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Research Article

Weak cell cycle dependency but strong distortive effects of transfection with Lipofectamine 2000 in near-physiologically synchronized cell culture

Previously, we reported a method to generate and validate cell cycle-synchronized cultures of multiple mammalian suspension cell lines under near-physiological conditions. This method was applied to elucidate the putative interdependencies of the cell cycle and recombinant protein expression in the human producer cell line HEK293s using Lipofectamine 2000 and the reporter plasmid pcDNA3.3 enhanced green fluorescent protein, destabilized using PEST sequence. A population-resolved modeling approach was applied to quantitatively assess putative variations of cell cycle dependent expression rates based on the obtained experimental data. We could not confirm results published earlier by other groups, based on nonphysiological synchronization attempts, reporting transfection efficiency being strongly dependent on the cell cycle phase at transfection time point. On the other hand, it is demonstrated that transfection and protein expression distort the progression of the cell cycle.

Keywords: Cell cycle distortion / Cell cycle synchronization / Lipofection / Modeling / Recombinant protein expression

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1 Introduction

The mammalian cell cycle is one of the major factors orchestrating the behavior of individual cells as well as the whole culture population [1,2]. In order to develop a more systematic understanding of cell cycle dependent effects, reliable experimental methods under physiological conditions are required. Unfortunately, many of the studies on cell cycle dependent effects utilized artifact prone chemical and/or physical whole-culture methods like double thymidine block, serum starvation, and temperature reduction, despite their intrinsic flaws [3–5].

On the other hand, a reasonable read-out for cell cycle related metabolism is needed. Susceptibility to transient transfection with reporter plasmids is rather straightforward, as it is easy to quantify and results can be applicable i.e. in process development. Previous studies have reported transfection efficiencies to be strongly cell cycle dependent, exhibiting differences in efficiency by orders of magnitude [6–8].

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Abbreviations: d2eGFP, enhanced green fluorescent protein, destabilized using PEST sequence; NC, negative control

We have developed a method [9,10] which allows to generate synchronized cultures of mammalian suspension cell lines, amongst those the human producer cell line HEK293s, under near-physiological conditions. In this study, we investigate if the earlier reported cell cycle dependence of transfection efficiency can be confirmed using near-physiologically synchronized human HEK293s cultures.

2 Materials and methods

Unless indicated otherwise, materials and methods are as published earlier [10].

2.1 Cell line and culture

The cell culture procedures were further optimized. HEK293s cells from FreeStyle 293 medium cultures were used to inoculate 293 SFMII medium (both Thermo Fisher Scientific, Waltham, MA, USA) 44 h prior to elutriation at a density, calculated to yield a cellular density of about 2 Mio/mL at the time of elutriation, in order to avoid cell aggregation. Directly after elutriation, fractionated cells were resuspended in prewarmed and pH-adjusted

FreeStyle 293 medium at an initial density of 0.5 Mio/mL. No intermediate cultivation in 293 SFMII medium was employed. Eighteen hours after incubation at 37°C, 200 rpm and 8% CO₂ in a constantly humidified atmosphere, transient transfection using Lipofectamine 2000 [11] was conducted. No antibiotics were used at any point.

2.2 Transient transfection using d2eGFP as reporter protein

Transient transfection was performed as described in the manufacturer's protocol [11] and published previously [10]. In order to maximize reproducibility, all "shaking" steps were performed for 5 s using a *Vortex Genie 2* (Bender and Hobein AG, Zürich, Switzerland) at the speed denoted as "Vortex 1" on the control panel.

2.3 Time-dependent transfection efficiency and vesicle functionality

The following experiments were performed with nonsynchronized cultures, to estimate the interdependency of incubation time and transfection efficiency. Furthermore, the functionality of Lipofectamine 2000 DNA complexes over time was estimated.

2.3.1 Incubation time

Forty milliliters culture of HEK293s cells was adjusted to 0.5 Mio/mL in prewarmed Freestyle293 medium and transfected as described in Section 2.1. Subsequently, the culture was divided into four subcultures of 10 mL each (t_0). Three, 6, 9, and 24 h after transfection medium including all potentially remaining DNA Lipofectamine 2000 complexes were exchanged for fresh medium. Forty-eight hours after each transfection, cell viability and viable cell density were determined and remaining cells were fixated as described [10].

2.3.2 Vesicle stability

Complementary and in parallel to the incubation time experiment, four 10 mL cultures of HEK293s cells were prepared at the same time (t_0) and density. The DNA Lipofectamine 2000 complexes were kept under cultivation conditions (37°C, 200 rpm, 8% CO₂, and humidified atmosphere) in a sterile membrane tube. Three, 6, 9, and 24 h after t_0 , transfection was conducted.

As described in Section 2.3.1, 4 h after each transfection, cell viability and viable cell density were determined and cells fixated.

2.4 Flow cytometry

The flow cytometry method previously published [10] was applied in an optimized version. For the incubation time and vesicle stability experiments, 10.000 events were detected per sample to determine the eGFP expression levels. For the experiment targeting cell cycle dependency of transfection, 30.000 events of each sample were detected to determine eGFP expression levels

as well as the cell cycle state. Shortly, the cell cycle was determined according to the distribution of Propidium Iodide bound DNA fluorescence (detected in the PerCP-A channel) with simple cutoffs to distinguish G1, S, and G2/M phases; an adaptive peak determination was employed to compensate for inevitable staining intensity drifts. In this study, enhanced green fluorescent protein, destabilized using PEST sequence (d2eGFP) expression (detected in the FITC-A channel) and cell cycle distribution were determined from the same samples. No compensation was applied for PerCP-A/FITC-A as the crosstalk is moderate and negligible in the context of the standardized quantification method.

2.5 Elutriation and synchronization

Cell cultures were synchronized using centrifugal counter flow elutriation as described in [10]. In brief, an Avanti J 26S XP centrifuge and a JE-5.0 elutriation system with a 5 mL standard chamber (Beckman Coulter, Brea, CA, USA) were used with a rotor speed of 1500 rpm. After loading at 5 mL/min, fraction X1 was collected by slowly increasing the flow rate from 10 to 17 mL/min. For fraction X2 17–34 mL/mL were utilized. Of both fractions 150 mL were collected.

After elutriation, these two synchronized cultures were further processed. In parallel, a nonelutriated but similarly treated negative control (NC) was cultivated. From each of the three cultures, subvolumes were separated after predefined time points (T1, T2, and T3) and transfected (Overview in Fig. 1). In total, this yielded six transfected synchronized cultivations (denoted as X1...2–T1...3) and three transfected nonsynchronized NCs (NC-T1...3), which are used to assess potential systematic errors due to culture handling and transfection treatment.

2.5.1 Calculation and application of the normalized cell cycle position

The method to calculate the normalized cell cycle position described in [10] was employed to represent the cell cycle distribution information, obtained from the FACS analysis, in a one-dimensional value. Based on knowledge from previous studies, points in time were calculated to yield evenly distributed t_{cc} values between 0 and 1.

2.6 Cell cycle model

A strongly simplified combined variant of the population balance model described in [1] and a population based transfection model [12] has been set up to quantify the expected impact of putative cell cycle dependent plasmid uptake rate on overall measured data. It distinguishes three cell cycle states: G1, S, and G2/M. The plasmid uptake rate can be varied between these phases; the remaining parameters are fixed to be cell cycle independent and intracellular conversion or loss processes are lumped for simplicity. The model is kept as simple as possible to minimize the number of parameters that need to be adapted, while still allowing to assess the potential influence of cell cycle dependency of transfection. The general outline of the model approach is sketched in Fig. 2; the details are described in the following.

Figure 1. Schematic representation of synchronous transfection procedures including numerical representation of the different cell cycle states (t_{cc} values) at the time of transfection. Synchronous cultures X1 (high in G1 = high t_{cc} value) and X2 (high in S and G2/M phase = low t_{cc} value) were generated using elutriation. In parallel an NC, a culture of unaltered cell cycle distribution was kept; 19.5, 22, and 27.5 h after elutriation, transient transfection with the reporter plasmid (pcDNA3.3_d2eGFP) was conducted. Samples for quantification of the reporter protein (d2eGFP) were taken exactly 48 h after transfection.

The progress of the normalized cell cycle position, t_{cc} , is modeled, similarly as given in [1], as sawtooth pattern with a fixed period time ($t_{cc,p}$) and a shift ($t_{cc,s}$) that depends on the startup conditions of the synchronized culture, for which elutriation fraction is used:

$$t_{cc} = \frac{(t - t_{cc,s})}{t_{cc,p}} \text{mod} 1 \quad (1)$$

with cultivation time t given in hours. The corresponding amplitude I_m is kept constant at an initial level (I_{m0}) before the transfection time point t_{trans} and afterward decays exponentially with the rate $k_{i,decay}$:

$$\frac{dI_m}{dt} = \begin{cases} 0 & t < t_{trans} \\ I_m k_{i,decay} & \text{else} \end{cases}; I_m(t=0) = I_{m0} \quad (2)$$

G1 and S phase contributions, reversely as in [1], are correspondingly approximated as:

$$G1(t) = COG_{G1} + \cos(2\pi(t_{cc} + 0.75)) I_m \quad (3)$$

$$S(t) = COG_S + \sin(2\pi(t_{cc} + 0.75)) I_m \quad (4)$$

$$G2M(t) = 1 - G1(t) - S(t) \quad (5)$$

The centers of gravity (COG) for both G1 and S phase distribution are needed for the calculation of the normalized cell cycle position [1], and have been computed directly from the experimental data.

The change of viable cell density $X(t)$, which is given in 10^6 cells/mL, is defined as follows:

$$\frac{dX}{dt} = X(t) \mu; X(t=0) = X_0 \quad (6)$$

Cell death is not explicitly considered. The amount of active (potentially transfectable) complexes in the medium n_c is 0 at $t=0$. It is set to the normalized value (density) of n_{tr} ($=1 \text{ mL}^{-1}$) at transfection time point $t = t_{tr}$ and is afterward subject to Eq. (1) a linear decay according to Fig. 3 with linear decay time

$t_{c,decay}$, and Eq. (2) the uptake by cells, the latter being summarized in $u(t)$:

$$\frac{dn_c}{dt} = -\frac{n_{tr}}{t_{c,decay}} - u(t); n_c(t = t_{tr}) = n_{tr}; n_c \geq 0. \quad (7)$$

Note that n_c is maintained to be not smaller than 0. The summarized uptake $u(t)$ is defined as the sum of cell cycle specific uptake rates (split to absolute rate k_{up} and relative rates $k_{up,\Phi}$), multiplied with the respective viable cell densities belonging to a specific cell cycle at a given time point (split to absolute density $X(t)$ and relative fractions $\Phi(t)$). The considered cell cycle phases are denoted as = {G1, S, G2M}:

$$u(t) = u(t, X(t), \Phi(t)) = X(t) k_{up} n_c(t) \sum_{\Phi=\{G1, S, G2M\}} \Phi(t) k_{up}; n_c(t) > 0 \quad (8)$$

The total amount of further processable intracellular complexes or plasmids within cells $n_p(t)$ is consequently a combination of uptake $u(t)$, intracellular decay—including dilution by cell proliferation—depicted by $k_{p,decay}$ and further conversion $c(t)$. The latter is modeled as a lumped first-order conversion step with rate $k_{p,convert}$ that comprises intracellular vector trafficking, unpacking, nuclear incorporation, transcription, etc., without further distinguishing the single steps and/or cell cycle for simplicity:

$$\frac{dn_p}{dt} = u(t) - k_{p,decay} n_p(t) - c(t) \quad (9)$$

with

$$c(t) = n_p(t) k_{p,convert} \quad (10)$$

The normalized amount of converted genetic material (e.g. mRNA) $n_g(t)$ that is subsequently available for the gene expression machinery, again considering a specific decay rate

Figure 2. Workflow of the applied simplified cell cycle resolved transfection model. The only assumed influence of cell cycle applies during the uptake phase of complexes from the medium. Cell death is neglected. Notation: n_c = number of active complexes in medium; n_p = number of further processable intracellular complexes/plasmids; n_g = normalized amount of converted genetic material that is subsequently available for the gene expression machinery; n_{gfp} = amount of active/detectable green fluorescent protein.

$k_{g,decay}$, is therefore:

$$\frac{dn_g}{dt} = c(t) - k_{g,decay} n_g(t) \quad (11)$$

and the combined expression and decay rate of the reporter protein (d2eGFP), with its amount denoted as n_{gfp} , is

$$\frac{dn_{gfp}}{dt} = k_{gfp,build} n_g(t) - k_{gfp,decay} n_{gfp}(t) \quad (12)$$

The used parameters are summarized in Table 1. They were approximated such that cell cycle distribution around the transfection time points, cell numbers, and overall reporter protein expression time course roughly agree with the average experimental data of all three X1, three X2, and three NC experiments (see Fig. 4 for example). The model has been implemented in Microsoft Excel due to its simplicity and for the sake of easy portability; it is available upon request.

Figure 3. Transfection efficiencies of different transfection conditions. Incubation time: HEK293s cells were incubated with Lipofectamine 2000 DNA complexes for 3/6/9/24 h, subsequently medium was exchanged. Unchanged level of highly positive eGFP expression indicates interaction takes place within the first 3 h. Vesicle stability: Identical Lipofectamine 2000 DNA complexes were stored under culture conditions for 3/6/9/24 h before being added to HEK293s cells. Decreasing levels transfection indicate a loss of functionality over time.

Table 1. Summary of parameters used for the simplified transfection model

Parameter	Value	Unit	Parameter	Value	Unit
dt	1	h	n_{tr}	1	1/mL
$t_{cc,p}$	30	h	$t_{c,decay}$	6 or 24	h
$t_{cc,s}$	−6 (X1)	h	$k_{up,G1}$	Variable	—
	9 (X2)	h	$k_{up,S}$	Variable	—
I_{m0}	0.15	—	$k_{up,G2M}$	Variable	—
$k_{l,decay}$	−0.2	h	k_{up}	Variable	$1/(10^6 \cdot h)$
t_{trans}	Variable	h	$k_{gfp,build}$	0.05	1/h
COG_{G1}	0.547	—	$k_{gfp,decay}$	0.346	1/h
COG_S	0.234	—	$k_{p,decay}$	0.005	1/h
X_0	0.85	$10^6/mL$	$k_{p,convert}$	0.01	1/h
μ	−0.009	1/h	$k_{g,decay}$	0.05	1/h

Variable parameters are given for specific conditions in the Results section.

COG = centers of gravity.

3 Results and discussion

3.1 Flow cytometry analysis

Expression level of the reporter protein d2eGFP was detected in the FITC-A channel. In line with the manufacturer's protocol [11], maximum expression levels were detected 48 h after transfection. Only high producers were defined as transfected. PerCP-A and FITC-A crosstalk was moderate and negligible in the context of the described, standardized quantification method (above and [10]), its accuracy, and approach to limit the experimenters subjectivity.

Figure 4. Time courses of the nine transfection experiments (three times X1, X2, and NC, respectively). One single outlier sample at $t \approx 23$ h was excluded from analysis due to an erroneous staining and subsequent inaccurate gating. Overlaid is an example (X1-T3 with $k_{up,G1} = k_{up,S} = k_{up,G2M} = 1$) of simulated transfection course of the model described in Section 2.6.

3.2 Incubation time and complex stability

One key element in studies using Lipofectamine 2000, self-evidently, is the stability of Lipofectamine 2000 DNA complexes. A previous version of the manufacturer's protocol [13] states that the Lipofectamine 2000 DNA complexes can be removed after 4–6 h without diminishing the transfection efficiency. Furthermore complexes are described as stable for 6 h.

In fact, the experiments conducted as described in Sections 2.3.1–2.3.2 confirmed that the transfection efficiency does not correlate with the incubation time. The maximum transfection efficiency can be obtained after 3 h or less; longer incubation times did not increase the efficiency (Fig. 3). However, Lipofectamine 2000 DNA complexes, stored under suspension culture conditions without cells, could transfect cells even after 6 h or more, although transfection efficiency declines over time and largely diminishes within 24 h.

From this, we can draw the conclusion that certain amounts of Lipofectamine 2000 DNA complexes itself remain stable for up to approx. 24 h, if kept under suspension culture conditions.

These experimental data provide some substantiation to the hypothesis that a certain percentage of Lipofectamine 2000 DNA complexes could linger in the cell culture medium until cells enter cell cycle phases that are more susceptible to transfection. This has to be considered when drawing conclusions on the potential cell cycle dependency of plasmid uptake rate.

3.3 Distortive effects on cell cycle

Previously, we established a method to create near-physiologically synchronized cultures of HEK293s cells [10]. Here, such synchronized cultures were used for transfection studies. Cell cycle data prior to transfection confirmed synchronous growth as expected and shown before [1]. However, subsequent to the transfection, the cycle synchronization declines (Fig. 5). This is in line with observations made on extended analyses of earlier data [1] which consistently show a flattening of originally oscillating cell cycle distribution approx. 24 h after transfection (Fig. 6). It is identifiable from the

direct comparison of the graphs representing the G1 percentages of the nontransfected culture (G1 NT) to the transfected culture (G1). While both graphs display a decrease in amplitude over time, their courses differ. The nontransfected culture (NT) follows the expected damped oscillation, while the transfected culture (G1) displays a vigorous response to the transfection, including reduced G1 percentage and very low oscillation.

3.4 Cell cycle dependent transfection of HEK293s

Transfection of branches of the oscillating cultures was conducted at precalculated points in time, covering well distributed t_{cc} values. These t_{cc} values originate from the X1 as well as X2 fraction, both transfected within 19.5–27.5 h after elutriation (Fig. 1). In theory, all t_{cc} values could also be obtained using a single synchronized culture over a longer period of time. But due to the fact that synchronicity decreases over time (Fig. 6, [10]), here a combination of X1 and X2 was chosen and used in a shorter time frame.

The time courses of all transfection experiments (three times X1, X2, and NCs, respectively), are given in Fig. 4, with time scale relative to the transfection time point. Before the transfection peak, which is around 48 h posttransfection, the data vary merely between the source cultures (X1 vs. X2), but not with respect to the transfection time point (T1...T3). Peak values, around $t \approx 48$ h, are very similar in all cases. The decay of fluorescence after the peak scatters to some extent.

In contrast to studies previously published in literature [6–8], the peak transfection efficiency did not correlate with different cell cycle states (here represented as t_{cc}). Neither with the t_{cc} in general, nor with the course of time in single synchronized cultures (i.e. X1). There was also no systematic difference between the synchronized cultures and the NC. This is also true for the mean brightness values of positive cells, detected 48 h after transfection, at their maximum levels (data not shown). While peak transfection efficiencies in all cases yielded $48.1 \pm 1.5\%$ ($n = 9$), mean brightness values were 8750 ± 750 A.U (Fig. 7).

Figure 5. Synchronized cell cycle progression after transient transfection using Lipofectamine 2000. All cell cycle phases (G1, S, and G2/M) cease to oscillate after transfection, in line with data from Fig. 4.

Figure 6. Comparison of general effect of lipofection on synchronized cell culture, example for G1 distribution: nontransfected (G1 [NT]) and unaffectedly oscillating culture in light blue (data of “X1” culture in [1]) versus branch of the same culture (G1), transfected at $t \approx 18$ h (red line), denoted in dark blue dotted curve. The transfection was briefly evaluated in [1], however cell cycle effects were not shown. The original oscillation remains further for max. 24 h and then diminishes totally.

3.5 Model-based estimation of upper limit of potential cell cycle dependency of transfection

The obtained results showed no obvious cell cycle dependency of the peak transfection efficiency or time point, however, the data are affected by two inevitable experimental shortcomings: one, the cultures are never perfectly synchronized, i.e. their behavior at all the time points is the result of a (variable) mixture of multiple cell cycle populations. Two, the complexes remain active in cell culture medium for relatively long time, as shown in Section 3.2. This enables to hypothesize that complexes could remain unaffected and “wait” until a cell is able to incorporate them. In a theoretical extreme case, if the complexes showed no inactivation at all, potential cell cycle variable uptake rates would show no effect on the result in the given experimental setup. However, since inactivation occurs within 24 h or faster (Section 3.2), some effect of potential cell cycle uptake variability is expected to be seen in the experimental data.

To quantify this expected effect, a strongly simplified cell population model has been set up (Section 2.6). This model

assumes potential cell cycle dependent variations of the plasmid uptake process; however after that, no further variations are considered for simplicity.

Assuming no cell cycle dependent variations (i.e. $k_{up,G1/S/G2M}$ all set to 1) and after manual parameter adaptation, the model reproduces the experimental data well (see Fig. 4 for visualization of one example) and shows a comparable variance in results (relative standard deviation SD_{sim} of 3.0% with $n = 6$, compared to relative experimental variation SD_{exp} of 3.1% with $n = 9$) as in the experimental data. In the model, this variance is caused by variable cell densities at the different transfection time points; in the experimental data, the same effect holds true but is additionally overlaid by experimental noise.

With variation of cell cycle specific parameters (i.e. at least one parameter $k_{up,G1/S/G2M} \neq 1$), the simulated peak transfection efficiency varies depending on the transfection time point, however not always very strongly. For example, a variation of either parameter by a factor of 2, and considering the measured long-term complex activity of 24 h, yields only a very marginal

Figure 7. Transfection efficiency and mean brightness 48 h after transfection. Percentages of transfection efficiency vary minimally ($48.1 \pm 1.5\%$) showing no systematic correlation with t_{CC} , X1, X2 fraction, or the NC. Likewise mean brightness values (8750 ± 750 A.U.) display no correlation.

Figure 8. Visualization of expected variations of transfection efficiency compared to the experimental observations, depicted by factor F . Data according to the model, assuming transfection (time point, cell cycle position, etc.) according to our setup (Fig. 1), given assumed specific ratios of cell cycle dependent uptake rates: (A) for $k_{up,G1}/k_{up,S}$ versus $k_{up,G1}/k_{up,G2M}$; (B) for $k_{up,G1}/k_{up,G2M}$ versus $k_{up,S}/k_{up,G2M}$. Note the logarithmic scale of the ratios. For ratios of 1 versus 1, the variations are trivially low ($F \approx 1$, black). The positions reported in literature are depicted by arrows. The interpretation is as follows: with any arrow located at a high (orange to red, $F \geq 2.5$) position, indicating the assumption of a certain ratio of uptake rates, one would expect strong (i.e. significantly higher than observed) variations of transfection efficiency in our experiments, even given the experimental uncertainties of our setup and the slow inactivation of vesicles. This is the case for the two literature data points; indicating that such strong k_{up} variations are not in line with our experimental data. Hypothetical k_{up} variations located in the black to yellow areas however would not be detectable given our setup, hence these are the regions that can neither be excluded nor confirmed by our data. Surface plots were done with Gnuplot version 4.4 (Interpolation: 15×15 gridding, $qnorm = 5$).

increase of SD_{sim} to $\approx 3.2\%$, a difference which would very likely not be possible to determine experimentally given the underlying experimental variations.

We consider a simulated variation to be significant compared to the experimental data only if the maximum deviation of at least one single simulated point is at least three-fold higher than the SD_{exp} (with $n = 9$); this fold factor is denoted as F . The simulated F , depending on (pairwise ratios of) combinations of $k_{up,G1}$, $k_{up,S}$, and $k_{up,G2M}$ is depicted in Fig. 8. Strong variations, i.e. $F > 3$, correspond to a red to white color in Fig. 8. Low significances, i.e. little expected variations during the cell cycle are depicted in black to gray colors. Mediocre regions ($F \sim 2$) are in yellow. Generally, when all k_{up} are similar, i.e. all given ratios are around 1, F is close to 1 (black), as expected.

Apparent overall transfection efficiency variations given in literature are much larger than 2 for lipofection, e.g. $k_{up,G1} : k_{up,S} : k_{up,G2M}$ ratios of roughly 0.2:1:3 [6] or 30:5:1 [7]. These and some other examples are summarized in Table 2; the corresponding positions in the response map of Fig. 8 are given by arrows. In the first case, the simulated expected maximum variation is 10.6%, hence $F > 3$, which leads to the conclusion that the ratio of 0.2:1:3 is likely to be overestimated; this corresponds to the red color in Fig. 8 at this point. In the second case, the simulated maximum variation is only 7.6%, hence $2 < F < 3$, so we consider this result only slightly significant, corresponding to yellow to orange color at these points in Fig. 8. Consequently, the finding of a ratio of approx. 30:5:1 slightly contradict our results, however not very significantly.

Table 2. Simulated effects of assumed cell cycle dependent transfection variations (uptake rates) compared to experimental data

Parameter	Variants									[7]	[5]
$k_{up,G1}$	1	0.5	1	1	0.1	1	0.1	0.1	1	3	0.2
$k_{up,S}$	1	1	0.5	1	1	1	0.1	1	0.1	0.5	1
$k_{up,G2M}$	1	1	1	0.5	1	0.1	1	0.1	0.1	0.1	3
k_{up}	0.012	0.015	0.013	0.012	0.021	0.014	0.036	0.035	0.02	0.006	0.01
Result											
Maximum single deviation	3.54%	5.26%	4.49%	4.97%	7.96%	6.37%	11.72%	10.64%	7.29%	7.61%	10.65%
Fold sigma (F)	1.1	1.7	1.4	1.6	2.6	2.1	3.8	3.4	2.4	2.5	3.4
F, if $t_{c,decay} = 6$ h										4.1	7.1

Significance of simulated (expected) variations w.r.t. the measured low experimental variations is indirectly given by the fold sigma factor (F); if $F > 3$ then it is assumed that a significantly stronger peak transfection efficiency would be expected than measured ($SD_{exp} = 3.1\%$, $n = 9$). k_{up} is a scaling factor given for information; it varies to adapt absolute values of the simulation to experimental data (transfection peak height); however, it has no effect on the relative results used for further analysis. Visualization of the same (interpolated) data is given in Fig. 8.

Supposed that the complex inactivation takes place within 6 h, which is stated by the vendor but could not be confirmed by us, both simulations would yield strongly significant results, meaning that the given ratios do significantly not fit our experimental data and hence should be smaller. In the context of Fig. 8, it means that the whole response area is raised by a certain amount, leaving only a very small black to gray to yellow region (very close to [0,0]) that indicates low expected variations.

Generally, strongly elevated transfection efficiencies can be excluded rather safely for sole S or G2/M phases, corresponding to high-level, red to white regions in Fig. 8. However, similar putative elevation in G1 or combined G1/S phases could not be safely detected with our setup, given the apparent long complex stability, corresponding to the grayish to yellow areas in Fig. 8.

Considering the fact that this is the first published study of transfection efficiencies using unperturbed, i.e. freely oscillating synchronized cells under physiological conditions, the cell cycle dependency of the transfection process under these conditions for the used cell line is likely to be much smaller than conventionally assumed.

4 Concluding remarks

This study did not support the suggested strong cell cycle dependency of transfection reported in literature [6–8], using Lipofectamine 2000. Transfection efficiencies of HEK293s cells, for the first time transfected after physiological synchronization and in freely oscillating state, showed no obvious cell cycle dependency. Modeling further showed that the variations of transfection efficiencies assigned to single cell cycle phases are less pronounced than expected; in our case it is likely that the relative efficiency at the end of the cell cycle (S and/or G2/M) is smaller than 10-fold the efficiency at the beginning (G1). This factor is significantly smaller compared to previously reported factors of 15–500 [6]. If Lipofectamine 2000 DNA complexes inactivation rate under cultivation conditions is as high as stated by the manufacturer (6 h), which we could not observe in comparison experiments, the obtained experimental results could be considered to be more accurate and hence the corresponding upper limits of the derived relative efficiency factors would be even lower.

Our work, being the first one using controlled freely oscillating cultures under near-physiological conditions for transfection [10], is a notable contrast to previous studies where cells have been elutriated using low temperatures (15°C) and stored at 4°C until immediate transfection [6]. Other studies used chemical methods for synchronization [7,8], an approach intrinsically flawed [5,14]. Our work further demonstrated the strong effect of the transfection procedure on the synchronicity of the culture, as synchronization is lost rapidly (within approx. 24 h) after transfection, while it is well maintained otherwise.

Practical application

Previous studies, using chemical or physical whole culture methods to force cultures into synchronized growth, reported a strong cell cycle dependence of transfection efficiency of mammalian cells. This could have impacts on process development. In contrast to the previous reports, our studies, utilizing controlled synchronized cultures of HEK293 cells under near-physiological conditions, do not support such a conjecture. We show that cell cycle dependency of transfection is not as pronounced as previously assumed. On the other hand, we demonstrate that functional studies based on recombinant reporter protein expression by transfection with reporter plasmids and Lipofectamine 2000, are associated with inherent limitations. The transfection procedures distort the synchronous progression of the cell cycle.

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