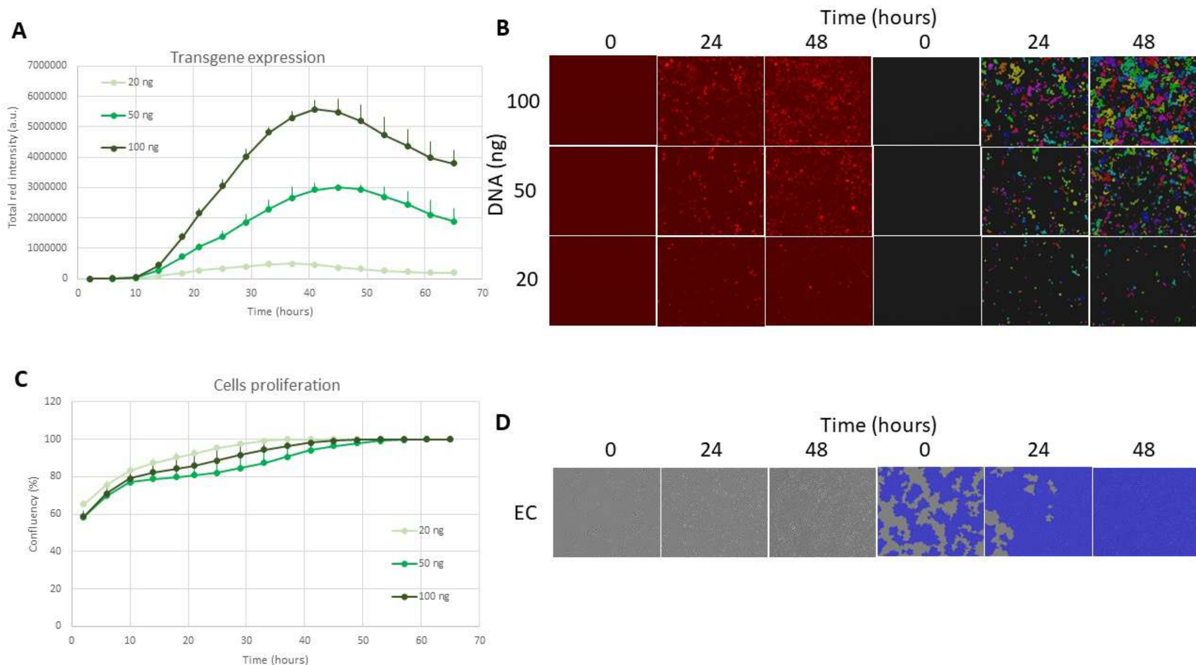

Figure 2. *Representative images and co-culture study analysis of MDA-MB-231 cells expressing either GFP or mCherry. (**A**) The proliferation of a heterogeneous mixture of MDA-MB-231 cells stably expressing either GFP or mCherry was monitored by acquiring the indicated timepoint images in the green and red fluorescent channels (top row) using 100 ms of exposure 0 dB of gain and 350 ms of exposure and 5 dB of gain, respectively. (**B**) Quantitative analysis of the cell confluence in EC without consideration of fluorophores expression (black line) or for each individual fluorescent channel. The same mask was used to compute the fluorescent object count metric (**C**) and the fluorescent intensity metrics (**D**, **E**, **F**). Cell population average fluorescence intensity calculated for each field of view in the respective channel (**E**), and population fluorescence intensity plot at the individual cell level at 72 hours (**F**). All data are shown as representative averages ± SEM of 2 FOVs. In (**F**), all cells detected in 2 FOVs were included.*

Kinetic assessment of transfection expressivity and penetrance

There are different ways to assess the efficiency of a transgene expression, and the CELLCYTE Studio software can help answer those questions. One possible way is to consider its relative magnitude in relation to the number of cells at a given time point. In genetic studies, this is referred to as expressivity, namely the degree of expression of a phenotype in a single cell with a given genotype. In this experiment, not all cells were transfected, and the ones that did express the transgene (i.e., the population of interest) tended to do so with some degree of heterogeneity. If different numbers of cells are transfected under the same conditions, the transgene expression will not scale proportionally down to the single cell. To better understand and represent this metric of relative single cell signal intensity, we can refer to it as transfection expressivity.

A quantitative dose-response understanding of transfection expressivity normalized to the cell number is necessary in situations where, for example, the transgene product has a recessive character. The expression of the transgene can be directly proportional to a phenotype of interest, such as a pathological state as seen for many haplosufficient loss of function mutant proteins like p53 (Sabapathy, 2018). Conversely, haploinsufficient genes, such as gain of function mutants of p53, might display a more complex genotype to phenotype relationship that can only be dissected and understood using the metric of transfection expressivity (Luo, 2021; Johnson, 2019). This metric can be calculated by dividing the total intensity data by the cellular confluency, obtaining a ratio that can then be normalized according to the user's preference. In this case, the 48-hour time point of 20,000 cells was chosen for normalization because it was the maximum signal output **(Figure 3A)**.

Indeed, dynamic transfection expressivity highlights how both 10,000 and 20,000 cells display a comparable relative transgene expression with slightly different kinetics reflecting on the respective cellular proliferation kinetics **(Figure 2C)**. On the other hand, 40,000 cells showed an almost biphasic growth in signal despite their full confluence throughout the whole experiment. This behavior might reflect a more complex biology of interest, where transgene expression was decoupled from cellular proliferation.

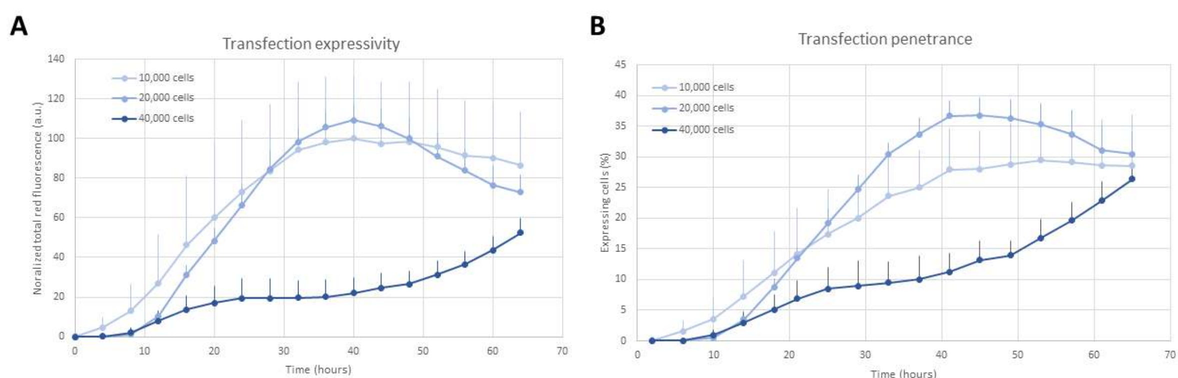

Figure 3. *Relative transfection penetrance and expressivity. Dynamic analysis of relative transfection efficiency with 0.4 µL of Lipofectamine 2000 per well and 100 ng of DNA vector per well with the indicated number of HEK 293T/17 cells. A) Transfection efficiency, or expressivity, calculated as a ratio between total fluorescent signal intensity and Enhanced Contour cellular confluency. The value was normalized to the value displayed by the 20,000 cells at 48 hours set as 100%. B) Proportion of transfected cells, or penetrance, calculated as the ratio between red fluorescence confluency and Enhanced Contour cellular confluency. Averages are shown with error bars as SEM of 3 wells.*

Additionally, researchers can maximize the relative proportion of cells expressing the transgene, independently of the degree of expression. This second scenario is referred to as penetrance and can be a parameter of interest in cases where the transgene

displays dominant traits, for example, gain of function mutant oncogenes. In the latter biological model, a minimal expression of the transgene is sufficient to achieve the phenotype of interest and could be used to maximize the transfected proportion of cells irrespective of the degree of expression.

The transfection penetrance can be calculated by using the surface area metric occupied by the fluorescent signal and dividing it by the total area occupied by all cells. This new metric yields a ratio that indicates what proportion of cells is expressing the transgene by any degree (Figure 3B). In our experiment, up to 35% of all cells could be seen expressing mCherry 48 hours after being transfected with 0.4 µL of Lipofectamine 2000 and 100 ng of DNA per well. Higher expression penetrance could be achieved by screening different experimental variables, including DNA vector types or lipofection reagents more appropriate to the cell of interest.

Conclusions

This application note has demonstrated

- The CELLCYTE X enabled the nondisruptive, high-content imaging acquisition and analysis necessary to quickly and economically optimize DNA transfection in mammalian cells using 96- or 384-well plates.
- The CELLCYTE Studio software offered extensive flexibility, making it possible to further refine the biological model of interest by independently studying transfection penetrance or expressivity, using either the total intensity (a.u.) or the confluence percentage and total area metrics.
- Cellular health and proliferation were evaluated throughout the experiment using confluency (%) and total area (mm2) metrics.

References

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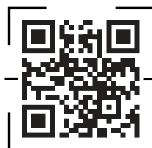

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