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Transient Gene Expression is a fast, flexible, and cost-effective approach to produce high-quality products that circumvents the time and cost required for the generation of stably transfected cell lines. However, the levels of recombinant protein produced by TGE, tend to be significantly lower than those of stable cell lines. Despite the continued interest in transient gene expression approaches, little is known about the transfection process at intracellular level, particularly for complex products such as VLPs. The kinetics of PEI-mediated transient transfection was studied with the aim of characterizing and understanding the complete process leading to VLP generation, and identifying important events to drive process improvement. For this purpose, DNA/PEI polyplexes were tracked using Cy3 DNA staining and the production of Gag-GFP VLPs was monitored by flow cytometry, confocal microscopy, and fluorometry. Flow cytometry and confocal microscopy assays show that using a standard transfection protocol DNA:PEI polyplexes interact with the cell membrane from time point zero. A linear increase in transfection efficiency is observed until 60 minutes of contact between cells and polyplexes. No change in transfection efficiency (percentage of GFP positive cells) or VLP production levels is obtained when additional contact time is allowed, reaching a maximum transfection efficiency of 60% and VLP production of $10x10^9$ VLPs/mL harvested at 48 hours post transfection (hpt). After 1.5 hpt, polyplexes are detected in the cytoplasm of transfected cells and reach the nucleus around 4 hpt. Of note, all cells show the presence of DNA/PEI complex in the cytoplasm after transfection but only a fraction of cells express the fluorescent Gag protein. By flow cytometry analysis of isolated nuclei, it was determined that polyplexes are only present in 60% of the nuclei at 6 hpt (concomitant with the GFP expressing cells), suggesting that the entrance of polyplexes to the nucleus is one of the limiting steps of the transfection process. After 10 hpt, GFP fluorescence is detected homogenously inside the cells, but generalized budding of VLPs is not observed until 48 hpt. As mentioned before, a unique population of cells Cy3+ (with a polyplex inside) appears from the very beginning of the transfection. A new population of cells that do not contain any polyplex inside (Cy3-) and do not express the protein (GFP-) appears at 24 hpt suggesting plasmid loss after this time point. The VLP production kinetics was also studied, observing that fluorescence in the supernatant is always 40% less than total fluorencense (supernatant plus pellet). Maximum VLP levels in the cell culture supernatant, while keeping cell culture viability still high, are observed at 72 hpt, which was determined to be the optimal harvest time. Three bottlenecks in VLP production could be identified in this work: polyplexes entry into the nucleus, plasmid loss during the production phase and VLP budding.

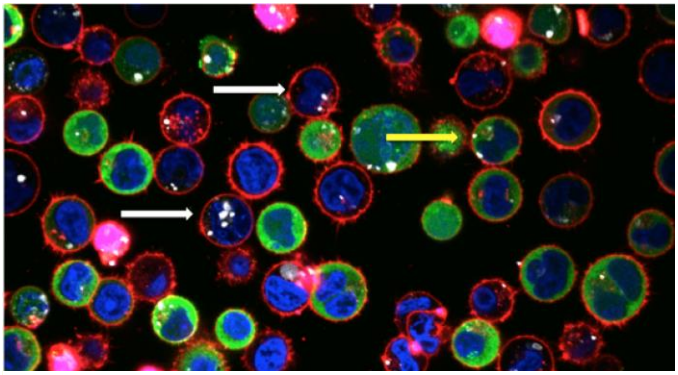

Figure 1. Confocal microscopy image of Gag-GFP expressing cells after 48 hpt. Cell membrane is stained using cell mask (red), nucleus is stained with Hoescht (cyan blue), Gag-GFP fluorescence is observed in Green and polyplexes can be observed in white due to DNA dye with Cy3. Highlighted with white arrows are cells with a polyplex inside, non expressing the Gag-GFP polyprotein and with white arrows, cells with a polyplex inside expressing the gag-GFP polyprotein.