CORRELATION BETWEEN CELL CYCLE AND CALCIUM PHOSPHATE TRANSIENT TRANSFECTION OF CHO CELLS

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In memory of Dr. Pascal Batard

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Résumé

La transfection par la méthode du calcium phosphate est une méthode largement utilisée pour produire des protéines recombinantes dans des cellules mammifères. Cependant, les mécanismes impliqués dans le transfert de l'ADN plasmidique dans le noyau des cellules transfectées sont encore mal compris et cela résulte en une grande variabilité de l'efficacité de transfection. Le rôle du cycle cellulaire dans la transfection par la méthode du calcium phosphate a donc été étudié en utilisant des cellules dérivées d'une lignée d'ovaire de hamster (CHO) comme modèle.

Des cultures de cellules adhérentes ont été synchronisées à la frontière entre les phases G1 et S grâce à l'utilisation de la mimosine. Une fois la drogue de synchronisation retirée, les cellules ont été transfectées à différents moments suivant la réinitiation du cycle cellulaire avec des plasmides exprimant soit la protéine fluorescente verte (EGFP) soit une protéine fluorescente rouge (DsRedExpress). L'expression de ces protéines fluorescentes a été suivie par fluorométrie ou par imagerie en temps réel et le cycle cellulaire a été analysé par cytométrie en flux.

Il a été montré que le cycle cellulaire a des implications majeures au moins à deux niveaux. La corrélation entre le pourcentage de cellules en phase S au moment de l'addition du complexe de transfection et l'expression de gène rapporteur résultante a démontré que le transfert efficace d'ADN exogène dans les cellules mammifères était dépendant du cycle cellulaire. Un protocole de transfection plus rapide, développé pendant ce travail, a montré que le moment optimal pour ajouter le complexe de transfection était dépendant du système de transfection utilisé. D'autres investigations ont démontré que la mitose jouait un rôle clé, sans doute dû à la rupture de la membrane nucléaire, pour fournir un accès aux molécules d'ADN plasmidique dans l'environnement nucléaire.

Le choc glycérol réalisé à la fin de la transfection a montré qu'il jouait aussi un rôle important pour le transfert efficace de gène rapporteur dans les noyaux. Ce choc osmotique important résulte en une diminution du volume cellulaire d'environ 55%. Comme il n'y a pas de perte de plasmide intracellulaire pendant ce choc, cela résulte en une augmentation significative de leur concentration intracellulaire. Le choc glycérol doit être effectué pendant que les cellules progressent à travers la mitose, probablement car la désagrégation de la membrane nucléaire

et l'augmentation de la concentration intracellulaire en plasmides facilitent l'accès à l'environnement nucléaire.

La synergie entre le choc glycérol et les cellules progressant à travers la mitose était une nécessité pour un transfert efficace de gènes rapporteurs dans les noyaux. De très faibles niveaux d'expression de gènes rapporteurs ont été obtenus lorsque l'un ou l'autre de ces événements était omis ou s'ils ne se produisaient pas au même moment.

Comme il a été montré que l'ADN plasmidique permettait une expression de gène rapporteur efficace même après un délai de 45 minutes suivant leur internalisation par les cellules, il a été possible de les transférer dans des sous-populations cellulaires additionnelles, lorsque celles-ci passaient à travers la mitose, à l'aide de chocs glycérols supplémentaires. Ces chocs répétés ont résulté en une augmentation de cellules positives parmi les populations transfectées.

En conclusion, les résultats obtenus pendant ce travail ont permis de définir un modèle expliquant l'importance du cycle cellulaire dans la transfection par la méthode du calcium phosphate, mettant en évidence la mitose et le choc glycérol comme des événements clé pour le transfert de molécules d'ADN plasmidique dans les noyaux.

Abstract

Calcium phosphate transfection is a widely used method to produce recombinant proteins in mammalian cells. However, the mechanisms involved in plasmid DNA transfer to the nucleus of the transfected cells remain poorly understood and result in great variation of transfection efficiency. The role of the cell cycle in transfection by the calcium phosphate method was studied using Chinese Hamster Ovary (CHO) cells as a model system.

Adherent cell cultures were synchronized at the G1/S boundary using mimosine. At various time points after the removal of the drug the cells were transfected with reporter plasmids that expressed either the enhanced green fluorescent protein (EGFP) or a red fluorescent protein (DsRedExpress). Fluorescent protein expression was monitored by fluorometry and live imaging. Cell cycle was monitored by flow cytometry.

It was shown that the cell cycle had major implications on at least two levels. The correlation between the percentage of cells in S phase at the time of transfection complex addition and the resulting reporter gene expression demonstrated that efficient transfer of exogenous DNA in mammalian cells was dependent on the cell cycle. A faster transfection protocol, developed within this work, showed that the optimal timing for the transfection complex addition was different depending on the transfection set-ups used. Further investigations demonstrated that mitosis played a key role, probably due to the nuclear membrane disruption, to provide access to the nuclear environment for the plasmid DNA molecules.

The glycerol shock performed at the end of the transfection was also shown to play an important role for efficient transfer of reporter gene in nuclei. This strong osmotic shock resulted in a cellular volume decrease of approximately 55%. As no intracellular plasmids were lost during the process, this resulted in a significant increase in intracellular plasmid concentration. Glycerol shock has to be performed while cells proceed through mitosis. The nuclear membrane disruption and the increasing intracellular plasmid concentration probably facilitate the access to the nuclear environment.

The synergy of glycerol shock and cells proceeding through mitosis was a requirement for efficient reporter gene transfer in the nuclei. If one of those two was omitted, or if they did not happened at the same time, very low reporter gene expression levels were achieved.

As it was found that plasmid DNA was driving reporter gene expression even after a delay of 45 minutes post cellular uptake, it was possible to efficiently target additional sub-populations of cells as they proceeded through mitosis with additional glycerol shocks. Those repetitive glycerol shocks resulted in an increase of positive cells among the transfected populations.

In conclusion, the results obtained in this work permitted to define a model explaining the importance of the cell cycle in the calcium phosphate transfection, highlighting mitosis and glycerol shock as key events in plasmid DNA molecules transfer to the nucleus.

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Aim of this work

Various methods exist to transfect mammalian cells in culture. It is generally accepted that individual methods have to be optimised for each of the cell lines or cell types used. Despite the fact that calcium phosphate transfection is widely used, many questions remain about its mode of action. Repetitive transfections in apparently identical conditions may results in significant day-to-day variation efficiency, despite the use of optimised protocols.

Some parameters can be controlled such as the cell density, the pH of the medium, the pH of the transfection solution or the incubation time in presence of the precipitate. But many parameters are difficult to control such as levels of plasmid uptake, calcium phosphate DNA complex morphology and internal plasmid degradation in the transfected populations. All those parameters play an important role in efficient transfer of plasmid DNA into nuclei of host cells.

Studying the cell cycle implication in transient transfection with two powerful methods (flow cytometry and live imaging) provided ways to better understand the mechanisms implicated in the transfer of plasmid DNA in the nuclei of transfected cells and tentatively fill gaps present in previous models.

This also has implications for stable cell lines production, as stable integration is a random event within the transfected cell population. If phenomena leading to transient transfection are better controlled, the efficiency of stable transfections may be improved as well.

A better understanding of the calcium phosphate transfection is expected to result in a more efficient ratio between plasmid DNA input and recombinant protein output.

Introduction

1. Calcium phosphate transfection

By definition, transfection is the introduction of a foreign DNA molecule into a eukaryotic cell and the subsequent expression of one or more genes in the transfected cell (Alberts et al., 1994). Many different chemical agents that facilitate transfection are commercially available. Calcium phosphate-DNA coprecipitation was first described in 1973 and is one of the most widely used transfection methods (Graham and van der Eb, 1973). It works with many different cell lines, and it is simple and cost effective. During calcium phosphate transfection, DNA is complexed with calcium and phosphate to form small insoluble particles that can be endocytosed by cells (Loyter et al., 1982a; Orrantia and Chang, 1990). The DNA is released into the cytoplasm by an unknown mechanism and eventually ends up in the cell nucleus where it is transcribed. Movement of plasmid DNA from the cytoplasm to the nucleus probably occurs by diffusion, a slow process during which it may be degraded by DNases (Loyter et al., 1982b).

Variation of reporter gene expression in individual cells post-calcium phosphate transfection is not due to inability of the complex to enter the cells because most cells take up plasmid DNA when transfected by this method (Batard et al., 2001) (**Figure 1**).

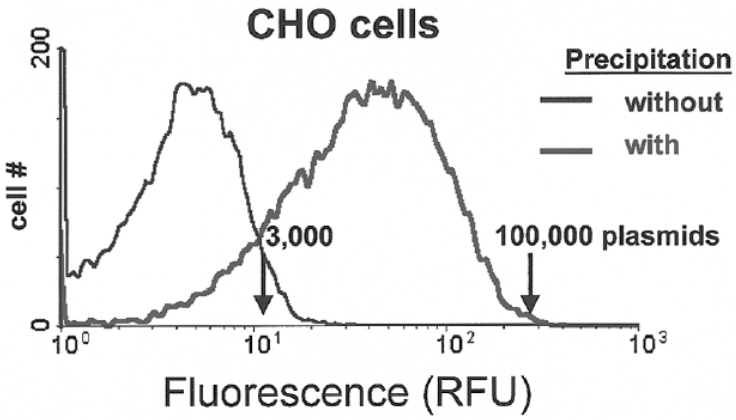


Figure 1. Calcium phosphate-mediated plasmid uptake is efficient. Fluorescein-labeled plasmid uptake was monitored by flow cytometry after 4 hours incubation. Left curve represents cells incubated without precipitate (negative control). Right curve represent cells incubated in the presence of calcium phosphate stained-DNA precipitate. The whole curve is shifted towards higher fluorescent values (compared to auto-fluorescence shown with the negative control) meaning that almost all the cells did actually take up plasmid during the 4 hours incubation. (Adapted from Batard *et al.*, 2001)

The time period of calcium phosphate-DNA coprecipitation prior to addition to cultured cells is an important parameter for efficient transfection (Jordan et al., 1996; O'Mahoney and Adams, 1994; Wake et al., 1984). Efficient transfection is also highly dependent on the pH of the calcium phosphate solution (Loyter et al., 1982a). The optimal pH ranges from 7.1 to 7.5. A higher pH results in the formation of large calcium phosphate complexes that do not enter cells. Calcium phosphate precipitate morphology plays an important role in transfection efficiency (**Figure 2**). Yang *et al.* (Yang and Yang, 1997) also reported effects on calcium phosphate precipitate morphology due to the culture medium (DMEM) and the presence of serum. They showed that precipitates underwent several steps of conversion in the presence of other components in the cell culture medium. The medium components participating in the calcium-phosphate complex formation were not identified, however.

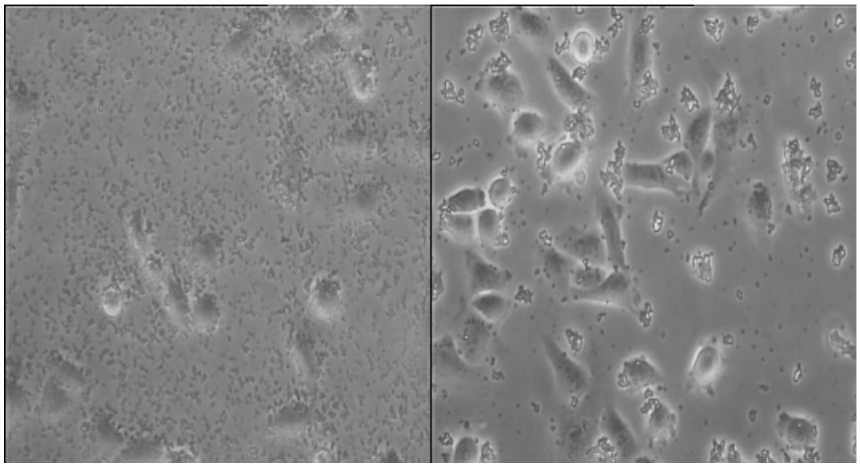


Figure 2. Small calcium phosphate-DNA precipitates (left) results in higher reporter gene expression post-transfection than larger precipitates (right). Pictures were taken three hours after addition of the transfection complex to the cells.

Many factors related to the physical properties of the calcium phosphate-DNA coprecipitate itself help to achieve an efficient gene transfer (Loyter et al., 1982b). These include:

- Precipitate formation increases the plasmid DNA concentration on the cell surface.
- The precipitate protects plasmid DNA from digestion by DNases.
- The precipitate enters cells through endocytosis (calcium phosphate precipitate without DNA is also taken up by cells)

- Electron microscopic data showed that the entire calcium phosphate DNA complex enters the cell and the nucleus

It is not the concentration of plasmid DNA on the cell surface that affects the level of plasmid uptake, but more likely the morphology of the calcium phosphate-DNA coprecipitate. Precipitate formation in presence of high plasmid DNA concentration results in inefficient precipitate uptake (Loyter et al., 1982b). Calcium phosphate alone also enter cells, suggesting the absence of a system on the membrane surface facilitating the entry of DNA into a cell (Loyter et al., 1982a). Controlled cells incubated in the absence of calcium phosphate precipitate had much less endocytotic vesicles as compared with the cells exposed to the precipitate (Loyter et al., 1982a).

Calcium phosphate may play a role in escape of DNA from endosomes. In vitro studies have demonstrated that it promotes fusion between biological membranes and phospholipid barriers (Fraley et al., 1980; Zakai et al., 1977). Within cells it may cause local solubilization of endosomal membrane allowing passage of plasmid DNA into the cytoplasm. Plasmid DNA was shown to access the nucleus still complexed with calcium phosphate (Loyter et al., 1982a). Using DAPI-stained plasmid DNA, the same authors also shown that the first signal of plasmid DNA inside the cell occurred after 2 hours of exposure to calcium phosphate-DNA complexes and increased for the next 8 hours. All exposed cells ended up with internalized calcium phosphate-DNA complexes in the cytoplasm, but only a few of them had detectable transfection complexes in the nucleus. It appeared that most of the calcium phosphate-DNA complexes present in the endocytotic vesicles were eliminated by exocytosis (Loyter et al., 1982a).

Movement of the coprecipitate complexes from the cytoplasm to the nucleus and their entry into the nucleus are the most significant barriers to gene transfer. It was shown that transfected cultures transferred less than 10% of internalized plasmid DNA into nuclei (Orrantia and Chang, 1990). Another 20% was recovered in the plasma membrane fraction as well as in the vesicular organelles fraction, and 50% was recovered from cytosolic fractions but was completely degraded into oligonucleotides of less than 100 base pairs. Intact plasmids as well as small DNA fragments were found in the nuclei; organelles fractions were enriched with intact plasmids and contained very little small fragments. These observations were confirmed by Lechardeur *et al.* (Lechardeur et al., 1999) who showed that plasmid DNA, both

single-stranded and double-stranded, has a half-life of approximately 50 – 90 minutes in the cytoplasm where it quickly got degraded by nucleases. The instability of plasmid DNA caused by cytosolic nucleases constitutes an additional barrier to the efficient translocation to the nucleus of the precipitate. These authors also showed that it was unlikely that lysosomal nucleases were responsible for the rapid turnover of plasmid DNA because dissipation of the acidic pH of the lysosomes with ammonium chloride or chloroquine did not delay the degradation of injected DNA (Lechardeur et al., 1999).

It was also shown that diffusion of free DNA from the cytoplasm to the nucleus is a very inefficient process (Capecchi, 1980). Therefore, transport to the nucleus of free DNA would be rather long, risking degradation by nucleases. One can assume therefore that most of the plasmid DNA in the cell is not in the form of free DNA but rather complexed with calcium phosphate, the latter providing protection against nucleases. Nuclear entry has been reported to be a major limiting step in non-viral gene transfer ((Dowty et al., 1995; Escriou et al., 1998; Mirzayans et al., 1992; Zabner et al., 1995). Plasmid size also plays a role in its intracellular diffusion following microinjection into the cytoplasm (Neves et al., 1999). Large plasmid (7Kbp) did not readily diffuse in the cytoplasm and remained at the site of injection. The low diffusion rate of plasmid and its degradation in the cytoplasm may be two of the most important factors limiting non-viral gene transfer efficiency (Lechardeur et al., 1999; Pollard et al., 1998).

Other obstacles to nuclear accumulation include slow internalization process of transfection complex in the cells, entrapment of plasmid DNA in the endolysosomal compartment, diffusion inside the cytoplasm and nuclear envelope barrier (Capecchi, 1980; Ciftci and Levy, 2001; Coonrod et al., 1997; Loyter et al., 1982a; Loyter et al., 1982b; Neves et al., 1999; Pouton and Seymour, 1998).

Orrantia *et al*. (Orrantia and Chang, 1990) hypothesized that plasmid DNA does not pass through the cytosol before it enters the nucleus but is transported directly from the endosomes-lysosomes to the nucleus through an intermediary vesicle (**Figure 3**). They demonstrated that inhibitors of lysosomal functions increased the efficiency of calcium phosphate DNA delivery to the nucleus, presumably by inhibiting lysosomal degradation of internalized plasmid DNA.

Other authors reported that plasmid DNA is released from vesicles before nuclear entry (Lechardeur et al., 1999). Small linear DNA (<1 Kbp) can enter the nucleus through the nuclear pore complex (NPC), but larger DNA (>1 Kbp) can not diffuse through the NPC and remains in the cytoplasm and thus requires an alternative route into the nucleus (Hagstrom et al., 1997)

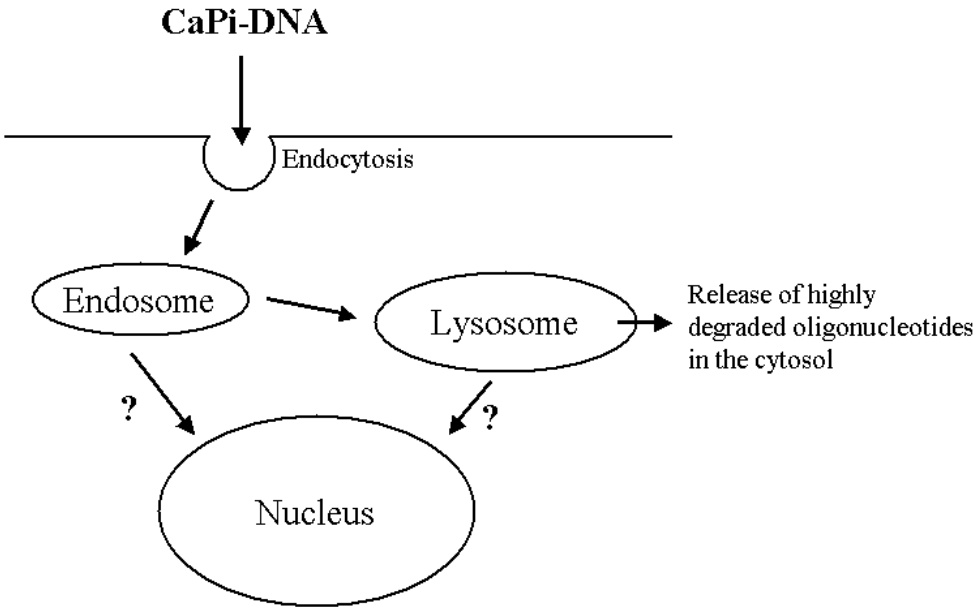


Figure 3. Proposed model for transport of calcium phosphate DNA complex to the nucleus (Adapted from Orrantia *et al.* (Orrantia and Chang, 1990)).

Liberation of plasmid DNA from the transfection complex must occur within the nucleus before transcription can proceed. If the precipitate is too stable then no, or very little, transcription will occur; if it is not stable enough then degradation will occur before the plasmid DNA reaches the nucleus (Luo and Saltzman, 2000b).

1.1 Transient transfection

The goal of transient transfection is to introduce foreign DNA into an entire population of host cells in order to transiently produce a recombinant protein without having to isolate a stable cell line (Wurm and Bernard, 1999). Therefore, this approach does not require the long selection, amplification and cloning procedures necessary to achieve a high producing stable cell line. Plasmid DNA is transfected into the host cells and the recombinant protein is

expressed for a few days. The plasmid DNA is then either degraded or diluted among each new generation of daughter cells, and the recombinant gene expression eventually diminishes. Both intracellular and secreted proteins have been expressed in this way. Transient transfection is not only a rapid tool to investigate the biological properties of a given recombinant protein, but it has also been used to produce recombinant protein at large scale (Girard et al., 2002). One drawback of this method is the need for large amounts of plasmid DNA especially when used in cell culture volumes of tens or hundreds of liters. Often, transient transfection is performed using the cost effective calcium phosphate-DNA coprecipitation method. A better understanding of the calcium phosphate transfection is expected to result in a more efficient ratio between plasmid DNA input and recombinant protein output.

Many different approaches can be considered to improve reporter gene expression in transient transfection. The recombinant gene can be codon optimized for the respective host cell system. Secondly, different eukaryotic promoters have differing strengths in any particular mammalian cell line (Ray and Gage, 1992; Wenger et al., 1994) (**Table 1**).

Table 1. Promoter strength in CHO cells

	Relative promoter strength in CHO cells
CMV	643
RSV	71
SV40	100

(Adapted from Invitrogen Life Technologies informative poster)

Transient transfection also depends on the transfection method. Many different transfection reagents exist which yield different transfection efficiencies depending on the cell line transfected. Calcium phosphate is relatively cost effective as compared to other methods (**Table 2**). This method works with many different cell lines and in different systems (adherent cells, cells in suspension, small and large scale cultures) (Girard et al., 2002).

Table 2. Cost comparison for different transfection reagents (cost for a transfection in a one milliliter volume) (Adapted from (Girard, 2001))

Transfection reagent	Cost per well [CHF/ml]
CalPhos Max	0.1
CaPi	0.01
CLONfectin™	1.23
DOSPER	1.22
DOTAP	0.66
Fugene™	1.55
LipofectAMINE™	2.79
PEI	0.01
Superfect™	1.24
Transfectam®	3.00

The condition of the cultured cells is another important parameter for transient gene expression. Not every cell in a transfected population will efficiently take up plasmid and/or support the movement of the plasmid into the nucleus. In this work it is shown that the cell cycle plays a crucial role in the efficient nuclear uptake of plasmid in CHO cells using calcium phosphate as a transfection agent.

1.2 Green Fluorescent Protein (GFP) as reporter gene

The green fluorescent protein (GFP) was isolated from *Aequorea victoria* (Shimomura et al., 1962). It is widely used as a reporter protein in gene expression studies since it can be easily detected using fluorescent microscopy in a non-invasive manner. Measurement of the transfected cells can be repeated as often as required, and the GFP level can be measured quickly by fluorometry (CytoFluor 4000) or by flow cytometry.

Many different variants of GFP are commercially available. Most of them are modified versions of wild-type GFP (wtGFP) with either a stronger emission signal in mammalian cells, as for the enhanced GFP (EGFP), or with fluorescence emissions of different wavelengths such as the enhanced blue (EBFP), enhanced cyan (ECFP) and enhanced yellow (EYFP) fluorescent proteins (**Table 3**). EGFP has 35 times the intensity of wtGFP (Clontech, Living Colors™ User Manual). Its excitation wavelength of 488nm makes it particularly interesting, since most flow cytometers are equipped with a 488nm laser.

A fluorescent protein that shifts from green fluorescence when initially expressed to red fluorescence when fully maturated has also been reported (Terskikh et al., 2000). Another family of fluorescent molecules has been isolated from *Discosoma sp* and provides a red shifted variant of GFP called DsRed (Matz et al., 1999). DsRedExpress, a variant of DsRed, is similar to EGFP. The resulting fluorescent proteins are in both cases intracellular. They have a comparable maturation rate, and one can be detected as quickly as the other. DsRedExpres has spectral properties (**Table 3**) that are ideal for dual-color experiments with EGFP (Bevis and Glick, 2002).

Table 3. Excitation and emission maxima for different variants of GFP

	Excitation maxima	Emission maxima
wtGFP	395 nm	508 nm
EGFP	488 nm	507 nm
EBFP	380 nm	440 nm
ECFP	433 nm	475 nm
EYFP	513 nm	527 nm
DsRed	557 nm	579 nm

(source: www.clontech.com)

1.3 CHO DG44 cells in biotechnology

Animal cell lines are widely used for the production of recombinant proteins such as hormones, enzymes, antibodies and vaccines. Animal cells are generally capable of secreting functional proteins that are correctly folded and are biological active. For drug development, the recombinant protein may require a human glycosylation profile if biological activity is necessary in the patient. Mammalian cells have the advantage over plant, insect, and prokaryotic cells, because they often provide such post-translational modifications.

The Chinese Hamster Ovary is popular for expression of recombinant proteins as its glycosylation machinery resembles that found in human cells (Jenkins and Curling, 1994). CHO cells are well characterized, stable, and able to produce proteins efficiently.

The CHO DG44 cell line lacks the enzyme dihydrofolate reductase (DHFR) and thus needs to be provided with both hypoxanthine and thymidine (Urlaub et al., 1986). If the cells are

transfected with a plasmid vector that encodes the DHFR gene, only transfected cells will actively grow in the absence of hypoxanthine and thymidine.

2. Cell cycle

Each new cell originates from another cell by a process called *cell division*. The dividing cell is called the *mother cell* and the two cells issued from the division are called the *daughter cells*. The mother cell transmits its genetic information to the two daughter cells. Each new cell division corresponds to a new cell cycle. Cells going through the actual division process are in the *mitosis* (M) phase and non-dividing cells are in *interphase* (the portion of the cell cycle between periods of cell division). Interphase may last for different periods of time (hours, days, weeks, or longer) depending on the cell type. It is divided into three phases: G1, S and G2. (**Figure 4**)

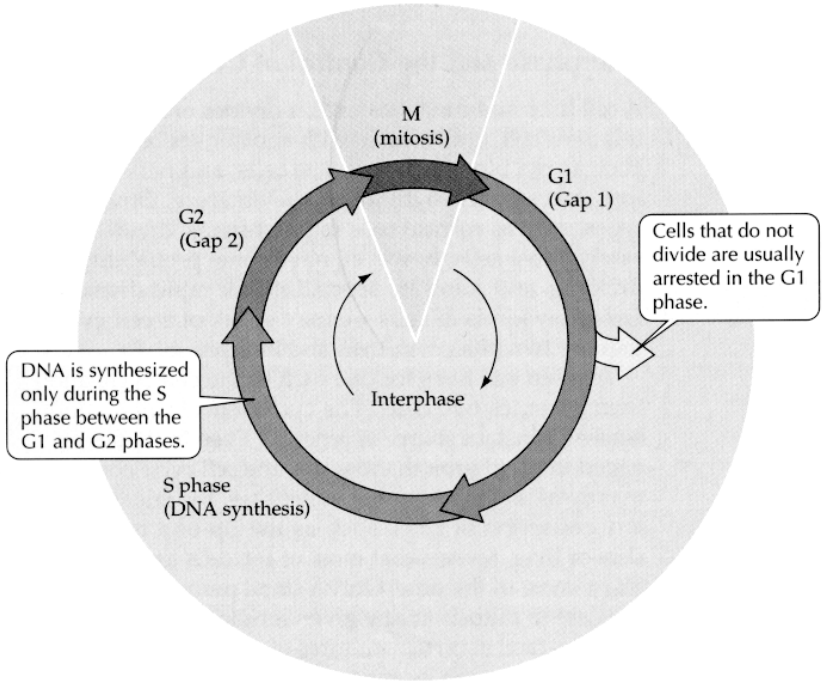


Figure 4. Schematic representation of the cell cycle. Adapted from Purves *et al.* (Purves et al., 1999)

Cell division (*cytokinesis*) actually occurs only when the genetic material has been fully duplicated and then segregated as two complete sets of chromosomes in distinct nuclei during mitosis. This segregation and cellular division is known as the M phase. Mitosis (prophase, metaphase, anaphase and telophase) is distinguishable from interphase by the condensation of the chromosomes. The chromosomes are highly condensed and very compact during mitosis, thus allowing an easier separation into the two daughter cells.

To provide a complete set of genetic material for each of its two daughter cells, the genetic information of the mother cell has to be duplicated, resulting in a cell with twice the normal amount of DNA. This duplication of the genome is performed during the synthesis (S) phase. Once mitosis has occurred, the two daughter cells each have a full copy of the DNA duplicated during the S phase. The S phase and mitosis are separated by the G2 phase where a checkpoint for completed DNA synthesis occurs. Mitosis and the S phase are separated by the G1 phase in which a checkpoint for DNA damage occurs.

Cells can reversibly enter a resting phase (G0) during G1 phase, if environmental conditions are not sufficient for growth and division. Under the appropriate conditions, cells resting in G0 will proceed through G1 phase.

2.1 Study of the cell cycle

Different approaches exist to study the cell cycle such as monitoring the cell concentration with time or visually following cells under a microscope. The use of specific stains, combined with flow cytometry, provides various means of estimation of cellular growth, as well as determination of doubling time and of percentage of cells present in the different phases of the cell cycle.

2.1.1 Growth rate

Following the cell growth of a culture (number of cells per milliliter as a function of time) is the simplest way to determine the average length of a cell cycle in a population. With every starting cell culture, there will be a lag phase in which little cell growth occurs. Cells will then grow and divide in an exponential manner until they reach a plateau phase where growth is inhibited. This may be due to a high cell density or a lack of one or more essential nutrients. By plotting the log of cell concentration versus time and then calculating the slope of the curve during the exponential growth phase, the *growth rate factor* (μ) is determined. The doubling time (i.e. the average cell cycle duration of the population, t_D) can be calculated from the growth rate factor using the following formula:

$$t_D = \ln 2/\mu$$

2.1.2 Microscopic analysis

In adherent cultures, only two types of cells can be distinguished from one another microscopically without the use of chemical stains: actively dividing and non-dividing cells. Cells tend to spread once they adhere to the cultivation vessel. Upon reaching mitosis they round up, detach and eventually (in most case) divide into two cells that subsequently settle down and adhere to the surface of the culture vessel and spread. Cell division occurs in a rapid time frame (30-60 minutes from rounding to actual division). Cell cycle duration can be determined, on a cell-by-cell basis by calculating the time between two successive mitotic events.

2.1.3 Analysis by flow cytometry

The DNA content of a cell increases during S phase from 2n to 4n (**Figure 5)**. It is possible, using specific fluorescent DNA stains such as DAPI (4', 6-diamidino-2-phenylindole), to quantify the relative amount of DNA in each cell of a population using flow cytometry. The result is a *cell cycle profile* of the analyzed population, a histogram reflecting the distribution of cells according to their DNA content (**Figure 6**). Cells with 4n DNA (G2 and M) will have twice the fluorescent signal than cells with 2n DNA (G1). Cells actively duplicating their DNA will have a fluorescent signal that falls in between those for 2n and 4n. The x-axis of the histogram represents the relative fluorescence measured per cell, and the y-axis indicates the number of cells with such fluorescence. The x-axis is divided into channels corresponding to an identical amount of detection window for increasing fluorescence intensity.

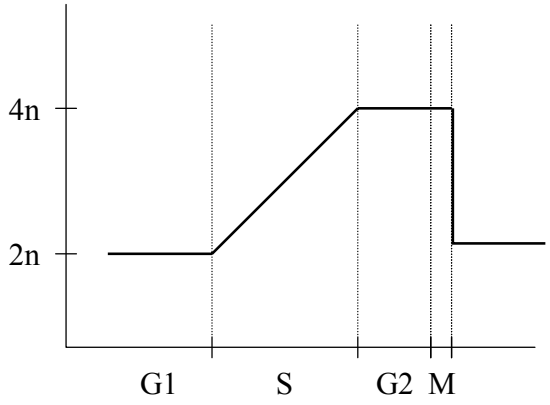


Figure 5. Schematic representation of DNA content per cell during cell cycle.

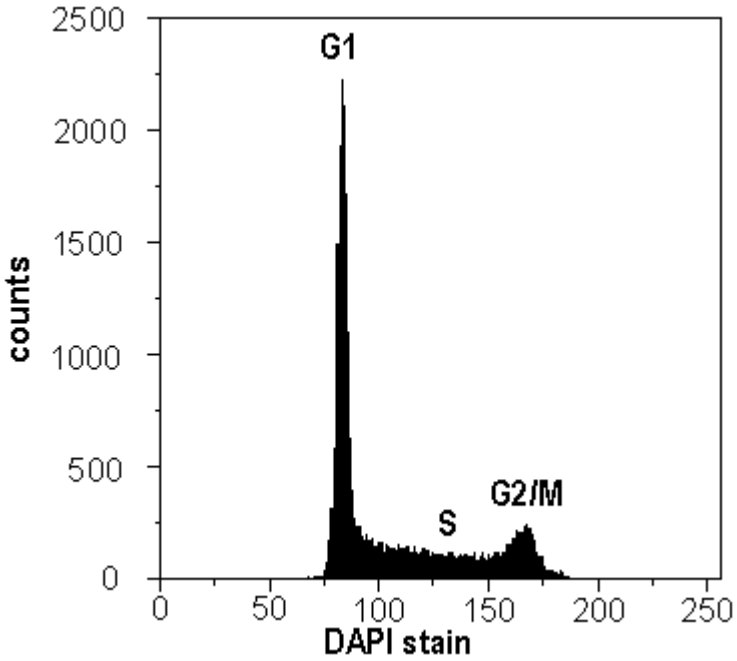


Figure 6. Cell cycle profile with DAPI staining. The fluorescence levels for cells in G1, S and G2/M are indicated. Note that the fluorescence ratio between the G1 and G2/M peaks equals 2. Cells in G2/M have 4n DNA content and G1 cells have a DNA content of 2n.

2.1.4 DNA stains

Many different fluorescent stains specific for DNA are available. Most of them are excitable at 488nm, which corresponds to the emission wavelength of the most commonly used lasers in flow cytometry. One interesting molecule for cellular DNA staining is DAPI (4', 6-diamidino-2-phenylindole) (**Figure 7**) (Tarnowski et al., 1991). It associates with the minor groove of double-strand DNA preferentially to AT clusters (Lin et al., 1977). It is an intercalating molecule that binds to DNA through hydrogen bounds and charge interactions. No ultra-structural changes in cells stained with DAPI have been observed compared to the appearance of non-stained cells (Tarnowski et al., 1991). Upon excitation of DAPI with UV light (360nm), it emits fluorescence at 460 nm (blue light) (Kapuscinski, 1995)

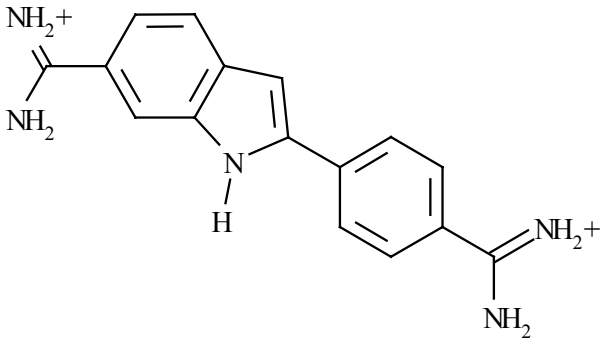


Figure 7. 4', 6-diamidino-2-phenylindole (DAPI)

Once bound to double-strand DNA a 20-fold fluorescence enhancement appears, compared to the unbound DAPI, due to the displacement of water molecules from both DAPI and the minor groove (**Figures 8** and **9**). It does not exhibit fluorescence enhancement when bound to single stranded DNA or to GC base pairs (**Figure 10**). Other molecules, such as Hoechst 33258, Ethidium bromide (EtBr) and Propidium iodide (PI), are also commonly used to stain DNA.

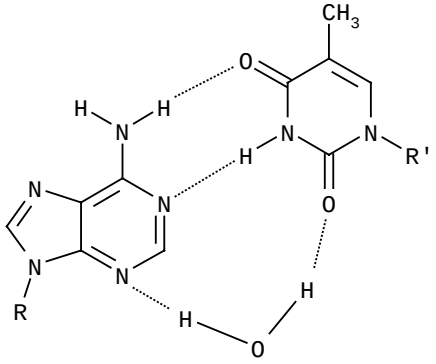


Figure 8. Water molecule binds to AT (or GC) base pair by hydrogen bounds (dotted lines)

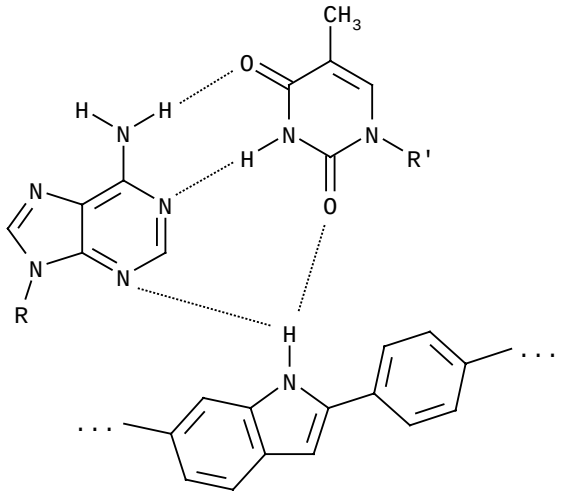


Figure 9. Water molecule is removed and replaced by DAPI (dotted lines correspond to hydrogen bounds) in the minor groove of double-strand DNA.

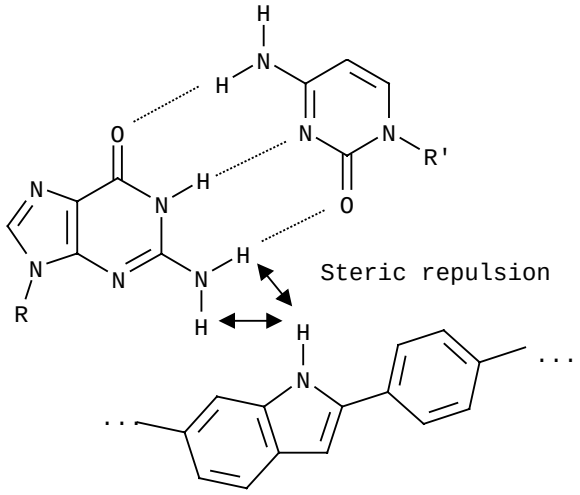


Figure 10. Base pair – DAPI interaction is much less effective with GC base pair, due to important steric repulsion.

2.2 Cell synchronization

Under normal culture conditions each cell will proceed through the cell cycle independently of the cells surrounding it. Such a population is called asynchronous, as cells are distributed randomly throughout the different phases of the cell cycle. To study the effect of a specific cell cycle phase on an experimental procedure, it is important to have all the cells cycling in a synchronous manner. Many different approaches have been published to achieve cell synchronization among a given population in culture. The two main approaches to obtain synchronized cells are by using a drug that inhibits the progression of cells at some point in the cell cycle and results in an accumulation of cells at that point (cycling resumes upon drug removal) and by physiological means such as contact inhibition or nutrient deprivation.

Reversible inhibitors of cellular DNA synthesis are useful tools for synchronization. However when an asynchronous cell population is treated, only cells reaching the point in the cell cycle inhibited by the drug will get affected by the synchronization agent and accumulate at that point. The other cells will continue to cycle until they reach that point. To achieve a high synchronization, cells must either be pre-synchronized in other phases prior to treatment with the drug or they must be repeatedly treated with the DNA synthesis inhibitor. Cells must be exposed to the synchronization agent for more than the cell cycle length to ensure that every single cell will reach the point in the cycle that is inhibited by the drug. If asynchronous cells

are treated for a shorter period, one can easily understand that not all the cells (randomly distributed through the cell cycle) will reach the synchronization point and will not be synchronized if the drug is removed.

Asynchronous, as well as synchronous, cell cycle analysis requires controls to ensure that cells do actually cycle and to prevent misinterpretation of the analyzes due to a static cell culture in which no growth is occurring.

2.2.1 Chemical synchronization

- *Double thymidine block* takes advantage of the observation that a high concentration of thymidine suppresses DNA synthesis in S phase. Cells in G2, M and G1 phases are not affected by excess of thymidine. The concentration of thymidine required to inhibit more than 90% of cell division in asynchronous cell cultures, allows a considerable degree of DNA synthesis and therefore slow progression through S phase. Double thymidine block is done to minimize this effect (Studzinski and Lambert, 1969).

- *Aphidicolin* is a molecule obtained from the culture filtrates of *Cephalosporium aphidicola* and other fungi. It inhibits cell growth by selectively inhibiting the DNA polymerases α and δ, the two major polymerases involved in DNA synthesis during the S phase (Ikegami et al., 1978). It prevents cells in G1 from entering the S phase, and cells in G2 and M continue to cycle and accumulate at the G1/S border. To achieve a well-synchronized cell population, pre-synchronization of the cell culture either in G2, M or G1 is required. Re-initiation of the cell cycle is accomplished by washing the cells with PBS and returning them to aphidicolin-free medium (Mortimer et al., 1999; Pedrali-Noy et al., 1980; Spadari et al., 1985).

- *Mimosine* is a rare amino acid derived from *Mimosa* and *Leucaena* plants. It is a reversible inhibitor of DNA synthesis. It arrests cell cycle at the boundary between the G1 and S phases, before the onset of DNA synthesis. As for aphidicolin, it requires a pre-synchronization of cells. Once removed from the cell culture medium, mimosine is quickly cleared from the cells (Orren et al., 1995). It also

reportedly has no effects on DNA metabolism post-synchronization (Petersen et al., 1995). After removal of the drug from the medium, cells proceed quickly and synchronously into S phase. The duration of S phase after mimosine treatment appears to be unaffected by the synchronization procedure and is identical to the time normally taken to traverse S phase. Albeit its mechanism of action has not been clearly defined, mimosine seems to inhibit the action of ribonucleotide reductases, a class of enzyme involved in the synthesis of deoxyribonucleotides (dNTPs) (Dai et al., 1994; Gilbert et al., 1995; Reichard, 1993). Lowering the intra cellular levels of dNTPs prevent entry into the S phase (Dai et al., 1994; Gilbert et al., 1995; Hoffman et al., 1991; Krude, 1999; Kulp and Vulliet, 1996; Lalande, 1990; Mosca et al., 1992; Watson et al., 1991).

- *Hydroxyurea* also inhibits the conversion of ribonucleotides to deoxyribonucleotides and has been used to synchronize cell populations. However, its effectiveness is cell line dependent. Exposure of S phase CHO cells to 10^{-3}M hydroxyurea for one hour results in cell death after 20 hours (Chang and Baserga, 1977).

2.2.2 Non-chemical synchronization

- *Mitotic shake-off* takes advantage of the observation that mammalian cells grown as a monolayer have a reduced attachment to the surface when they are rounded up during mitosis. Gently shaking the growing vessel will detach those mitotic cells that can be further used to start a synchronized cell population. The main drawback of this method is the low yield of mitotic cells and its applicability to adherent cells only (Terasima and Tolmach, 1961).

- *Baby machine* also uses the fact that mitotic cells have a reduced attachment to the surface of the growing vessel. Cells are grown on a porous membrane that rests on the top of an inverted funnel. Medium is gently pumped through the funnel, and cells rounding up for mitosis get detached and are washed away with the passing medium. These cells provide a synchronous cell culture. This method also yields a low level of synchronous cells (Thornton et al., 2002).

- *Centrifugal elutriation*, also known as counter flow centrifugation, is based on the separation of subpopulations of cells from a mixed population on the basis of cell size. As the cell size increases from G1 phase to M phase, the cells obtained are synchronized with respect to their position in the cell cycle. Separation is rapid and can be performed in isotonic media such as culture media and phosphate buffered saline (PBS). A disadvantage of this method is that only a small proportion of the original cell culture (~5%) is available for the subsequent experiments (Futcher, 1999; Kauffman et al., 1990).

- *Sorting cells* by flow cytometry. If cells are stained with a DNA probe (Hoechst, DAPI, PI, EtBr) their position in the cell cycle can be estimated according to the DNA level. A histogram representation of DNA fluorescence will enable the user to selectively choose cells that are either in G1 (small cells with 2n DNA), S (medium-sized cells with DNA amount between 2n and 4n) or G2/M (large cells with 4n DNA). Flow cytometry, as described further in this work, enables the operator to sort cells of interest in the cell population. Therefore, with adequate gating synchronous cells can be obtained in the different phases mentioned above. One can also gate cells in early, middle or late S phase. It is also possible to separate G2 and M phase cells with bi-virate analysis, but this requires expensive fluorescent probes. (Grdina et al., 1984).

- *Nutrient deprivation* is another way to prevent cells growth and induce synchronization of certain cell lines. When a nutrient source such as serum becomes limiting for cell division, cells will tend to stop growing and enter the G0 phase, where little metabolic activity takes places (Tobey and Crissman, 1972; Tobey and Ley, 1970). Upon changing of the culture medium to provide the limiting nutrient, the cells will resume cycling synchronously.

- *Contact inhibition* also allows cell synchronization for certain cell lines. When the concentration of adherent cells becomes too high and cells do not have enough space to spread, they will enter in the G0 phase. They will resume synchronized growth upon cell passage.

2.2.3 Disadvantages of synchronization

Synchronization over more than one or two cell cycles is difficult to achieve due to the fact that as soon as the synchronization factor is removed, each cell will again cycle on an individual basis, independently of the state of the cells surrounding it. The cause of de-synchronization is not understood but has been attributed to intracellular fluctuations in the number of key molecules necessary for the initiation of DNA synthesis (Spudich and Koshland, 1976). This conclusion is supported by the observation that the decay of synchronization correlates with increasing cell density due to an increase in the distribution of generation times (Murphy et al., 1978).

Depending on the synchronization approach used, cellular metabolism may be affected resulting in a longer cell cycle. When working with synchronized cells, it is important to have controls to ensure that the synchronization protocol did not affect the cells for the parameter(s) being tested.

3. Tools for Analysis

3.1 Flow Cytometry

Flow cytometry is a laser-based technology that is used to measure many cellular parameters, on an individual cell basis. It uses specific fluorescently labeled compounds that react with the cellular component of interest. Using fluorophore-linked antibodies targeted to a specific intracellular or membrane protein, it is possible to quantify that protein in every labeled cell being analyzed. Antibodies are not a prerequisite for flow cytometric analysis since other fluorescent molecules that specifically bind to cellular components are available.

Flow cytometers permit sampling large amounts of suspension cells for many parameters at the same time on a cell-by-cell basis. Thousands of cells can be sampled per second. Some flow cytometers are also equipped with a sorting device which permits the selection of individual cells from a population on the basis of the presence or absence of a specific molecule(s) or on the basis of a physical parameter such as cell size or shape. This type of instrument is termed a fluorescence-activated cell sorter (FACS). Not all flow cytometers have sorting capabilities, however.

3.1.1 Set-up

Flow cytometry takes advantage of laminar flow conditions in capillary tubes to bring cells one-by-one into the flow cuvette for analysis (**Figure 11**). Cell suspensions (permeabilized and/or stained as needed) are injected into a flow channel where a carrier fluid (*sheath fluid*) surrounds the cells. Sheath fluid draws the cell suspension into a thin laminar line through a process called *hydrodynamic focusing*. The fluids enter a *flow cuvette* channel made out of quartz glass where individual cells are illuminated by laser beam (usually at 488nm) and/or UV-light.

Cells pass the analysis point where they are illuminated to excite the fluorochrome(s). The emitted light is scattered into different directions with intensities depending on the refractive index, size and shape of each cell. Light scattered in small angles (*Forward Scatter*, FSC) is

proportional to the cell size; more light scatter correlates with increased cell size. Scattered light that is collected perpendicularly to the laser beam is called *Side Scatter* (SSC). It also is an indicator of cell size but is mainly influenced by surface and internal structures of the cell. These two parameters are independent of the presence of any fluorescent marker on or in the cell. Appropriate photo-detectors measure the magnitude of the pulse of light scattered. The magnitudes of these pulses are sorted electronically into channels that are represented as histograms of the number of cells possessing a certain quantitative property.

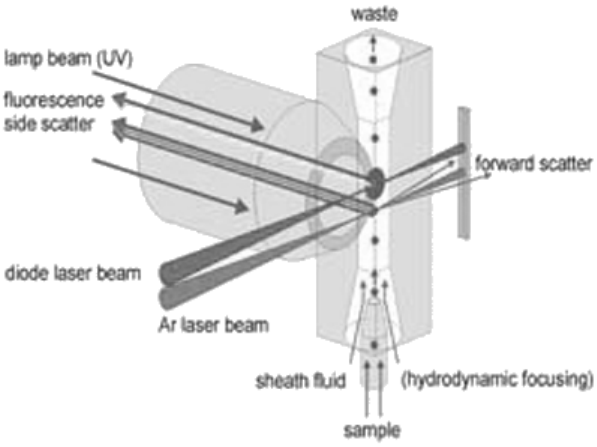


Figure 11. Flow cuvette with laser and UV excitation beams, hydrodynamic focusing of sample, Forward Scatter and Side Scatter (Adapted from www.partec.de/partec/flowcytometry.html)

Fluorescent markers within the cell or on its surface will be excited and will emit light at a specific wavelength. This emitted light is measured through sets of mirrors, filters and different photo-multipliers, each with a specific detection window (wavelength range) (**Figure 12** and **Table 4**).

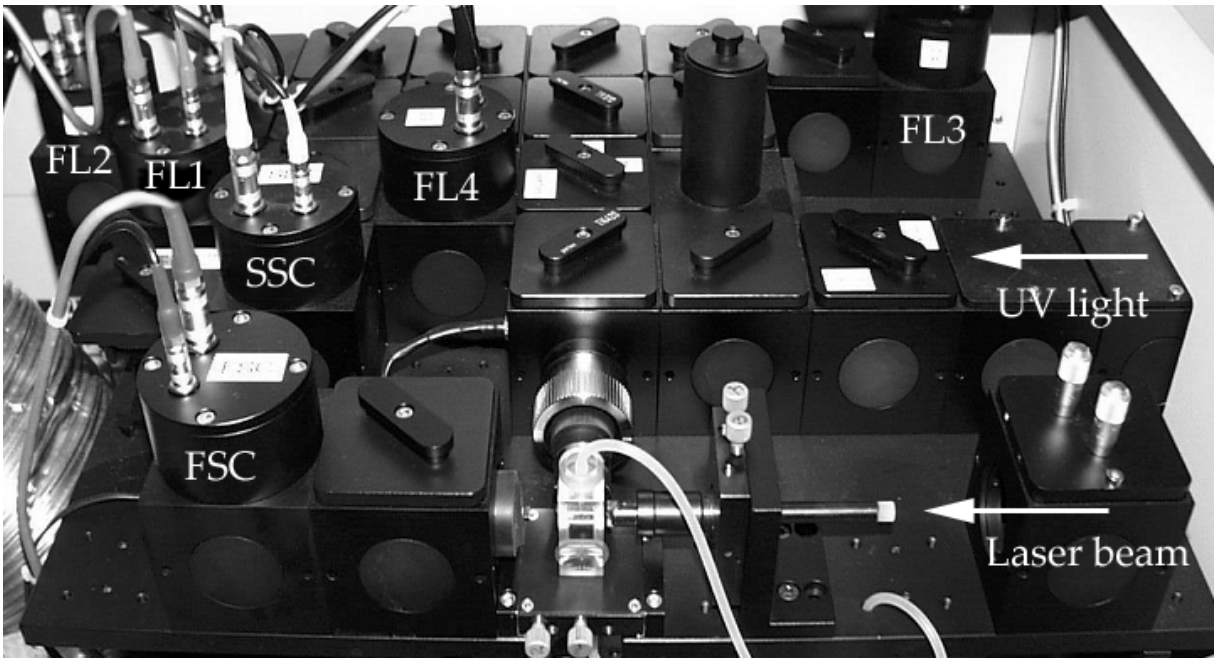


Figure 12. Optical part of the PAS III flow cytometers (Partec GmbH, Germany). Laser and UV-light sources are shown. FSC, SSC and FL1 to FL4 correspond to the 6 different photo-detectors installed in the machine. The wavelength sensitivities of the photo-detectors are given in **Table 4**.

Table 4. Wavelengths sensitivities of the different photo-detectors

Photo-detector	Wavelengths detection	Corresponding color
FL1	520 – 560 nm	Green
FL2	570 – 610 nm	Yellow
FL3	610 – 750 nm	Red
FL4	420 – 470 nm	UV, blue

Bi-virate representation of FSC versus SSC (also known as a dot plot) allows the identification of cell debris, cell aggregates and individual cells. Usually a clear cell population is distinguishable from the debris and aggregates (**Figure 13**). Cells with a strong FSC and a low SSC are considered viable cells, whereas cells with a low FSC and a high SSC are dead cells (small cells with granulated intracellular structures and/or polylobed nucleus).

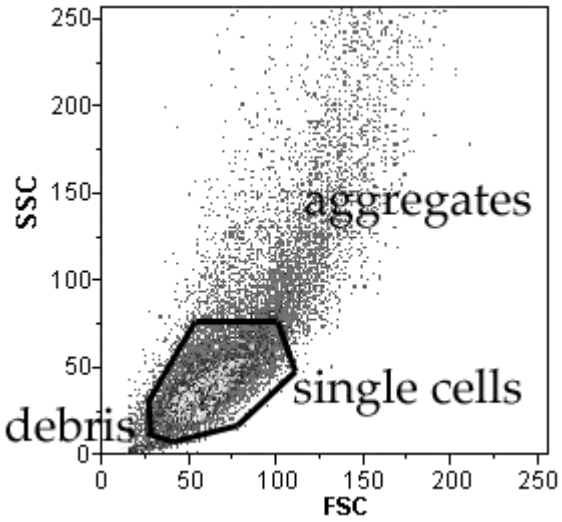


Figure 13. Distinction between single cells, aggregates and debris in bi-virate representation of FSC versus SSC.

As long as the fluorescent markers have an emission spectra distinct from one another, it is possible to combine them for simultaneous analysis. Up to 7 parameters are now commonly available on flow cytometers. These include FSC, SSC, and time plus a given number of fluorescence sensitivities, depending on the inner settings of the machine (filters, mirrors, photo-detectors). The filters and mirrors can separate the emitted light on independent photo-detectors for each cell. Optical (scatter, fluorescence) signals are sequentially generated by each single cell. Signals are detected and displayed for cell distribution analysis. Information for every cell is stored in a computer for further analysis.

The time parameter is useful when testing new staining conditions to ensure that the incubation time in the presence of the stain is sufficient to saturate the cell (**Figure 14**). During analysis post-staining, the time parameter is still useful as it monitors the fluorescence intensity of the parameter being studied as a function of time. If staining went well, intensities will not move towards brighter fluorescence (**Figure 15**). If the incubation time during staining was not long enough, a shift towards brighter events will appear during acquisition, indicating the staining problem. For instance, if a DNA stain is used and the staining incubation was not long enough, remaining stain molecule in the solution will keep reacting with the cells during data acquisition, resulting in an increase in fluorescence intensity for cells in a given phase of the cell cycle (i.e. G1 cells peak will start to shift towards S phase population because cells in the population being analyzed keep incorporating more stain).

This would finally result in an inaccurate analysis with a high CV% (coefficient of variation), making data interpretation difficult.

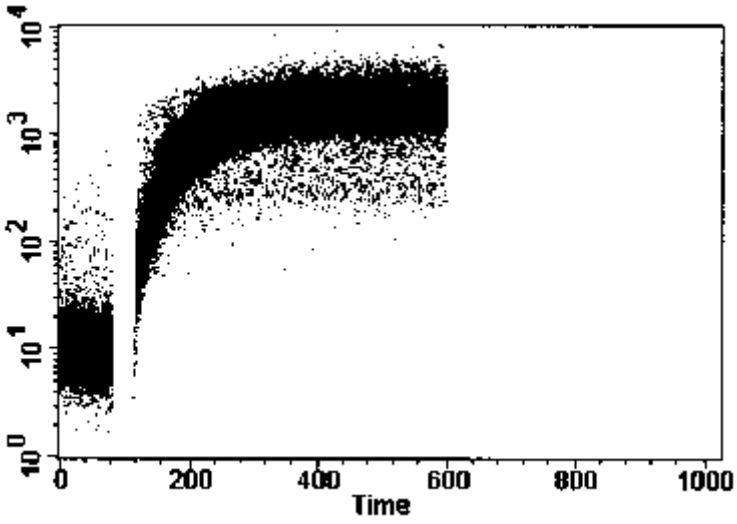


Figure 14. Stain incorporation as function of time. Y-axis represents fluorescence intensity. The first fluorescence intensity corresponds to the auto-fluorescence of the cells. The gap in fluorescence corresponds to the time where the stain was added to the analyzed cells. An increase in fluorescence is clearly visible (compared to auto-fluorescence) as soon as the stain is added. Fluorescence later reaches a plateau, meaning that the cells are saturated with the stain.

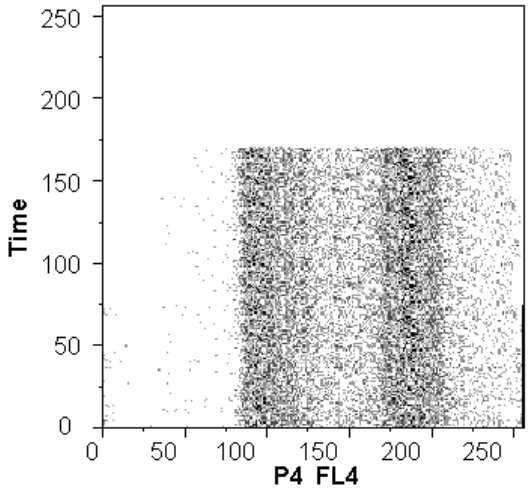


Figure 15. No intensity shift towards higher fluorescence (x-axis) as function of time (y-axis) occurs when cells are saturated with the stain.

Cell concentration determination is also possible by flow cytometry by adding beads to a given volume of cell solution as internal control of known concentration. Knowing how many beads were analyzed during the acquisition, it is possible to calculate the cell concentration of the sample.

3.1.2 Advantages

- *Multi-parameter data acquisition*: Appropriate combinations of stains with differing excitation and emission wavelengths permit the estimation of multiple parameters for each cell. After data acquisition it is possible to combine these parameters in every possible combination.

- *High-speed analysis*: Hundreds of cells per second can be analyzed. High-grade flow cytometers can easily go up to thousands of cells analyzed per second.

- *Sorting*: data treatment during acquisition can be electronically processed rapidly enough to sort the detected cells of interest in the overall population being analyzed. If a cell specification falls within a defined window, it is physically possible to separate that cell from the population.

3.1.3 Disadvantages

- The cost of the machine is relatively high. A basic instrument without sorting capability costs around one hundred thousand Swiss francs. A high grade FACS instrument costs about 700'000 Swiss francs.

- Optical alignments require time and expertise to achieve valid data acquisition in a reasonable period of time. Alignments must be done in a meticulous way using standardized beads as control to ensure a low CV in the detection windows that are to be used for a particular analysis.

- With flow cytometers it is not possible to follow a given cell as a function of time. Once a cell has been through the flow cuvette, it is either sorted into a collecting tube or a multi-well plate or is discarded. Flow cytometry provides a powerful tool to study one or more parameters among a very large cell population, but it is a poor approach for a cell-by-cell analysis as function of time.

3.2 Live imaging

Live imaging microscopy is an excellent tool to study transient transfection using fluorescent proteins as markers. Cells can be monitored visually before, during and after transfection. Counting fluorescent and non-fluorescent cells gives the transfection efficiency as well as information on cell proliferation when correlated with time.

It has the advantage over flow cytometry of monitoring cells on an individual basis as a function of time. Live imaging also provides information on intracellular distribution and localization of cellular components, which is not possible with flow cytometry.

Cell can be monitored over long periods of time, thus providing a tool for studying cellular kinetics. To ensure that all the conditions are met for cells to grow as they would in an incubator, a chamber with controlled temperature, CO_2 and humidity is fit onto the microscope.

3.2.1 Set-up

A live imaging microscope system is a microscope controlled by a computer. Every single parameter can be monitored and changed from the computer. Magnification lenses can be permuted during acquisition, normal light and/or UV light can be switched on and off at will, with exposition time set by the operator. It is possible to use different filter sets for the detection of diverse fluorophores during acquisition. The operator can also determine the time between each new acquisition. It is also possible to define different regions of interest that will be monitored as a function of time with all the conditions set by the operator (**Figure 16**)

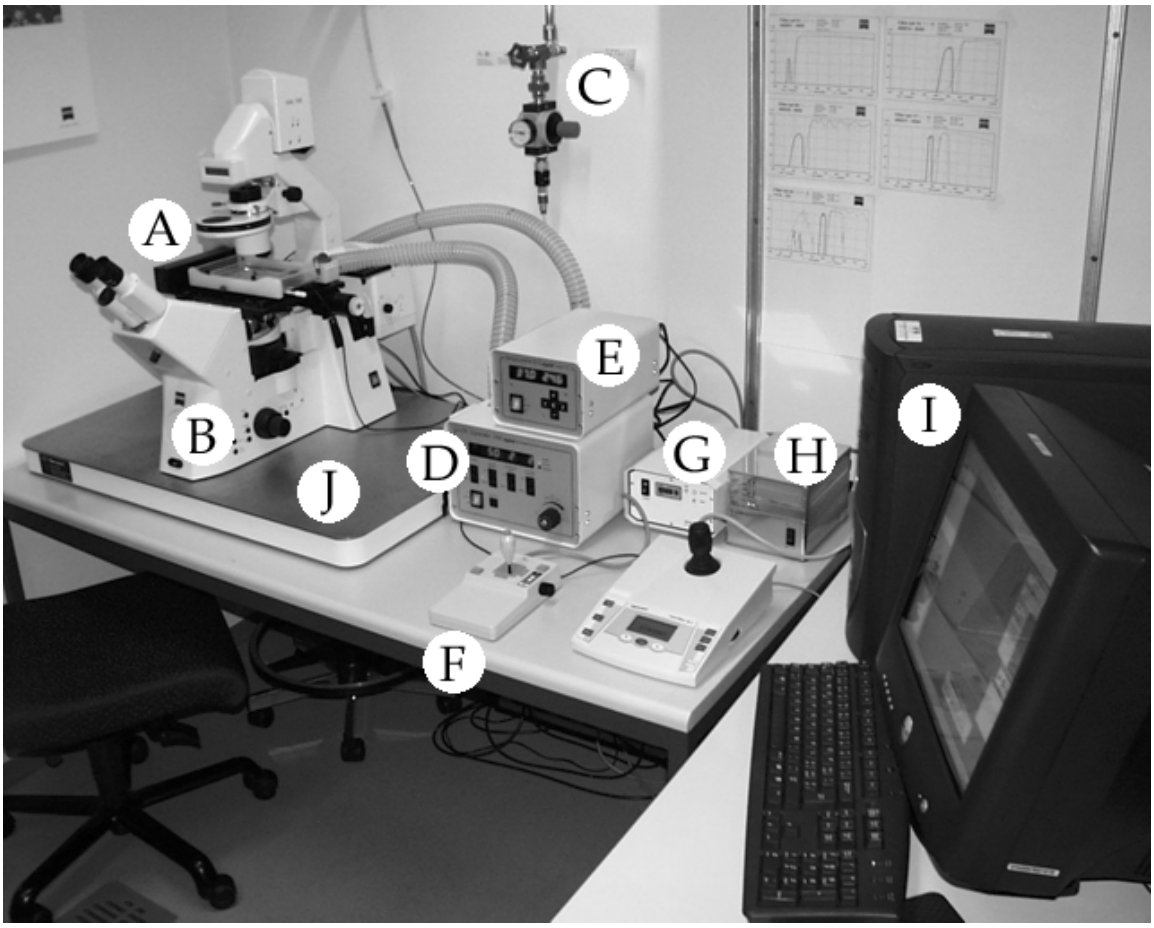


Figure 16. Live Imaging Setup. **A**: incubation chamber, providing incubator like conditions for the growing cells being monitored. Cells are grown in a multi-well plate placed inside this chamber. **B**: Motorized microscope, with sets of magnification lenses (10X, 20X, 40X and 63X), sets of filters (for fluorescence and normal light observations), motorized plate holder (x- and y-axis), motorized z-axis, motorized light and UV-light shutters, visible light and UV light source and a video camera for acquisition. **C**: CO_2 line to supply the incubation chamber through a controller. **D**: CO_2 controller. **E**: Temperature controller. **F**: plate holder positioner joystick (x- and y-axis). **G**: UV-light controller. **H**: plate holder x- and y-axis controller. **I**: Computer controlling the installation settings and storing acquired pictures. **J**: Anti-shock, air-suspended stabilization table to avoid vibrations in the system.

3.2.2 Advantages

- *Cell-by-cell analysis*: Live imaging has the advantage over flow cytometry of allowing morphological and time-dependent cell-by-cell analysis of adherent cell populations. If a given cell shows features of interest, it is possible to observe it over a period of time.

- *Simple cell cycle analysis*: It is possible to analyze the cell cycle since cytokinesis is a well-defined event that is easily monitored when the mother cell divides in two daughter cells. Distinctive observable guideposts of the cell cycle can be used to

determine the effect of the cell cycle on other events such as the expression of a fluorescent reporter protein as described below. It can also be used to determine the cell cycle duration for individual cells.

- *Multi-channel acquisition*: For each filter set used during an acquisition, the pictures are saved as layers (each fluorescent channel saved as a layer), permitting the superimposition of every possible combination of the different fluorochromes.

- *Co-localization*: It is possible to test whether or not two (or more) independent fluorescent signals in a cell are localized in the same sub-cellular compartment.

- *Time-lapse*: Each defined acquisition setting (with or without the multi-channel option) can be applied as a function of time repeatedly, thus providing moving videos of visual events (fluorescent and non-fluorescent) among the monitored cell population. It also provides information on the time required for specific, visually detectable events to occur in a given cell.

- *Gradient detections*: Using stains sensible to a given molecule or ions, it is possible to see a gradient in a cell.

- *Deconvolution*: Some computer programs provide reconstruction in 3D of objects observed through the Z-axis (with a single or multiple filter sets), thus facilitating co-localization of cellular structures and/or proteins.

3.2.3 Disadvantages

- *Reduced sample size*: One of the major drawbacks is, of course, that a much smaller cell population is being monitored over time compared to the tens of thousands of cells that can be analyzed in a few minutes with flow cytometry.

- *Non-automated analysis*: Since many different parameters can be monitored simultaneously, the software often runs short on possibilities to have automated analysis.

- *Computer power*: A lot of memory and a fast processor are required to handle the huge data files provided by live imaging in a reasonable period of time. For instance, monitoring cells expressing a fluorescent protein post transfection during 24 hours, with two pictures taken every 10 minutes (one with normal light and one with UV light) creates a file of many hundreds of megabytes that does not fit on a CD-Rom. If time-lapse acquisition has to be analyzed, the computer should be able to store the whole file in its active memory (not hard disc) and must have powerful video handling capacities to play all the pictures one after the other in a fluid way by superimposing pictures from acquisition under normal and UV light. The more fluorophores being monitored, the bigger the file saved during the acquisition and the more computer power required to analyze it post-acquisition. As files are too large in their raw format they cannot be saved on CD-ROMs and thus require a more powerful backup option such as DVD-ROMs or DAT streamers. If deconvolution is needed, a fast processor is required to avoid hours and hours of calculation.

- *Price*: A complete live imaging set-up costs more than one hundred thousand Swiss francs. The associated computer power cost is proportional to the degree of complexity of the analyses required. For instance, deconvolution requires a fast processor as well as calculation software costing tens of thousands of Swiss francs.

Experiments, results and discussion

4. Materials and Methods

4.1 Plasmid preparation

pEGFP-N1 and pDsRedExpress-N1 (Clontech, Palo Alto, CA, USA) plasmids were produced in DH5α bacteria. Extraction and purification of plasmid DNA was done using a Nucleobond® AX10000 kit (Machery and Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions. Purified plasmid DNA was then resuspended in TE (10mM Tris, 1mM EDTA, pH 7.4) to achieve a final concentration of 1 mg/ml.

4.2 Cell culture

CHO DG44 adherent cells were passaged three times a week in T-75 culture flasks (TPP, Wohlen, Switzerland) in DMEM/F12 medium (Life Technologies, Basel, Switzerland) supplemented with 2% Foetal Bovine Serum (FBS, JRH Biosciences) and incubated in an humidified 5% CO_2 atmosphere in a 37°C incubator (Cytoperm, Heraeus).

CHO DG44 cells are dhfr negative (i.e. they lack the enzyme **d**i**h**ydro**f**olate **r**eductase), so the culture medium had to be complemented with hypoxanthine and thymidine.

Three times a week, cells were detached from the culture vessel by trypsin-EDTA (Life Technologies, Basel, Switzerland), counted with a CASY® 1 TTC cell counter (Schärfe System, Reutlingen, Germany) and sub-cultured with a seeding density of 0.5e+5 c/ml (if passaged two days later) or 0.2e+5 c/ml (if passaged three days later). These conditions were determined to result in cultures with approximately 80% - 90% confluence for each new passage and a good fibroblastic shape, without a significant number of small and round cells. This passaging routine yielded cells with good growth conditions and avoided entry of cells into a G0 resting phase with partial synchronization.

For asynchronous transfection, cells were passaged in 12-well plates (TPP, Wohlen, Switzerland) with a seeding density of 1.5e+5 c/ml in 1 ml of DMEM/F12 supplemented with

2% Foetal Bovine Serum and incubated for 18 hours in an humidified 5% CO_2 atmosphere in a 37°C incubator (Cytoperm, Heraeus).

4.3 Cell number measurement

Cell number was measured during synchronized transfection and each time cells were passaged using either of the two methods described below.

4.3.1 Manual counting

Cells in suspension were counted using a haemocytometer (Neubauer, Poly-labo; depth = 0.100mm, square surface on the grid = 0.0025 cm^2) under an inverted phase contrast microscope (Zeiss, Germany). Cell concentration was calculated as follows:

$$\text{Cell concentration [cell/ml]} = (\text{number\_cells} * d * 10^4)/\text{number\_square}$$

(number_cells = number of counted cells; d = dilution factor;
number_square = number of squares taken into account on the haemocytometer grid)

4.3.2 Automated counting

Automated cell counting was performed with a CASY® 1 TTC cell counter. Briefly, 100ul of the cell sample were added to 10ml of CASYTON, an isotonic saline solution, and gently mixed by inverting the solution. 200ul of this solution was measured by the CASY.

4.4 Viability assessment

Viability assessment was performed with haemocytometer on cultures stained with Trypan blue (T-8154, Sigma Chemical Co., St. Louis, MO, USA).

4.5 Cell volume measurement

Cell volume determination was done with a CASY® 1 TTC cell counter. When estimating cell volume decrease, PCV (packed cell volume) was preferred over the CASY® 1 TTC measurements.

4.6 Cell synchronization

Cell synchronizations were performed using mimosine (Sigma, St. Louis, MO, USA), a cell cycle inhibitor that blocks cells at the G1/S border. To control that no side effects arose from the use of the synchronization drug, mimosine-free synchronizations were performed in repeat experiments to confirm the observations done with the mimosine-synchronized populations.

4.6.1 Chemical synchronization

Cultured cells were synchronized with mimosine (**Figure 17**) at the G1/S border prior to transfection, directly in 12 well plates. Prior to mimosine treatment, a pre-synchronization step was performed to enrich the amount of cells in the G0/G1 phase by incubating the cells in low 0.2% serum-containing medium for 48 hours. The medium was then changed for DMEM/F12 complemented with 10% FBS and 100 μM mimosine. The cells were incubated for an additional 14 hours, the minimum incubation time to allow all treated cells to cycle to the growth inhibition point. Incubation in the presence of mimosine has to last for at least a period of time equal to the duration of the complete cell cycle in order to achieve synchronization. Subsequently, to reinitiate the cycle, the medium was changed to mimosine-free medium complemented with 2% serum. Flow cytometry analysis (PAS III, Partec GmbH, Münster, Germany) of the cycle distribution showed that progression through the cycle restarted after mimosine was removed.

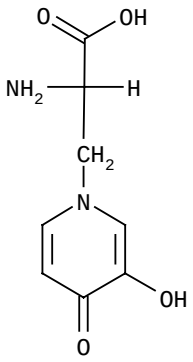


Figure 17. Mimosine

4.6.3 Non-chemical synchronization

Contact inhibition was used as a synchronization control. Cells were grown to confluence in T-75 flasks. Using these conditions CHO cells became synchronized in the G0/G1 phase, until passaged at a lower cell density where they resumed growth.

4.7 Cell cycle analysis

For each transfection, 12 well plates were prepared with the required cell density per well. At the time of transfection, 2 – 3 of those wells were fixed for cell cycle analysis while the remaining wells on the plate were transfected.

4.7.1 Cell fixation and staining

After trypsinization, cells were centrifuged for 5 minutes at 800 rpm in a Labofuge 400 centrifuge (Heraeus Instruments GmbH, Hanau, Germany) and fixed by resuspension in 70% ethanol for 1 minute. Cells were subsequently centrifuged to remove ethanol, resuspended in PBS 1X, and stored at 4°C until further treatment.

Fixed cells samples were centrifuged to remove PBS followed by the addition of 2 ml DAPI solution (FLUKA, 9471 Buchs, Switzerland) (1:1000 in MeOH) to each sample (~1.5E+6 cells per sample). Cells were incubated for 15 minutes at 37°C, centrifuged, resuspended in PBS, and analyzed within 6 hours post-staining.

4.7.2 Flow cytometry analysis

Forward scatter, side scatter and UV-fluorescence (FL4) were acquired for each sample. Cell cycle analysis was performed on the acquisition of minimum 30'000 events in order to have well defined profiles. Determination of the percentage of cells in the different phases of the cell cycle was done through software analysis (Flomax 2.3, Partec GmbH, Münster, Germany) when well distinguishable G0/G1 and G2/M peaks were present. For synchronized populations, when G0/G1 and G2/M peaks were not present (or not significantly enough to be recognized as such by software analysis), gating of regions of interest was performed to calculate the percentage of cells in the different phases. Regions gated manually were compared with software analysis for well-defined profiles to ensure that the estimation was correct.

4.8 Calcium phosphate transfection

Two different protocols for calcium phosphate transfection were used. The classical transfection corresponds to the protocol published by Jordan *et al.* (Jordan et al., 1996) and the fast transfection protocol was developed during this work. All the transfections were performed in 12 well plates.

4.8.1 Classical transfection protocol

CHO cells were transfected using an optimized calcium phosphate method (Jordan et al., 1996) with pEGFP-N1 plasmid (Clontech, Palo Alto, CA, USA). Briefly, for transfection in 12-well plates, 100 µl of transfection solution (0.125 M Ca^{2+}, 0.7mM PO_4^{3-} and 2.5 µg DNA per 100 µl of solution) was added per well. Cells were then incubated for 3 hours at 37°C under a 5% CO_2 humidified atmosphere before a glycerol shock was applied for one minute by changing transfection medium with a 10% glycerol solution in PBS at 37°C. Glycerol-PBS solution was quickly removed and fresh medium containing 2% serum was added onto cells. Plates were incubated until reporter gene expression was assessed. When preparing the transfection mix, the calcium solution (0.25 M, 50µl/well) and DNA (2.5µg/well) were first mixed together. As soon as the phosphate solution (1.4mM, 50µl/well) was added, the precipitate was left to maturate for one minute before the transfection complex was added

onto the cells. For asynchronous cell transfection, cells are plated 18 hours before transfection at a seeding density of 1.5e+5 cells per ml.

4.8.2 Fast transfection protocol

Exponentially growing cells were seeded at a concentration of ~6.5e+5 cells per ml in 12-well plates. Cells were incubated for 4 hours in a 5% CO_2 humidified atmosphere at 37°C. Just before adding the calcium phosphate DNA precipitate onto the cells, two thirds of the culture medium were removed from each well. The final volume left per well was 330 µl. 100 µl of transfection solution (0.125 M Ca^{2+}, 0.7mM PO_4^{3-} and 2.5 µg DNA per 100 µl of solution) was added per well. Cells were then incubated for one hour at 37°C in a 5% CO_2 humidified atmosphere before a glycerol shock was applied as described above. The 10% glycerol solution in PBS was quickly removed and the cells were washed once with 5mM EGTA in PBS and then with PBS. Fresh medium containing 2% serum was then added onto cells. Plates were incubated until reporter gene expression was assessed.

4.9 Reporter gene detection and relative quantification

4.9.1 Fluorescent microscopy

Using a fluorescent inverted microscope (Axiovert 100, Zeiss, Feldbach, Switzerland) with an HBO 50 lamp as the fluorescence excitation source, transfection efficiency was judged by the number of EGFP-positive cells. Excitation and emission wavelengths were selected with Zeiss filter set #25 (excitation: TBP 460, 495, 570nm; beamsplitter: FT 410, 505, 570nm; emission: TBP 460, 530, 610nm). Digital images were captured on a computer running the Axiovision 3.0.6.1. software (Carl Zeiss Vision) a color video camera (Sony 3CCD) connected to a camera control unit (AVF-Horn, Aalen, Germany)

The kinetics of expression by live imaging was done using a motorized fluorescent microscope (Axiovert 200M, Zeiss, Feldbach, Switzerland) equipped with an incubation chamber and an HBO 100 lamp as fluorescence excitation source. Image acquisition was performed under normal and UV light (with corresponding sets of filters) every 10 minutes for 24 hours post-transfection with a Zeiss AxioCam HRm camera and a computer running the Axiovision 3.1 Software (Carl Zeiss Vision). Excitation and emission wavelengths were

selected with Zeiss filter set #17 (excitation: BP 485/20nm; beamsplitter: FT 510nm; emission: BP 515 – 565nm) for EGFP and filter set #14 for DsRedExpress (excitation: BP 510 – 560nm; beamsplitter: FT 580nm; emission: LP 590nm)

Laser scanning confocal microscopy was performed using a Zeiss LSM510 microscope (Zeiss, Feldbach, Switzerland).

4.9.2 Fluorometry

Relative fluorescence of the transfected population was assessed using a multiwell plate reader (Cytofluor™ 4000; PerSeptive Biosystems Inc., Farmingham, MA, USA) equipped with a 50W tungsten-halogen lamp. The following excitation/emission filters were used to measure EGFP and DsRedExpress:

	Excitation [nm]	**Emission [nm]**
EGFP	485/20	530/25
DsRedExpress	530/25	590/35

Fluorescence intensity is reported in relative fluorescence units (RFU). Every measurement was made with a gain set to 80. For each transfection condition, a non-transfected well was used as a blank to subtract the auto-fluorescence signal of cells and medium from the raw RFU signal measured in each transfected well.

Reporter gene RFU = Raw RFU signal – blank RFU signal

4.9.3 Flow cytometry

Relative intensity of EGFP positive cells was also monitored through flow cytometry. Forward Scatter, Side Scatter and corresponding fluorescence sensitive channel (FL1, 520 – 560 nm, for EGFP and FL2, 570 – 610 nm, for DsRedExpress) were acquired. A 488nm air-cooled argon laser was used as an excitation source. For each sample, 30’000 events were acquired. Peak localization in the FL1 window gave information on the homogeneity of the reporter gene expression among the transfected population.

5. Calcium phosphate transient transfection efficiency is cell cycle related

To study cell cycle effects on calcium phosphate transfection, a batch of synchronized CHO cells was divided in half after release from mimosine block. One half of the cell population was transfected at given times after cycle recovery, while samples from the other half were fixed and further analyzed (**Figure 18**).

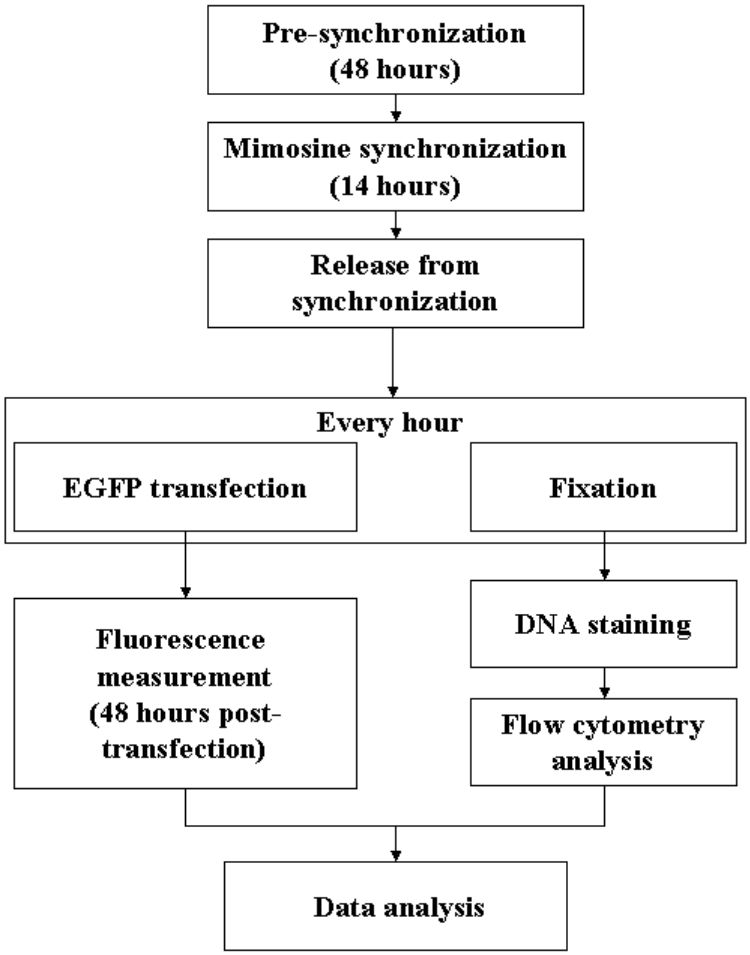


Figure 18. Experimental approach for analysis of EGFP expression and cell cycle status upon transfections at various time-points during cell cycle.

After mimosine synchronization, approximately 80% of the cells proceeded through S phase in a synchronous way. Higher concentrations of mimosine (more than 100μM) gave higher percentages of cells arrested at the G1/S boundary, but once released from such concentrations, a large percentage of cells remained blocked irreversibly at the G1/S

boundary. **Figure 19** shows, based on analysis by flow cytometry, the progression of the synchronized cell population through the cell cycle. The histograms in **Figure 19** represent the amount of cells in each category defined by their relative DAPI fluorescence. The peak at ~100 represents the population of cells with 2n DNA (G0/G1), the 4n DNA population is represented by the peak around 200 (G2/M) and the events in-between those two peaks correspond to the synthesis phase (S). The relative percentage of events in each of these three categories enables to follow the cell cycle progression. The sequence of histograms from 1 hour to 11 hours (post-release) shows that DNA synthesis and subsequent mitosis occurs with a high degree of synchrony with the vast majority of cells. As growth rate was not affected post-release, cell cycle phases duration were approximated from the profiles (**Table 5**).

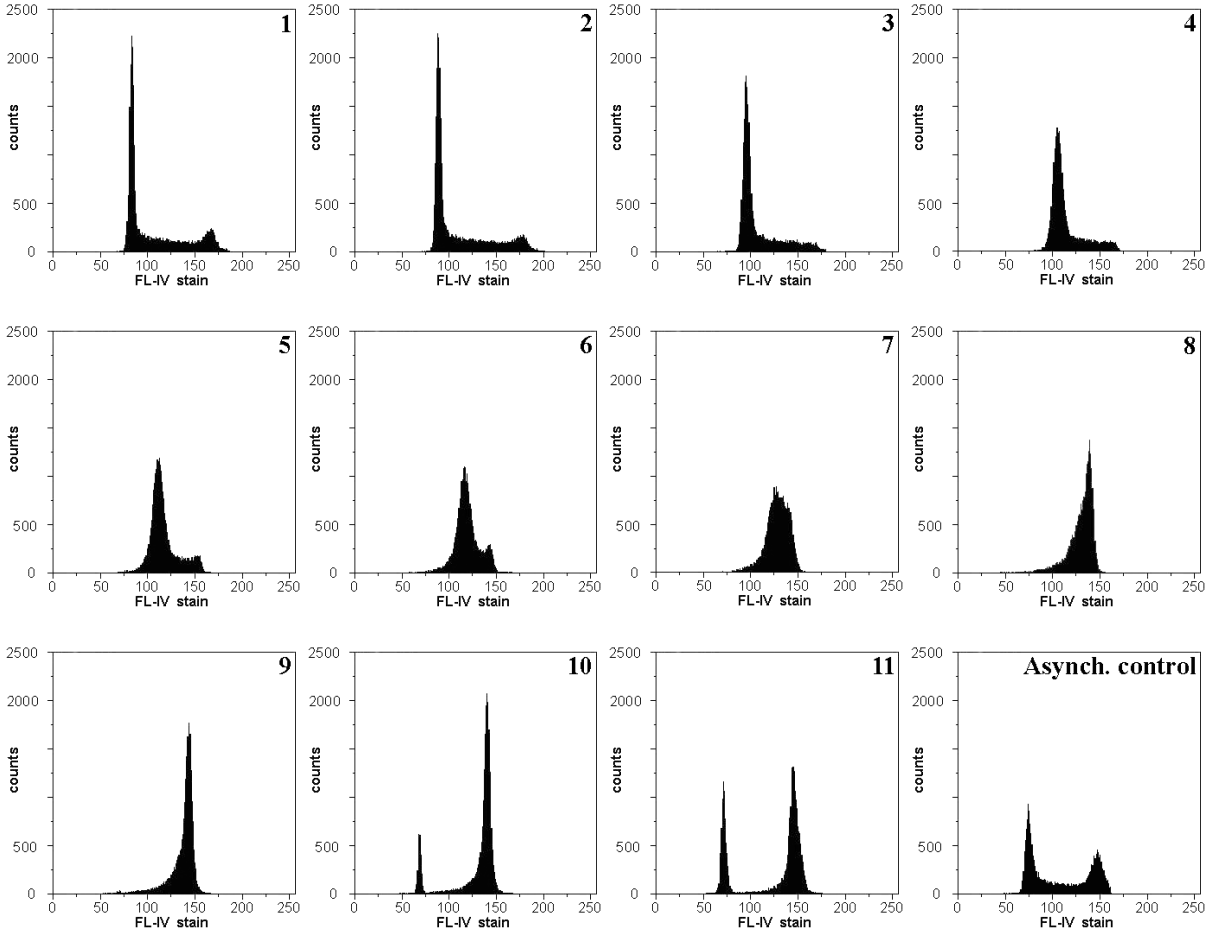


Figure 19. Progression through the cell cycle. Cells were pre-synchronized for 48 hours in 0.2% FCS containing medium and then synchronized with mimosine for 14 hours. Samples were taken for cycle analysis from 1 to 11 hours after mimosine release (time in upper right corner of each profile), stained with DAPI and analyzed by flow cytometry. Y-axis was kept constant for each analysis. Each acquisition totalizes 30'000 cells. A typical asynchronous cell population profile is shown for comparison.

Table 5. Duration of the different cell cycle phases of the CHO DG44 cells.

	Phase length
G1	~ 3 hours
S	~ 6 – 7 hours
G2	~ 3 hours
M	~ 1 hour

The duration of theses phases correlates with the doubling time of CHO cells (in DMEM/F12 medium supplemented with fetal bovine serum (FBS) in a 5% CO_2 atmosphere) of 14 hours reported by Clough (Clough, 1998).

5.1 Calcium phosphate transfection is S phase-related

Results presented in **Figure 20** show EGFP expression levels from cells transfected at various time points post-mimosine treatment and the corresponding cell cycle distribution of cells analyzed by flow cytometry and DNA labeling. A strong correlation was observed between the percentage of cells in S phase at the time of calcium phosphate DNA complex addition and the EGFP signal measured 48 hours post transfection. The more cells in S phase at the time of transfection, the higher was the signal for transient EGFP expression 48 hours later. (**Figure 20** and **Figure 21**).

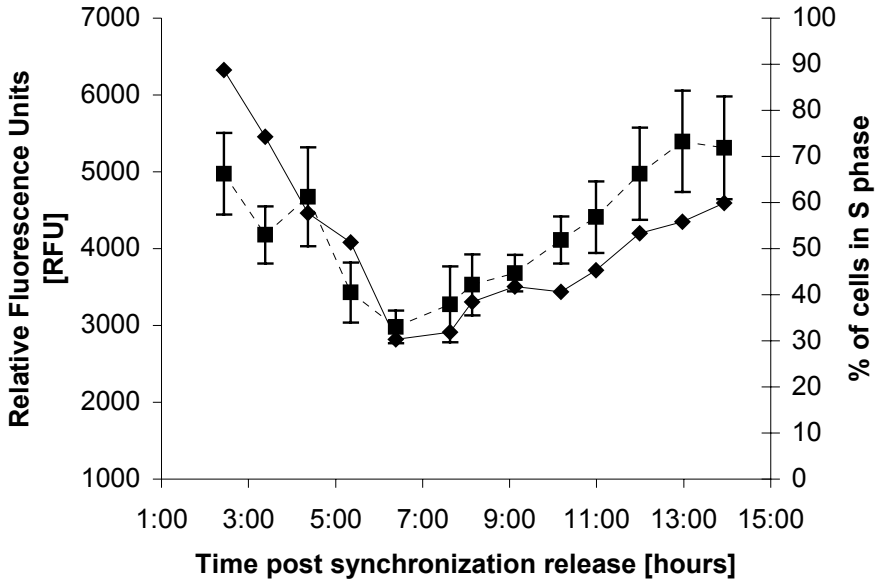


Figure 20. EGFP expression correlates with percentage of cells in the S phase. Other phase distributions (G0/G1 and G2/M) are not shown for clarity. Transfections were performed on new samples (from the same synchronized batch of cells) every hour for 14 hours after release from mimosine. Dotted line represents the EGFP expression 48 days post transfection. Full line represents the percentage of cells in S phase at the time of transfection.

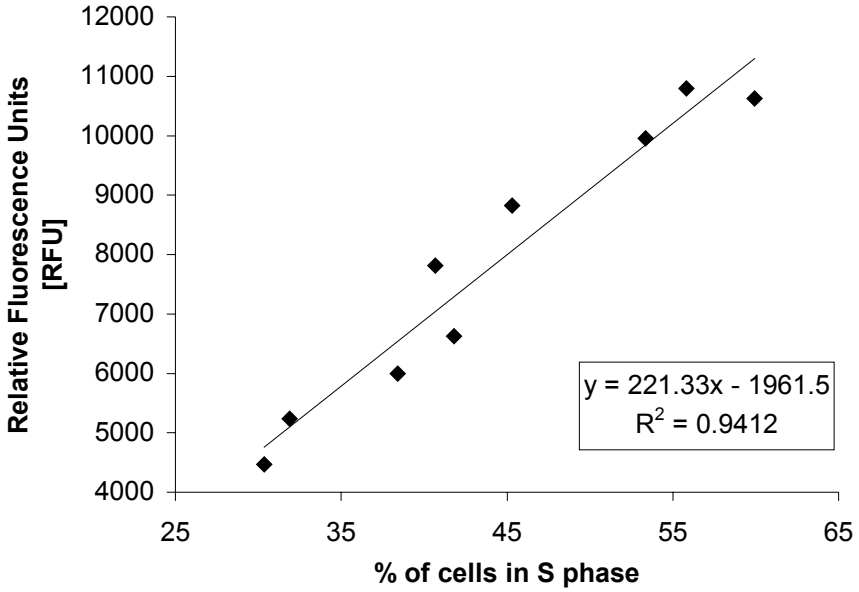


Figure 21. Correlation between fluorescent signal and the increase of cells in the S-phase. More cells in S-phase at the time of transfection resulted in higher EGFP expression. The correlation factor obtained for the linear regression was equal to 0.9412. Values are not from the same experiment as those presented in figure 20.

To exclude a direct effect of mimosine treatment on the transfection efficiency, cell synchronization was then induced by allowing cells to grow to confluence. This pool of cells was then trypsinized and divided into aliquots, to re-initiate the cell cycle, which were in turn transfected at 1-2 hours intervals (**Figure 22**). A high percentage of S-phase cells (~70%) was observed at about 15 hours post seeding (re-initiation of cell cycle). Transfection of the cell population at this time point yielded the highest GFP expression 48 hours later.

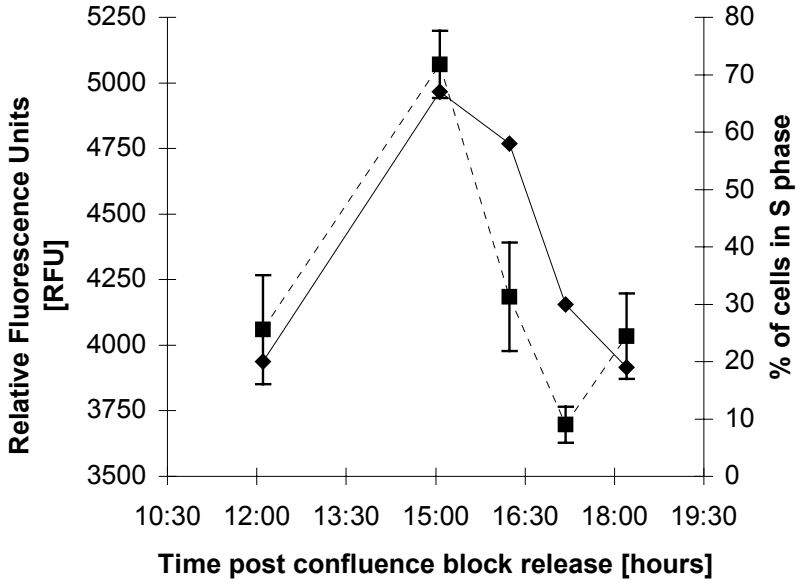


Figure 22. S phase and reporter gene expression level correlation was also achieved with mimosine free synchronization protocol. Dotted line is the EGFP relative fluorescence signals. Full line is the percentage of cells in the S-phase at the time of the transfections.

Maximum S phase percentage approximately 15 hours post seeding is in agreement with the mimosine synchronization results. After the cells were seeded at lower density to re-initiate their cycle, the first peak of S phase probably occurred ~3 – 4 hours post seeding (during cell incubation). Transfections were performed from 12 hours to 18.5 hours post-seeding. As the CHO cell cycle is approximately 12 – 13 hours long, cells proceeded through a second cycle while being transfected, and proceeded through the second S phase then.

Confluence synchronization required replating in order to reinitiate the cell cycle and an additional incubation step for cells to adhere again. Mimosine-synchronized cells could be transfected as soon as the cell cycle was reinitiated by medium exchange. This synchronization procedure was performed directly in multi-well plates, with a cell density low enough to avoid confluence upon re-initiation of the cell cycle.

Visual observation under fluorescent light also showed that significantly more positive cells were achieved if transfected while they were in S phase (**Figure 23**). The positive cells observable when transfection was performed either in G0/G1 or G2/M phase may result from the fact that synchronization was not 100% efficient and that some cells proceeded through the different phases of the cell cycle independently of the synchronized population.

Figure 23. Addition of transfection complex on cells while in S phase clearly resulted in the most efficient reporter gene expression. GFP expressing cells transfected in the different parts of the cell cycle, 24 hours after transfection.

These results were surprising as one might expect mitosis to play a key role for efficient nuclear entry of the calcium phosphate complexed plasmid DNA, as reported for other transfection methods such as cationic liposomes DOTAP and DOPE in HeLa cells (Tseng et al., 1999) or in immature human epithelia (Fasbender et al., 1997), lipofection with Lipofectamine, polyfection with PEI in K562 cells and HeLa cells (Brunner et al., 2000), lipoplexes with DODAC:DOPE in SK-OV-3 cells (human ovarian tumor) (Mortimer et al., 1999), and peptide-based gene delivery (a 12 amino acid DNA binding peptide) (Wilke et al., 1996).

5.2 A faster transfection protocol as a novel tool

Considering that the S phase represents approximately 6 – 7 hours of the ~14 hour cell cycle and that the transfection procedure lasts for 3 hours, it was still difficult to identify the precise time point (within a 30-60 minute resolution) for efficient transfection. In order to improve the resolution of the analysis, a faster protocol was developed in which cells were only exposed to calcium phosphate DNA complex for one hour (instead of three).

The fast transfection protocol was based on the assumption that a smaller volume of culture medium on top of cells during transfection would result in a more rapid sedimentation of the precipitates on the adherent cells (based on the work of Luo *et al* (Luo and Saltzman, 2000a)). These authors used dense silica nanoparticles to concentrate DNA-vector (i.e. DNA-transfection reagent) complexes at the surface of cell monolayers. The culture medium volume was lowered to its limit (330 µl per well, in 12 well plates) and this immediately prior to the addition of the transfection complex (ensuring that cells still remain submerged in medium and not become exposed to air where they would dry out and die). The number of

cells per well remained the same as in normal transfection as cells were plated with the same cell density, and no (or very few) cells were removed when part of the medium was taken away. The amount of calcium phosphate DNA complex added onto the cells also remained the same, thus providing a ratio of precipitates per cells equal in both methods. The incubation time in the presence of transfection complex under these conditions was determined to be approximately 1 hour (instead of three) and resulted in the same reporter gene expression level as the normal transfection when assessed 48 hours post transfection (**Figure 24**)

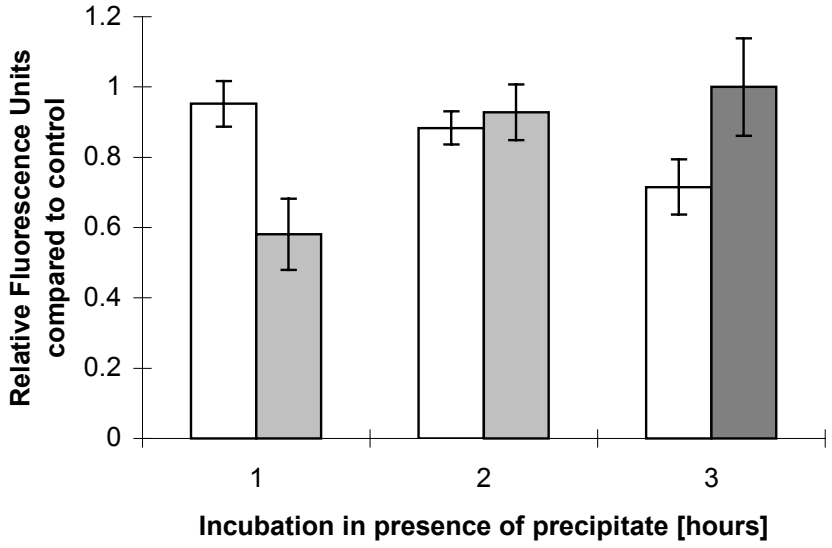


Figure 24. Transfection in reduced medium volume for one hour is as efficient as transfection for three hours in normal medium volume. Incubation in reduced and normal medium was tested for one, two and three hours. White bars represent the reporter gene expression level for reduced medium. Grey bars are the values for normal medium volume. Dark gray bar represents transfection for three hours in normal medium volume and was used as control. Incubation time for less than an hour resulted in low reporter gene expression even with the reduced medium volume.

The 16 to 18 hours incubation time prior to transfection could be shortened as well. Such a long incubation time was considered necessary for (a) the cells to adhere to the well, (b) the cells to reach a higher confluence and therefore lose less plasmid DNA during transfection and (c) to stabilize the system (medium temperature, pH…). All these steps could be improved resulting in an overall process faster than 16 – 18 hours.

- The cells have to adhere to the well:

The cell adhesion does not require 16 hours: monitoring, every ten minutes, the cell behavior post seeding, showed that cells started to have their fibroblastic morphology again after incubation for 4 hours post-seeding.

- The cells have to reach a given confluence:

Increasing the cellular density at seeding and following the cell growth (CASY) enabled to obtain the desired confluence (~80%) after the 4 hour incubation required by the cells to become fibroblastic again.

- The system has to stabilize:

After four hours in the incubator, the medium temperature has reached 37°C. The pH of the medium is comparable to what is achieved after 16 – 18 hours of incubation (~7.4).

These observations led to a shortened incubation time prior to transfection of 4 hours instead of 16 to 18 hours. The reporter gene levels achieved after these shorter incubations were comparable to those obtained with a longer classical incubation time and this for a precipitate exposure time of only one hour.

Different cell seeding densities were tested to find the optimal condition for this fast transfection protocol in 12 well plates. Those densities ranged from 3.0e+5 c/ml to 7.5e+5 c/ml. The reporter gene expression levels were measured 48 hours post transfection (**Figure 25**):

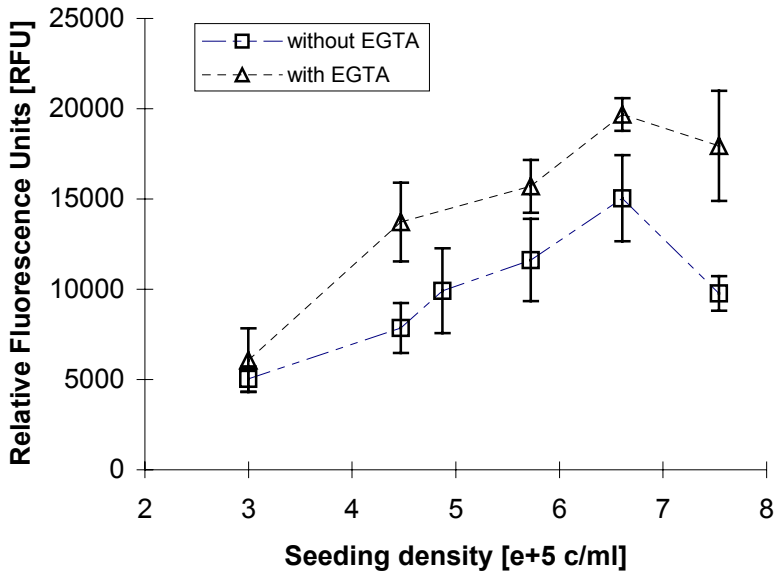


Figure 25. Fast transfection cell seeding determination. Transfections were performed either with (triangle) or without (square) additional EGTA wash-step post glycerol shock (see chapter 7: additional EGTA wash-step).

A seeding density of 6.5e+5 c/ml led to the best reporter gene expression, a higher concentration was probably too high for the cell to go through an unaffected cell cycle post transfection, and the high confluence achieved prevented growth of the cells (contact inhibition) thus preventing a higher level of reporter gene expression.

Combining the reduced medium volume over the cells during transfection and the improved cell seeding density, lead to an overall transfection done, from seeding to the end of transfection (glycerol shock), in 5 hours, instead of 20 – 22 hours with the "classical" approach.

This new transfection approach was then used to test if a different set-up would still result in a correlation between S phase and reporter gene expression levels as it did with the 3 hours exposure. These two different set-ups (classical and fast transfection) provided a way to test if the transfection efficiency was only related to the cell cycle (i.e. correlation between S phase and reporter gene expression should remain unchanged, whatever the set-up of transfection used), or if the transfection set-up also influenced it.

5.3 Transfection efficiency is shifted with a faster transfection protocol

Previous results had shown that while using the classical transfection protocol (i.e. 3 hours exposure to transfection complex in 1 ml) a maximum reporter gene expression level, at 48 hours post transfection was obtained, when calcium phosphate DNA complex was added onto cells in S phase, regardless of the synchronization method used (mimosine or contact inhibition). After being synchronized in T150 flasks, cells were seeded in multi-well plates at release from mimosine synchronization, and transfected following the shorter protocol. Cell samples were fixed for further flow cytometric analysis each time a transfection was performed. With the new system, the highest reporter gene expression level, 48 hours post transfection, was shifted by approximately two hours closer to mitosis (**Figure 26**).

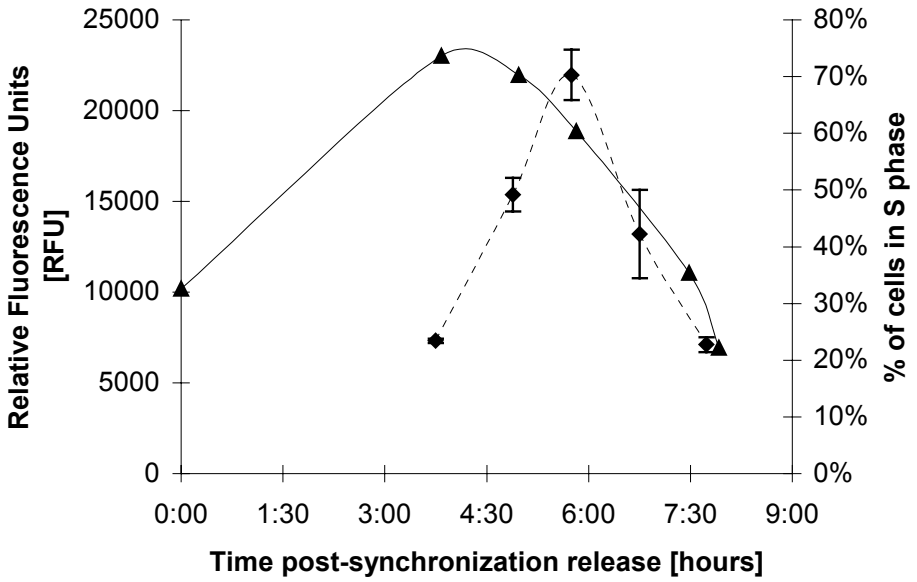


Figure 26. EGFP expression 48 hours post transfection (dotted line) is shifted in comparison to S phase profile of the cell cycle at the time of transfection (full line) using the fast transfection protocol. Repeats of the experiment showed similar results.

These results showed that the role of the cell cycle could be influenced by the transfection protocol, since with a faster transfection protocol, the S phase correlation was shifted.

Depending on the transfection set-up, the optimal time to add the precipitate on the cells may be different. Therefore, the S phase may not play the key role in efficient reporter gene

expression post transfection. Another important consideration is of physical nature: a larger medium volume on the cells results in a longer time for the calcium phosphate precipitate to settle down on cells, since the gravitational settling occurs over a longer distance. With a smaller medium volume, the transfection complex has to be added to cells earlier.

The observed two-hour shift for high expression may be due to the shorter time of exposure to plasmid DNA (one hour instead of three, with the fast transfection protocol used), however, it may also reflect another phenomena in the cell cycle that could play important roles for efficient reporter gene expression.

These results also showed that the kinetics of uptake was relatively fast and dependent on the transfection set-up. One hour of calcium phosphate DNA complex exposure time was enough to achieve reporter gene levels as high as with three-hour exposure, simply by lowering the volume of medium present onto cells during transfection. This is in contradiction to Loyter *et al*. (Loyter et al., 1982a) who had reported that no detectable uptake was achieved after one hour exposure in Petri dishes, and that only weak uptake was seen after 2 hours.

6. Live imaging

With live imaging, it is possible to see the precipitates, to identify cells going through mitosis, as well as to monitor the expression of EGFP, on individual cells. It provided then a perfect tool to further investigate the role of cell cycle during calcium phosphate transfection.

Cells were monitored from plating, through the complete transfection process (calcium phosphate added onto cells and glycerol shock) until 24 hours post-transfection. Positive cells were easily distinguished from negative cells using acquisition under UV light exposition in parallel with normal light acquisition. Calcium-phosphate precipitates were observable outside the cells, as dense crystals.

6.1 The fate of calcium phosphate precipitate post glycerol shock

Early observations showed that calcium-phosphate-DNA precipitate remained on cells post glycerol shock. Approximately 90 minutes of incubation time was required for the precipitate to dissolve again in the fresh culture medium added onto cells post-glycerol shock. (**Figure 27**)

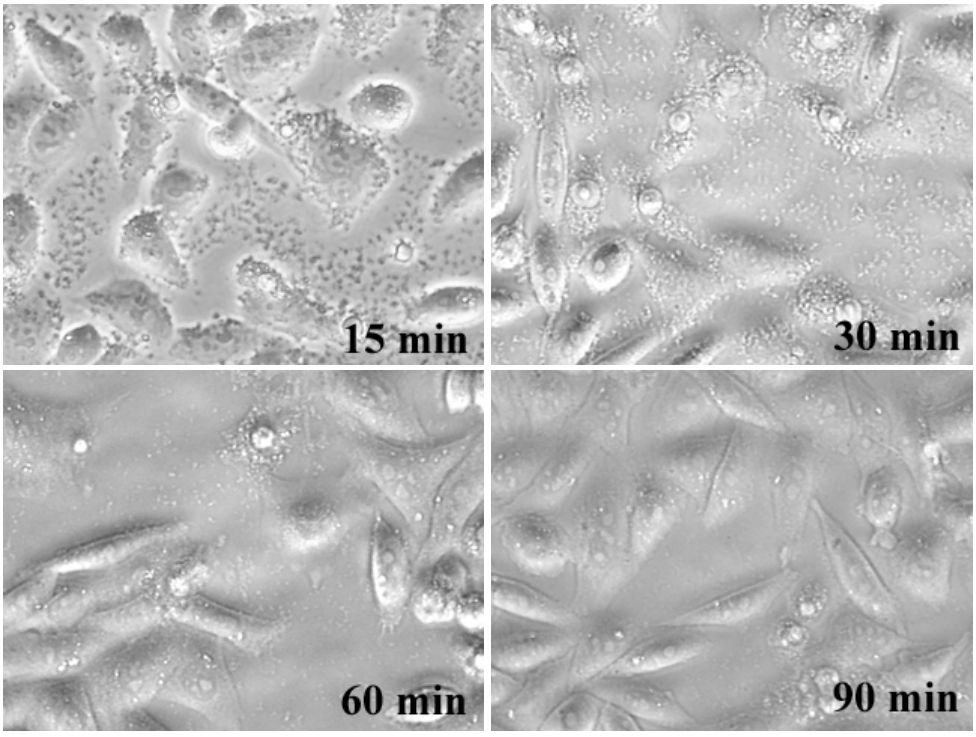


Figure 27. Calcium phosphate precipitate remained on cells for approximately 90 minutes post glycerol shock before fully dissolving in the fresh culture medium (times indicates incubation time post glycerol shock).

Remaining calcium concentration on the cells was monitored during and after transfection by Nova bio-analyzer (Nova Biomedical, Waltham, MA, USA). An increase in the amount of residual calcium was measured even 24 hours post transfection. The calcium concentration was measured pre-transfection, during transfection, immediately post-transfection and 20 hours post-transfection (**Table 6**)

Table 6. Calcium concentrations in the medium pre-, during and post-transfection

	Calcium concentration [mM]
Pre-transfection	0.8
During transfection	11.6
Post-transfection	1.0
20 hours post-transfection	1.0

During transfection in a 12 well plate, the amount of calcium present in 1 milliliter of medium (DMEM/F12, GibcoBRL) is equal to $1.0*10^{-6}$ moles; the amount of phosphate present is equal to $9.5*10^{-7}$ moles. The quantity of calcium added through the transfection complex is equal to $1.25*10^{-5}$ moles and phosphate is equal to $7.0*10^{-8}$ moles. This calcium addition corresponds to approximately 10 times more calcium than what is already present (phosphate added corresponds to approximately a tenth of what is present). The remaining calcium phosphate precipitate on the cells post glycerol shock is due to calcium more than phosphate.

As there might be negative effects due to calcium toxicity, it was important to ensure the complete removal of the calcium phosphate precipitate from the medium post-transfection. This also prevented additional calcium phosphate-DNA uptake by the cells post-glycerol shock.

To completely remove the precipitate, an additional EGTA (5mM in PBS) wash-step was performed just after glycerol shock as done by Loyter *et al* (Loyter et al., 1982b). This was then followed by a PBS wash-step to remove all the EGTA present. After the EGTA wash-step, no more precipitate was visible under the microscope. Non-complexed naked plasmid DNA, as control, did not provide any reporter gene expression. (*See chapter 7: Additional EGTA wash-step*)

6.2 Importance of mitosis

In asynchronously transfected cell populations, the first EGFP expressing cells were detectable approximately 2.5 hour post-glycerol shock. Up to 20% of the transfected cells were detected as positive, based on EGFP expression. Among these cells, about 90% either underwent mitosis or appeared to prepare for mitosis (rounding up) but did not divide before they started to express EGFP. The ratio of executed and non-executed mitosis in the early EGPF-expressing cells was around 3 to 5.

The first EGFP-positive cells usually became highly fluorescent, indicative of extensive intracellular accumulation of large quantities of EGFP. None of these cells underwent a second mitosis. They all became round after 7 to 15 hours post first observation of EGFP and some of them lost fluorescence after a few hours. Only cells that proceeded through mitosis post glycerol shock resulted in two EGFP-positive daughter cells (**Figure 28**). None of the cells undergoing mitosis between the time of DNA addition and before treatment by a glycerol shock (first visible EGFP-positive cells) produced two EGFP-positive daughter cells – only one of them was positive. A non-homogeneous plasmid distribution within the cell at mitosis was probably responsible for the asymmetry in expression.

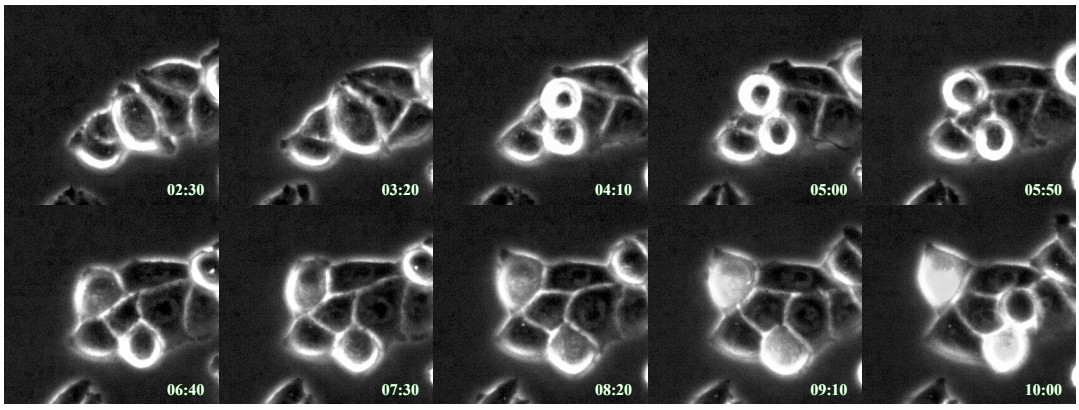


Figure 28. EGFP-positive daughter cells appear post mitosis. ~90% of the positive cells express EGFP post-mitosis or post-aborted mitosis. The time passed since glycerol shock is indicated in hours and minutes in the lower part of each image. GFP-positive cells appear brighter than the others. First detectable signal appeared two and a half hours post-mitosis (position 06:40).

These results support the notion that mitotic activity is a requirement for different transfection methods. Disruption of the nuclear envelope during mitosis permits plasmid entry in the nuclear environment and its following transcription and translation into protein.

If mitosis provides access for the plasmids to the nuclear environment, then an alternative way of entry in the nucleus must exist to explain the 10% of positive cells that did not show a clear mitosis (or aborted mitosis) prior to expression of EGFP. A direct entry of plasmid in the nucleus without mitosis may explain those, as reported by Coonrod *et al*. (Coonrod et al., 1997) for linear plasmid DNA and by Escriou *et al.* (Escriou et al., 2001) for circular plasmid DNA microinjected in the cytoplasm. The 10% of positive cells that did not go through mitosis may come from partially degraded DNA that diffused inside the nucleus. Diffusion of DNA in the nucleus with micro-injection studies, showed that short linear DNA rapidly diffuses in the nucleus (up to 1.5Kbp), whereas large plasmid DNA does not (Chin et al., 1990; Clarenc et al., 1993; Leonetti et al., 1991; Zabner et al., 1995). The size of the promoter and the gene encoding for the fluorescent protein does not exceed 1.4Kb in both case.

6.3 Occurrence of positive cells appearing with time

As degradation of the plasmid occurs in the cytoplasm (Lechardeur et al., 1999) appearance of reporter gene expression was followed in individual cells for a period of 17 hours post transfection, to see if expression occurs preferentially in cells close to glycerol shock at the time of mitosis or if expression randomly appears unaffected by the mitosis localization in time.

Analysis of approximately one hundred positive cells, demonstrated that more than 80% of these appeared within the first 6 hours post-glycerol shock (**Figure 29**). These observations showed that glycerol shock is another key parameter for efficient gene transfer into the nucleus of the cells.

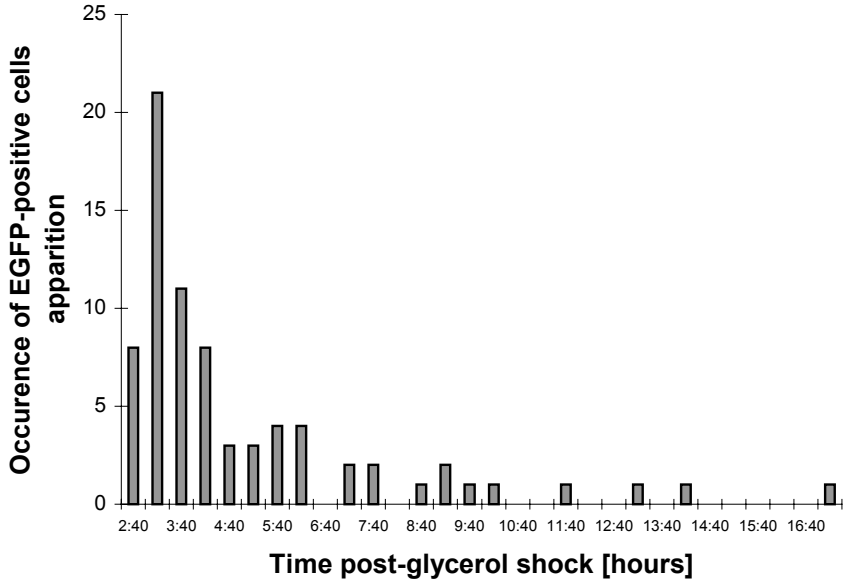


Figure 29. More than 80% of positive cells appeared within the first 6 hours post-glycerol shock as reflected by the occurrence of new EGFP-positive cells (either post mitosis or aborted mitosis) as function of time. Late detectable EGFP-expressing cells all went through mitosis 2:30 to 14:00 hour post-glycerol shock.

The positive cells could also be analyzed using their last mitosis as a reference. Among all the positive cells (independently of where they are localized relative to the glycerol shock time point), 92% were observed between 2:30 to 4:40 hours following mitosis. Only 2% of the EGFP-positive cells were observed in less than 2 hours after mitosis, and only 6% of the EGFP-positive cells were first observed more than 7:30 hours post-mitosis . This indicates that for the large majority of positive cells, the fluorescence signal appears quickly post mitosis. Even if mitosis is far from the glycerol shock, if it results in positive cells afterwards, they will quickly express EGFP.

Rapidity of expression post mitosis seems unaffected by the localization in time relative to the timing of the glycerol shock: if a cell becomes positive post mitosis, it will do so quickly regardless of its localization in time post-glycerol shock. However, the occurrence of new positive cells reduces in frequency when distant in time from the glycerol shock.

6.4 Transgene expression affects cell growth

It has been recently shown (Batard et al., 2001) that cells can take up a large number of plasmid molecules, from 3'000 up to 100'000. Such high copy number of plasmid not only correlated with expression levels but also exhibited reduced cell proliferation and decreased survival. Live imaging studies support these results since EGFP-positive cells had an extended cell cycle compared to negative cells (**Table 7**). Cells with very high levels of EGFP often rounded up and detached after a few hours. The occasional loss of fluorescence in these cells may have been indicative of membrane leakage and eventual cell death as blebbing was frequently observed.

Table 7. EGFP-positive cells have a longer cell cycle compared to negative cells.

	Doubling time [hours]
EGFP-negative cells	12 - 14
EGFP-positive cells	18 - 22

First visible EGFP expression in cells was observed as early as 2.5 hours post-glycerol shock. **Figure 30** represents the number of cells counted by microscopy within a surface area of 0.34 mm^2. EGFP-positive cells reached a maximum of about 20% of the population by 14 hours after the glycerol shock (**Figure 31**). Both EGFP-positive and negative cells increased in number over a 24-hour time frame, with the growth of EGFP-negative cells outpacing EGFP-positive cells. For this reason, the EGFP-positive subpopulation of cells fell to about 15% at 24 hours post-glycerol shock.

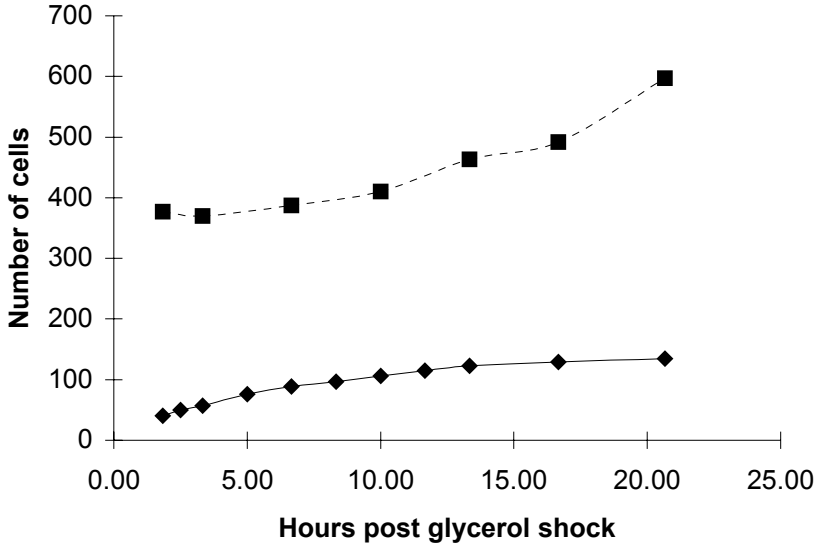


Figure 30. Number of EGFP-positive and negative CHO cells post-transfection. Full line corresponds to the number of GFP-positive cells. Dotted line corresponds to the number of GFP-negative cells.

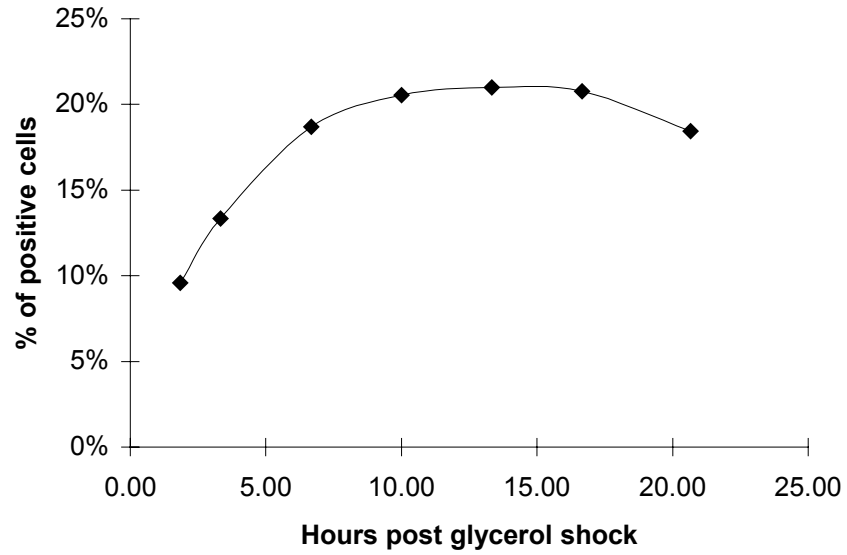


Figure 31. Transfection efficiency. The percentage of GFP-positive cells post-transfection reached a maximum after approximately 14 hours due to their longer doubling time (compared to negative cells, **Table 6**).

For these transfections the fast transfection protocol was used, requiring just one hour of interaction between cells and DNA. Since maturation of EGFP to its fully fluorescent state takes about one hour in mammalian cells (Tsien, 1998), processes for transfer to the nucleus, transcription and translation took approximately 2.5 hours as cells were in contact with calcium phosphate DNA precipitate for at best 3.5 hours before the first positive signal appeared, denoting a rather fast transfer to the nucleus. In this case again, this is significantly

faster than the observations of Loyter *et al*. (Loyter et al., 1982a) using 60mm Petri dishes, where only a small uptake occurred 2 hours after adding the precipitate.

7. Additional EGTA wash-step

An EGTA wash-step was added post-transfection to ensure that reporter gene expression only occurred from plasmid DNA taken up by the cells during the time between the addition of the transfection complex onto the cells and the osmotic shock. No efficient plasmid uptake occurred after calcium phosphate complexes were washed away.

Visual controls under the microscope showed the complete removal of all remaining calcium phosphate complexes on the transfected cells post-EGTA wash-step, whenever this wash step was applied (before, during or after the glycerol shock).

Tests were performed to check if the EGTA wash-step would affect reporter gene expression if performed before, after, or at the same time as the glycerol shock or one hour and two hours after the latter (**Figure 32**).

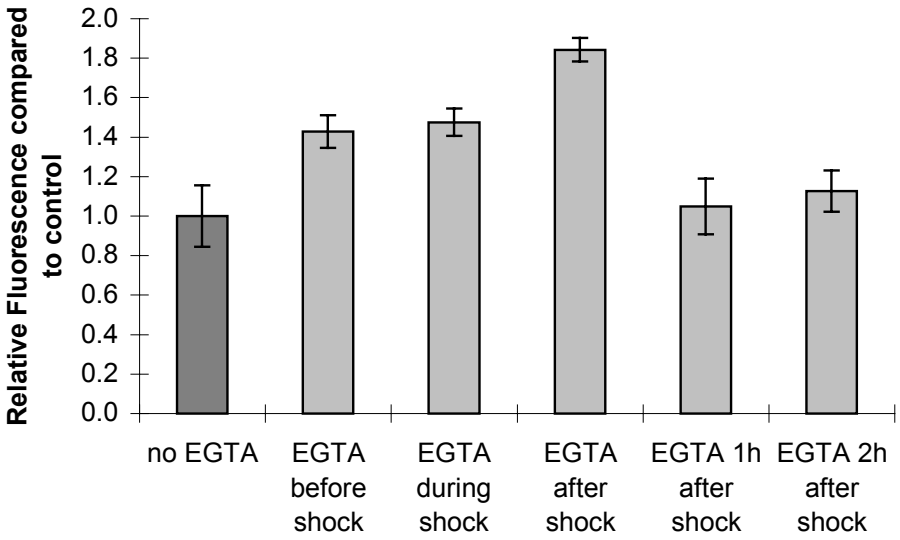


Figure 32. EGTA wash step provided a reporter gene expression increase only if performed close to the osmotic shock. EGTA additional wash step was performed either just before, at the same time, just after, one hour after and two hours after glycerol shock. Cells not exposed to the EGTA wash-step post-transfection (darker bar) were used as control.

The EGTA wash-step did not show any negative effects on reporter gene expression. On the contrary, higher levels of reporter gene expression were achieved when the remaining calcium phosphate precipitates were fully removed from the cells shortly before or after the glycerol shock. If performed far from the glycerol shock, in spite of removing all the extra-cellular

calcium, no significant increase in reporter gene expression was observed. This was probably related to additional calcium uptake by the cells (as in control), due to a longer exposure to the precipitate and subsequent toxic side effects. Less precipitates uptake occurs if it is quickly removed from the cells. Lower intracellular calcium concentrations are most likely achieved with the EGTA wash-step performed close to glycerol shock, and thus fewer side effects due to calcium.

7.1 EGTA treatment does not affect viability or growth rate

The growth performance of EGTA treated cells was measured. Growth curves and doubling time were similar for control and EGTA treated cells (**Figure 33**), as well as the viability of EGTA-washed cells (**Figure 34**).

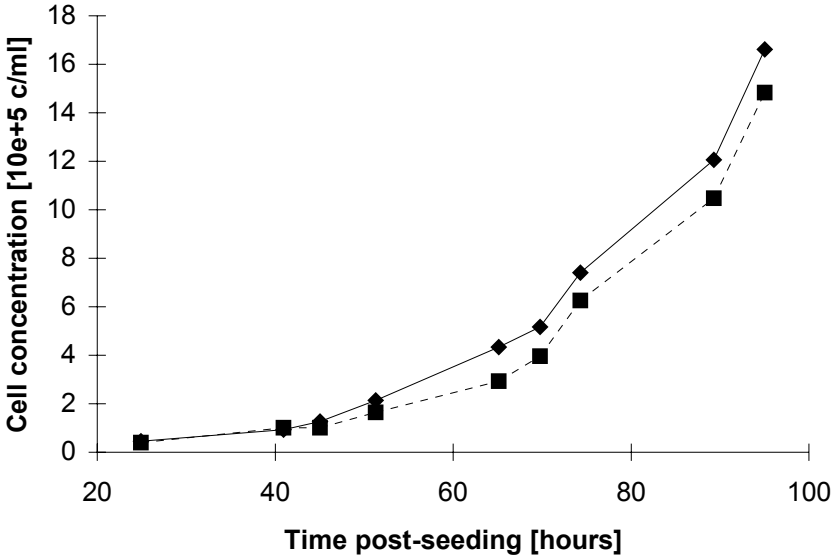


Figure 33. Growth curve for EGTA washed cells (dashed line) and control cells (full line). Cells were seeded at 0.2e+5 c/ml and growth was monitored for 4 days. Doubling time calculated from these data points was respectively 13:24 hours and 13:18 hours for EGTA washed cells and control cells.

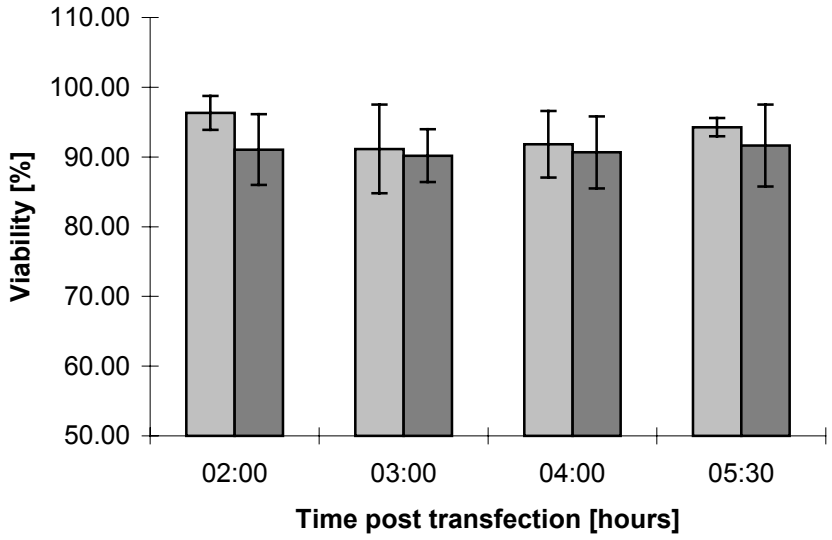


Figure 34. Viability was comparable for EGTA washed cells (light gray bars) and non-washed control cells (darker gray bars) following transfection.

7.2 EGTA wash-step increases overall expression

When the EGTA wash-step was tested with the fast transfection protocol (**Figure 35**), an increase in reporter gene expression measured 48 hours post-transfection was also observed.

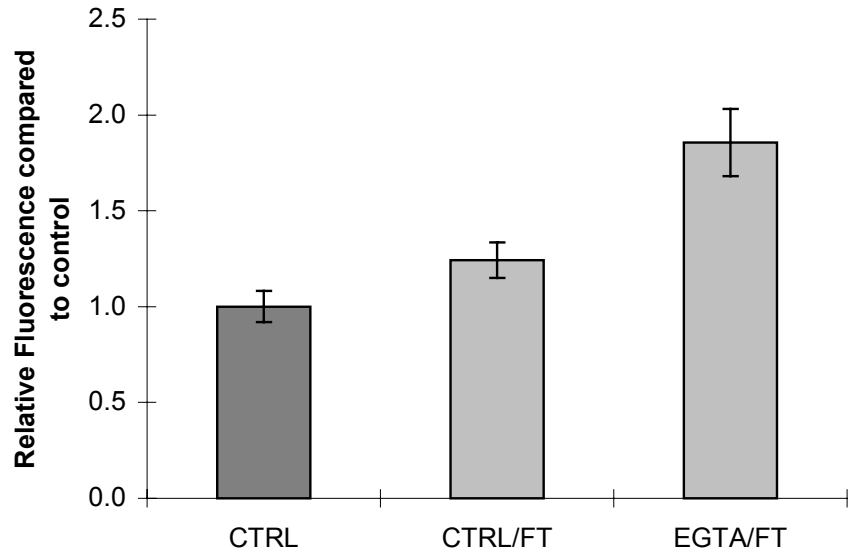


Figure 35. EGTA wash-step also increases reporter gene expression level with the fast transfection protocol compared to normal transfection without EGTA wash-step (CTRL). CTRL/FT = fast transfection without an EGTA wash-step; EGTA/FT = fast transfection with an EGTA wash-step. EGFP expression was measured 48 hours post-transfection.

This additional wash-step post-osmotic shock showed increased reporter gene expression compared to non EGTA-washed control cells. When measuring the EGFP expression in the

first hours following transfection, instead of 48 hours post-transfection, the difference in the fluorescence signals was already visible (**Figure 36**).

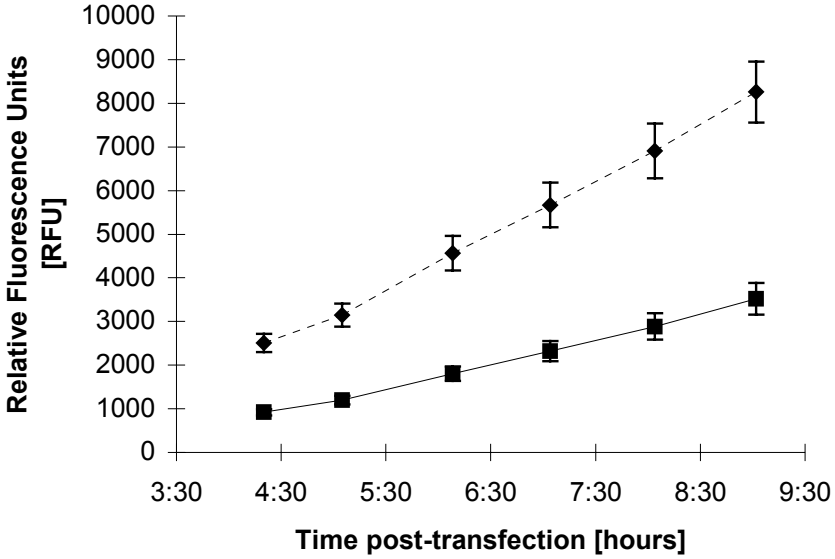


Figure 36. EGTA additional wash-step increases reporter gene expression signal already in the first hours post-transfection. EGTA-washed cells (dotted line) showed a clear higher reporter gene expression level compared to non-washed control cells (full line) already in the first hours following transfection.

It was studied whether the increase in reporter gene expression arose from a larger number of positive cells post-transfection or from boosting the expression of a constant number of positive cells. Microscope observations clearly showed (**Figure 37**) more positive cells in the EGTA-washed population than in the control.

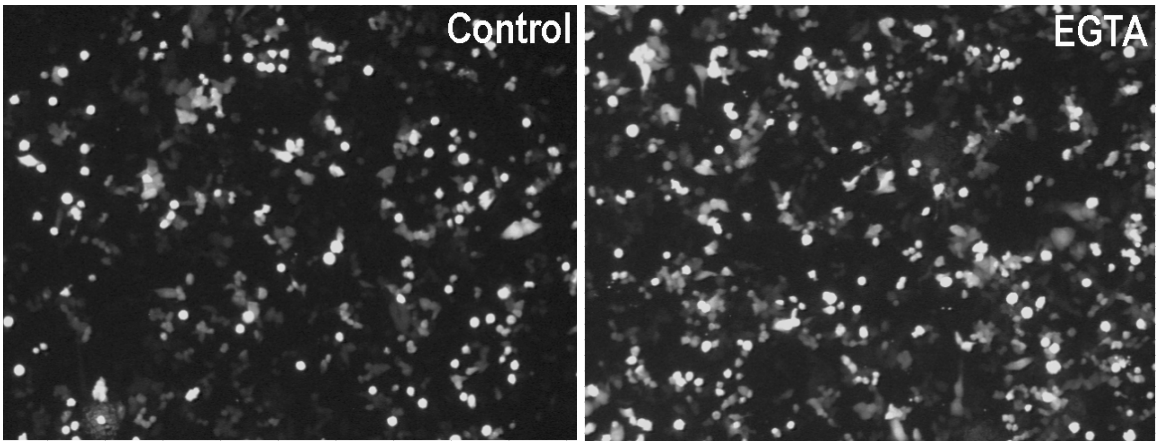


Figure 37. EGTA wash-step post-transfection (right) showed more positive cells in the transfected cell population than non-washed control (left). Both samples were transfected at the same time, with the same transfection mix. Cell confluence was equal in both samples. Pictures taken 24 hours post-transfection.

8. Role of glycerol shock

Glycerol shock did not show an increase in plasmid uptake (Batard et al., 2001) post transfection, with the amount of intracellular plasmid remaining constant prior to and post-glycerol shock. Thus its role is not to load more plasmids into cells. The same authors proposed that destabilization of endosomal and lysosomal membranes by glycerol would result in temporary pH neutralization within these compartments. Apparently this increases the plasmid stability and favors its release in the cytosol. Sabelnikov (Sabelnikov, 1994) proposed that glycerol affected the membrane fluidity.

As live imaging observations suggested, glycerol shock plays a strong role, because less positive cells appeared when far from the time point where it was applied. Whenever transfecting CHO DG44 cells with the calcium-phosphate method, high expression levels were only obtained when cells were treated by an appropriate glycerol shock. It is important that this shock is applied three hours (classical transfection protocol) or one hour (fast transfection protocol) after the precipitate has been added onto the cells. It is essential for an efficient reporter gene expression post transfection, and avoiding it results in low, or no expression.

8.1 Visual effect of glycerol on cells

The one-minute glycerol shock provokes significant changes in the cellular morphology. The most striking visible effect is rapid shrinking in volume of the cells. As shown in **Figure 38**, glycerol solution acts rapidly upon its addition. In a matter of 10 seconds, cells flattened and remained in this state until the glycerol solution was removed and cells were returned to culture medium. One minute after returning into standard culture medium, no morphological change was detectable compared to non-treated cells. Most cells are attached to the culture vessel surface and thus the shrinking of the volume results in a flattening of the cells.

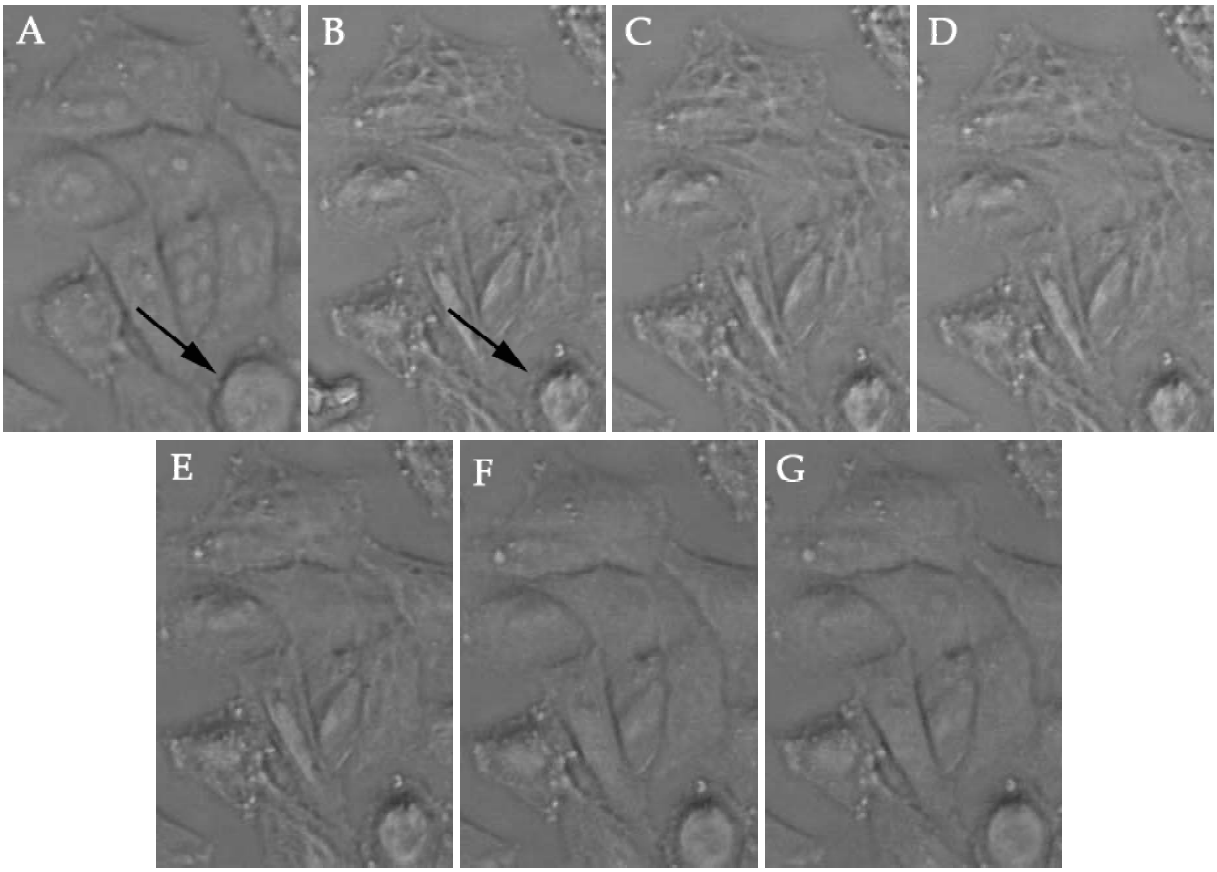


Figure 38. Visual effect of glycerol shock on adherent cells. **A**: cells prior to addition of glycerol. **B**: cells in glycerol after 10 seconds. **C**: cells in glycerol after 30 seconds. **D**: cells in glycerol after 60 seconds. **E**: cells 10 seconds after return in culture medium. **F**: cells 30 seconds after return in culture medium. **G**: cells 60 seconds after return in culture medium. Arrows indicate a detaching cell where a clear shrinkage is visible upon addition of the 10% glycerol solution.

Rounded, detached cells (cells going through mitosis) shrink upon addition of glycerol, thus visibly reduce their diameter, and regain their original size/shape once glycerol is removed and they are returned in culture medium.

8.2 Osmolarity effect of glycerol

This "shrinkage" of cells is most likely a purely osmotic effect induced by the high, non-physiological, osmolarity of glycerol. The osmolarity of solutions used during culture and/or transfection is given in **Table 8**.

Table 8. Osmolarity of the different solutions used during transfection

	Osmolarity [mOsm]
DMEM/F12 + 2% serum	326
DMEM/F12 without serum	328
Trypsine	296
PBS 1X	282
10% glycerol (in PBS 1X)	1976

Using different glycerol concentrations (0, 1, 2, 5, 10, 15, 20, 25 and 30%) (**Table 9**) showed that only 10 and 15% glycerol gave good transfection efficiency. Higher concentrations had negative effects on viability, whereas lower concentrations showed no effect at all. These different glycerol concentrations were tested on synchronized cells while in S phase as a comparison and the results obtained were similar (**Figure 39).**

Table 9. Osmolarity of the different solutions with increasing glycerol percentage

	Osmolarity [mOsm]
0 % glycerol	282
1 % glycerol	454
2 % glycerol	628
5 % glycerol	1086
10 % glycerol	1978
15 % glycerol	2788
20 % glycerol	3618
25 % glycerol	4449
30 % glycerol	5279

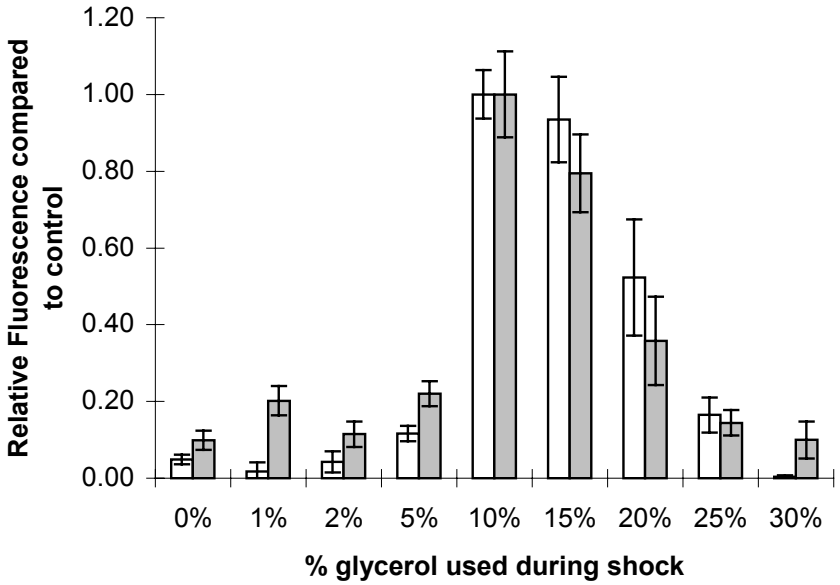


Figure 39. No improvement was achieved using higher (or lower) glycerol concentrations, on both synchronized (gray bars) and asynchronous cells (white bars). Reporter gene expressions were assessed 48 hours post-transfection, relatively to the standard concentration (10% glycerol).

The reduced expression for glycerol concentration higher than 15% is probably due to toxic osmotic effects of glycerol, as was observed by Wilson *et al.* (Wilson and Smith, 1997) with three different cell lines (African green monkey kidney cell (BSC-40), NIH 3T3 fibroblasts, and AtT-20/D-16v rat cell line) transfected with calcium phosphate.

8.3 Quantifying the size change upon glycerol shock

As no references were found in the literature about the effect of glycerol shock on cellular volume at the end of transfection, different approaches were tested to quantify it. The first two approaches used involved flow cytometry and CASY^{TM} measurements. Forward scattering in flow cytometry, as mentioned in the introduction, gives a representation of the size of the analyzed cells. CASY^{TM} gives cell concentrations as well as a profile corresponding to the size distribution of the cells. Both methods were tested; both have a mean of measurement that may interfere with the parameter being analyzed as will be explained briefly. The third approach was PCV (*packed cell volume*). All the measurements were performed in 10% glycerol solutions, as cells rapidly regain their original size in solutions with medium-like osmolarity (**Figure 38**)

8.3.1 Flow cytometry approach

Forward scatter (FSC) was monitored in correlation with time for a population of cells exposed to a 10% glycerol solution in PBS. As previously monitored under microscope, a direct effect was seen on cell size distribution. FSC signal shifted towards smaller events (meaning cells were decreasing in diameter) within the one-minute time frame used for a glycerol shock (**Figure 40**).

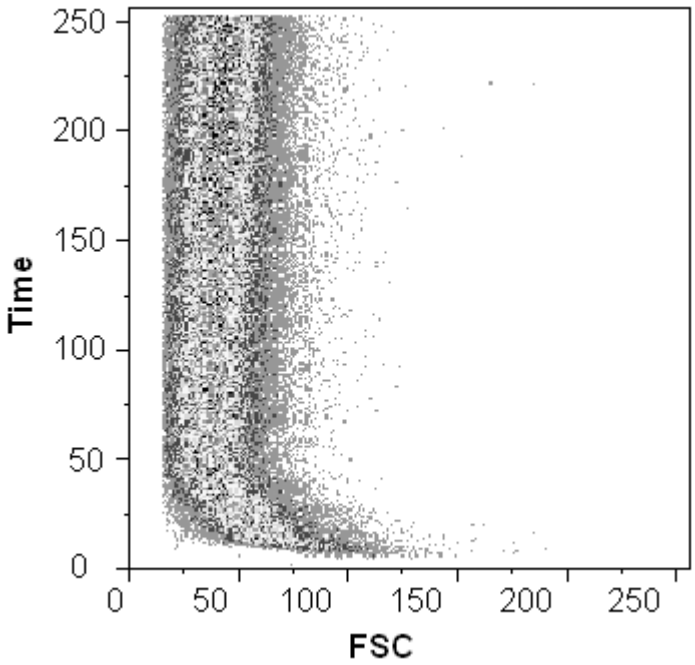


Figure 40. Forward scatter monitored as a function of time for a cell population exposed to a 10% glycerol solution in PBS. Y-axis represents the time; scale is so that 50 corresponds to one minute. Cells reached the smallest size less than one-minute after their exposure to the glycerol solution. An average 50% decrease was observable. Non-treated cells had an average FSC peak around 110. With the same acquisition settings, upon addition of glycerol, FSC peak shifted towards an average of 46.

Unfortunately forward scatter measurement on a flow cytometer is dependent on light diffraction; adding glycerol to the samples changes this index, and effects coming from glycerol have to be considered as well. To check this, the same measurement protocol was done with beads (2.5µm polystyrene beads (Molecular Probes, Leiden, The Netherlands)). A shifting towards the left was also observed. The lack of standard beads with different sizes did not allow determining the optical correction factor for glycerol.

8.3.2 CASY approach

CASY^{TM} counts particles and measures their volume. Each and every event that passes in the analyzing capillary is measured and transferred in a histogram of event size. Cells exposed to glycerol solution also showed a change in the CASY profile (**Figure 41**).

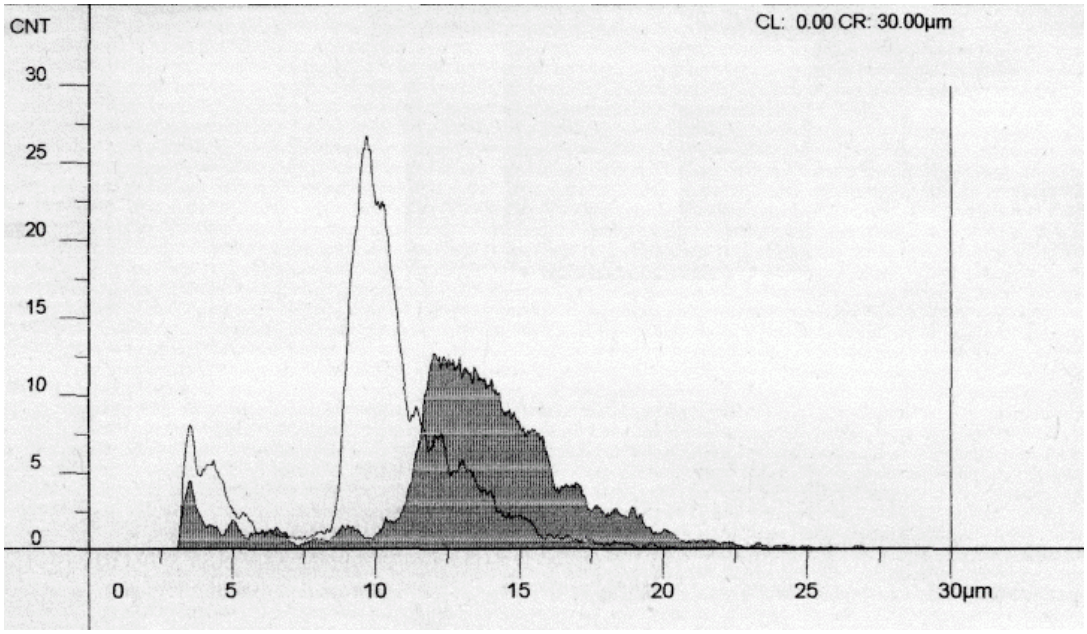


Figure 41. CASY profile superimposition of cells before (black histogram) and after (white histogram) glycerol treatment.

The profile for the cells exposed to glycerol was also shifted toward smaller events. Using these values, it was determined that the cell's mean diameter shifted from 14.11µm (untreated cells) to 11.00 µm (glycerol treated cells). This 3.11 µm diameter shift corresponds to a total volume decrease of approximately 53% (1470 μm^3 for the untreated cells; 695 μm^3 for the glycerol treated cells).

CASY measurements are based on change of conductivity of the sampling solution each time an event (cell, debris, aggregate) passes into the analyzing capillary. If the salt concentration of the sampling solution is changed, the results will be affected. Tests were also done with 2.5 µm polystyrene beads, and they showed a slight shift towards smaller events (**Figure 42**).

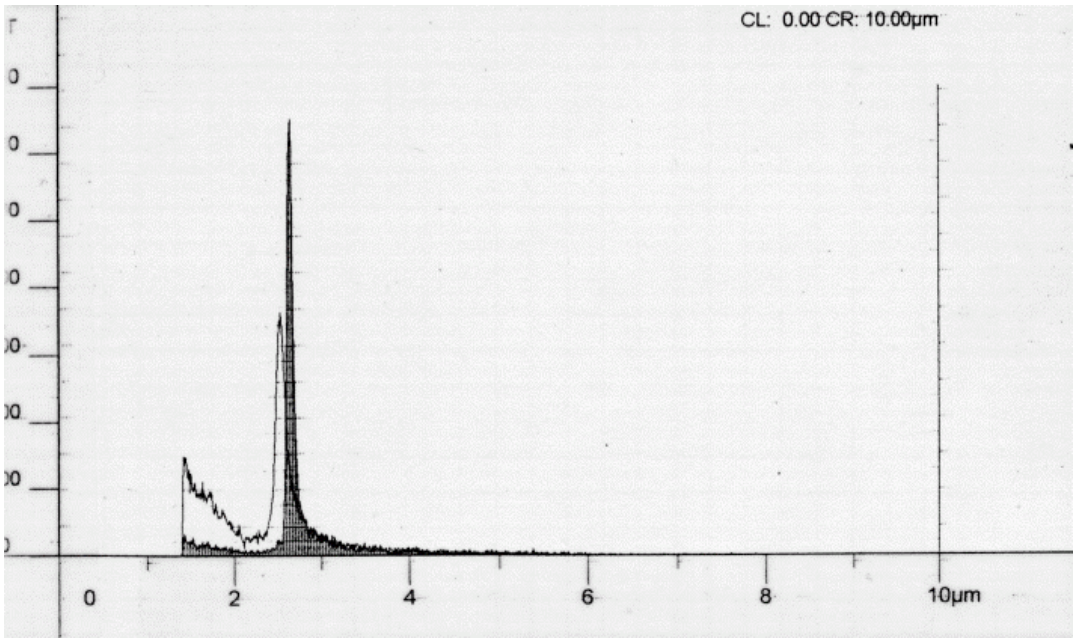


Figure 42. Beads tested before (black histogram) and after (white histogram) glycerol treatment. Beads shifted from 2.64um to 2.52um.

Beads shifted from 2.64 µm (untreated) to 2.52 µm (treated with glycerol). This corresponded to a volume decrease of approximately 14%. Therefore, glycerol affected the measurement system by its presence in the sampled solution due its different conductivity compared to CASYTON. No other beads sizes were available to quantify the influence of glycerol on measurements and thus determine indirectly the real size shift.

8.3.3 PCV approach

With PCV (packed cell volume), cell sizes can be compared in presence or absence of glycerol, as its presence does not affect the measurement principle. With this method a size decrease of approximately 55% was estimated for the cells in the presence of 10% glycerol (**Figure 43** and **Figure 44**). Mini-PCV tubes were used (developed in our lab in collaboration with TPP (TPP, Wohlen, Switzerland), not available on the market yet) with an Eppendorf 5417C centrifuge. The PCV assessment can be done within one minute (5'000 rpm for one minute), corresponding therefore to the normal exposure time to glycerol during transfection.

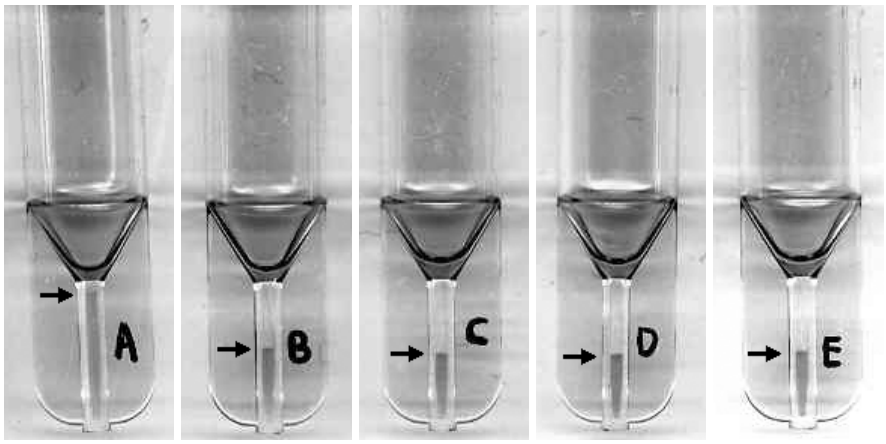


Figure 43. Mini-PCV of cells treated with increasing concentrations of glycerol. **A**: untreated control; **B**: 5% glycerol; **C**: 10% glycerol; **D**: 15% glycerol, **E**: 20% glycerol. Arrows indicate the PCV.

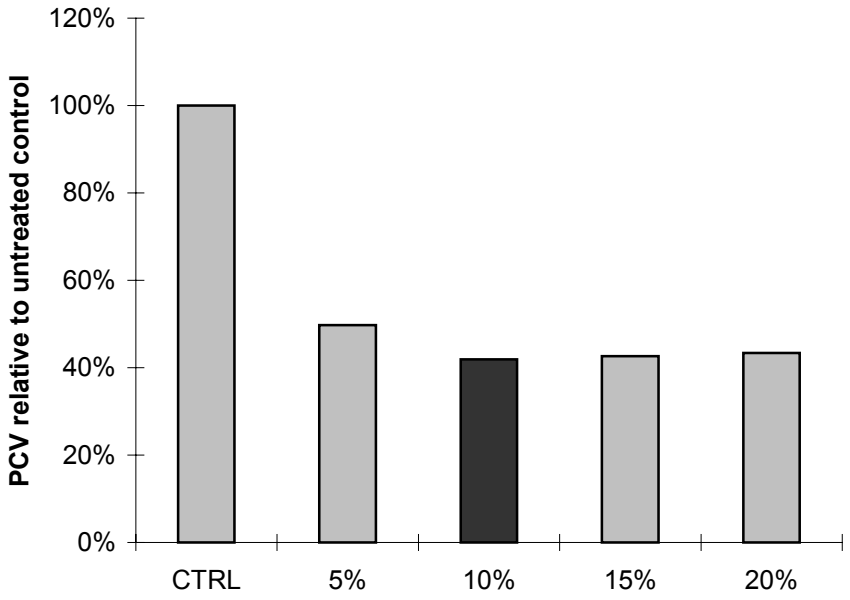


Figure 44. Corresponding PCV compared to non-treated control cells for increasing glycerol concentrations during shock. Cell volume decreases of approximately 55% (drops to 45% of the original size upon 10% glycerol exposure (darker bar))

With PCV measurements, it was estimated that cell volume decreased of approximately 55% during the one-minute exposure to the 10% glycerol solution. These results are comparable with those obtained with CASY measurements. The slight difference between both methods may indicate that CASY measurements may not be too affected by the glycerol solution when measuring a sample (even though beads shifted towards smaller events). Flow cytometry analysis also showed similar relative size shift (average FSC peak ~ 110 $\Rightarrow$ ~ 46 post glycerol addition), meaning that the optical effect due to the glycerol might not have been too significant either.

Monitoring the reporter gene expression level as a function of the cell cycle state at the time of glycerol shock, showed a correlation between increase in reporter gene expression and the percentage of cells proceeding through mitosis (increasing amount of G1 cells in a synchronized cell population) (**Figure 45**).

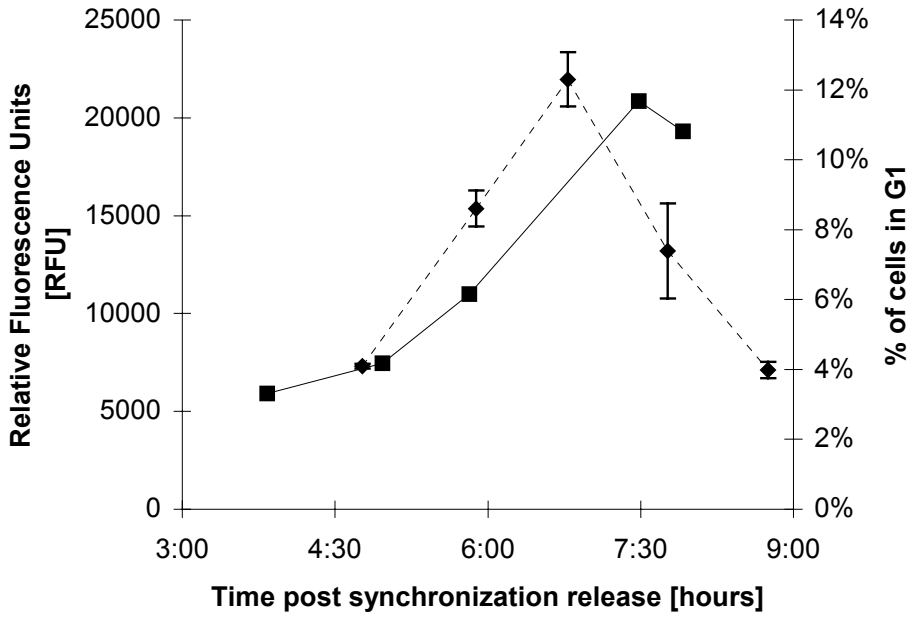


Figure 45. Cell cycle profile at glycerol shock correlates with cells going through mitosis. Dotted line represents reporter gene expression 48 hours post transfection. Full line represents the percentage of cells in G1 (out of mitosis) at the time of glycerol shock. The shift between both curves maximum is probably due to the fact that it was not possible to measure the percentage of cells in mitosis, and that an alternative option was used (G1 cells in a synchronized population)

9. Osmotic shocks with different solutions

Other solutions with osmolarity close to that of10% glycerol were tested after transfection in order to check whether the glycerol shock effect is related to glycerol in particular or to the osmolarity of the solution.

9.1 Effect of osmotic shock on cellular volume

Three different solutions were tested: PBS 10X, NaCl 1M solution and DMSO 10% (**Table 10**). Cells exposed to PBS 10X needed to be washed with PBS 1X to fully remove the PBS 10X trace, as it contains salt concentrations too high for cells (control without wash-step showed mostly dead cells when GFP was assessed).

Table 10. Osmolarity of the different solutions tested for osmotic shock

	Osmolarity [mOsm]
Glycerol 10%	1978
PBS 10X	2690
DMSO 10%	1910
NaCl 1M	2000

PCV was also measured for every conditions and showed similar results for glycerol 10%, PBS 10X and NaCl 1M. Only DMSO 10% did not show significant reporter gene expression post transfection (**Figure 46** and **Figure 47**).

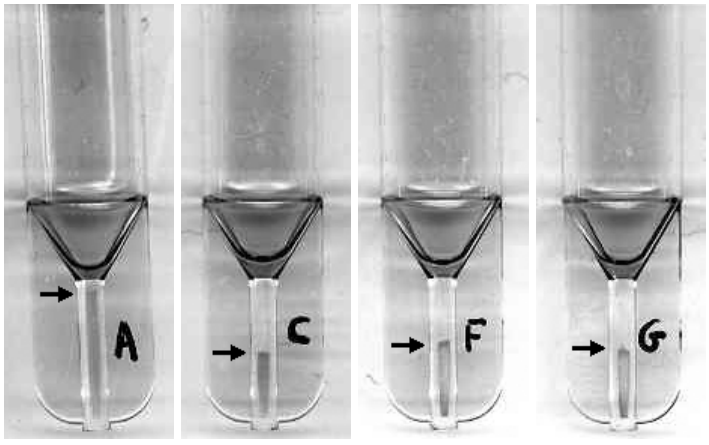


Figure 46. The same PCV decrease was measured with glycerol-free solution **A**: untreated control; **C**: 10% glycerol; **F**: PBS 10X; **G**: NaCl 1M. Arrows indicate the PCV.

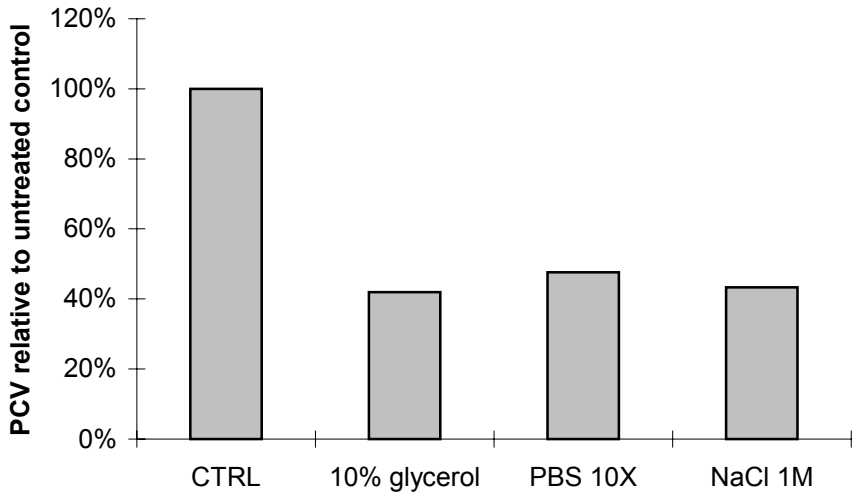


Figure 47. Effect of hyperosmolarity on PCV. Corresponding PCV for different solutions compared to 10% glycerol and non-treated control. Equal volume decreases were observable.

The osmotic effect of DMSO is temperature dependent. PCV on cells with DMSO at 0°C, 12°C, RT or 37°C gave different results (**Figure 48**). Cold DMSO had a stronger impact on size decrease than DMSO at room temperature. The latter was used during all experiments and resulted in almost no reporter gene expression (its PCV resulted in a 20% decrease in volume). As the DMSO effect was temperature sensitive, it was not kept for further testing to avoid eventual side effects due to temperature drops during the osmotic shock.

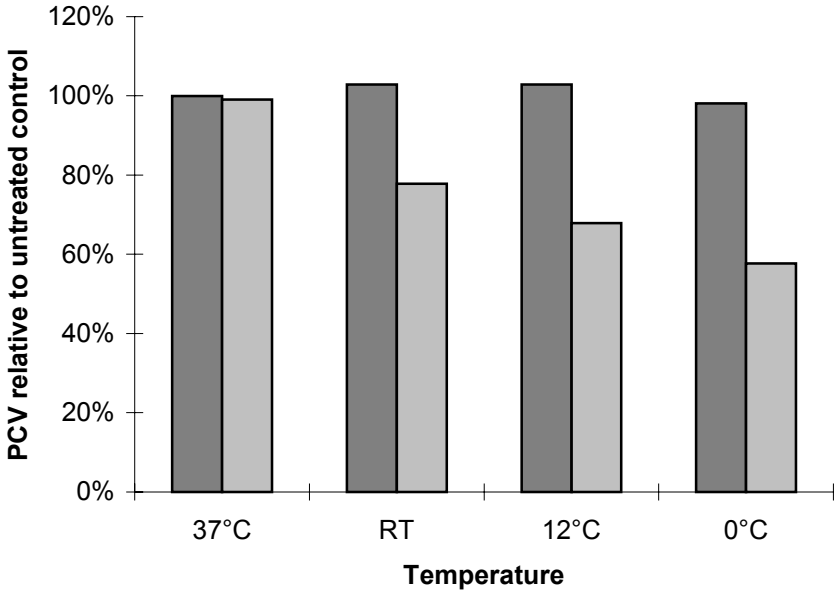


Figure 48. Osmotic effect of DMSO is temperature dependent. PCV measured using DMSO 10% (light gray bars), as osmotic agent, gave almost no volume decrease when used at 37°C. Effect on volume was more significant for lower temperature. Controls were performed with untreated cells (darker bars) to ensure that no effect arose from the temperature. Data kindly provided by Dr. M. Jordan (unpublished).

9.2 Effect of osmotic shock on reporter gene expression

Cells were transfected using the fast transfection protocol and an osmotic shock was applied with glycerol 10%, PBS 10X or NaCl 1M. Reporter gene expression was measured 48 hours post transfection (**Figure 49**).

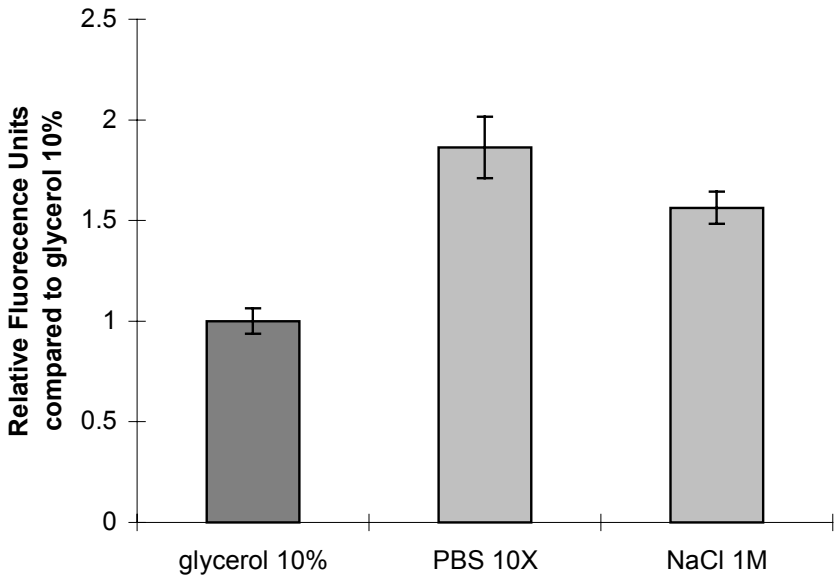


Figure 49. Relative fluorescence 48 hours post transfection for different osmotic shocks. PBS 10X and NaCl 1M solution were compared to glycerol 10% (darker bar).

These results showed that it was not a uniquely glycerol that improves transfection, but rather an osmotic event that can also be obtained by salts.

Effects other than osmotic ones cannot be fully excluded at this point. 5% glycerol had the same effect on volume decrease as the 10% glycerol solution, but not on reporter gene expression post transfection. 15% glycerol provided almost the same level of reporter gene expression as 10% glycerol, but increasing amounts of glycerol (even if they produced an identical volume decrease) had a negative impact on the reporter gene expression (**Figure 39** and **Figure 44**). PBS 10X (with an osmolarity a little below that of glycerol 15%) provided a signal increase of about 1.8 compared to glycerol 10%, and this with a similar volume decrease. NaCl 1M provided an osmolarity a little above glycerol 10% and a reporter gene expression level 1.5 times higher, also with similar volume decrease (**Figure 47** and **Figure 49**). It is possible that an osmotic shock could provide a means to escape endosomes by

destabilizing their membrane *(Batard et al., 2001; Sabelnikov, 1994)*, while at the same time the cellular volume decreases (resulting in an increase of cytoplasmic plasmid concentration and an easier entry in the nucleus).

10. Intracellular plasmid residence time for efficient reporter gene expression

To test how long the internalized plasmids remain effective for reporter gene expression, asynchronous cells were transfected for one hour, before the calcium phosphate transfection complex was removed by EGTA wash-step. At this point the plasmid is within the cell, but not within the nucleus, as it seems that nuclear uptake is triggered by the osmotic shock. Cells were left in the incubator for different periods of time before osmotic shock was applied (**Figure 50**). It appeared that the osmotic shock could be delayed for approximately 30 minutes without losing much expression. A clear decrease in efficiency was measured for cells incubated for more than half an hour before osmotic shock was applied. These data are in agreement with the observations of Lechardeur *et al.* (Lechardeur et al., 1999) of an intracellular DNA half-life of approximately 50 - 90 minutes.

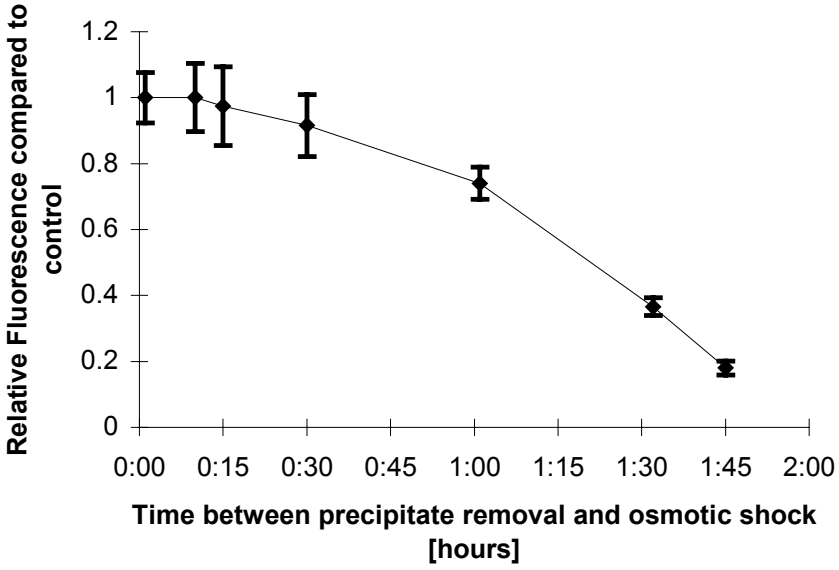


Figure 50. Intracellular plasmid efficiency for transfection quickly decreases with time. Asynchronous cells were exposed to transfection complex for three hours before its complete removal by an EGTA wash-step. Cells were left to incubate for different periods of time before osmotic shock was applied. Reporter gene expression was measured 48 hours later. Control corresponds to cells where osmotic shock was applied one minute after the transfection precipitate was removed. $T_{1/2}$ calculated from those values, for "efficient plasmid" transfer to the nucleus, was equal to 80 – 85 minutes.

The data also provided evidence that internalized plasmid DNA was still driving reporter gene expression for at least 30 - 45 minutes after the uptake had finished (controls, where no osmotic shock was applied, had no significant reporter gene signal measured).

11. Possibilities to target more cells during transfection

Cells apparently have to pass through mitosis to efficiently take up plasmid into the nuclear environment and to express the reporter gene post-transfection. To further check the impact of mitosis in cell expression post-transfection, cells were transfected twice with two plasmids encoding different fluorescent reporter genes: pEGFP-N1 and DsRedExpress-N1 respectively (both from Clontech). Both plasmids have the same promoter (CMV) and an equivalent size, thus avoiding size- or promoter-related side-effects (**Table 11**)

Table 11. pEGFP-N1 and pDsRedExpress-N1 comparison. (Adapted from www.clontech.com)

	Promoter	**Plasmid size**	**Excitation**	**Emission**
PEGFP-N1	CMV	4.7 Kbp	488 nm	507 nm
pDsRedExpress-N1	CMV	4.7 Kbp	557 nm	579 nm

The first transfection was performed with EGFP coding vector, using the fast protocol and the EGTA wash-step after glycerol shock to prevent any transfection complex entering the cells post-osmotic shock. An hour and a half later, a second transfection was performed but this time using DsRedExpress as a reporter gene (**Figure 51**). The 90-minute incubation between both transfections was chosen based on the previous experiments (**Figure 50**). This time period should ensure that no EGFP plasmids from the first transfection remained in the cytoplasm when the second osmotic shock was applied. Under these conditions, the second shock was performed two and a half hours after the first one. Based on Lechardeur *et al*. observations of cytoplasmic plasmid half-life equals to 50 – 90 minutes (Lechardeur et al., 1999). Controls were performed with both plasmids transfected at the same time (**Figure 52**). Reporter gene expression was monitored 24 hours post-transfection and no co-localization was visible, meaning that both vectors were expressed by two distinct sup-populations.

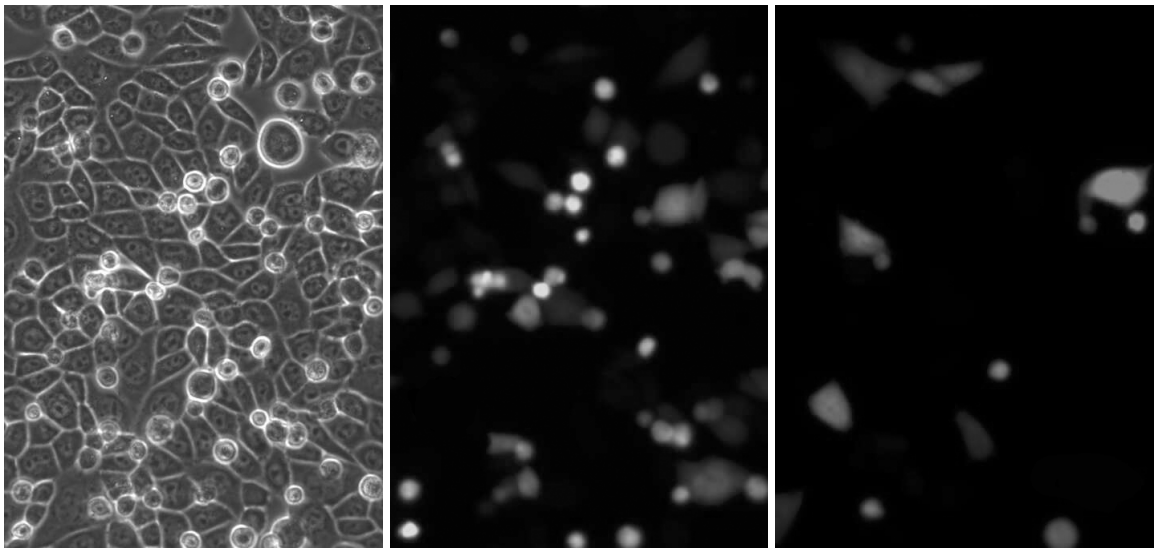


Figure 51. Two successive transfections, using first EGFP (middle) and then DsRedExpress (left), targeted different sup-populations of cells. Both vectors were transfected independently and no reporter gene co-localization was visible.

These results support the importance of mitosis for efficient plasmid transfer into the nucleus. Once a cell has gone through mitosis, it will take another 12 hours before it goes through the next one. If a second transfection is performed, cells that went through mitosis during the glycerol shock at the end of the first transfection will not provide access to their nuclear environment for the second plasmid molecules. If the time between two successive transfections is shorter than the duration of the cell cycle, then no double positive cells will be obtained. Half-life of cytoplasmic plasmid DNA is not long enough for them to subsist through a complete cell cycle. Controls with both plasmids transfected at the same time, on the other hands, showed co-localization of both reporter genes (**Figure 52**).

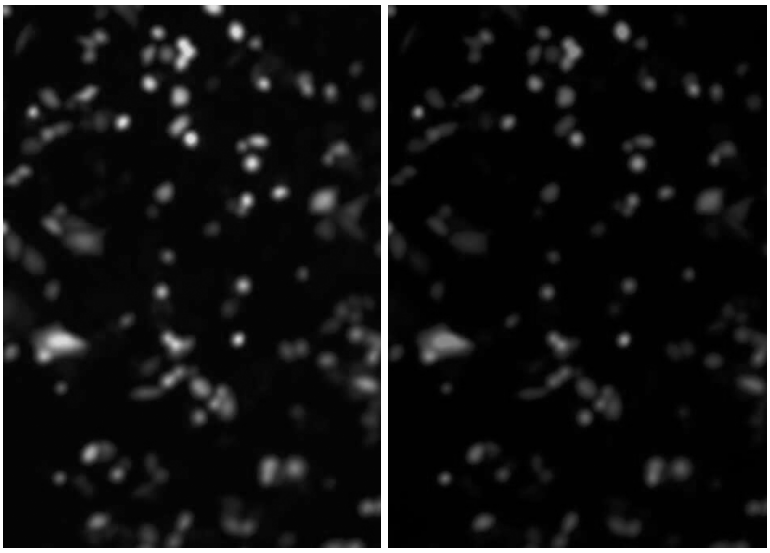


Figure 52. Co-localization of EGFP (left) and DsRedExpress (right) expression when transfected at the same time (mixed 1:1 prior to transfection complexation with calcium and phosphate)

Those results strongly indicated that whenever two or more plasmids have to be transfected in cells (i.e. IgG, heavy and light chain sequences on two distinct plasmids), transfection must be done in the presence of both plasmids if co-expression is required. Transfecting plasmid, one after the other, with calcium phosphate, will not result in co-expression and therefore will only provide poor expression titers (if any) in the transiently transfected population, unless a stable expressing population is re-transfected with a second plasmid.

11.1 Multiple transfections

In theory, repetitive transfections should target different sub-populations, but when it was first performed on an asynchronous cell population, without an EGTA wash-step, it did not result in any signal increase (**Figure 53**). This was probably due to calcium accumulation inside the cells. It was reported that calcium concentrations higher than 50mM had a negative impact on cell viability (Lam and Cullis, 2000). Repetitive transfection, even if only exposing cells to 12.5mM calcium each time (normal transfection) could increase the intracellular pool of calcium, finally reaching the same negative impact that would occur due to an exposure to calcium with a concentration greater or equal to 50mM. The additional transfections were performed after more than 3 hours incubation post-first transfection to ensure that no residual intracellular plasmid from the first transfection would take part in the second transfection procedure (**Figure 50**).

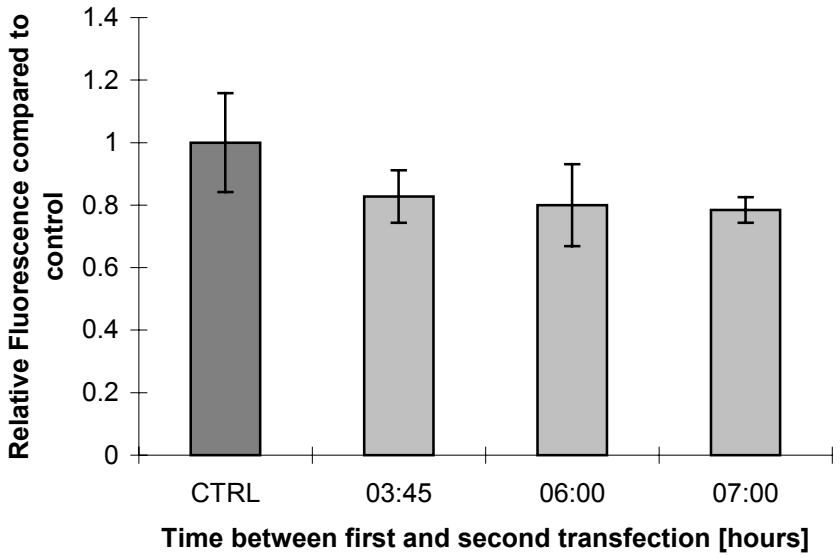


Figure 53. Repetition of transfection on the same asynchronous cell population does not increase reporter gene expression when cells are not washed with EGTA post transfection. Cells were transfected a second time after different incubation periods (x-axis). Reporter gene expression was measured 48 hours later and compared to control (darker bar) where cells were transfected only once. First transfection was done at the same time for all the different tests.

When the EGTA treatment was included in the transfection protocol, asynchronous cell populations showed an increase in reporter gene expression levels upon re-transfection with both normal and fast transfection protocols (**Figure 54**). Cells were also incubated for 3 hours (or more) before second transfections were performed.

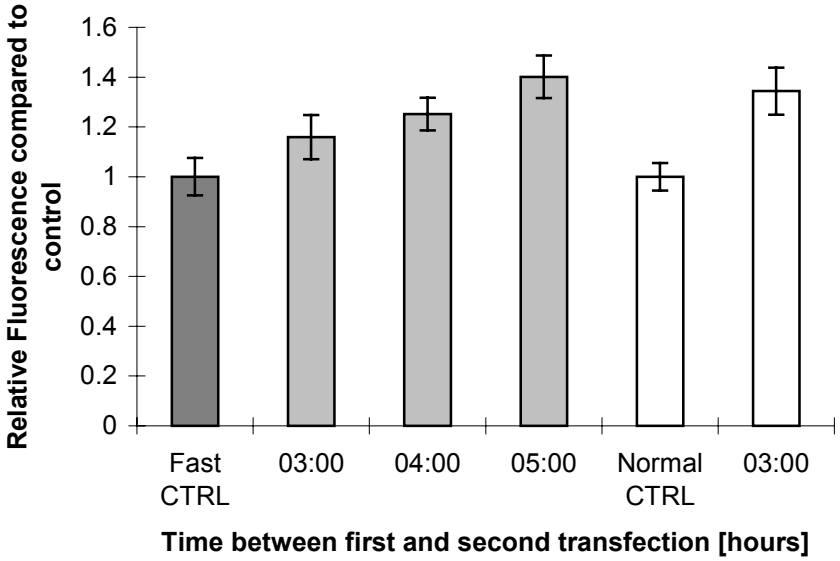


Figure 54. Increased reporter gene expression was achieved with repetitive transfection when EGTA wash-step was performed post-exposure to the transfection complex. Cells were transfected with the fast protocol (gray

bars) or the normal protocol (white bars) for their respective exposure times (i.e. one hour for the fast transfection, 3 hours for the normal transfection). Once the osmotic shock was performed, residual transfection complex was removed and cells were re-incubated for different periods of time (X-axis) before being transfected again. Both fast and normal protocol showed an increase in reporter gene expression when re-transfected compared to control.

This signal increase upon re-transfection with both normal and fast protocols indicated that this phenomenon was not dependent on the transfection set-up. The difference between the classical transfection and the fast transfection was probably due to the toxic effect of calcium accumulation in cells and thus resulted in no signal increase upon normal re-transfection, whereas the additional EGTA wash-step in the fast transfection protocol enabled reporter gene increase upon re-transfection.

11.2 Repetitive osmotic shocks

Intracellular plasmid DNA was shown to drive reporter gene expression even after a delay of 45 minutes post cellular uptake, therefore tests were performed to check if more plasmid molecules could reach nuclei of different subpopulations with repetitive osmotic shocks post-transfection.

Assuming that plasmid DNA has access to the nuclear environment only during mitosis and that DNA is rapidly degraded once it has been released into the cytoplasm, one could expect to target the nuclei of different cell sub-populations, as they pass through mitosis, with additional osmotic shocks performed while no significant plasmid degradation has occurred. To test this hypothesis, cells were transfected with the fast protocol, and washed with EGTA after the first osmotic shock, to prevent any additional precipitate to enter cells. Those same cells were then subjected to additional osmotic shocks within the hour following the EGTA wash-step. Reporter gene expression levels measured 48 hours post transfection (**Figure 55**) showed that expression was higher for cells that had been exposed to repetitive osmotic shocks compared to those where only the first osmotic shock was performed.

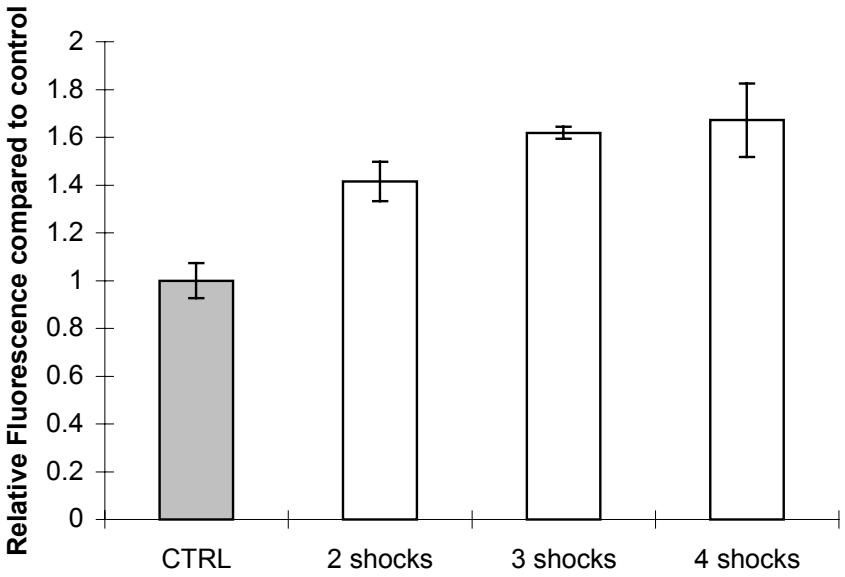


Figure 55. Repetitive osmotic shocks post transfection increase reporter gene expression compared to control. Cells were washed with EGTA post transfection to prevent entry of additional calcium phosphate DNA complex between the repetitive osmotic shocks. For each condition a first osmotic shock was performed one hour after addition of the precipitate and cells were washed with EGTA (control, darker bar). A second shock was applied 20 minutes after the first one, a third one 35 minutes after and a fourth 45 minutes after. **2 shocks** indicates that the two first shock were applied on cells, **3 shocks** means that the 3 first shocks were applied on cells, **4 shocks** means that all the four consecutive shocks were applied on the cells.

Cell cycle analysis showed that cells kept passing through mitosis during the whole process, as the percentage of cells in G1 increased (meaning that more cells went through division) during the consecutive osmotic shock period (**Figure 56**). The signal still increased for the two last time points, even though the G1 cell percentage decreased. This may result from the cumulative effect of multiple osmotic shocks performed on the cells. The G1 cell percentage does not reflect the exact number of cells that went through mitosis, as cells keep on dividing (and thus enter the G1 peak) and proceed further away in the cell cycle (entering S phase, and thus leaving the G1 peak). It does reflect however, that there are still a significant number of cells in G1 phase at the time of repetitive osmotic shock treatment.

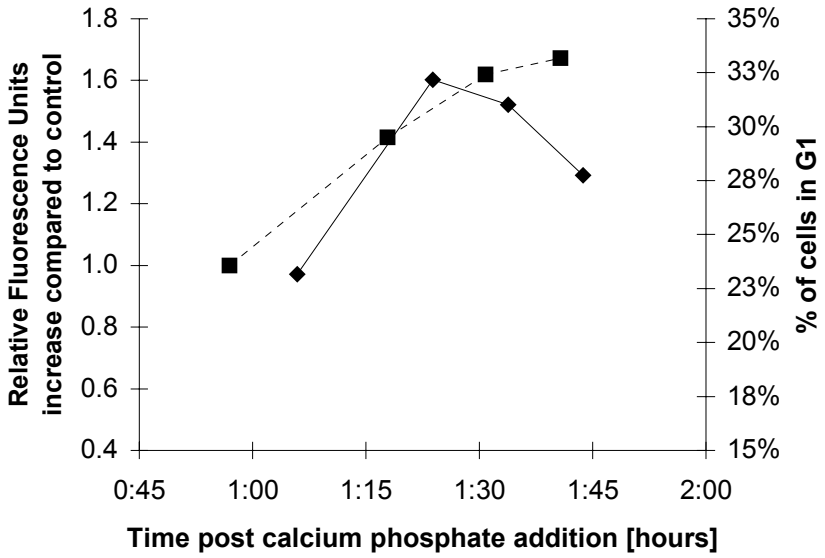


Figure 56. Repetitive glycerol shocks affected the reporter gene expression level. Fluorescence values are expressed relative to the time point of the corresponding osmotic shocks (and not of calcium phosphate addition). Calcium phosphate DNA complex remained on cells for one hour and was washed away by EGTA after the first osmotic shock. Dotted line corresponds to fluorescence compared to control (first point, only one osmotic shock post transfection). Full line represents the G1 cells profile progression during the whole manipulation.

Microscopic studies showed an increase in the number of positive cells when a second osmotic shock was performed 10 minutes after the first one (used as control). To exclude any DNA uptake from extra-cellular transfection particles, these residual transfection complexes were washed away with EGTA after the first osmotic shock. For both situations, the number of positive cells was counted in three random localizations under the microscope, 24 hours post transfection, and compared to the relative fluorescence units measured with the Cytofluor (**Table 12**).

Table 12. Comparison between number of positive cells with one or two osmotic shock(s) and resulting reporter gene expression level measured.

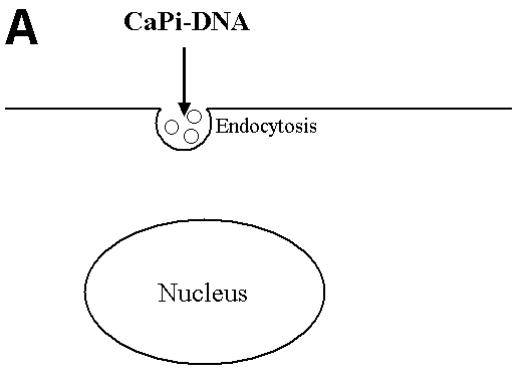
	Control	Additional osmotic shock
Number of positive cells	804	1091
Number of negative cells	2416	2112
% positive cells	24%	33%
Relative fluorescence units	16119	23753

For each situation, the total number of cells counted in the three random localizations was comparable (approximately 3200 cells). The increase in positive cells with the second osmotic shock was equal to ~1.35 and the reporter gene level increase was equal to ~ 1.45. Thus the

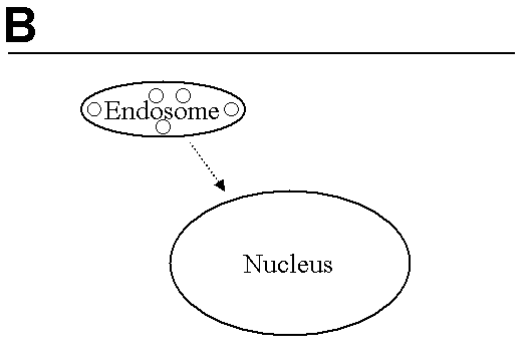
signal increase resulted from a larger amount of positive cells among the transfected population rather than positive cells being more positive.

12. Cell cycle related model for calcium phosphate transfection

As was shown before, calcium phosphate plasmid DNA is taken up by the cells, independently of the presence of plasmid, by endocytosis after settling down on the membrane (**Scheme A**) (Coonrod et al., 1997; Loyter et al., 1982b; Orrantia and Chang, 1990). This uptake event is rather fast once the precipitate has settled down on the cells. The fast transfection protocol demonstrated that cells exposed to the calcium phosphate precipitate (with remaining complexes washed away by EGTA) for just one hour achieved reporter gene expression levels as high as those achieved with a three hour exposure during a normal transfection.



Endosomes tend to migrate towards the center of the cells, resulting in enrichment in late endosomes/lysosomes in the perinuclear region (Coonrod et al., 1997) (**Scheme B**). This enrichment was confirmed by confocal microscopy. Cells transfected with pEGFP-Endo (Clontech, Palo Alto, CA, USA), a fusion protein targeted to the membrane of endosomes, showed an accumulation of the endosomes in the perinuclear region (**Figure 57**).



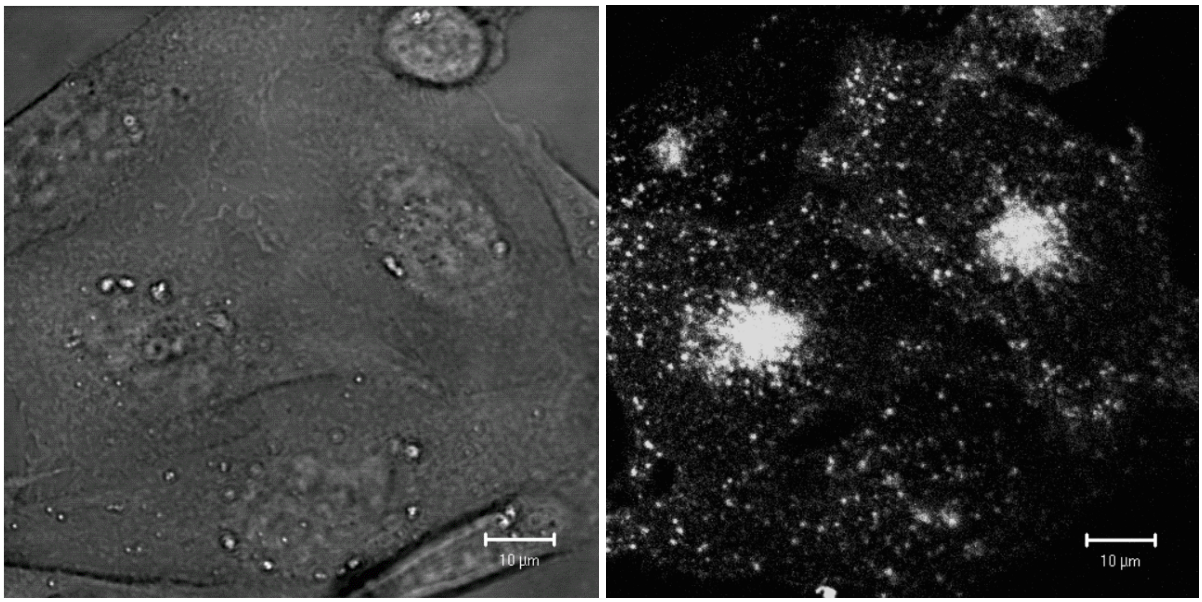
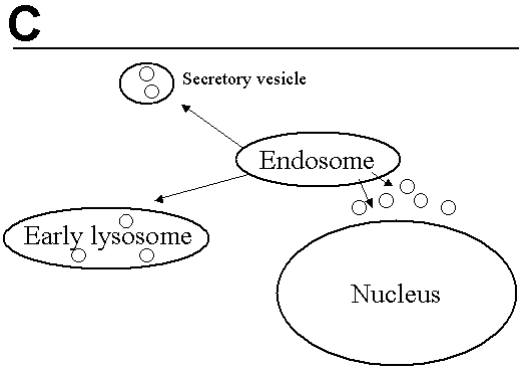


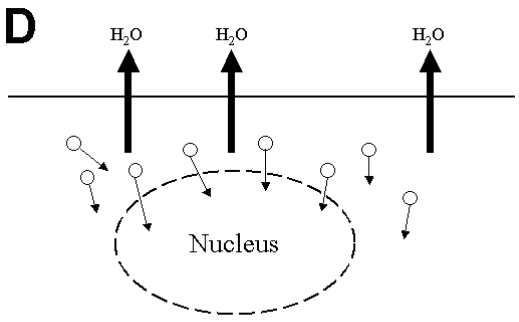
Figure 57. Endosome enrichment in the perinuclear region (right). Acquisitions were done 13 hours post-transfection. Contrast phase picture clearly showed the nucleus localization (left). Bars represent 10 µm.

The transfection complex must then escape endosomes before it can enter the nucleus. Haberland *et al.* suggested that the presence of micro-precipitates of calcium phosphate, as well as soluble Ca^{2+}, could play a role in mechanisms of membrane lysis and escape from endosomal/lysosomal compartments (Haberland et al., 1999). This effect even seemed to be increased with precipitate compared to soluble Ca^{2+}; Loyter *et al.* also mentioned that calcium phosphate may cause local solubilization of phagocytic vacuoles, allowing leakage of DNA into the cytoplasm (Loyter et al., 1982a). Zakai *et al.* reported local solubilization of phospholipid bilayers due to calcium phosphate precipitates (Zakai et al., 1977). This could provide a way out of endosomes for the calcium phosphate DNA complex (**Scheme C**). Some plasmid DNA molecules may be lost through fusions of endosomes with other vesicles (such as early lysosomes or secretory vesicles).



This is where the cell cycle plays a key role for efficient transfer of plasmid DNA in the nuclear environment. First on the transfection level: cell cycle is important for plasmid uptake through endocytosis in order to have DNA entry at the right time frame to prevent too much degradation after its release in the cytoplasm and before it has access to the nucleus. This access to the nucleus is then greatly facilitated when the cells go through mitosis (i.e. nuclear membrane gets disrupted, thus facilitating diffusion of plasmid DNA in the nuclear environment).

On top of that membrane disruption, if osmotic shock (such as glycerol 10%) is applied at the right moment in the cycle (mitosis), the significant cell volume decrease that will occur (approximately 55%) without loss of plasmid already taken up by the cells, as shown by Batard *et al*.(Batard et al., 2001). This cell volume reduction will increase the intracellular concentration of plasmid DNA molecules, thus increasing the probability to diffuse in the nucleus through its disrupted membrane and this before plasmid DNA gets degraded by nucleases in the cytoplasm (Loyter et al., 1982b; Orrantia and Chang, 1990) (**Scheme D**).



Electron microscopic data obtained by Loyter *et al.* (Loyter et al., 1982b) showed that plasmid DNA enters the nucleus as part of a calcium phosphate complex, hence still protected from nucleases. If glycerol shock is performed far from mitosis, little reporter gene expression level will be achieved, because most of the plasmid DNA molecules will become degraded before they can access the nucleus. The protective effect of calcium against plasmid degradation was also shown by Lam *et al.*, where additional calcium was mixed with liposome complexes for transfection, and yielded significantly more intact plasmid DNA in the cells in comparison with liposome complexes used alone (Lam and Cullis, 2000). These authors showed optimal calcium concentration for transfections ranging from 5 to 25mM (calcium phosphate transfection protocol used here falls in between those values, as cells are exposed to a calcium

concentration of 12.5mM during transfection). Higher concentrations of calcium induced cytotoxicity.

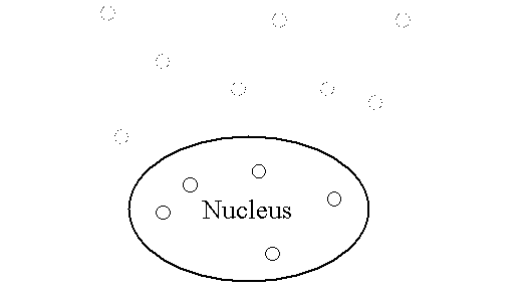
Some partially degraded plasmid may enter the nucleus by diffusion, without the need for mitosis. Escriou *et al.* suggested that plasmid DNA could cross the nuclear envelope of CV-1 cells with very low efficiency, but that mitosis considerably enhanced expression of a transgene (Escriou et al., 2001). According to Coonrod *et al*., all cells are competent for linear plasmid uptake in the nucleus, but the bulk of transfected DNA is rapidly eliminated from the nucleus, probably by a protective mechanism involved in nuclear elimination of exogenous DNA (Coonrod et al., 1997). The efficiency of such a mechanism may also give insight on the better transfectability of certain cell lines. Expression may occur in cells whose nuclei are heavily enough loaded with plasmid DNA molecules that they cannot eliminate all of them. This could also explain why not all the cells that were in mitosis, during the osmotic shock, expressed the reporter gene afterwards.

Repetitive osmotic shocks also increase the level of reporter gene expression achieved, because it provides plasmid entry in other subpopulations of cells going through mitosis, which were prevented nuclear access during the first shock. These repetitive osmotic shocks are efficient as long as plasmid DNA is not degraded in the cytoplasm but rapidly became ineffective because of increasing cytoplasmic plasmid degradation.

The duration of the mitotic phase is approximately 1 hour. Therefore, even with transfection complexes that are efficiently internalized and released into the cytoplasm, the window of opportunity, within a typical cell cycle of approximately 12 – 14 hours, to efficiently enter the nucleus is limited to 1 hour during the mitotic breakdown of the nuclear membrane.

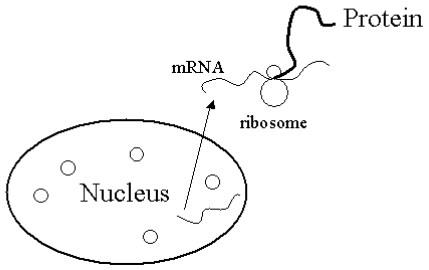
Once the nuclear membrane does reform (post-mitosis), plasmid DNA will be enclosed inside the nucleus of the two newly divided daughter cells (**Scheme E**). Plasmids remaining in the cytoplasm will get degraded.

E



Plasmid DNA will then get transcribed into mRNA, exported to the cytoplasm and translated into protein (**Scheme F**). This process also is rather fast. Detectable reporter gene expression (EGFP) was already observable two and a half hours post mitosis. Knowing that EGFP needs one hour to maturate to its fluorescent form (Tsien, 1998), this means that transcription into mRNA was done during the 1 – 1.5 hour following mitosis (and plasmid localization in the nuclear environment).

F



This hypothetical mechanism of action for calcium phosphate mediated transfection implies three distinct roles played by calcium phosphate:

- It provides a way for the plasmid DNA (once complexed) to enter the cells through endocytosis (Loyter et al., 1982a)
- Once inside endosomes, it affects the membrane, creating local solubilization and thus enables release in the cytoplasm (Zakai et al., 1977).
- It protects the plasmid DNA from nuclease activity in the cytoplasm (Lechardeur et al., 1999).

Conclusion

Complexation of plasmid DNA with calcium phosphate has many advantages for transfection. It enables plasmid DNA internalisation through endocytosis and probably also triggers endocytosis as cells exposed to calcium phosphate showed more endocytotic vesicles than control cells (Loyter et al., 1982a). It also protects plasmid DNA from intracellular degradation by nucleases present in the cytoplasm (Lechardeur et al., 1999), probably by preventing reaction with the catalytic sites of the enzymes due to steric obstruction provided by the precipitate. Calcium phosphate may even create local solubilization of the endosome membrane (Zakai et al., 1977), allowing leakage of plasmid DNA into the cytoplasm. Electron microscopy studies showed that the entire calcium phosphate DNA complex entered the nuclei (Loyter et al., 1982b) thus providing protection during the whole transfer from the cellular membrane to the nucleus. However, this is probably a relatively rare event as only 10% of the internalised plasmid DNA molecules were found in the nuclei of transfected cells (Orrantia and Chang, 1990). This low yield in nuclear internalisation is probably due to the fact that cytoplasmic plasmid DNA has a half-life of approximately 50 – 90 minutes (Lechardeur et al., 1999). If not internalised rapidly, plasmid DNA molecules are degraded in the cytoplasm (Lechardeur et al., 1999; Orrantia and Chang, 1990). No plasmid degradation was shown to occur in the endosomes (Wattiaux et al., 2000).

The cell cycle was monitored to see if it had any implication for efficient transfer of plasmid DNA molecules in the nuclear environment. Using mimosine synchronized cells, it was possible to show that it does, indeed, play an important role. The first observations led to the conclusion that the cell cycle state at the time of addition of the transfection complex onto the cells was important. A clear correlation was observed between the percentage of cells in S phase at the time of addition of the calcium phosphate precipitate onto the cells and the subsequent reporter gene expression measured 48 hours later. Using a faster transfection protocol, requiring a shorter exposition of the cells to the transfection complex, showed that this correlation with the cell cycle was still present but also showed that the optimal timing for the transfection complex addition was dependent on the transfection set-up used.

Knowing that plasmid DNA molecules are rapidly degraded in the cytoplasm if they are not internalised into the nucleus, live imaging studies were performed to check if it was possible to distinguish any specificity in the cells that expressed the reporter gene post-transfection compared to those which remained negative. The results suggest that mitosis is an important event required for subsequent reporter gene expression, probably due to disruption of the

nuclear membrane occurring during mitosis, which would facilitate access to the nuclear environment for the plasmid DNA.

The optimal timing for transfection complex addition onto the cells had to be optimised according to the transfection set-up, as shown with the classical transfection protocol and the fast transfection protocol, in order to achieve plasmid release in the nuclear vicinity when the larger amount of the transfected cells passed through mitosis. If released too remote from mitosis, plasmid DNA molecules will get degraded before they can have access to the nuclear environment. When using the fast transfection protocol, calcium phosphate DNA settled down on cells more rapidly than with the classical approach, thus the optimal timing for the addition of the transfection complex was closer to mitosis. Once internalised with either one of the two transfection methods, the time required to reach the nucleus vicinity was probably identical, the only difference for the addition of the transfection complex relative to the cell cycle being the settling time.

Monitoring the apparition of positive cells post transfection also demonstrated the importance of the glycerol shock. The more remote from the glycerol shock, the less mitoses resulted in daughter cells expressing the reporter gene. The glycerol shock was shown to have a drastic effect on the cell volume. During the one-minute exposure to the 10% glycerol solution, cellular volume decreased by approximately 55%. Cells regained their original size quickly upon returning them to culture medium. Since no plasmids are gained or lost during this strong osmotic shock (Batard et al., 2001), it resulted in an increase of the intracellular plasmid concentration.

The synergy of glycerol shock and cells proceeding through mitosis was a requirement for efficient reporter gene transfer in nuclei. If one of the two was omitted, or if they did not happen at the same time, very low reporter gene expression was achieved. The role of the glycerol shock was shown not to rely on glycerol itself, but rather on the osmotic strength, since different other solutions (PBS 10X, NaCl 1M) producing similar volume decreases, yielded reporter gene expressions as high as those achieved with glycerol 10%, if not higher.

The osmotic shock probably has two different roles. The first being to reduce the cellular volume, thus helping plasmid DNA molecules transfer into nuclei of mitotic cells, by increasing their intracellular concentration. The second being to destabilize the endo-

lysosomal membranes, thus providing leakage of plasmid DNA in the cytoplasm (Batard et al., 2001; Sabelnikov, 1994). Both effects play a role in the overall transfer of plasmid DNA molecules from the endosomes to the nuclei. The second effect was probably also reflected in the fact that even if it produced a similar volume decrease as glycerol 10%, the 5% glycerol solution only resulted in very low reporter gene expression, whereas glycerol concentrations higher than 15% resulted in increased cell death, in agreement with Wilson *et al.* (Wilson and Smith, 1997).

As it was found that plasmid DNA was driving reporter gene expression even after a delay of 45 minutes post-cellular uptake, it was possible during that time to efficiently transfer them in nuclei of different sub-populations of cells, by applying repetitive osmotic shocks, each targeting successive sub-populations of mitotic cells.

In theory, cells that went trough mitosis close to the osmotic shock at the end of a first transfection will not provide access to their nuclear environment for a second plasmid transfected shortly after the first one. This was shown to be true when transfecting a cell population once with EGFP and 90 minutes later with DsRedExpress, as no co-localization of both reporter genes was observed, whereas complete co-localization was achieved when both were transfected at the same time. These results strongly indicated that whenever two plasmids have to be transfected in cells, transfection must be done in presence of both plasmids at the same time if co-expression is expected.

Removing the excess of calcium phosphate precipitate post-glycerol shock with an additional EGTA wash-step was shown to be positive for subsequent reporter gene expression, probably by lowering the surrounding calcium concentration and thus avoiding toxic effects due to high calcium concentrations. This small additional step post-transfection resulted in reporter gene increase of about 1.5 to 2 times compared to control.

Live imaging also provided information on doubling times of both positive and negative cells. The fact that cells expressing a reporter gene have a longer doubling time (18 – 22 hours) compared to negative cells (12 – 14 hours) may explain why it is difficult to achieve high percentages of positive cells in a transiently transfected population, as negative cells rapidly outgrow positive cells.

Controlling the cell cycle is apparently not enough to achieve 100% efficiency of transfection. Levels of plasmid uptake, calcium phosphate DNA complex morphology and internal plasmid degradation in the transfected population also play important roles that are difficult to control.

Nonetheless, all these results taken together permitted to define a model explaining the importance of both mitosis and osmotic shock for efficient plasmid DNA molecules transfer into nuclei, due to the increased intracellular concentration in presence of disrupted nuclear membrane that they provide when occurring at the same time. This model may also explain why certain non-dividing cell lines (i.e. neurons or muscle cells) are difficult to transfect, due to the absence of nuclear membrane disruption.

This model does not fit with the hypothesis of Orrantia *et al.* that plasmid DNA does not pass through the cytosol before it enters the nucleus but is transported directly from endosomes-lysosomes to the nucleus through an intermediary vesicles (Orrantia and Chang, 1990), unless such vesicles would selectively interact with mitotic cell nuclei only, and plasmid degradation would take place in those vesicles as it does in the cytoplasm (Lechardeur et al., 1999)

Perspectives

As demonstrated in this work, cell cycle plays an important role in the transfer of plasmids in the nucleus of CHO cells. Those observations can be used, not only for transient transfection but also in stable integration of plasmid DNA. By increasing the probability to have plasmid DNA in the nucleus, the probability to have a random integration resulting in a stable expression should also increase (selection and amplification of the positive clones would still be required afterwards).

Cell cycle analysis of cells in suspension (in spinners, or bioreactors) by flow cytometry prior to transfection, should help to determine the best time to add the transfection complex onto these cells. This would also avoid low efficiencies due to partial synchronization of the culture, where more plasmid would be degraded in the cytoplasm than efficiently accessing the nucleus.

Cell culture conditions for large-scale transfection (i.e. bioreactor) can certainly be optimized to partially synchronize them (without need for medium exchange as with mimosine). Monitoring the cell cycle after re-initiation would then help determining the appropriate time to add the transfection complex. Such large-scale synchronization could be achieved, for instance, by using nutriment deprivation, and re-initiation of the cycle could be done upon nutriment complementation of the medium. Such synchronization would prevent the high costs involved with large-scale mimosine synchronization, due to medium exchanges.

A means to synchronize cells just prior to entry in mitosis should enable to achieve higher positive CHO cells post transfection, ensuring that such a synchronization would not affect plasmid uptake. Mimosine synchronization is an interesting tool, but the loss in synchrony and the fact that it was difficult to achieve a highly synchronous passage through mitosis is probably responsible for the fact that 30 – 40 % positive cells at maximum could be observed post transfection. This non-synchronous passage through mitosis was also demonstrated by the signal increase resulting from multiple osmotic shocks post-transfection.

EGTA wash-step also demonstrated a positive side effect when used on transfected cells, as an increased reporter gene level was achieved (1.5 to 2.5 times, see annex) compared to controls. Even if the cellular effect of such an additional wash-step at the end of transfection is not fully understood, it should be added to the protocol of calcium-phosphate transfection. Further studies to fully understand why it does result in higher reporter gene expression

should provide interesting information about calcium phosphate and maybe the limit of this transfection method (possibly due to the high calcium concentration used during the process). Indications of a "negative" role for the calcium concentration used were found with EGTA wash step (increase the reporter gene level, maybe due to the removal of an inhibition due to large amount of calcium).

The fast transfection protocol developed during this work may provide an interesting alternative way for transfection of adherent CHO cells. The whole process from seeding to osmotic shock at the end of transfection is performed in 5 hours instead of 22 hours with the protocol defined by Jordan *et al*. ((Jordan et al., 1996) and can therefore be done in less than a day

Monitoring stained-plasmid DNA uptake with flow cytometry combined with cell cycle and osmotic shock effects on different cell lines (HEK, BHK…) may provide information that could explain why some cells seem more transfectable than others. This could also be used to determinate the plasmid uptake rate of those different cell lines.

Better monitoring of the mitotic cells through flow cytometry (using stains specific to mitotic cells, such as marker for histone H3 phosphorylation (Darzynkiewicz et al., 2001; Hans and Dimitrov, 2001; Hendzel et al., 1997; Juan et al., 2001; Juan et al., 1998; Van Hooser et al., 1998; Wei et al., 1998)) would provide more precise tools for analysis and more precise information on the role played by mitosis during, for instance, repetitive osmotic shock post-transfection.

Working with stained DNA (i.e. stained with Mirus Label IT® Tracker™ Intracellular Nucleic Acid Localization Kit, Madison, WI, USA) and stained endosomes could provide precious information on the hypotheses developed in the "cell cycle related model for calcium phosphate transfection", combined with live imaging. This would enable checking how many cells that uptake plasmid DNA have it localized in the nucleus afterwards, and if it really gets in during mitosis. This could also validate/invalidate the exogenous DNA elimination system proposed by Coonrod *et al*. (Coonrod et al., 1997). This would also give more insights on the exact role of glycerol.

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Curriculum Vitae

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1994 – 1998 Study of **Biochemistry** in the Chemistry Departement of the University of Geneva

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1991 Diplôme de culture générale, type scientifique, at the CESSOUEST high-school in Nyon

Projects

Teaching assistant at EPFL since 1999, planning and implementation of new student experiments within the biotechnology field.

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Publications

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Other publications and presentations

Wurm, F. M., Hunt L., Hacker D., El Abridi H., Grosjean F., Jordan M., DeJesus M. (2003). Pausing of mammalian cells by cold exposure, limits and opportunities. Poster presentation at the 18th ESACT meeting in Granada, Spain. Proceedings to be published.

Grosjean F., Jordan M., Wurm F.M. (2003). Analyzing CaPi transfection of adherent CHO cells using microscopic live imaging. Poster presentation at the 18th ESACT meeting in Granada, Spain. Proceedings to be published.

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Grosjean F., Batard P., Wurm F.M. (1999). Calcium-phosphate transfection efficiency throughout the CHO cell cycle. Poster presentation at the SPP Minisymposium at ISREC in Lausanne.