siRNA Transfection Enhancement on Callisto System

To achieve desired efficiency in cell culture applications on the Callisto™ system, siRNA transfection must be optimized empirically for each cell type, especially for hard-to-transfect cells and confluent cell cultures. This technical note describes how to optimize siRNA transfection efficiency for cells loaded on Callisto.

Technical Background

In a transfection with lipid vesicles, a complex containing siRNA is delivered to cells. With commercially available kits, RNA complexes are added directly to the cells in culture flasks or well plates. Callisto is fundamentally different because lipid siRNA complex reagents are added to reagent inlets, where they can sit for extended periods prior to being delivered to the cells. In Callisto, cells are cultured in microchambers in the integrated fluidic circuit (IFC). Due to the lower volume (100 nL) relative to the cell culture surface (1.25 mm^2) of chambers, typically reagents and media need to be delivered more frequently than in the traditional well plate. For siRNA transfection, multiple doses over a longer period are used to transfect cells efficiently; hence adsorption and settling of the lipid-based siRNA complex in the inlet wells may reduce transfection efficiency.

Experiment

Transfection efficiency enhancement was studied on human induced pluripotent stem (hiPS) cells seeded on Matrigel®-coated surfaces in Callisto Adherent Cell Culture IFCs. *GAPDH* and *POU5F1* knockdown efficiency was evaluated using two orthogonal methods: qPCR and immunofluorescence.

Materials and Methods

Experiment Planning and Run Time

Experiments were run on the Callisto user interface (v3.2.2) and designed in Callisto Experiment Planner software (v1.0). Experiments were run on the Callisto Adherent Cell Culture IFC (PN 100-9996) using Callisto reagent kits (100-8682 and 100-8688). Operation of the instrument was performed according to instructions available in the Callisto System User Guide (100-7598).

Required Materials

The following materials were used in these experiments.

Material	Manufacturer
Callisto system	Fluidigm
Callisto Adherent Cell Culture IFC	Fluidigm
Callisto Experiment Planner software (v1.0)	Fluidigm
ABI 7900HT	Thermo Fisher Scientific
Inverted microscope	Leica®
MetaMorph® Microscopy Automation and Image Analysis Software	Molecular Devices

Required Reagents

The following reagents were used in these experiments.

Material	Manufacturer
Callisto reagent kits	Fluidigm
Callisto Suspension Reagent	Fluidigm
Callisto Lysis Plus reagent	Fluidigm
Matrigel	Corning
StemPro® Accutase®	Thermo Fisher Scientific
mTeSR™1 medium	STEMCELL™ Technologies
1X Penicillin-Streptomycin	Thermo Fisher Scientific
Thiazovivin	Stemgent®
Stemfect™	Stemgent

Material	Manufacturer
Stealth RNAi siRNAs	Thermo Fisher Scientific
RNAse Inhibitor	Clontech®
Reverse Transcription Master Mix	Fluidigm
DNA Suspension Buffer	TEKnova
SsoFast™ EvaGreen®	Bio-Rad
ROX™ Reference Dye	Biotium
Forward and reverse primers	IDT®
Paraformaldehyde solution	Electron Microscopy Sciences
Triton™ X-100	Thermo Fisher Scientific
Normal donkey serum	Thermo Fisher Scientific
Primary Goat Anti-OCT3/4 antibody	R&D Systems
Alexa Fluor® 555 Donkey Anti-Goat IgG (H+L)	Thermo Fisher Scientific
DAPI	Thermo Fisher Scientific

IFC Preparation and Cell Seeding

The IFC was pretreated using the procedure described in the Callisto System Getting Started Guide (PN 100-7599). Culture chambers were pretreated with 5% hESC-certified Matrigel prior to seeding cells. hiPS cells were singulated using StemPro Accutase from live culture and seeded in mTeSR™1 medium containing 1X Penicillin-Streptomycin (P/S) and 2 µM Thiazovivin at a loading density of ~15 million cells/mL, resulting in a density of ~750 cells/mm^2. Cells were cultured overnight at a feeding interval of 2 hr. After initial attachment and growth, medium was changed to mTeSR1 with 1X P/S prior to dosing cells with RNA complex.

Dosing Reagent Preparation

RNA knockdown experiments were performed using the Stemfect RNA Transfection Kit. Stemfect was chosen due to the stability of RNA complex at ambient temperature over days. Briefly, cationic-lipid siRNA complexes were created by mixing Stemfect reagent with Stealth RNAi siRNAs at a ratio of 80 pmol RNA to 1 µL Stemfect reagent, then diluted in medium (Table 1). Unless otherwise stated, prior to adding reagents to the IFC inlets, Callisto Suspension Reagent was added to the diluted siRNA complexes to a final concentration of 2% (v/v). siRNAs used in this experiment were targeted against *GAPDH* and *POU5F1*. A negative control siRNA—a scrambled

sequence nonspecific to any gene—was also used. Reagents were dosed into cell culture chambers at four different concentrations for siRNA complexes against *GAPDH* and *POU5F1* and at a single concentration for both negative and medium controls. Replicate groups were set up in the experimental plan software and chambers were dosed for 16 hours at 2 hr intervals. The replicate group setup, by chamber number, is summarized in Table 2. After dosing, visualization of complex delivery was performed for the Cy3-labeled siRNA complex against *GAPDH* conditions by Cy3 fluorescence imaging.

Table 1. Instructions for generating the Stemfect reagent complexes with Stealth RNAi siRNAs used in this study

Reagents	**Materials and Amounts**		
Complexation mix	14 µL Stemfect Reagent + 91 µL Stemfect Buffer		
Reagent	**siRNA-Negative Control**	**Cy3-siRNA-GAPDH**	**siRNA-POU5F1**
RNA	8 µL*	16 µL*	16 µL*
Buffer	7 µL	14 µL	14 µL
Complexation mix	15 µL	30 µL	30 µL
	Wait 10 Minutes		
mTeSR1 + 1X P/S	87.6 µL	175.2 µL	175.2 µL
Suspension Reagent	2.4 µL	4.8 µL	4.8 µL
Total volume	120 µL	240 µL	240 µL
[RNA] in wells	1.33 µM	1.33 µM	1.33 µM

*Stock concentration 20 µM

Table 2. Setup of dosing groups by chamber. Transfection with siRNA against *GAPDH* and *POU5F1* was tested at four concentrations (n=3 replicates each), and medium control and negative control were tested at a single concentration (n=4 replicates).

Condition	Concentration	Chamber Numbers
Medium control	--	1, 11, 21, 31
siRNA-negative control	670 nM	2, 12, 22, 32
Cy3-siRNA-*GAPDH*	670 nM	3, 13, 23
Cy3-siRNA-*GAPDH*	335 nM	4, 14, 24
Cy3-siRNA-*GAPDH*	168 nM	5, 15, 25
Cy3-siRNA-*GAPDH*	67 nM	6, 16, 26
siRNA-*POU5F1*	670 nM	7, 17, 27
siRNA-*POU5F1*	335 nM	8, 18, 28
siRNA-*POU5F1*	168 nM	9, 19, 29
siRNA-*POU5F1*	67 nM	10, 20, 30

Cell Lysate Analysis

Culture chamber contents were lysed using the Callisto Lysis Plus reagent containing 2 U/µL RNAse Inhibitor. Collected lysate from the IFC was reverse-transcribed to cDNA using Reverse Transcription Master Mix (100-6298) (Table 3). A no template control was also run in parallel with the samples substituting PCR water for the lysate volume.

Table 3. Reaction setup for reverse transcription on cell lysate from culture chambers

Component	Volume (µL)
Fluidigm Reverse Transcription Master Mix	1
PCR-certified water	3
Lysate sample (2.5 pg to 250 ng)	1
Total	**5***

*Samples vortexed, centrifuged, and thermocycled:
25 °C for 5 min → 42 °C for 30 min → 85 °C for 5 min → 4 °C for ∞

Samples were diluted with DNA Suspension Buffer 1:10 after reverse transcription and then combined with appropriate qPCR master mix. qPCR mix was created by combining PCR water, SsoFast EvaGreen with low ROX, ROX Reference Dye, and forward and reverse primers against the gene of interest (*ACTB*, *GAPDH,* or *POU5F1*) (Table 4). Completed samples were vortexed, centrifuged, and loaded into 384-well plates for analysis in a standard qPCR system (e.g., ABI 7900HT). A standard run (40 cycles) was performed and Ct

values determined for each sample relative to each gene of interest. *ACTB* was used to normalize the Ct value for *GAPDH* and *POU5F1*. A delta-delta-Ct value was then calculated to determine the relative knockdown versus the control condition in the IFC (Schmittgen and Livak 2008).

Table 4. Reaction setup for qPCR on diluted reverse transcription (RT) sample. Samples were analyzed on an ABI 7900HT system.

Component	Volume per Reaction (µL)
SsoFast EvaGreen with low ROX	5
RNase-free water	2.5
10X ROX Reference Dye	0.5
10 µM forward and reverse primers	1
1:10 Diluted RT sample	1
Total	**10**

Fixation and Immunostaining Analysis

Image analysis of expression knockdown was performed using OCT4 (encoded by *POU5F1* gene) as the target protein of interest. After cells were dosed, they were fixed in 2% paraformaldehyde solution for 20 minutes and permeabilized with 0.2% Triton X-100 for 20 minutes. Chambers were incubated with blocking solution containing 5% normal donkey serum and 0.2% Triton X-100 in PBS. After blocking, a primary goat anti-OCT3/4 antibody at 10 µg/mL was raised against the fixed cells followed by a secondary antibody of Alexa Fluor 555 Donkey Anti-Goat IgG (H+L) at 10 µg/mL. DAPI counterstaining at 5 ng/mL was also performed on all cell culture chambers. Imaging was performed by epifluorescence using an inverted microscope in Cy3 and DAPI channels. Analysis for protein expression knockdown was performed using MetaMorph Microscopy Automation and Image Analysis Software and a custom analysis journal. Knockdown by image analysis was performed by normalizing the total intensity of the Cy3 channel (OCT4 signal) to the number of nuclei counted in each chamber.

Results

Delivery Enhancement with Callisto Suspension Reagent

Transfection efficiency with an siRNA complex can significantly impact experimental outcome. Cationic lipid siRNA complexes made with Stemfect reagent are denser than water and will sink in an aqueous solution. This is not a problem—and is advantageous—in adherent cell culture because cells are grown on the bottom of a flask or well plate. The Callisto Adherent Cell Culture IFC presents new considerations of geometry and adsorption because reagent inlets in the IFC carrier are orthogonal to the cell culture chambers. Because the Callisto Suspension Reagent changes the buoyancy and adsorption properties of the siRNA complex, the reagent facilitates delivery of the complex in the IFC.

To track the delivery of siRNA complex from the reagent inlets to culture chambers, we used a Cy3-labeled siRNA against *GAPDH*. Adsorption of siRNA complex to reagent inlets and fluidic channels was tested using two conditions: siRNA complex with and without suspension reagent added. Without addition of suspension reagent, incubation of siRNA complex in reagent inlets shows dramatically higher fluorescence intensity than when suspension reagent is added [Figure 1(a) and (b)]. To track the downstream effect of adding suspension, reagent cells were dosed with different concentrations of Cy3-siRNA (670, 335, 168, and 67 nM) and imaged for Cy3 fluorescence. The cells show higher levels or Cy3 fluorescence when suspension reagent is present during transfection in all concentrations tested [Figure 1(c) and (d)]. Thus, suspension reagent keeps siRNA complex in solution and facilitates more efficient delivery to the cells.

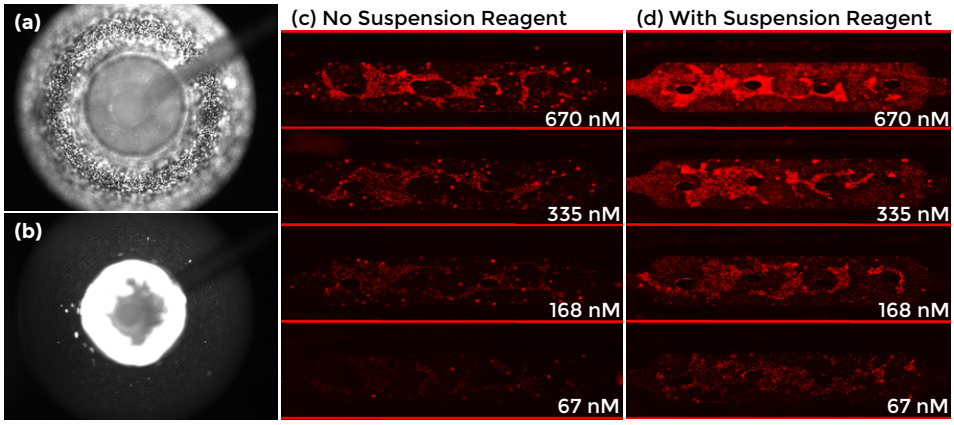

Figure 1. Fluorescent images showing the effect of including Callisto Suspension Reagent with siRNA-complex. Shown are reagent inlets without (a) and with (b) suspension reagent following incubation with Cy3-labeled siRNA against *GAPDH,* and images of chambers after four concentrations without (c) or with (d) 2% suspension reagent added.

To test that delivery of siRNA complex is uniform across cell culture chambers, we used the same Cy3-labeled siRNA-*GAPDH* as in the previous experiment. Cells were dosed using the same concentrations in the presence of suspension reagent (2% volume). Cy3 fluorescence was collected and total intensity was measured for each cell culture chamber, using a custom journal in MetaMorph software. Over three replicates, Cy3 fluorescence intensity was similar for each concentration tested. The maximum variation, occurring in the 670 nM condition, was 4% on average and below 2% for the three other conditions (Figure 2). This indicates that reagent delivery is optimal and repeatable on the Callisto system.

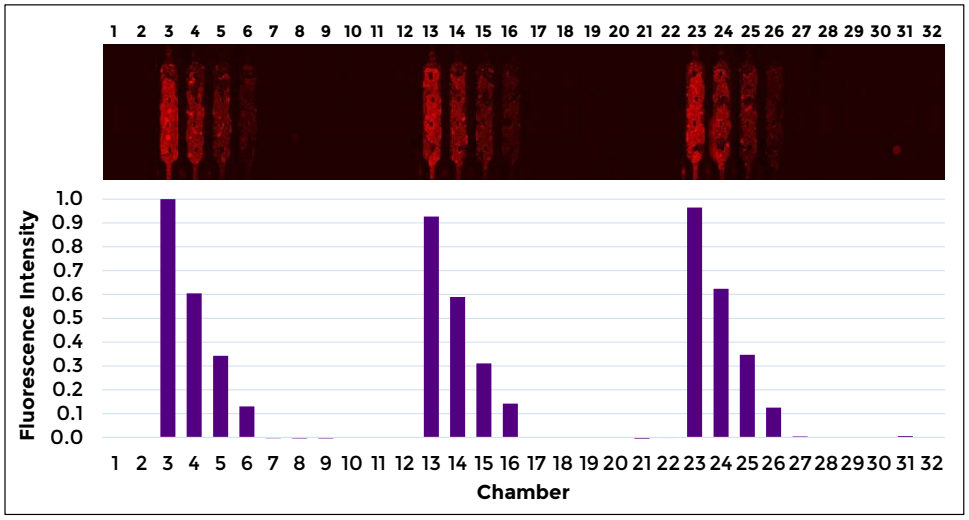

Figure 2. Background-corrected and normalized fluorescence intensity across all 32 cell culture chambers after delivery of the Cy3-tagged siRNA-*GAPDH* complex with 2% Callisto Suspension Reagent. Inset is a montage image over all culture chambers.

Efficient Gene Expression Knockdown of hiPS Cells

Enhancing delivery is especially critical for hiPS cells because as colonies reach confluency, it becomes substantially more difficult to transfect cells. This occurs because cells divide in tight colonies and the relative surface area to transfect lessens.

To assess that the siRNA complex is having the correct knockdown effect on the targeted genes, we knocked two biologically relevant genes, *GAPDH* (a housekeeping gene) and *POU5F1* (a stemness gene). After incubation with transfection reagent, cell lysate was harvested and analyzed by qPCR. Two concentrations of siRNA complex (670 and 67 nM) were used for both gene targets, without and with suspension reagent. Gene expression analysis demonstrates that the presence of suspension reagent improves the knockdown level of the target for the two genes studied. At high concentration (670 nM), there is ~10% improvement in gene expression knockdown when comparing conditions without and with suspension reagent (68% vs. 79% and 86% vs. 95% for *GAPDH* and *POU5F1,* respectively). At the same time, the coefficient of variation (CV) dramatically decreases when suspension reagent is used (21% vs. 2% and 9% vs. 1% for *GAPDH* and *POU5F1,* respectively) (Table 5). At low concentration (67 nM), the benefit of suspension reagent is less clear when only examining the gene expression knockdown levels without and with suspension reagent (41% vs. 26% and 56% vs. 70% for *GAPDH* and *POU5F1,* respectively). Again, the CV is markedly less when suspension reagent is used (68% vs. 3% and 37% vs. 10% for *GAPDH* and *POU5F1,* respectively) (Table 5). This data suggests that in conditions without suspension reagent, gene expression knockdown may be higher, but with high CV the data is not reliable. Overall, expression knockdown variability is decreased when suspension reagent is added. Therefore, adding suspension reagent for siRNA complex delivery will give more accurate targeted effect, even at high cell density, when transfection can prove more difficult.

Table 5. Comparison of knockdown efficiencies without (n=33) and with (n=9) adding Callisto Suspension Reagent to siRNA complex reagents prior to dosing

	siRNA-GAPDH				siRNA-POU5F1			
Condition	**Without SR**		**With SR**		**Without SR**		**With SR**	
[Complex]	670 nM	67 nM	670 nM	67 nM	670 nM	67 nM	670 nM	67 nM
Knockdown	68%	41%	79%	26%	86%	56%	95%	70%
Knockdown CV	21%	68%	2%	3%	9%	37%	1%	10%

Protein Expression Knockdown Correlates to RNA Levels

Suspension reagent improves delivery, uniformity, and targeted effect of siRNA complexes in transfection experiments. To confirm that suspension reagent does not affect gene translation cellular machinery, we evaluated the expression of OCT4 protein following knockdown of *POU5F1* gene by transfection. Cells were transfected using siRNA against *POU5F1* at four different concentrations (670, 335, 168, and 67 nM), a negative control (scrambled sequence) siRNA (670 nM), and a medium control (no transfection). Cells were fixed post-transfection and immunostained for OCT4 protein and counterstained for nuclei with DAPI. Total intensity of OCT4 staining normalized to the total number of nuclei was measured to evaluate the protein expression knockdown.

Compared to control conditions, the OCT4 signal decreases as the concentration of siRNA against *POU5F1* increases [Figure 3(a)]. Cell-loading density and viability are similar across all culture chambers, as shown respectively by nuclear staining [Figure 3(b)] and phase contrast imaging [Figure 3(c)]. Quantitative analysis of the OCT4 expression knockdown mirrors closely the expression knockdown of *POU5F1* by qPCR [Figure 3(d)]. Thus, the amount of available RNA transcripts following siRNA knockdown is directly correlated to the amount of protein detected. This result strongly indicates that addition of suspension reagent is functionally inert to protein expression.

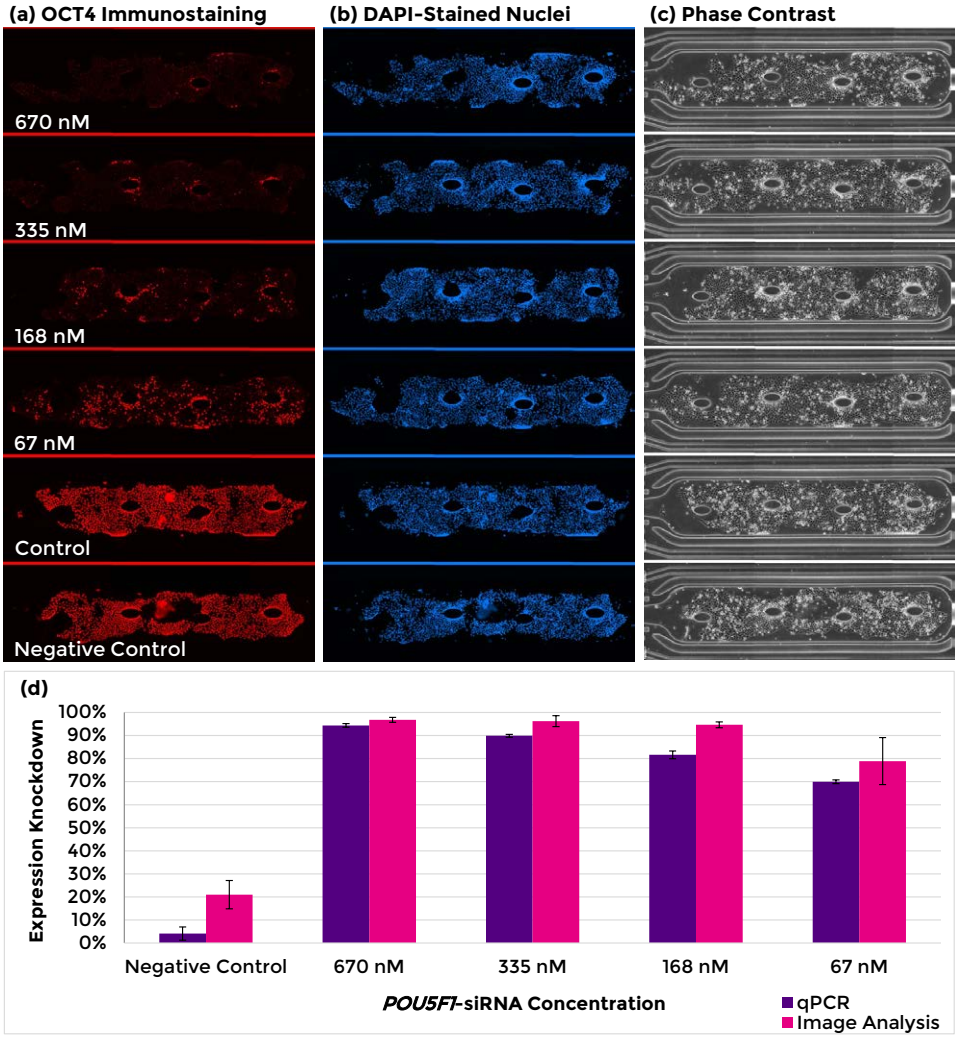

Figure 3. (a)–(c) Representative fluorescence and phase contrast images of immunostained chambers following dosing with siRNA-*POU5F1* complex. (d) Graph comparing data from qPCR (n=9) expression knockdown of *POU5F1* and image analysis (n=3) knockdown of OCT4 protein.

Conclusions

The procedures and data here outline an efficient knockdown method of endogenous genes using siRNA on Callisto optimized for hiPS cells. Callisto Suspension Reagent has been proven to be an effective additive for the dosing of siRNA-complex reagents. Complex adsorption to carrier walls is lower and delivery to the cells is enhanced when suspension reagent is used. When suspension reagent is added, the results show more efficient knockdown with dramatically lower variation compared to identical conditions where suspension reagent is not added. Additionally, Callisto Suspension Reagent is inert to the cellular translation machinery because expression levels of mRNA transcripts following siRNA knockdown mirror closely the

amount of protein expressed. For improved delivery of cationic-lipid RNA complexes in the IFC for transfection, Fluidigm recommends adding Callisto Suspension Reagent prior to dosing.

Reference

Schmittgen, T.D., and Livak, K.J. “Analyzing real-time PCR data by the comparative C(T) method.” *Nature Protocols* 3(6) (2008): 1101–1108.

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