Standardized Establishment of a Viral Transduction Protocol

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Application Note
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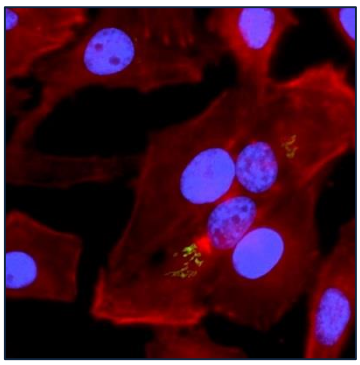
Abstract



Transfection is a common method in protein research (transient transfection) and cell line development (stable transfection). Despite the standard use of this method, it is associated with many difficulties. Everyone knows how challenging and time consuming it may be to establish an ideal transfection protocol. Even if the desired vector is designed and cloned, the way to a reliable transfection is still far!

In this application note, we introduce a standardized method for protocol establishment with the NYONE® Cell Imager, SYNENTEC's image analysis YT-software®, and a modulated BacMam virus. For this purpose the viability of the Hek-293 culture was initially determined with the NYONE®. After subsequent seeding of the cells into the desired number of cells per well and adding the virus particles, it was monitored by the NYONE® if the expected cell density per well can be found. Subsequently, the toxicity was determined non-invasive by growth curve and the transfection efficiency over a period of 8 days.

With this NYONE®-assisted protocol we could exactly determine the optimal number of virus particles per cell and the expression peak for our Hek-293 with our culture conditions.

KEYWORDS: BACMAM, CELL LINE DEVELOPMENT, FLUORESCENCE MICROSCOPY, GFP, GOLGI, HEK-293, PROTEIN CHARACTERIZATION, TRANSFECTION, TRANSIENT VIRAL TRANSDUCTION

Introduction

Baculoviruses are enveloped, rod-shaped viruses with a circular, double-stranded genome of 80-180 kbp. The typical diameter of a Baculovirus is 40-50 nm with a length of about 200-400 nm. Transcription, DNA replication and the formation of the nucleocapsid take place in the nucleus of the infected host cell. During the productive infection in permissive cells there is no integration of the viral DNA in the host genome (transient transduction) [O'Really et al. 1992].

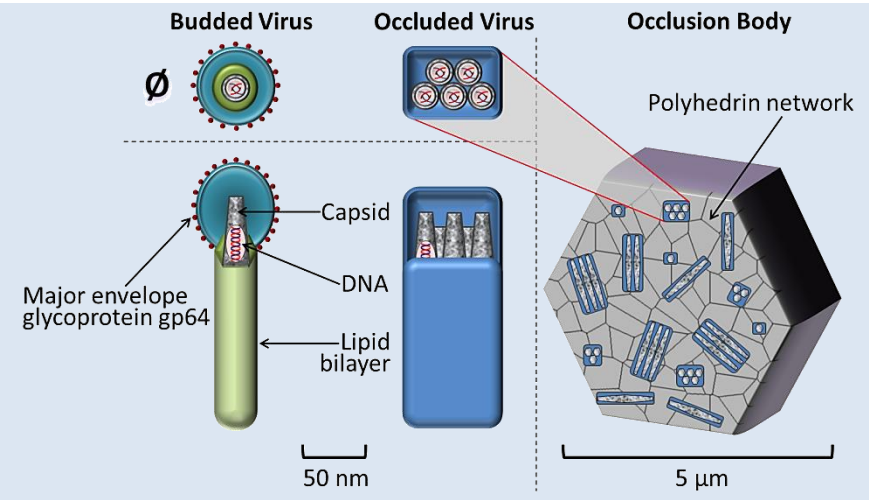


Fig. 1: Viron-phenotypes over infection period
Budded virus (BV) with gp64-fusion proteins (red) and filled with 1 capsid containing the modified viral DNA. Occluded virus including a few capsids are generated in the host-cell nucleus (modified from: Lynn 2006).

Characteristic for the cycle of infection is the formation of two different viron-phenotypes: in the early time of infection (6-8 h after infection) the budded virus (BV) is formed at the cytoplasmic membrane and in the late stage, from 18 h after infection, the occlusion-derived virus (OV) migrates in the nucleus (fig. 1). BV is infectious in cell culture and provides the transfer from cell to cell in infected animals.

Its entry into the host cell takes place via receptor-mediated endocytosis. The viral gp64 is a membrane fusion protein and is at BV essential for the viral budding, the virus entry into the cell and cell to cell transfer [Hefferon 1999].

Recombinant Baculoviruses are widely used in the heterologous protein expression in insect and mammalian cells [Kost et al. 2002; Kost et al. 2005; Chondreay & Kost 2007; Ghosh et al. 2002; Chen et al. 2011]. Baculoviral expression systems permit a high gene-transfer rate and a quick expression of recombinant proteins and vaccines compared to other eukaryotic systems. In addition, the work with Baculoviruses presents a low security risk for users as the infection spectrum is limited to some invertebrates (infect mainly insect larvae from the order Lepidoptera) [Kost et al. 2005; Virtanen et al. 2009].

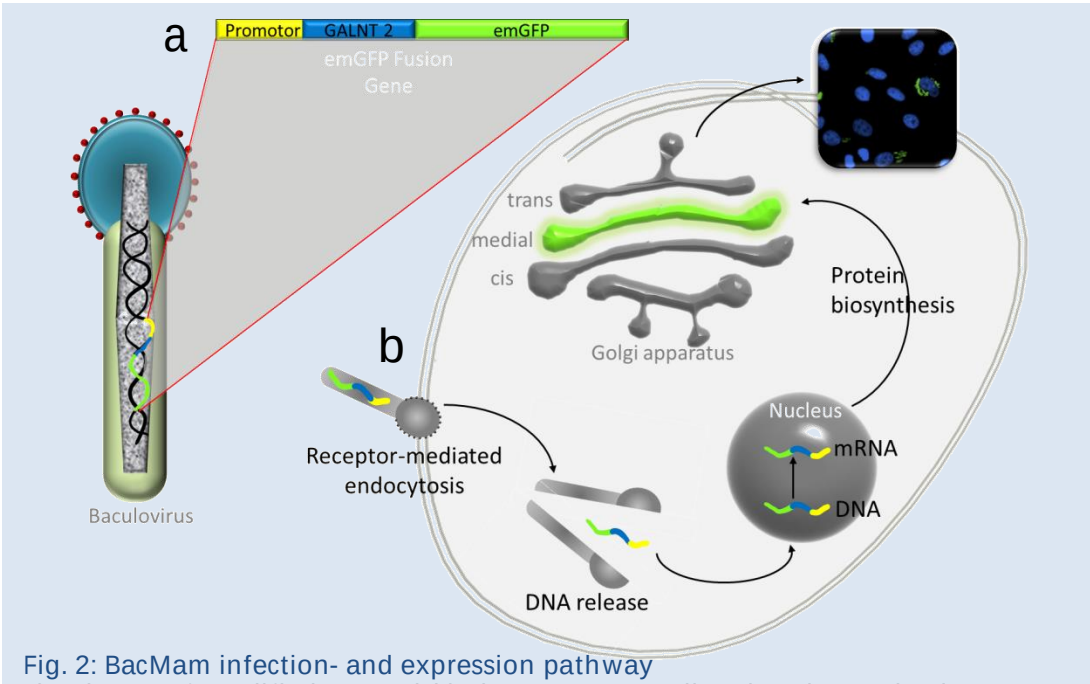


Fig. 2: BacMam infection- and expression pathway
a) Scheme of modified BV and b) the receptor-mediated endocytosis via gp64 membrane interaction. DNA release in cytoplasm, replication in the nucleus and protein biosynthesis with final location of GALNT-2-GFP-construct in the medial Golgi (modified from: ThermoFischer Scientific 2015).

In mammalian cells that have been inoculated with the wild-type virus, no cyto-genetic effects on the chromosomes of the host cell such as chromosomal aberrations and sister chromatid exchanges were found [Miltenburger & Reimann 1980].

To optimize the specific expression of a transgene in mammalian cells, the transgene expression is subject to the control of an RNA polymerase II-specific promoter, such as SV40 promoter or human cytomegalovirus major immediate early promoter (hCMV-MIE), and the SV40 polyadenylation signal (BacMam technology) [Boyce & Bucher 1996; Kost et al. 2005; Food & Condreay 2002].

This technology is also used for the staining of cell organelles for colocalization experiments of the target protein or specific cell compartments marked with fluorescent proteins. This is possible with the commercially available Cellular Lights™ reagents [Virtanen et al. 2009]. The construct of fluorescent protein and signal protein is cloned in this labeling method in a Baculovirus.

The reagent contains modified virus particles of an insect virus, which genes are regulated in part by a mammalian promoter. This means that at a transduction of mammalian cells, only the desired proteins are formed - in this case, the *green fluorescent Human Golgi resident enzymes N-acetylgalactosaminyltransferase 2 of the Golgi apparatus* [Virtanen et al. 2009].

This enzyme is part of the glycosylation of the proteins produced in the endoplasmic reticulum (ER) and is located in the medial Golgi. The glycosylation structure is essential for the effectiveness of *in vitro* produced proteins and antibodies and forms a wide field of research.

In addition to the modification and sorting of synthesized proteins (e.g. glycosylation), the golgi apparatus is also part of the stress response of a cell. In literature it can be found

that the Golgi induces both, apoptosis and it can enable the regeneration of the cell [Mukherjee et al. 2007]. This mechanism can be used in cancer research, for example, as well as in regenerative medicine [Wlodkowic et al. 2009].

Material & Methods

Material:

- Hek-293 (adherent monolayer forming cells; human embryonic kidney)
- DMEM/Ham's F12 1:1 with 10 % fetal bovine serum and 1 % Penicillin/ Streptomycin
- Phosphate buffered saline (PBS^{--}, w/o Ca^{2+} and Mg^{2+})
- Trypsin 0.25 %
- Trypan Blue 0.02 % stock
- Transparent 96-well half area plate (e.g. Corning cat. # 3690)
- Black 96-well plate (e.g. Greiner µclear cat. # 655096)
- Modified Baculovirus for transduction (e.g. CellLight® Golgi-GFP BacMam 2.0; ThermoFisher Scientific)
- One of SYNENTEC's imaging systems (here: NYONE® Imaging System)
- SYNENTEC's YT-software®

Methods:

Hek-293 cells were trypsinized to detach and transferred to a 15 mL centrifugation tube. For cell counting and viability determination a sample with a final dilution of 1:40 was set up with PBS^{--} and Trypan Blue. The sample was measured with the NYONE® Imaging System and the *Viability* image analysis algorithm.
With the determined cell density and the desired total cell #/per well (here: 6000 viable cells/well), the amount of particles per well can be calculated according to the manufacturers' specifications of CellLight® Golgi-GFP BacMam 2.0 (ThermoFisher Scientific).

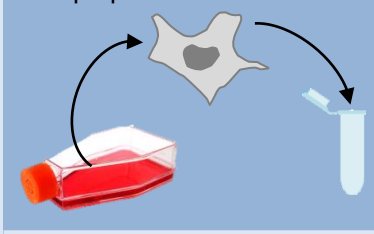
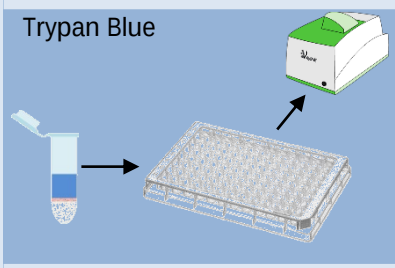
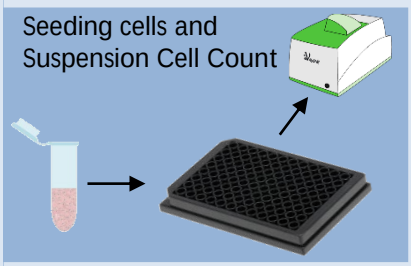
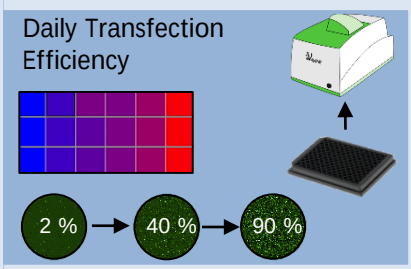
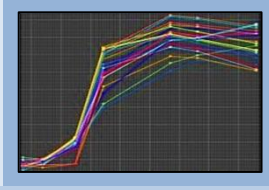
Here we discovered a dilution series of 1, 5, 10, 20, 30, 40, 50, 100 and no (=control) particles per cell to figure out the most sufficient PPC-concentration as well as any toxicity of the virus particles.
To control cell density after seeding in context with used PPC-concentration, a measurement with the *Suspension Cell Count*- application of the YT-software® was conducted.
Daily measurement with *Confluence 1F* application of the YT-software® over a period of 8 days was performed. For this measurement two channels were necessary - one for the cell detection in brightfield and one for the detection of the GFP-Golgi Apparatuses (tab. 1).
Transfection efficiency was displayed in the heat map with *BF AND Fluo Area/BF Area [%].* Here, it is assumed that on a statistical average - in relation to the large number of cells/well, all cells contain more or less the same amount of Golgi. For toxicity-study no new measurement is necessary!
For a first overview of toxic influences of the BacMam particles the non-invasive generation of a growth curve with the time chart tool (*Cell Confluence BF [%]*) is sufficient (tab. 2). If an influence is found, in further experiments the genotoxicity etc. can be examined.

Tab. 1: Optical settings for *Confluence 1F* application

Channel	Brightfield	Fluorescence
Objective	10x (default)	
LED	LED_Brightfield (default)	LED_Blue (default)
Emission Filter	Emi_Green (default)	Emi_Green (default)
Intensity	100 %	100 %
Exposure Time	30 ms	100 ms
Gain	0 %	10 %

Experimental workflow

Tab. 2: Overview of experimental steps carried out for this application note in combination with helpful

Step	Procedure	Information
Cell preparation 	• Remove medium from the cells, wash twice with PBS^{--} and detach cells via incubating with 500 µL Trypsin (5' @ 37°C) • Stop trypsin reaction by adding 4.5 mL full cell culture medium (containing serum)	• Step is just necessary for adherent cell lines • To prevent cell re-attaching during the following steps, place remaining cells in culture flask or 15 mL centrifugation tube on a rocker in the incubator or softly shake it from time to time
Trypan Blue 	• Dilute 25 µL cells with 225 µL PBS^{--} (1:10 dilution) and add 250 µL Trypan Blue stock solution [0.02 %] (1:20 final cell dilution) • Measure plate with the NYONE®-Viability Application	• For detailed information look at SYNENTEC's Technical Note for Trypan Blue measurements (TN-B004-XV-17) at synentec.com
Cell Density and Virus particle calculation 	• With the determined cell density and the desired total cell #/per well (here: 6000 viable cells/well), the amount of particles per well can be calculated • Here we discovered a dilution series of 1, 5, 10, 20, 30, 40, 50, 100 and no particles per cell	• Viability should not be less then 95 % • Equation to calculate particles per cell (PPC) in µL is: $\left(\frac{\text{number of cells x desired PPC}}{1 \times 10^8 \text{ CellLight® particles/mL}}\right) \text{X } 1000$
Seeding cells and Suspension Cell Count 	• Dilute cells to obtain desired cell density • Add required amount of virus particles to cell suspension • Seed cells to 96-well plate	• To control cell density in context with used PPC-concentration, measure the plate after seeding with the *„Suspension Cell Count"* application of the YT-software®
Daily Transfection Efficiency 	• Daily measurement with *Confluence 1F* application of the YT-software® (here: over 8 days) • To track the transfection efficiency choose *„(BF AND Fluo Area) / BF Area [%]"* in the Result table or the heat map	• Depending on the fluorescence of the co-transfected target you have to adjust the excitation-LED and the emission filter in the setup-window of the YT-software® • For the toxicity-study no new measurement is necessary
Daily Confluence for Toxicity 	• To track the toxicity of the virus particles choose *„Cell Confluence BF [%]"* and generate a time chart • Take the averaged *„Cell Confluence BF [%]"* values of the replicates to compare the differences between different PPC-concentrations	• The non-invasive monitoring of the growth curve of a culture is a first information about the toxicity of a substance • If influences were found the genotoxicity etc. can be examined in further experiments

Results & Discussion

It is possible to process the taken images during measurement or afterwards by switching to the evaluation mode of SYNENTEC's YT-software®. To inspect the experiment data later, open the YT-software®, choose evaluation (not measurement), open your experiment with *View Experiment* and press the *Evaluation* button in the YT-software® main window.

1. Trypan Blue viability determination

Previous to cell seeding for the transfection efficiency experiments it is re-commended to test the health of the cell culture of interest and furthermore the cell density. For this purpose we carried out SYNENTEC's Viability Assay (see TN-B004-XV-17).

The analysis of the Hek-293 sample replicates (n = 8) with the *Viability* image analysis algorithm resulted in a cell number of 1.71E+06 (±0.068E+6) cells/mL and an average viability of 96.2 % (±0.52 %) (fig. 4). The VCD per well was ~3000 cells in the half area plate. The figure 3 presents the cell density in the half area plate. This illustrated cell density (1:20 dilution) has been found suitable for the subsequent long term experiments – a sufficient cell number for a meaningful sample plus the cells have enough space to expand. Therefore the 96-well full area plate for transfection efficiency was set up with ~6000 viable cells/well (based on the ~3000 viable cells/well in the half area plate (fig. 3)).

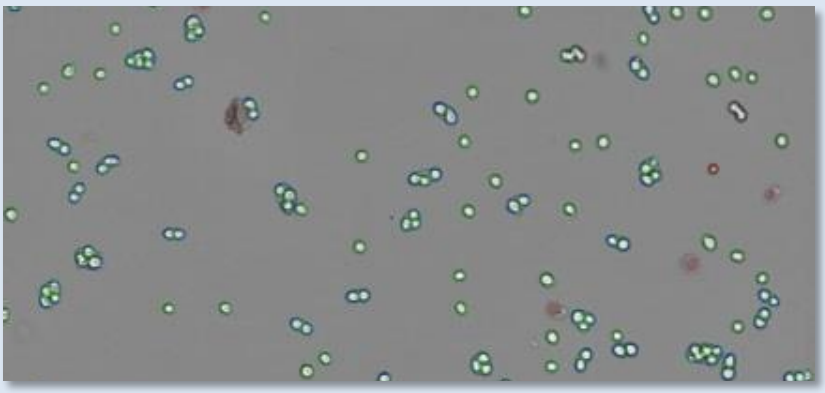


Fig. 3: **SYNENTEC's** *Viability* image analysis
Brightfield image of Trypan Blue stained Hek-293 cells. **SYNENTEC's** image analysis automatically marks viable cells (green), dead cells (red) and Aggregates (blue).

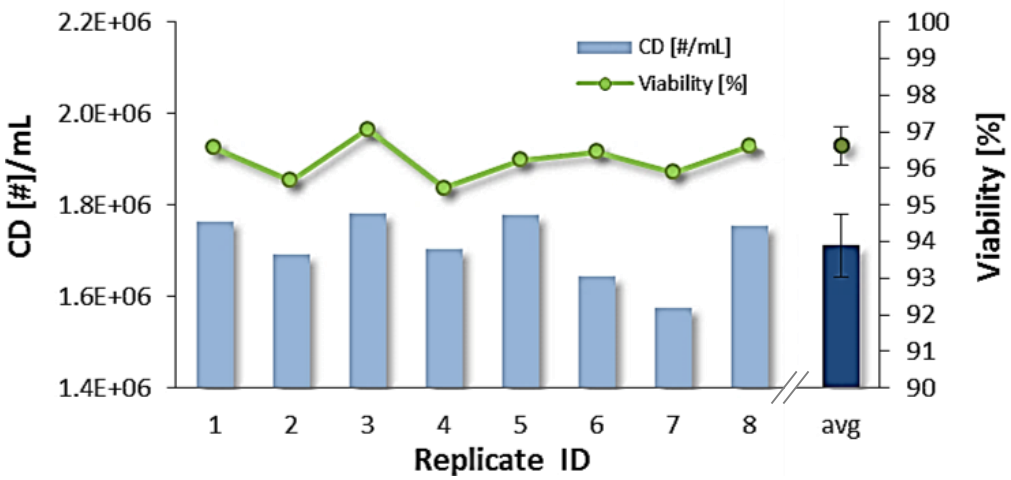


Fig. 4: Results of viability testing and cell density determination
The light blue columns show the cell density of used Hek-293 culture, whereas the dark blue column displays the average of the 8 replicates with the standard error of the mean (SEM). The light green spots indicate the viability per replicate and the dark green spot on the right hand of the diagram shows the average of 8 replicates with the standard error of the mean (SEM). The deviation between cell densities of each replica is ~5 % and the standard error of the measured viabilities is ~0.5 %. Thus, it is an actionable measurement. The investigated average viability of 96.2 % (±0.52 %) indicates a sufficient state of culture for further experiments.

2. Seeding control

After viability- and cell counting 9 different samples, made up of cells and virus particles, were set up. 3 replicates per sample were filled in a black 96-well full area plate. To check whether the ratio of *cells to virus particles* corresponds to the theoretical values, the 96-well plate was analyzed by *Suspension Cell Count* application after seeding of the 9 samples.

The analysis of the plate showed an average total cell count (TCD) of 6313 cells/well. If we include the previously determined viability of 96.2 %, we get a viable cell density (VCD) 6073 viable cells/ well (data not shown). This test has shown that the cell density is as theoretically determined. Thus, the experiment can be continued and the data can be evaluated in a meaningfully way.

3. Transfection efficiency

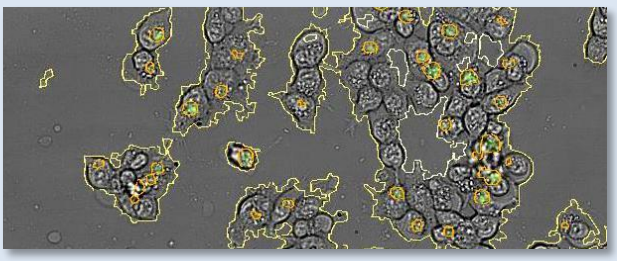


Fig. 5: **SYNENTEC's** *Confluence1F* image analysis e.g. for transfection efficiency analysis
Brightfield image of adherent Hek-293 cells (yellow image detection) and green fluorescent golgi spots (orange image detection).

After viral transduction conducted by a cell infection with modified BacMam viruses (CellLight® Golgi-GFP BacMam 2.0; ThermoFisher Scientific) which induces GFP-Golgi-Protein expression (fig. 2), daily measurements with *Confluence1F* application of the YT-software® were carried out over a period of 8 days .

To track the transfection efficiency *„(BF AND Fluo Area)/ BF Area [%]"* of the result table or the heat map was chosen. The results show the expression peak of the *"green fluorescent Human Golgi resident enzymes N-acetylgalactosaminyl-transferase 2 of the Golgi apparatus"* in each sample on day 1.5 after cell infection/viral transduction (fig. 8). Furthermore the PPC-concentration of 100 PPC shows the highest relative fluorescent area [%] per well (RFA) in all measurements which can be taken as an indicator of the transfection efficiency (fig. 6). In comparison, the manufacturer recommends 10-50 PPC (depending on the cell line). Whether there is a toxic amount of virus particles at 100 PPC or at the other concentrations, can not yet be seen from these results.

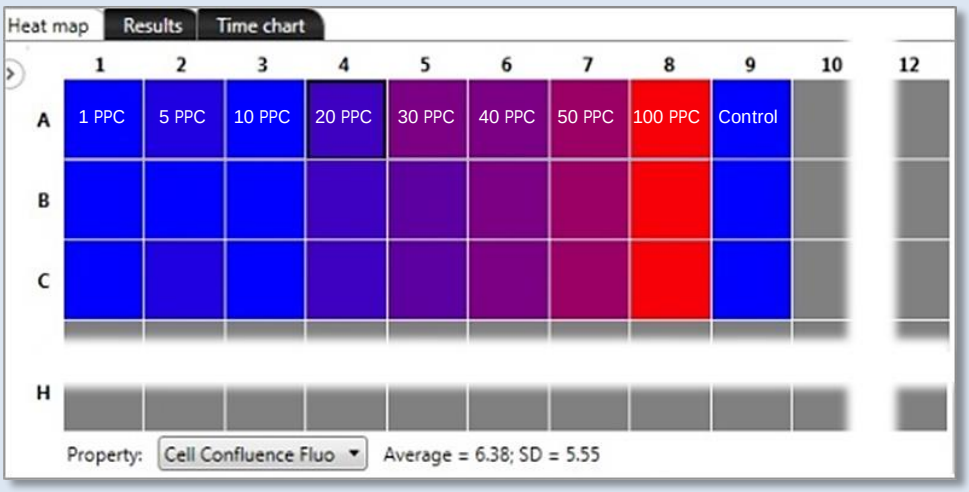


Fig. 6: Heat map of **SYNENTEC's** data analysis YT-software®
This heat map presents the fluorescent area per well on day 1.5 after cell infection (=expression peak). Column 1-8 have an increasing PPC-concentration with 3 replicates each, whereas column 9 indicates the control group. The color code of the heat map is from blue (low) over violet (medium) to red (high). From this it is seen that the samples with PPC = 100 have the highest transfection efficiency.

When monitoring the results with SYNENTEC's YT-software® it is always possible to visually examine the images made with the NYONE® System.

Furthermore it is also possible to determine whether the image analysis algorithm was applied precisely or if the default settings for object detection need to be modified. The various export functions of the YT-software® allow an easy evaluation of the data and an informative documentation of the experiments.

In fig. 7 a tendency can be visually recognized that we obtained the highest transfection efficiency with a viral particle concentration of 100 PPC. The pictures were taken at measurement 4 (= 1.5 days of incubation). Fig. 8 shows at 1.5 days of incubation the expression-peak of GFP-Golgi enzymes.

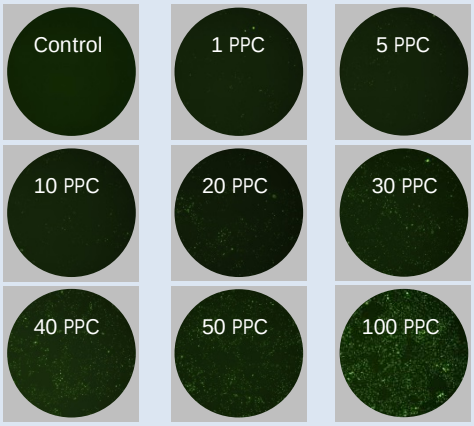


Fig. 7: Comparison of different PPC-concentrations
Fluorescence images of the different virus particle concentration taken from measurement 4 (= 1.5 days of incubation).

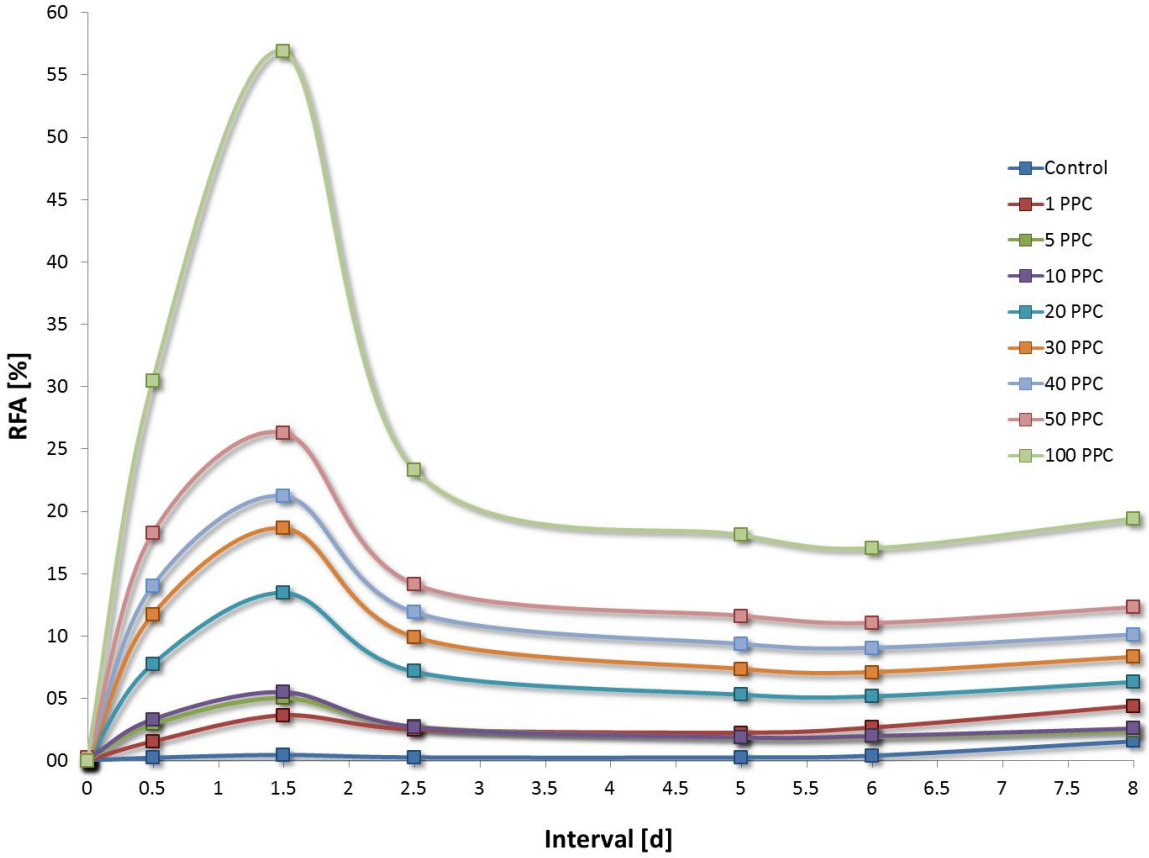


Fig. 8: Relative fluorescent area (%) based on the different virus particle concentrations over time
The different colored lines represent the development of the RFA of the used virus particle concentrations over time (control, 1 PPC, 5 PPC, 10 PPC, 20 PPC, 30 PPC, 40 PPC, 50 PPC, 100 PPC). The squares on each line indicate one conducted measurement with SYNENTEC's *Confluence1F* image analysis, displayed as mean value of 3 replicates each (SEM not calculated). Images were taken over a period of 8 days (d) (0d, 0.5d, 1.5d, 2.5d, 5d, 6d and 8d) after infection of Hek-293 cells with a modified Baculovirus. The transfection efficiency seems to be virus particle dependent. Furthermore it can be said that the highest transfection rate is achieved in samples infected with 100 PPC .

4. Non-invasive toxicity testing

With the conducted measurements for transfection efficiency it is also possible to get a first impression whether the infection with virus particles have an influence on the proliferation of Hek-293 cells.

To track the toxicity of the virus particles *„Cell Confluence BF [%]"* was chosen in the YT-software® and a time chart was generated (fig. 9). It can be seen that the cells of all samples exponentially proliferate up to the fifth measurement.

However, after measuring 5, it can be said that the cells at a concentration of 5 PPC - 100 PPC (column 2 - 8 of the plate overview in fig. 9) showed a reduction in the measured confluence (possibly due to growth inhibition or stagnation and lethal phase was reached).

It is striking that in the control group (column 9 of the plate overview) and in the samples with only 1 PPC (column 1 of the Plate overview) a slower cell proliferation can be seen. It seems as if the virus particles have an activating effect on cell physiology.

Interestingly, the reduction of confluence as previously described does not occur in these two samples. This could be due to the fact that the cells of these samples have not yet reached the period of stagnation and no confluence related growth inhibition took place at the time of measurement 5.

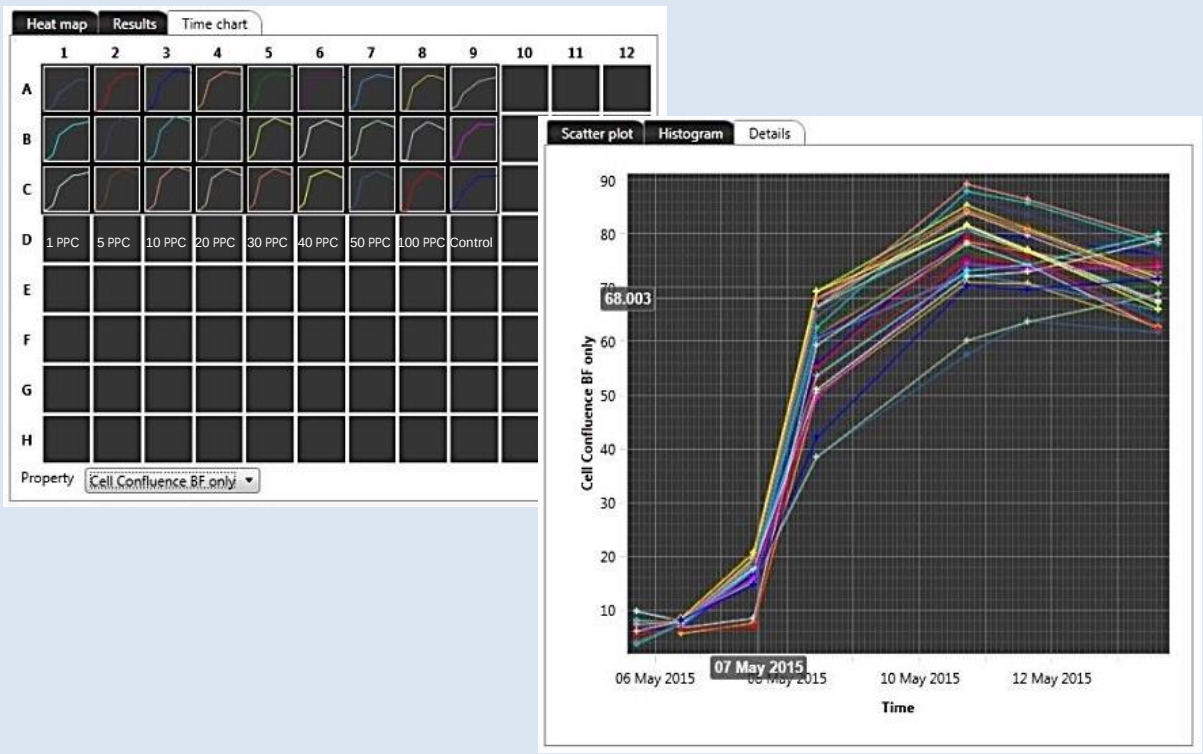


Fig. 9: Time chart generated with **SYNENTEC's** YT-software®

After measuring and processing all conducted images and measurements, the *Time Chart* function at the *Analyst*-Tab can be selected. The image top left shows the plate overview with all generated time charts started from the calculated confluence (in brightfield) per well. By marking the desired wells therein a detailed presentation of the confluence development over time is obtained as shown in the lower right diagram.

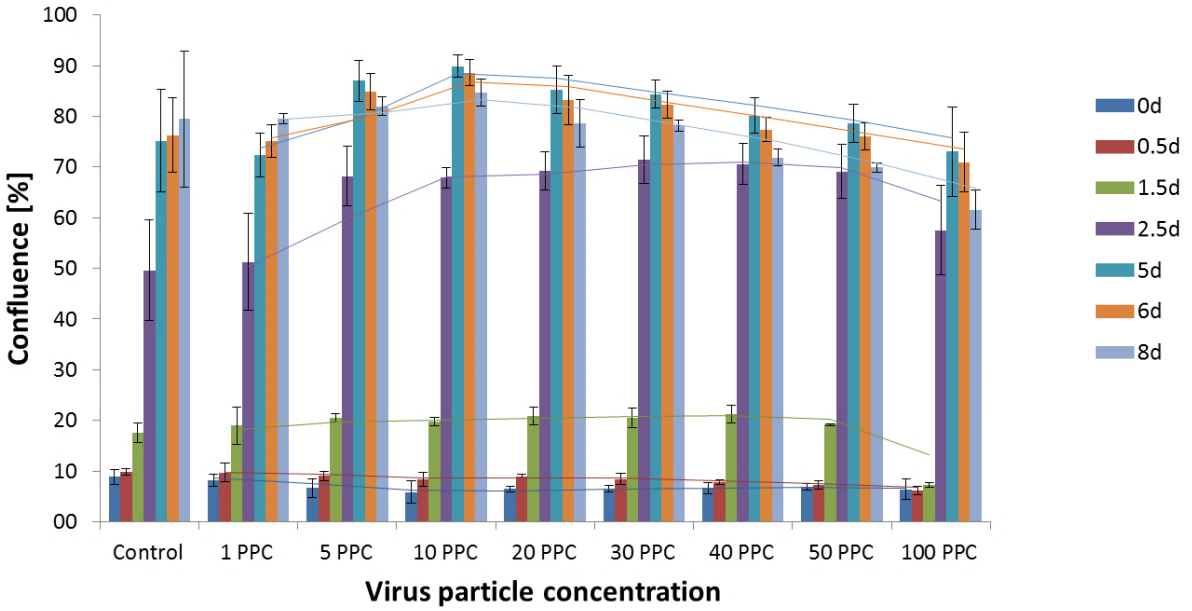


Fig. 10: Virus particle concentration and time-dependent influence on the confluence

The different colored columns represent the evaluated confluence (%) on different time points. All 9 sample groups are shown (control, 1 PPC, 5 PPC, 10 PPC, 20 PPC, 30 PPC, 40 PPC, 50 PPC, 100 PPC). The SEM of each concentration on each time point is displayed as black error bars on all columns. To get an overview of the differences between the individual concentrations, a trend line for each time point was added (period 2; average of 2 adjoining data points).

The averaged *„Cell Confluence BF [%]"* values of the replicates were taken to compare the differences of proliferation between different PPC concentrations.

The comparison of the confluence against the virus particle concentration shows the same tendency as the previous discussed time charts have shown (fig. 9, fig. 10). It also shows that there are virus concentration-dependent differences in the cell proliferation. The highest confluence is achieved in the samples with 10 PPC (fig. 10). The lowest confluence (on average) is achieved at a virus concentration of 100 PPC.

Also here it can be seen that no decrease of the confluence in the control group and in the samples with 1 PPC after 5 days of incubation occurs (fig. 10, fig. 11).

However, the implementation of the t-test has shown that the observed differences are not significant (p = 0.05).

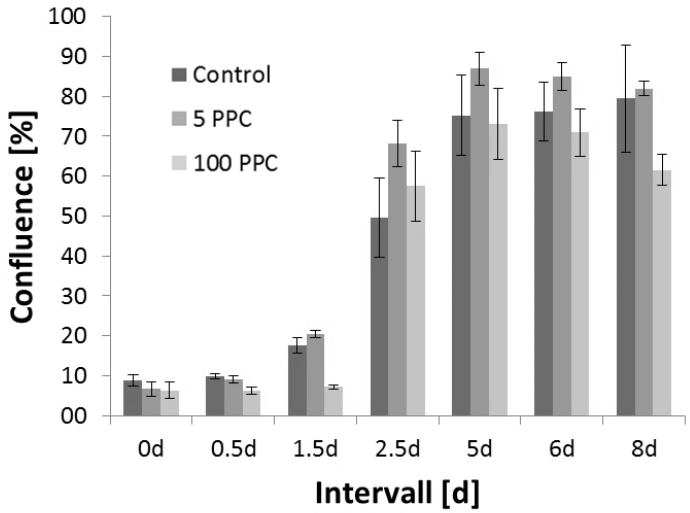


Fig. 11: Comparison of confluency against virus particle concentration over time

The different colored columns represent the evaluated confluence (%) at different virus concentrations over time. Only 3 of the 9 taken sample groups are shown (control, 5 PPC, 100 PPC). The SEM of each concentration on each time point is displayed as black error bars on all columns. The differences of proliferation between 5 PPC and 100 PPC can be seen, but the conducted t-test (p=0.05) shows no significant differences in the proliferation rate.

Conclusion & Outlook

The carried out experiments are a very potent ability to evaluate the transfection efficiency. The advantage is that all necessary measurements can be performed with the same workstation.

In addition to the control of the transfection efficiency and the analysis of the cytotoxicity of the virus particles, both the analysis of the cellular stock as well as the seeding control could be carried out with the NyONE®.

In relation to the transfection efficiency, we can say that the more virus particles are being used, the higher is the transfection efficiency on expression peak (range 1 - 100 PPC). However, a minimal influence of viral particles can be observed in the rate of cell division. This seems to be also virus concentration-dependent, but does not deviate significantly from the control.

The modified BacMam viruses are rare and precious, therefore the aim is to use as little as possible. Summing up all the obtained data, the samples with 5 PPC seem to have the maximum cost-benefit ratio. A detectable transduction can be achieved (fluorescence), an impact on the proliferation hardly exists and only a small volume of BacMam must be used.

To evaluate the toxic influences of the transduction further experiments can be done, e.g.:

Apoptosis assays

- JC-1 assay (based on mitochondrial membrane potential)
- Annexin-V assay (based on phosphatidylserine location)

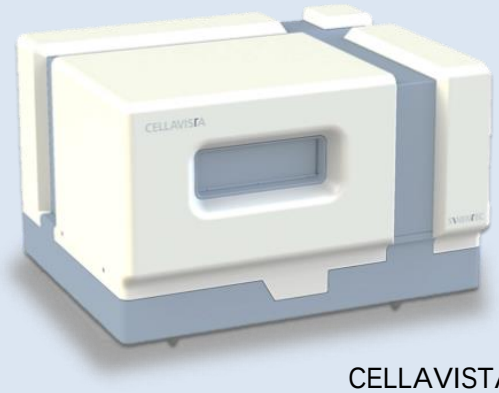
Genotoxicity studies

- H2AX-assay (based on antibody dependent analysis of DNA-damage indicator)

Viability testing's

- LIVE/DEAD assays (based on viable- and dead cell discrimination)
- Trypan Blue assay (based on membrane integrity)

This applications are measurable and evaluatable with all our devices and are implemented in the YT-software®.



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