Kinetic Assessment of Reporter Gene Expression with Live Cell Imaging

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Abstract

Gene expression manipulation is a critical tool for understanding the molecular biology of cells, in both physiological and pathological environments. Methodologies that leverage RNA interference have emerged and simplified gene manipulation, letting researchers easily achieve the transient post-transcriptional downregulation of targeted genes. Unlike conventional end-point microscopy, live cell imaging makes it possible to dynamically image live cells over long periods of time throughout the experiment, from within the incubator, and obtain multiparametric quantitative data of fluorescent reporter gene expression. In this application note, the CELLCYTE X was used as a versatile live cell imaging fluorescent microscope to kinetically quantify the expression of a reporter gene using enhanced green fluorescent protein (EGFP) over a period of 7 days. It was also demonstrated that live cell imaging can be used to obtain the robust transfection conditions necessary to achieve optimal small interfering RNA (siRNA)-mediated gene expression knockdown in human pancreatic cancer cells (PANC-1), with minimal user intervention.

Introduction

Recent advancements in genetic engineering have unveiled a much deeper level of complexity in gene regulation within animal cells and have, thus, furthered our understanding of multifactorial diseases such as diabetes (Ono, 2011) and cancer (Kumar, 2007). Differential gene expression, for one, is an essential molecular mechanism evolved by cells to maintain homeostasis. In more complex organisms, like humans, the regulation of gene expression evolved as a multilayered, self-regulating process that interacts with multiple environmental stressors. Understanding and manipulating these interactions have been key to bringing about many of the medical breakthroughs from the past century.

Today, to understand the biology of a gene of interest and establish functional causality, its physiological expression can be manipulated, either positively or negatively, to elicit a specific phenotype. In this setup it is also necessary to accurately and kinetically quantify the expression of the gene of interest. To acquire this information, cells or animals can be engineered to express reporter genes in place of the gene of interest. These reporter genes are genetic elements, of either natural or synthetic nature, often used to determine and quantify a function of interest, such as a metabolic state or environmental stressors (Fischer, 2020). In particular, reporter genes expressing fluorescent proteins under the transcriptional control of a target gene can be visualized and functionally tracked with quantifiable fluorescent microscopy.

This direct manipulation of gene expression can be easily achieved with the use of methodologies that leverage the phenomenon of RNA interference. A common approach in many labs, RNA interference begins by transfecting cells with synthetic small interfering RNAs (siRNAs) to achieve a transient, post-transcriptional downregulation of the gene of interest. siRNAs are powerful tools that work in a vast array of cell lines but often require lengthy experimenting to obtain robust conditions to ensure consistent optimal efficacy. Live cell imaging enables the dynamic and quantitative analysis of fluorescent reporter gene expression, significantly improving the ability to identify optimal conditions and maximizing the data output of each knockdown experiment. In this application note, the CELLCYTE X was used to achieve optimal siRNA-mediated knockdown of the reporter gene EGFP in the pancreatic cancer cell line (PANC-1).

Materials and methods

Cell culture

The PANC-1 cell line, which was engineered to constitutively express the EGFP transgene in its cytoplasm (CYTENA), was kept in culture using complete F-12 media [Ham's F-12 Nutrient Mix (Gibco, 11765054) complemented with 10% fetal bovine serum (Gibco, 10270-106), 2 mM GlutaMAX supplement (Gibco, 35050-038), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco, 15140-122)]. Cells were kept in their expo-nential growth phase through chemical detachment using TrypLE Select Enzyme 1X (Gibco, 12563029) solution and biweekly 1:2 to 1:4 passaging in T75 flasks. Viability was assessed by trypan blue exclusion and values over 90% were considered acceptable. Sixteen hours before the siRNA-mediated knockdown of EGFP expression, cells were detached, and the necessary number of viable cells were seeded in a 96-well culture

plate. To achieve homogenous seeding across the whole well surface, cells were seeded in 50 µL of complete F-12 media distributed on top of the previously dispensed 40 µL of complete media in the well. Cells were then left to sediment over 30 minutes at room temperature before being moved to a humidified incubator at 37°C 5% CO2.

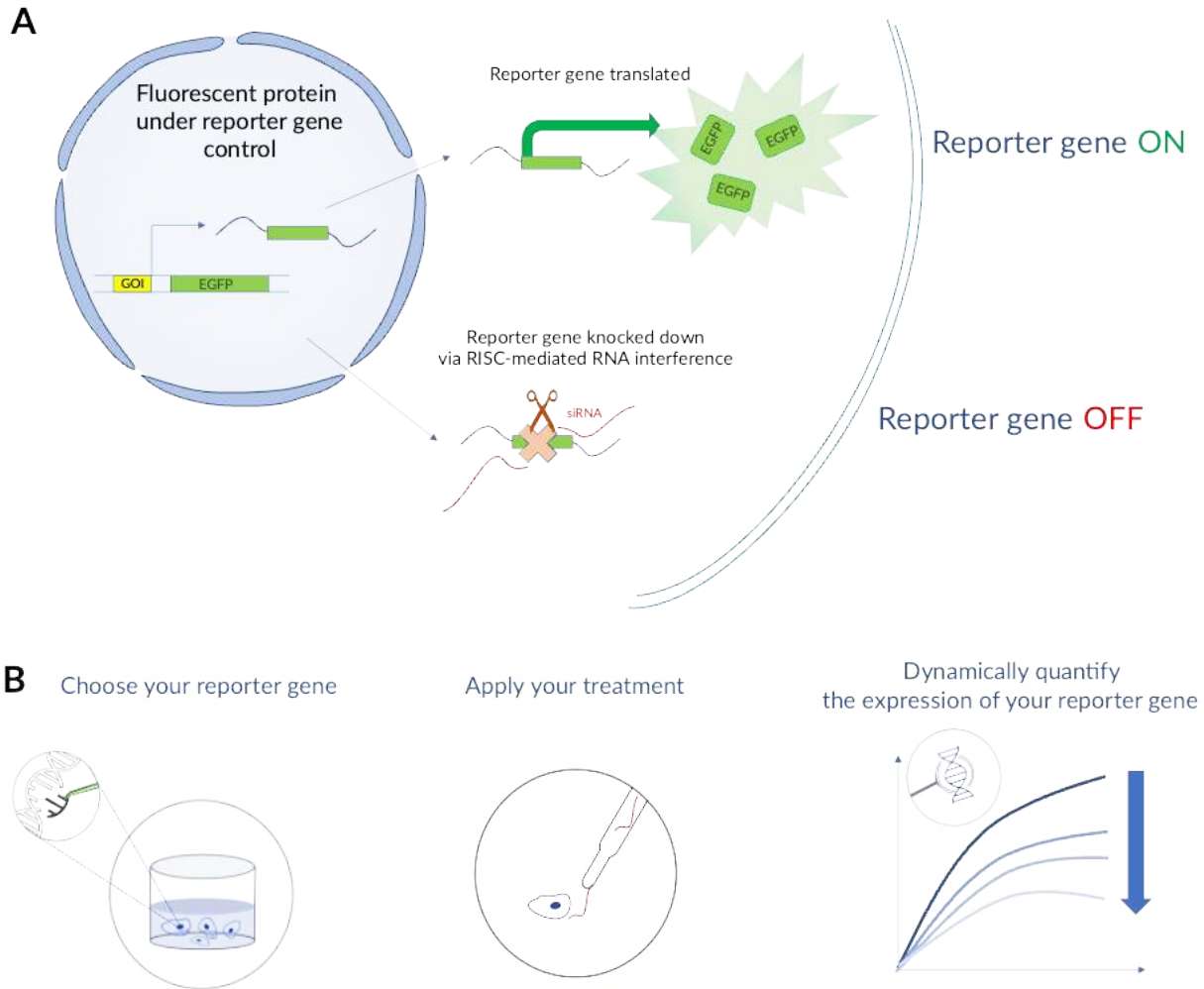

Figure 1. (A) *Cells can be engineered to constitutively express a reporter gene of interest. Using fluorescent proteins, like EGFP, it is possible to quantitatively and dynamically assess the efficacy of siRNA-mediated knockdown in cells of interest. (B) The workflow: Choose a cell model expressing a fluorescent protein under the control of a stimulus of interest, then apply experimental treatment and kinetically monitor the quantitative expression of the reporter gene.*

Lipofection of siRNAs

Positive control Silencer EGFP siRNA and nontargeting negative control siRNA (Thermo Fisher, AM4626) were transfected via lipofection into PANC-1-EGFP 16 hours after seeding. Specifically, 0.2, 0.3, 0.4 or 0.5 µL of Lipofectamine 2000 Transfection Reagent (Thermo Fisher, 11668027) was diluted in 5 µL of Opti-MEM I Reduced Serum Medium (Gibco, 31985-070) for every reaction well. In a separate set of tubes, both EGFP targeting and nontargeting control siRNAs were prediluted to a final working concentration of 5, 20 or 75 nM in 5 µL of Opti-MEM I Reduced Serum Medium per reaction. Equal volumes of each solution were mixed and incubated for 5 minutes at room temperature. Then, 10 µL of each type of lipoparticle solution was distributed per well of cells on top of the 90 µL of complete F-12 media. Triplicates were prepared for each condition. Cells were imaged using the green channel with four fields of view per well, every 4 hours at 200 milliseconds of exposure and 3 dB of gain with the CELLCYTE X in a humidified incubator at 37°C 5% CO2.ds of view (FOVs) per well.

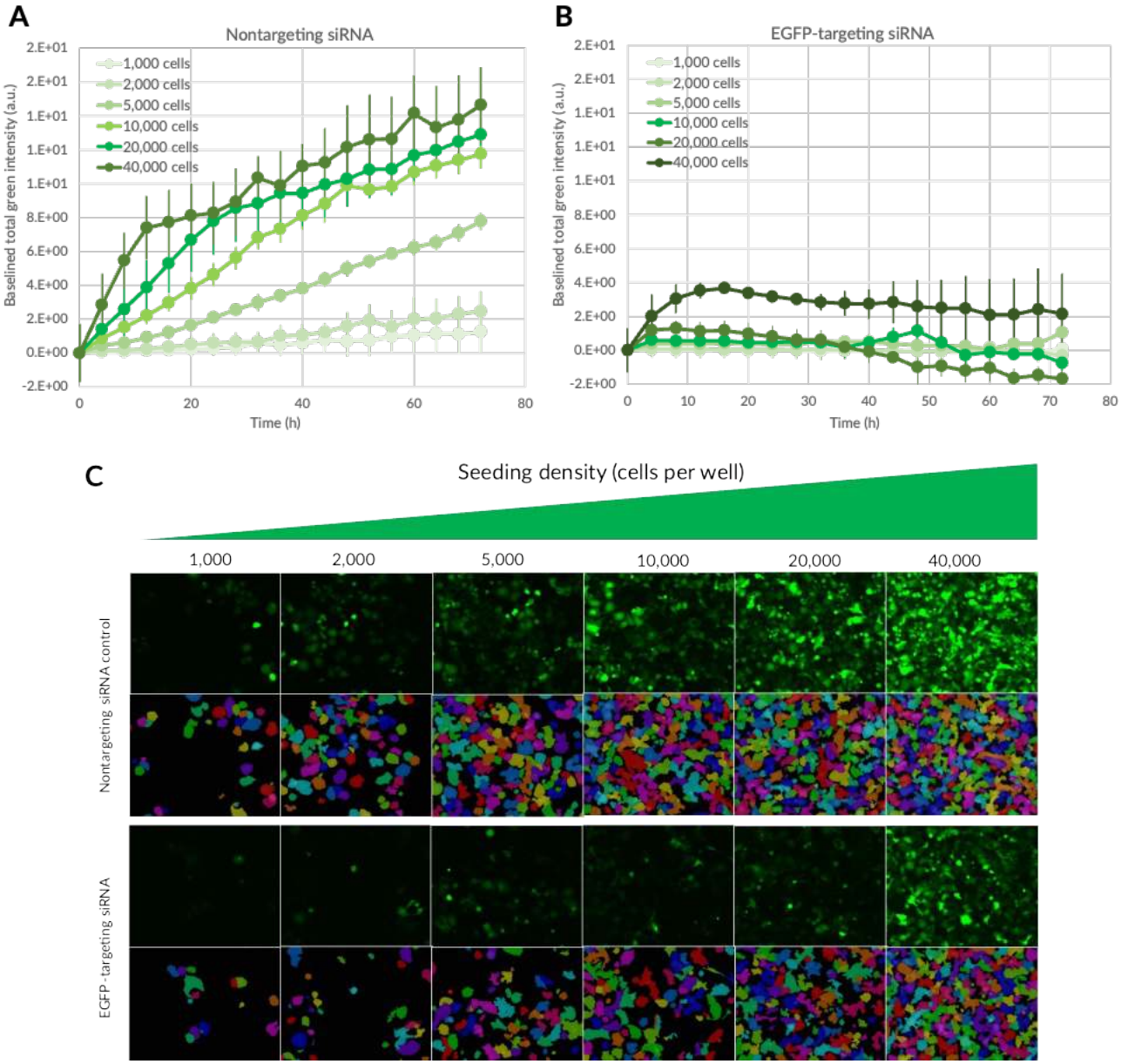

Figure 2. *EGFP expression quantitatively reports siRNA-mediated knockdown efficacy across a wide dynamic range. Kinetic and quantitative measurement of green fluorescent total intensity signal, baselined to 0 arbitrary units of PANC-1-EGFP cells, transfected with 20 nM of either nontargeting siRNA control (A) or EGFP-targeting siRNA (B). Each time point indicates averages of triplicate wells with error bars representing standard deviations. (C) Panels of representative green fluorescent images acquired 48 hours after transfection with either siRNA at the indicated cell seeding density. Below each image, the respective mask used for intensity measurement analysis is shown.*

Results and discussion

High-throughput live cell imaging of 96- or 384-well plates is a necessary tool to quickly obtain reproducible results in studies screening the effect of multiple experimental conditions. The three main factors affecting gene expression are seeding cell density, amount of siRNA and amount of Lipofectamine 2000.

Establishing the optimal transfection conditions requires determining the minimal amount of both Lipofectamine and siRNA needed to achieve the acceptable level of gene knockdown at a given time point. This is necessary not only to reduce the amount

of valuable consumables used but also to minimize cellular toxicity caused by lipofection and, lastly, to minimize off-target knockdown that siRNAs can display at higher concentrations. The CELLCYTE X enabled the identification of optimal experimental conditions and also enabled kinetic understanding of siRNA-mediated knockdown efficacy over 7 days.

This application note demonstrates how to quickly and efficiently establish and monitor siRNA-mediated knockdown of EGFP expression in PANC-1 cells. Working in 96-well plates, the kinetics of EGFP expression in PANC-1 cells **(Figure 2A)** and the activity of 20 nM siRNA, particularly at lower seeding densities **(Figure 2B)**, were evaluated. The total gene expression was quantified as the total fluorescence intensity signal measured directly from high resolution fluorescent images **(Figure 2C)**. The CELLCYTE Studio software algorithms analyzed the fluorescent signal intensity emitted only by cells actively expressing the reporter gene, excluding from the metric any background signal displayed by media components, increasing both signal sensitivity and specificity. With this approach, it was quickly and confidently established that the optimal conditions to achieve robust and stable knockdown of the EGFP gene expression in PANC-1 cells was with 0.2 µL per well of Lipofectamine 2000 and a concentration of 5 nM siRNA.

When compared to endpoint, or other disruptive, imaging methodologies, kinetic live cell imaging enables the validation with superior granularity of the reporter gene expression by imaging cells at regular intervals from within the incubator, under more stable environmental conditions. This study demonstrated that the knockdown of EGFP using the CELLCYTE X was consistent and stable up to a week, even with the lowest amount of siRNA **(Figure 3)**.

Furthermore, the degree of knockdown achieved with our chosen range of siRNA showed no significant difference up to 3 days. Unexpectedly, after 7 days, only the highest dose showed a marginal superiority, making 5 nM siRNA the recommended dose for these cells. Given the exceptional activity achieved with a minimal amount of siRNA, even lower quantities could be evaluated to further minimize off-target activity with an acceptable level of gene knockdown.

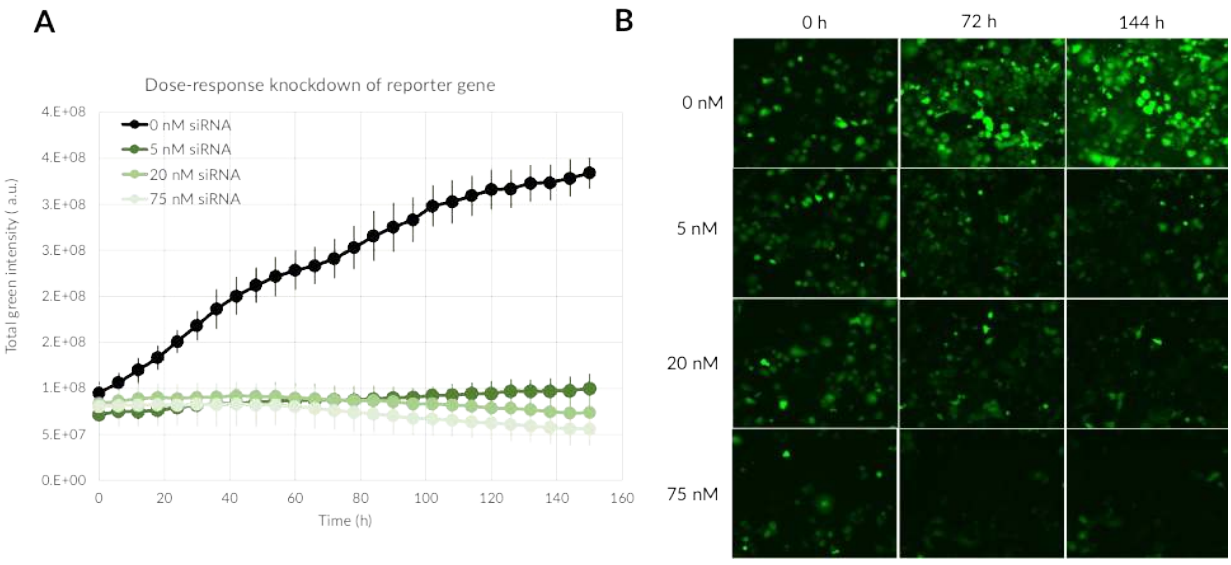

Figure 3. *The CELLCYTE X offers temporal high-throughput control of reporter genes. (A) Quantitative analysis of total EGFP expression in 5,000 PANC-1 cells transfected with variable amounts of siRNA and 0.2 µL of Lipofectamine 2000 per well. (B) Panel of representative images acquired in the same study at the indicated time points.*

Conclusions

This application note has demonstrated that minimal amounts of Lipofectamine 2000 and siRNA elicited robust EGFP knockdown in PANC-1 cells over 7 days using the CELLCYTE X. The versatile live cell imaging fluorescent microscope also enabled the acquisition of fluorescent images dynamically in a nondisruptive physiological environment.

In summary:

• The CELLCYTE X provided the kinetic visualization of the fluorescent protein EGFP used as a reporter of genetic manipulation techniques such as transient siRNA-mediated knockdown.

• The CELLCYTE X reduced the amount of valuable reagents needed to image 96- and 384-well plates.

• The CELLCYTE X's onboard analytics software provided versatile and high-throughput kinetic fluorescent signal intensity measurements superior to end-point microscopy or spectrophotometry.

• The CELLCYTE X enabled the quantitative assessment of the expression of the reporter gene with a high-dynamic range of seeding densities and signal over long periods of time.

References

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