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OPTIMIZED CONDITION FOR PEI-BASED TRANSIENT TRANSFECTION OF LIFEACT-GFP/NLS-MCHERRY EXPRESSING PLASMID USED AS CELL BARCODE FOR SYNCYTIA LIVE CELL IMAGING

KONDISI OPTIMAL UNTUK TRANSFEKSI TRANSIEN PLASMID PENGEKSPRESI LIFEACT-GFP/NLS-MCHERRY SEBAGAI BARKODE SEL UNTUK PENCITRAAN SEL HIDUP PADA SYNCYTIA

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ABSTRAK

Keberhasilan transfer DNA plasmid ke dalam sel dipengaruhi secara positif oleh efisiensi transfeksi dengan penekanan pada jumlah DNA plasmid dan rasionya terhadap reagen transfeksi. Polyethyleneimine (PEI) merupakan suatu reagen transfeksi cost-effective yang umum digunakan. Reagen transfeksi PEI memfasilitasi transfer DNA dengan membentuk kompleks DNA yang bermuatan positif. Kompleks bermuatan positif memungkinkan DNA untuk berinteraksi dengan permukaan sel yang bermuatan negatif kemudian mengalami internalisasi ke dalam sel secara endositosis. Dalam penelitian ini, kami menelusuri kondisi optimal untuk telakukan transfeksi transien plasmid pengekspresi lifeact-GFP/NLS-mCherry pada sel BHK-21 dan sel 293T menggunakan reagen transfeksi PEI. Plasmid ini dapat digunakan sebagai biosensor sitoskeleton dan nukleus yang memungkinkan pengamatan pencitraan langsung menggunakan mikroskop fluoresensi, salah satunya pada pengamatan syncytia. Pada penelitian, kami menggunakan dua variabel independen, yaitu jumlah DNA (0,5 dan 1 µg) dan rasio DNA-PEI (1:3 dan 1:4). Ekspresi GFP dan mCherry diamati pada 24, 48, dan 72 jam pascatransfeksi. Sebagai hasilnya, efisiensi transfeksi sel 293T yang dicapai menggunakan PEI lebih tinggi dibandingkan dengan efisiensi pada sel BHK-21, yaitu ~90% dan ~50%. Selain itu, di antara empat kondisi transfeksi yang berbeda pada kedua lini sel, sejumlah 1 µg DNA plasmid dengan rasio DNA-PEI 1:3 menghasilkan efisiensi yang paling tinggi dengan tingkat toksisitas yang lebih rendah. Kami menggunakan kondisi ini untuk mengamati syncytia pada sel 293T sebagai model SARS-CoV-2 cell-to-cell transmission. Syncytia yang terbentuk berhasil diamati dengan mendeteksi sel berukuran besar yang berfluoresens hijau/merah yang memiliki lebih dari satu inti.

Kata kunci: ***Lifeact-GFP/NLS-mCherry; polyethyleneimine; SARS-CoV-2; protein berfluoresens; efisiensi transfeksi***

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ABSTRACT

The transfection efficiency positively affects the successful plasmid DNA transfer into cells, with the highlight on the amount of plasmid DNA and its ratio to the transfection reagent. Polyethyleneimine (PEI) is a cost-effective transfection reagent that facilitates DNA transfer by forming positively charged DNA complexes. It allows DNA to interact with negatively charged cell surfaces and enter the cells by endocytosis. In this study, we optimized the condition for transient transfection of life act-GFP/NLS-mCherry-expressing plasmid in BHK-21 and 293T cells using PEI. This plasmid is helpful as a biosensor of the cytoskeleton and nucleus that enables live imaging observation using a fluorescence microscope, for instance, in the observation of syncytium. Here, we optimized two independent variables: the amount of DNA (0.5 and 1 µg) and the ratio of DNA-PEI (1:3 and 1:4). GFP and mCherry expressions were observed at 24, 48, and 72 h post-transfection. As a result, transfection efficiency achieved by using PEI in 293T cells is higher than in BHK-21 cells, which are ~90% and ~50%, respectively. Moreover, amongst four different transfection conditions, in both cell lines, 1 µg of plasmid DNA with a 1:3 DNA-PEI ratio yields the most efficiency with the least amount of toxicity. We used this condition for the syncytia observation in 293T cells as a model of the cell-to-cell transmission of SARS-CoV-2. Syncytia formation was successfully observed by detecting the giant cells expressing GFP/mCherry with multiple nuclei.

Keywords: ***Lifeact-GFP/NLS-mCherry; polyethyleneimine; SARS-CoV-2; fluorescence protein; transfection efficiency***

INTRODUCTION

Transfection efficiency measures the percentage of cells that express the transfected nucleic acid, typically DNA or RNA. It can be influenced by several factors that affect the uptake, processing, and expression of the transfected nucleic acid, which involves the origin of target cells, transfection reagent, nucleic acid, culture conditions, and plasmid design (Chong et al., 2021). Thus, transfection efficiency is an essential parameter for gene transfer experiments that indicate the expression level of a particular gene. As a preliminary study, a marker gene, such as green fluorescent protein (GFP), can quantify the percentage of cells that have taken up the gene of interest (Septisetyani et al., 2021).

Polyethyleneimine (PEI) is a cationic ethyleneimine polymer that can bind and form a complex with negatively charged nucleic acids and has been used for transfection (Sonawane et al., 2003). These positive charge complexes protect the nucleic acids from degradation, allowing them to bind to the negatively charged cell surface and enter the cells by endocytosis (Sonawane et al., (2003). Once inside the cell, the PEI-DNA complex can escape the endosome and enter the cytoplasm, where the DNA can be transcribed and translated to produce the desired protein (Sonawane et al., 2003).

Besides PEI, other transfection agents used in gene transfer experiments in mammalian cell cultures include liposomes, calcium phosphate, and nanoparticles (Chong et al., 2021). PEI has several advantages over other transfection agents, including its high transfection efficiency, cost-effectiveness, versatility in forming complexes with various types of nucleic acids, and low cytotoxicity (Pandey & Sawant, 2016). PEI is available in various molecular weights, and one commonly used form is PEI 25,000 or PEI 40,000. PEI 40,000 has a higher transfection efficiency than other PEI forms (Boussif et al., 1995).

In this research, we performed a ratio optimization of PEI and DNA for the transient expression of an F-actin and nuclei dual fluorescent reporter (pcDNA_Lifeact-GFP_NLS-mCherry) in two different cell lines, BHK-21 and 293T cells. This plasmid is inserted with the Lifeact and Nuclear Localization Signal (NLS) genes fused by a fluorescence protein. Lifeact is a short peptide with an affinity for F-actin microfilaments (Riedl et al., 2008). Meanwhile, NLS is a single cluster amino acid that plays a role in cytoplasm-nucleus protein transportation (Bernhofer et al., 2018). These fluorescent proteins can be applied as cell barcodes for live imaging to observe cytoskeleton dynamics under a fluorescence microscope.

Furthermore, in this study, we utilized this plasmid to observe the SARS-CoV-2

spike/hACE2-mediated syncytia formation. Syncytia is a fusion cell formed after a spike protein expressed by a donor cell interacts with hACE2 of the adjacent cell (Rajah et al., 2022). The fused cells were observed as giant green cells with multiple red nuclei using the cell barcode.

METHOD

Cell Culture

BHK-21/WI-2 (Kerafast EH1011) and 293T cells (ECACC 12022001) are collected from the Research Center for Genetic Engineering, National Research and Innovation Agency (BRIN, Bogor Indonesia) and maintained in Dulbecco's Modified Eagle Medium (Gibco, Billings USA) supplemented with 5% (BHK-21) or 10% (293T) fetal bovine serum (Sigma-Aldrich, St. Louis USA); 100 µg/ml streptomycin and 100 IU/ml penicillin antibiotics at 37°C in a humidified incubator under 5% CO_2 (Thermo Fisher Scientific, Waltham USA). Approximately 40,000 BHK-21 and 293T cells were seeded onto a 24-well plate (Corning, Corning USA) and incubated overnight in the CO_2 incubator.

Cell Transfection

BHK-21 and 293T cells were transfected with pcDNA_Lifeact-GFP_NLS-mCherry (a gift from Olivier Pertz; Addgene plasmid # 69058) by using PEI (PEI MAX®-Transfection Grade Linear Polyethylenimine Hydrochloride (MW 40,000). The cells were transfected with DNA/PEI with the ratios of 1:3 (0.5 µg DNA), 1:3 (1 µg DNA), 1:4 (0.5 µg DNA), and 1:4 (1 µg DNA). Each DNA-PEI ratio was prepared in Opti-MEM separately (Gibco, Billings USA) with a total volume of 50 µl per well. Each mixture was vortexed and incubated for 15 min at room temperature. Four types of DNA/PEI mixture were added to the cells in the different wells of the 24-well plate. The cells were incubated in the mixture-containing medium overnight, and then the medium was replaced with a fresh culture medium. GFP and mCherry expression were observed at 24, 48, and 72 h post-transfection using a fluorescence microscope (Olympus IX83, Japan).

Table 1.
Amount of DNA and PEI for Transfection Mixture

Ratio	DNA (µg)	1 mg/ml PEI (µL)
1:3	0.5	1.5
1:3	1	3
1:4	0.5	2
1:4	1	4

Source: Variables from the authors (2023)

Observation of Syncytia Formation

Forty thousand cells of 293T were seeded onto gelatin-coated coverslips on a 24-well plate and incubated overnight before transfection. The following day, the transfection was carried out utilizing plasmids pcDNA3.1-SARS-Spike and pcDNA3.1-hACE2 (gifts from Fang Li; Addgene plasmid #145031 and #145033) in addition to pcDNA_Lifeact-GFP_NLS-mCherry, with a DNA ratio to PEI 1:3. The next day, the cells were fixed using 4% PFA and mounted in a mountant containing DAPI. Fluorescence observation was investigated under an inverted fluorescence microscope (Olympus IX83, Japan).

RESULTS AND DISCUSSION

The efficiency of PEI-mediated Transfection in BHK-21 Cells

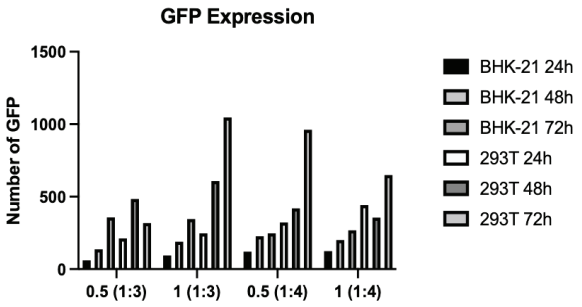

Figure 1.
GFP Expression on BHK-21 and 293T Cells at 24 h, 48 h, and 72 h after Treatment

BHK-21 cells were transfected using one plasmid DNA expressing GFP and mCherry with four different DNA/PEI ratios. GFP/mCherry-expressing cells were examined under a fluorescence microscope at 24 h, 48 h, and 72 h following transfection. Based on the GFP/mCherry intensity results, as shown in Figures 1 and 2, the highest fluorescence intensity was obtained with a 1:3 DNA/PEI ratio (1 µg DNA). At a 1:3 DNA/PEI ratio (0.5 µg DNA), the intensity of GFP/mCherry was lower and peaked after 72 h incubation. Meanwhile, at a 1:4 DNA/PEI ratio (0.5 and 1 µg DNA), the fluorescence intensity was high after 24 h incubation but decreased after 72 h. These results suggested that a 1:3 DNA/PEI ratio is the optimum transfection condition for BHK-21 cells.

The efficiency of PEI-mediated Transfection in 293T Cells

The 293T cells were transfected under the same conditions and variables as BHK-21 cells. Compared to BHK-21, the number of GFP/mCherry-expressing 293T cells was higher (Figure 1), with the transfection efficiency reaching 90% after 24 h incubation (Figure 3). The highest efficiency was achieved with the transfection of 1 µg plasmid DNA with a 1:3 DNA/PEI ratio. In a 1:4 DNA/PEI ratio, GFP/mCherry was expressed in 293T cells with a similar trend as in BHK-21 cells. At first, the 1:4 DNA/PEI ratio (0.5 and 1 µg DNA) showed higher fluorescence intensities. However, the signal was getting lower after 48 and 72 h incubation, which represents the cytotoxic effect of the transfection condition. Therefore, transfection of the lifeact-GFP/NLS-mCherry plasmid into 293T cells with the DNA-PEI ratio of 1:3 was chosen as an optimum condition for syncytia assay.

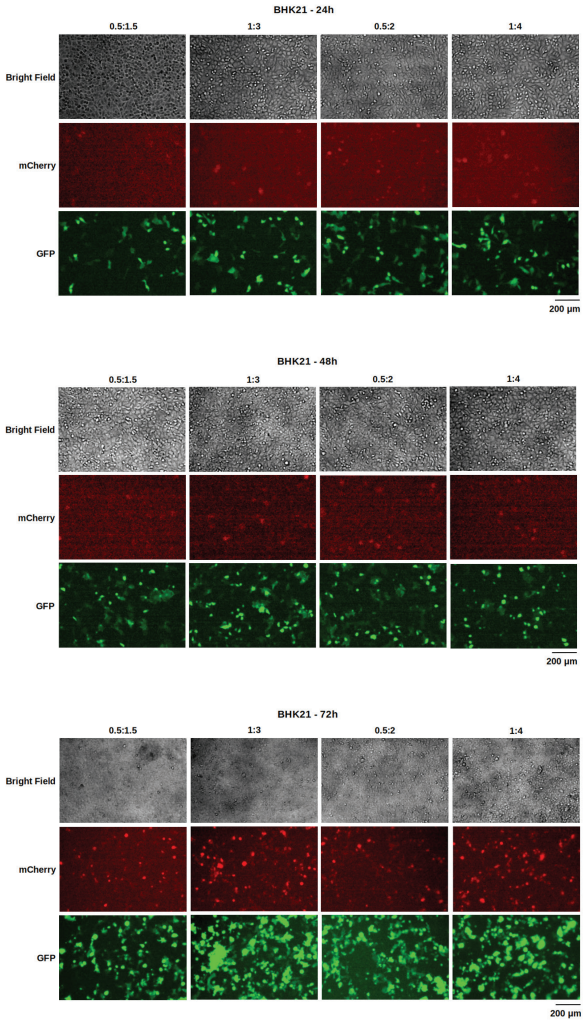

Figure 2.
Fluorescence Images of BHK-21 Cells Transfected with Lifeact-GFP/NLS-mCherry Plasmid by PEI-based Transfection Method. The 293T cells were transfected with DNA-PEI mixture at 0.5:1.5, 1:3, 0.5:2, and 1:4, then observed at 24 h, 48 h, and 72 h post-transfection.

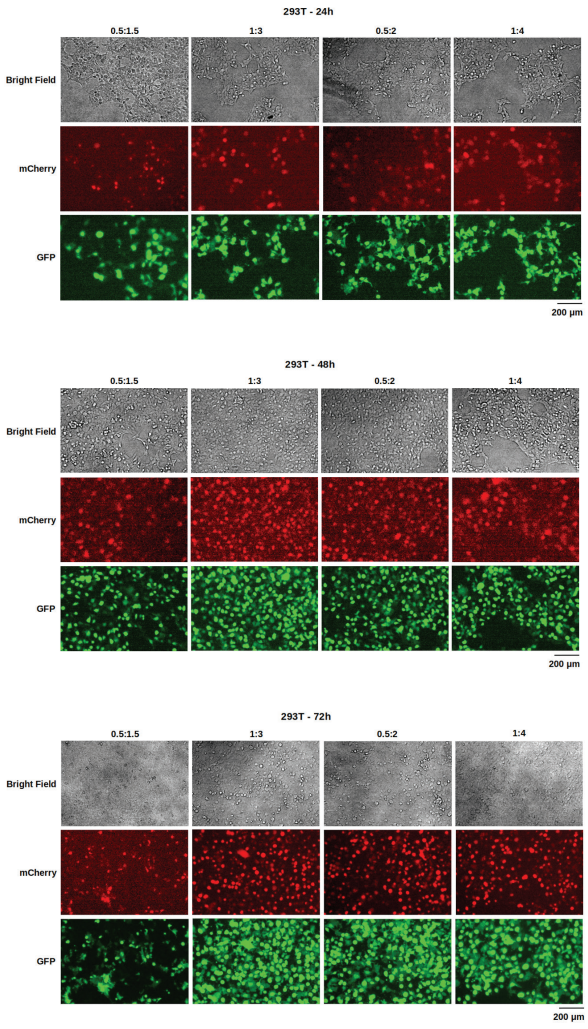

Figure 3.
Fluorescence Images of 293T Cells Transfected with Lifeact-GFP/NLS-mCherry Plasmid by the PEI-based Transfection Method. The 293T cells were transfected with DNA-PEI mixture at 0.5:1.5, 1:3, 0.5:2, and 1:4, then observed at 24 h, 48 h, and 72 h post-transfection.

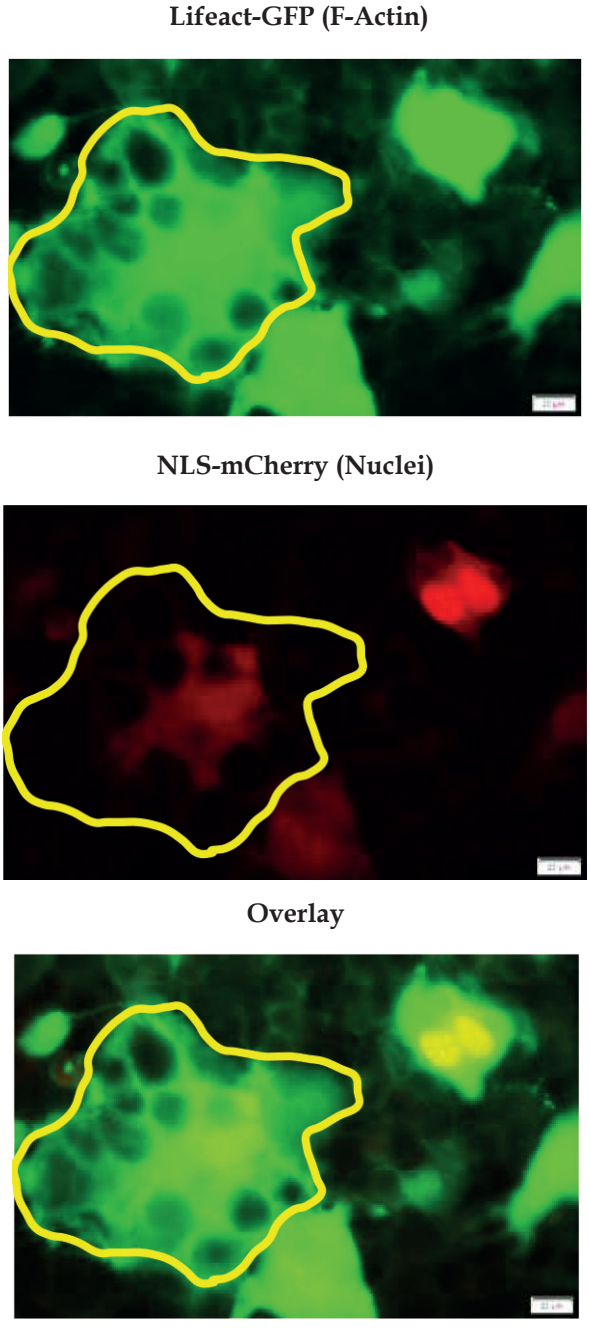

Figure 4.
Fluorescence Observation of Lifeact-GFP/NLS-mCherry during Syncytia Formation in 293T Cells Transfected with SARS-CoV-2 spike/hACE2/lifeactGFP-mCherry with a DNA-PEI Ratio 1 to 3. Bar = 20mm. The yellow area: syncytium.

Application of PEI-mediated Transfection for Syncytia Observation in 293T Cells

Syncytium is a multinuclear cell formed via the membrane fusion of mononuclear cells. A standard illustration of physiological syncytia is found in muscle cells. Additionally, syncytia formation is a pathophysiological effect of several virus infections, such as HIV, herpes simplex, and SARS-CoV-2 (Leroy et al., 2020).

Cell-to-cell transmission is one mechanism that mediates SARS-CoV-2 transmission. The mechanism involves interaction between the spike glycoprotein of SARS-CoV-2 with the target receptor hACE2. Spike expressed by the infected cells localizes in the plasma membrane, binds to hACE2 of the adjacent cell, and subsequently leads to cell-to-cell fusion. The lab can mimic this phenomenon by expressing spike protein and hACE2 in the cell models.

We used the ratio of total DNA-PEI 1:3 according to the optimized transfection condition. However, since we had to do co-transfection of lifeact-GFP/NLS-mCherry plasmid with spike and hACE2 plasmids, the amount of lifeact-GFP/NLS-mCherry plasmid was reduced to a ratio of 0.25, with spike plasmid 0.5, and hACE2 plasmid 0.25 to make a total of 1 portion of plasmid DNA. We could investigate the syncytia formation by detecting the green giant cells with multiple, red-stained nuclei (Figure 4). Therefore, the GFP/mCherry used here was suitable for live cell imaging without additional staining steps.

Discussion

BHK-21 and 293T are cell models generally used to express recombinant DNA. Here, we compared both cells for transfection of life act-GFP/NLS-mCherry plasmid to obtain better conditions for observation of fluorescence cell markers. Both cell lines, BHK-21 and 293T, were known to have a wide range of transfection efficiency, depending on the transfection reagents being used. We used PEI as the transfection reagent because PEI can facilitate higher transfection efficiency than the calcium phosphate method. Even though PEI transfection efficiency is lower than lipofection agents, PEI transfection is less toxic yet more cost-effective than lipofection agents. This study shows that 293T has a higher transfection efficiency than BHK-21.

As demonstrated in Figure 2 and Figure 3, the amount of GFP/mCherry-expressing cells in BHK-21 is much less than in 293T cells. After 72 h incubation, the massive difference of GFP/mCherry-expressing cells between BHK-21 and 293T cells is visible. This study achieves medium (~50%) and high (~90%) transfection efficiency in BHK-21 and 293T cells, respectively. The categorization refers to Horibe et al. (2014), ranging from high (more than 60%), medium (20–60%), and low (less than 20%) transfection efficiency. According to Thomas and Smart (2005), 293T cell lines are easy to grow and maintain with a high reproducibility. They can express exogenous receptors, which makes them amenable to many transfection procedures and the ability to express a wide variety of receptor proteins. These findings are consistent with a study by Tang et al. (2015). They discovered that 293T cells transfected transiently with PEI in the lentivirus production exhibited high consistency and repeatability. Another study published by Blackstock et al. (2020) employed the PEI-based transfection approach for transiently producing Chikungunya virus-like particles using 293T cells. According to the authors' report, the method yields high transfection efficiency (≥97%) in all tested samples.

PEI is commonly used as a transfection agent for many mammalian cell types, including human, mouse, rat, and other animal cells. However, the optimal transfection conditions, including the PEI/DNA ratio and incubation time, may vary depending on the specific cell type used (Fus-Kujawa et al., 2021). Some cell types might be more vulnerable to PEI's harmful effects, which could decrease cell viability and transfection efficiency (Fus-Kujawa et al., 2021). Overall, while PEI is a versatile and widely used transfec-

tion agent, the suitability of PEI for a specific cell type should be determined through careful optimization experiments.

To identify the most suitable conditions for transient transfection, we transfected lifeact-GFP/NLS-mCherry expressing plasmid into two cell lines, BHK-21 and 293T, using two DNA/PEI ratio: 1:3 and 1:4, and observed the fluorescence proteins expression 24, 48, and 72 h post-transfection. The ratio of PEI to DNA can affect the transfection efficiency, as it can influence the formation and stability of the DNA/PEI complexes. Typically, a higher PEI to DNA ratio can lead to higher transfection efficiency but may also increase the toxicity of the complexes. In this study, the DNA to PEI ratios of 1:3 and 1:4 have a similar GFP expression after 24 h incubation in BHK-21 (Figure 2) and 293T cells (Figure 3). The 1:4 ratio shows cell death higher than 1:3. Figure 2 and Figure 3 show rounded GFP signals with higher intensities, indicating dead cells, in cultures that were previously transfected with a higher concentration of PEI (1:4 ratio, either with 0.5 or 1 µg DNA). These results are by Xie et al. (2013) research that indicated a decline in cell viability and an increase in PEI to DNA ratio.

As shown in Figure 2 and Figure 3, higher transfection efficiency is achieved by the transfection of 1 µg DNA into BHK-21 and 293T cells, compared to that of 0.5 µg DNA. A higher amount of DNA resulted in a higher level of transfection, but it should be optimized as it can also contribute to cell toxicity. On the other hand, DNA in a lower quantity formed a smaller complex with the transfection agent (Sergeeva et al., 2018), in which a more petite size resulted in a higher viability (Sergeeva et al., 2018). In addition, the internalized single DNA, not in the form of a complex, can also be sensed by receptors and subsequently mediated cytokine production, resulting in inflammation and lytic cell death, or so-called pyroptosis (Briard et al., 2020).

According to research by He et al. (2021), transfection with an incubation time of under 24 hours shows good efficiency in 293T cells. However, the incubation time post-transfection should be considered when we expect higher expression of the recombinant protein of interest. Based on the results of the GFP intensity seen on the fluorescence microscope, the GFP intensity in BHK-21 was higher after 24 hours of incubation post-transfection. Whereas in 293T cells, the intensity of GFP was higher after 48 hours post-transfection.

Moreover, 78 hours post-transfection caused higher GFP intensity in both cells rather than shorter incubation times. However, as too high GFP intensity can upset the cell balance and viability, the recommended maximum incubation time is 48 hours post-transfection. Longer incubation times may increase the accumulation of the fluorescence proteins but also increase the cytotoxicity. Longer incubation time post-transfection of more than 48 hours is susceptible to cell death due to rapid cell proliferation and over-confluence. Whereas in BHK-21 cells, at 48 hours and 72 hours of incubation post-transfection, the cells had started to die. This is to a study conducted by Liu et al. (1999), which stated that at an incubation time of 48 hours post-transfection in BHK-21 cells, many cells expressing GFP had died. So, the maximum incubation time for BHK-21 cells is recommended to be about 24 hours.

We can investigate syncytia formation by a phase contrast inverted microscope to observe the multinucleated giant cells. However, it is sometimes difficult to quantify the number of nuclei without staining. Crystal violet and HE stains are often used to observe cell morphologies. However, the resulting staining contrast between nuclei and cytoplasm sometimes must be clarified. Therefore, using fluorescence reporters will help differentiate the presence of syncytia. In this study, we used a plasmid expressing the lifeact-GFP/NLS-mCherry. Zeng et al. (2022) visualized eGFP-based large syncytia formation by utilizing the target-donor co-culture system of 293T cells. The donor 293T cells were transfected with a lentiviral vector harboring intron-Gaussia luciferase (inGluc), SARS-CoV-2 spike, and eGFP encoding plas-

mid vectors. Then, these transfected cells producing SARS-CoV-2 spike pseudotyped HIV lentivirus were cocultured with the target cells, 293T/hACE2. However, using this method, the nuclei cannot be observed using a fluorescence microscope as the syncytia appear as green cells with dark nuclei.

In addition, Wünschmann and Stapleton (2000) have established fluorescence-based methods to distinguish and quantify HIV-1-induced syncytia based on flow cytometry utilizing propidium iodide as a DNA staining reagent and color fusion assay using two different fluorescence-based cytoplasmic dyes. In the flow cytometry method, PI will facilitate the analysis of nuclei number and cell size, representing syncytia occurrence. Moreover, in color fusion assay, staining of the target cells with green and red cytoplasmic fluorescence dyes followed by cell coculturing will visualize the syncytia as yellow-stained cells as the fusion products of green and red colors. The fluorescent-assisted assays allow feasible methods to evaluate transfection efficiency and fusogenic activity in the cell culture system. Using both cytoplasmic and nuclear fluorescence cell markers will assist in confirming the presence of nuclei besides staining the nuclei using fluorescence-based DNA labeling dyes.

CONCLUSION

PEI can be used as a transfection agent in 293T and BHK-21 cells with better transfection efficiency for lifeact-GFP/NLS-mCherry plasmid observed in 293T cells. We observed that the DNA ratio to PEI 1:3 was the optimum condition that could result in high GFP/mCherry expression with minimum cytotoxicity. This condition could be used to investigate syncytia formation mediated by SARS-CoV-2 spike/hACE2 in 293T cells. To clarify the occurrence of cell fusion, the co-culture method can be used by applying two different fluorescence proteins expressed by two cell populations to differentiate the resulting fused cells from the parental cells. However, this co-culture method is more difficult to carry out with more steps, which involve cell harvesting and seeding of two different cell populations in one well. This requires optimizing the number of cells seeded, homogeneous cell seeding techniques, and harvesting techniques to prevent declining of cell viability due to this treatment.

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