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Effect of Protein Concentration and Injection Pressure in Microinjection Delivery of Maltose Binding Protein into Breast Cancer Cells

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ABSTRACT

Microinjection is a powerful tool to deliver various substances, such as nucleic acids, proteins, peptides, RNA, and synthetic molecules into mammalian cells mechanically. Through microinjection, a controlled amount of protein can be delivered into the target cells to elucidate the specific functional effects *in vitro*. In this study, a series of protein microinjection optimization was performed in human breast cancer cells. The presence of Maltose Binding Protein (MBP) was microscopically monitored through indirect immunofluorescence assay. The optimization experimentation gave a high success rate when MBP protein was used at the minimum concentration of 1.5 mg/ml and at the injection pressures of 50 and 70 hPa. The average success rate of injections was 49.2±4.15% and 50.8±4.6%, while the average cell survivability was 50.98±4.67% and 49.72±5.48% at 50 and 70 hPa, respectively. The optimization of the MBP concentration and injection pressures successfully allowed an efficient delivery of precise protein dosage into breast cancer cells without any adverse effect. This microinjection optimization can be a practical guideline in any downstream applications of protein functional work.

Keywords: Cell microinjection, fluorescence microscope, immunofluorescence, MBP

ABBREVIATIONS

Maltose Binding Protein (MBP)
Mammary Carcinoma Cells (MCF7)
Fetal Bovine Serum (FBS)
Deoxyribonucleic acid (DNA)
Ribonucleic acid (RNA)
Milligram per milliliter (mg/ml)
Micrometer (μm)
Minute (min)
Hectopascal (hPa)
Carbon dioxide (CO_2)
Second (s)
Hour (h)
Fluorescein Isothiocyanate (FITC)

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INTRODUCTION

In biological research, microinjection is a prominent approach for delivering dissolved substance such as DNA, proteins, cellular organelles, enzymes, antibodies, genes, metabolites, ions, RNA, and various markers into living cells (King, 2003; Feramisco *et al*., 1999). This particular technique is complemented with live cell imaging to observe immediate phenotypic changes in the cells to study the effect of the injected protein on cell morphology or the localization of the injected protein. Thus, it elucidates the roles of specific proteins in cell function, specifically when studying severe or transient cellular responses in various applications, such as cytology, physiology, genetic engineering, molecular biology, virology, tumour biology, developmental biology, pharmacology, and toxicology (Viigipuu & Kalio, 2004).

As parameter requirements for microinjection such as protein concentration and injection pressure are different from cell to cell, validated optimization is necessary to ensure the survival of the cells and to minimize the mortality of the cells (Smaili *et al*., 2008; Derouazi *et al*., 2006).

This manuscript reports the optimization of microinjection for breast cancer cells using Maltose-Binding Protein (MBP), which includes the determination of success rates for penetration, the survival rate of the injected cells and the presence of the injected protein in the cells. The optimization of the microinjection of the targeted protein into adherent cell is needed to ensure that the morphology that changes in the cell is not due to drastic injection so that evaluation of the effect of the injected sample can be done.

The optimization of microinjection into adherent cells is crucial to exclude the interference of morphological changes in cells due to technical fault. An optimized approach will make way for a precise evaluation of the effect of injected substance.

MATERIALS AND METHODS

Cells

The experiments were performed on adherent cell line, human breast cancer cell MCF7 (provided by Professor Fauziah Othman, at the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia). The maintenance medium for MCF7 cell was RPMI media, which was supplemented with 10% fetal bovine serum (FBS) and antibiotic solution (100 units/ml penicillin, 100 mg/ml streptomycin). All the cell culture media and supplements were retrieved from Gibco Invitrogen Life Sciences (Paisley, UK). MCF7 cells were plated on cover slip (sized 22x22 mm^2) 24 h prior to microinjection.

Injection Substances

Maltose-Binding Protein (MBP protein) and PBS were used as injection substance. MBP protein was expressed using *in vitro* protein expression system, Rapid Translation System (Roche Diagnostic, USA). The protein was then purified by using amylose resin (New England Biolabs, US) and resuspended in PBS buffer (137mM NaCl, 2.7 mM KCl, 100 mM Na_2HPO_4, 2 mM KH_2PO_4). The protein concentration was determined using UV Visible Spectrophotometer (Cary UV DKSH, USA). Meanwhile, PBS (137 mM NaCl, 12 mM Phosphate, 2.7 mM KCl, pH 7.4) was used as a negative control for the injection.

Microinjection

A 0.5 µm diameter sterile microinjection needle (Femtotip, Eppendorf, USA) was loaded with protein sample, MBP protein by using Eppendorf microloader (Eppendorf, USA). The experimented protein concentrations were 0.8, 1.0, 1.5, 2, and 2.5 mg/ml, respectively. Prior to loading, the injection samples were centrifuged at 16,000xg at 4°C for 20 min to eliminate precipitates that might block the microinjection needles.

During microinjection, the cells were incubated in phenol red free RPMI 1640 medium (containing 25 mM Hepes, pH 7.2, 10% FBS, penicillin, and streptomycin). The temperature of the medium was kept at 37°C using an objective heater. Protein or PBS delivery into the cytoplasm of cells was carried out under an injection pressure ranging from 30-110hPa, with an injection time of 0.5 s. A total number of 50 MCF7 cells were injected on each cover slip and the injected cells were switched to fresh culture medium immediately after microinjection and incubated at 37ºC in 5% CO_2 incubator at different time points (1, 2, 5, 10, and 24 h).

Pressure Optimization

The microinjector is equipped with an inverted microscope (Axiovert 135 TV, Zeiss, Germany), a pressure injector, Femtojet (Eppendof, USA) and a joystick micromanipulator, InjectMan NI2 (Eppendorf, USA). The MCF7 cells were injected using Femtojet at various injection pressures (30, 50, 70, 90 and 110 hPa) for 0.5s, respectively.

Viability Observation

A light microscopic observation was performed periodically at various time points (1, 2, 5, 10, and 24 h), after microinjection of 50 cells per cover slip. Meanwhile, the attached viable microinjected cells were calculated prior to cell fixation.

Indirect Immunofluorescence Assay

At various time points (1, 2, 5, 10, and 24 h) after microinjection of 50 cells per cover slip, the cells were fixed with 100% ice-cold methanol for 30 min at 4ºC and washed 3 times with PBS for 5 min each. In order to detect the presence of MBP protein in the injected cells, anti-MBP mouse monoclonal (Chemicon, USA) was used. This monoclonal antibody recognized the epitope within the MBP protein. The fixed cells were incubated with this monoclonal antibody for 1h at room temperature, and this was followed by 3 washing steps. The FITC-labelled goat anti-mouse antibody (Chemicon, USA) was used as the secondary antibody. The cells were incubated with this secondary antibody for 30 min at room temperature and this was also followed by 3 washing steps. The stained cells were analyzed using fluorescence microscopy (Zeiss, USA). Images were captured using digital image analysis equipment.

RESULTS AND DISCUSSION

Delivery of Protein into Human Breast Cancer Cells

The injection tests were carried out to study the injection success rate into the cells. From a total of 50 cells which had been injected on each cover slip, 20-30 cells were successfully injected, giving an average success rate of 49.2±4.15% at the injecting pressure of 50 hPa and 50.8±4.6% at 70 hPa (Table 1). Successful protein injection was indicated by liquid flow transfer from the end of the microneedle into the cell upon microneedle penetration.

Nonetheless, penetration of elastic cellular membrane without causing any damage is indeed a challenge. Optimum injection pressure is an important factor to ensure success of cell injection. When the cell was injected with low pressure at 30 hPa, no flow was observed, and this raised doubts on intended protein delivery. However, cell injection with high injection pressures at 90 and 110 hPa caused the cells to burst immediately after the liquid had flowed into the cells (*Figs. 1m-1p)*, and thus, not possible for survival rate determination. Meanwhile, MBP protein transfer into breast cancer cells was readily attained at both 50 and 70 hPa injection pressures.

A common hurdle for effective protein microinjection is capillary clogging (Viigipuu & Kalio, 2004). Protein aggregates could easily block, specifically at the edge of fine microcapillary. Hence, protein has to be centrifuged at high speed for 15-20 min before loading the protein into the microneedles (King, 2003; Komarova *et al*., 2007). However, obstruction induced by cell debris or flowing particles in the cell culture medium could not be excluded (Viigipuu & Kalio, 2004).

There is a vast difference between microinjecting salt and protein. Protein has a tendency to form aggregates and its viscosity increases with concentration. Microinjecting protein is much more challenging as compared to salt substances, whereby the latter has been found to have higher injection success rate. KCl could be a safe and ideal substance for beginners to practice with, as it is a cellular constituent (Viigipuu & Kalio, 2004). Since injection skills had been acquired beforehand, the experimentation with MBP protein (negative control) was therefore relevant for future study on MBP-based proteins.

Survival Test of Microinjected Human Breast Cancer Cells

In this study, the survival rate of microinjected human breast cancer cells was examined. The nature and amount of injected substance should be of no harm to the cells. To encourage cell survival during and after the injection, a constant temperature at 37°C and pH 7.4 were maintained throughout the procedure. Injection time is another important factor in ensuring a good cell survival rate. The cells must be injected within 15 min, specifically in a harsh environment such as in the absence of CO_2 supply.

The injected cells were observed under a light microscope for morphological changes. In *Figs. 1a-1l*, the injected viable cells remained attached to the cover slip and showed no significant changes from their initial morphology after 10h of post injection, whereas at this time point, dying cells remained rounded, detached and failed to return to their original shape, as illustrated in *Figs. 1h* and *1l*.

The injected cells were also observed at 10h post injection and the survival rates are shown in Table 2. The average survivability of the MBP injected cells at 50 and 70 hPa was 50.98±4.67% and 49.72±5.48% respectively, while the PBS injection demonstrated 50.2±9.5% and 49.52±4.88% survival rates at 50 and 70 hPa injections, respectively. The MBP protein alone did not induce any adverse effect to the cells, as the differences in the success rate among MBP and PBS injections were negligible.

Eventually, viable injected cells from a total of 50 cells were only 24.8±4.15% and 25.2±3.35% for the MBP protein delivery and 24.4±4.15% and 24.8±3.35% for the PBS delivery at 50 and 70 hPa, respectively. These findings indicate the overall damage undergone by cells under microinjection procedure. Cells could die during post injection incubation time if delivery holes failed to shut. This could be due to prolonged penetrating time and delayed microneedle removal (Robert, 2003). Moreover, other external factors might also influence the low survival rate, such as pH of the culture medium, the molarity between the cells, as well as injecting substances and temperature changes. Furthermore, microinjection process should be limited within 15 minutes to prevent cell damage due to pH changes in the culture medium (Komarova *et al*., 2007).

TABLE 1
Success injection rates. The injection rates of microinjection into breast cancer cells were taken immediately after 1.5 mg/ml of MBP and 1M PBS injection. Success rate (percentage) was calculated based on a total number of 50 cells that had been injected. The average success rates of MBP were 49.2±4.15% and 50.8±4.6% and PBS 49.6±2.61% and 50±3.16% at 50 and 70 hPa, respectively

Plate	Success injection rates							
	PBS				MBP			
	Pressure 50 hPa		Pressure 70 hPa		Pressure 50 hPa		Pressure 70 hPa	
	Number of cell successfully injected	Percentage	Number of cell successfully injected	Percentage	Number of cell successfully injected	Percentage	Number of cell successfully injected	Percentage
1	23	46	26	52	26	52	25	50
2	26	52	25	50	27	54	23	46
3	26	52	23	46	25	50	26	52
4	24	48	24	48	22	44	29	58
5	25	50	27	54	23	46	24	48

TABLE 2

Cell survival rates. The survival rates of microinjected breast cancer cells were determined after 10 h of post injection. Cells survival rate (percentage) was calculated based on the number of cells that had successfully been injected. The average survival rates of the cells that were injected with MBP protein were 50.98±4.67% and 49.72±5.48% and the injection with PBS demonstrated 50.2±9.5% and 49.52±4.88% at injecting pressure 50 and 70 hPa, respectively

Plate	Cell survival rates											
	PBS						MBP					
	Pressure 50 hPa			Pressure 70 hPa			Pressure 50 hPa			Pressure 70 hPa		
	Number of cells successfully injected	Number of viable cells	%	Number of cells successfully injected	Number of viable cells	%	Number of cells successfully injected	Number of viable cells	%	Number of cells successfully injected	Number of viable cells	%
1	23	11	47.8	26	12	46.2	26	15	57.7	25	14	56.0
2	26	12	46.2	25	14	56.0	27	14	51.9	23	11	47.8
3	26	11	42.3	23	10	43.5	25	13	52.0	26	11	42.3
4	24	16	66.7	24	12	50.0	22	10	45.5	29	14	48.3
5	25	12	48.0	27	14	51.9	23	11	47.8	24	13	54.2

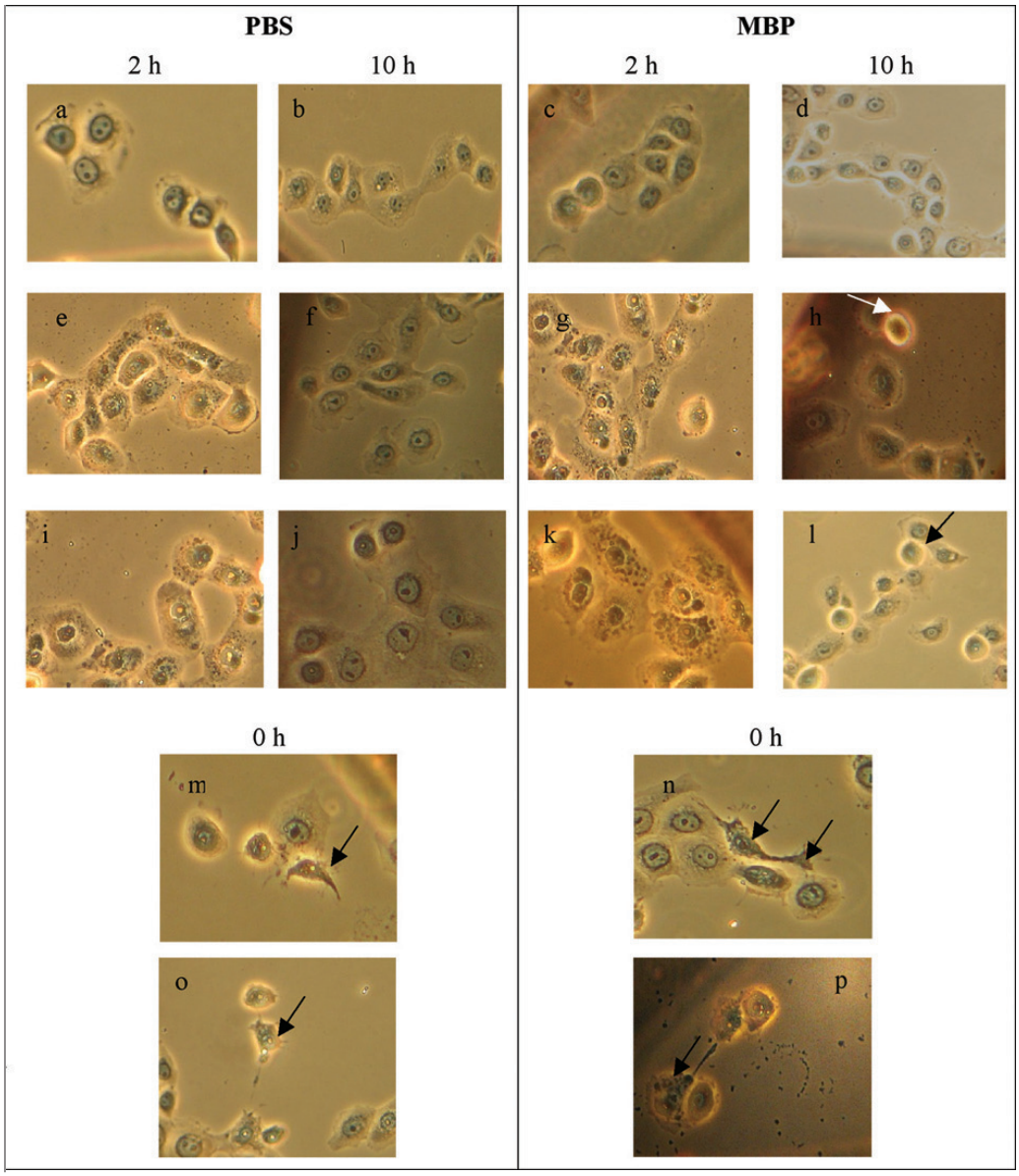

Fig. 1: Microinjection of the PBS and MBP proteins into MCF7 cells. PBS acted as a negative control. The microinjected cells were still adhering to the cover slip after 2 and 10 h of post injection. The arrows in figures m, n, o and p show cell burst after microinjected with high pressures (90 and 110 hPa), whereas the arrows in figures h and l show that the microinjected cells remained rounded, detached and could not attach back after 2 and 10 h of incubation, suggesting cell death

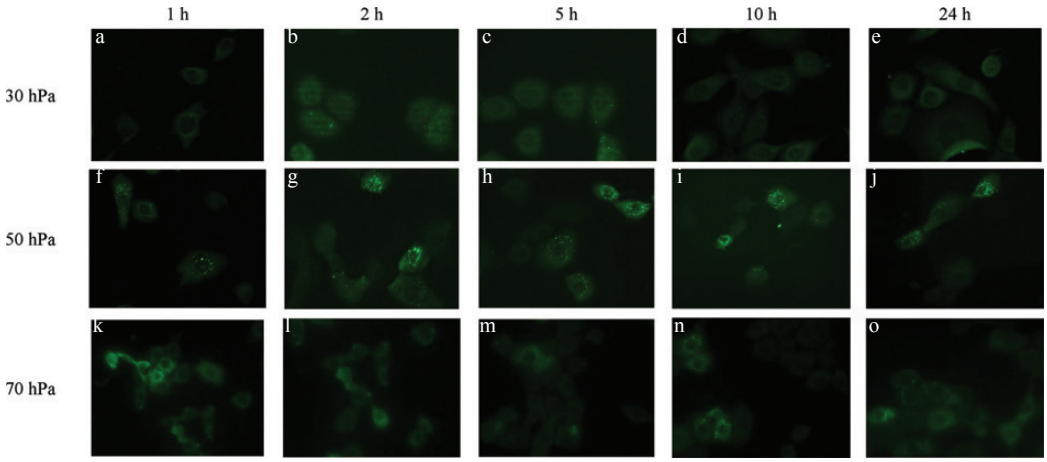

Fig. 2: Indirect immunofluorescence staining of the injected MCF7 cells. The cells were injected with 0.8 mg/ml of the purified MBP protein. The cells that had been injected (Figs. a-e) at 30 hPa showed no fluorescence signal, (Figs. f-j), whereas 50 hPa and (Figs. k-o) 70 hPa showed mild or very little fluorescence signal

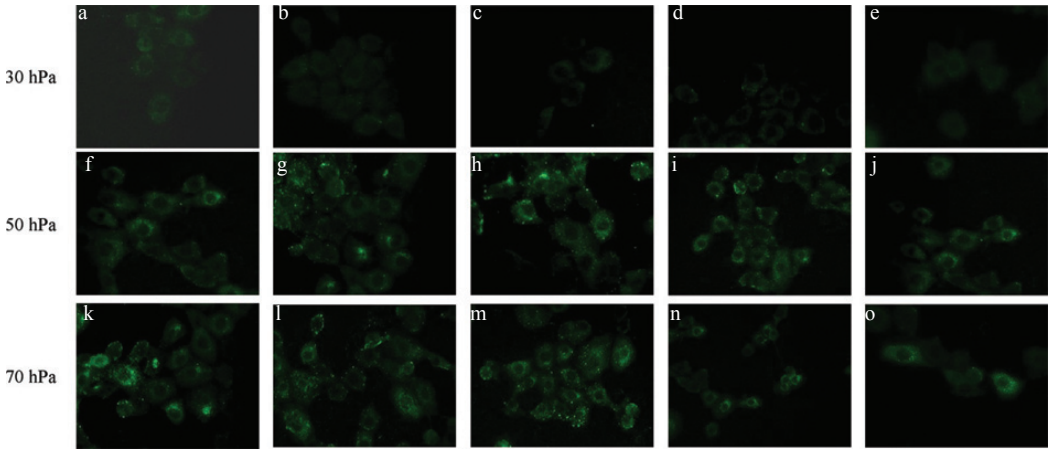

Fig. 3: Indirect immunofluorescence staining of the injected MCF7 cells with 1.0 mg/ml of the purified MBP protein. The cells which had been injected (Figs. a-e) at 30 hPa showed no fluorescence signal, whereas (Figs. f-j) 50 hPa and (Figs. k-o) 70 hPa showed mild or little fluorescence signal.

Fluorescent Tracking of the MBP Protein in the Injected MCF7 Cells

This microinjection experiment involved staining of the injected cells with fluorescent antibodies against the MBP protein and analysis using fluorescence microscopy. The indirect immunofluorescence was used to detect the presence of protein in the injected cells as the MBP protein was not fused with fluorochrome-conjugate.

In *Figs. 2a-2e* and *3a-3e*, the MCF7 cells that had been injected with low protein concentrations (0.8 and 1.0 mg/ml) at a low pressure of 30 hPa showed no fluorescence signal at the cytoplasm of the cells, whereas in *Figs. 2f-2o* and *3f-2o*, the increased pressures at 50 and 70 hPa respectively

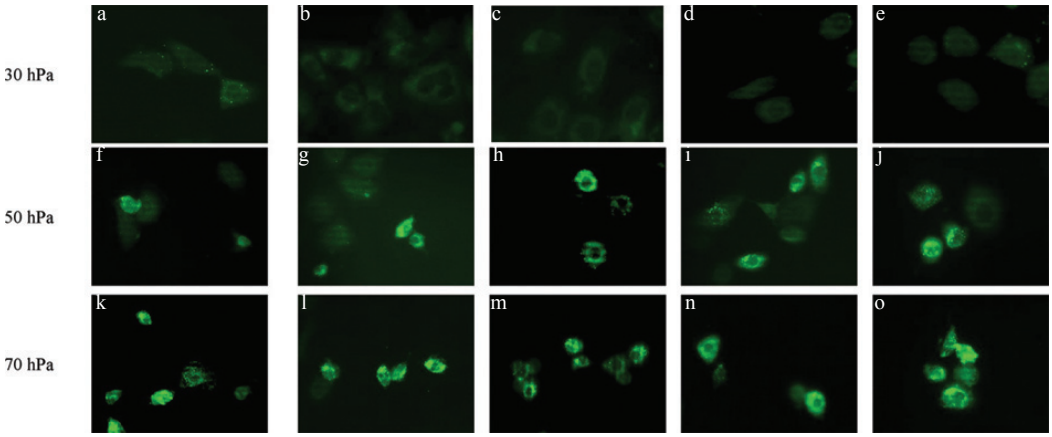

Fig. 4: Indirect immunofluorescence staining of the injected MCF7 cells. The cells were injected with 1.5 mg/ml of purified MBP protein. The cells that had been injected (Figs. a-e) at 30 hPa showed no fluorescence signal, whereas 50 hPa (Figs. f-j) and 70 hPa (Figs. k-o) showed strong fluorescence signals

showed mild or little fluorescence signals at the cell cytoplasm. Similarly, *Figs. 4a-4e* illustrate an increase in the MBP protein concentration to 1.5 mg/ml, indicating the absence in cytoplasmic fluorescence when injected at 30 hPa; however, fluorescence was detected when the pressure was increased to 50 and 70 hPa (*Figs. 4f-4o*). In *Figs. 5a-5e* and *6a-6e*, cell cytoplasm emitted a mild or little fluorescence signal when injected with 2 and 2.5 mg/ml, respectively, at 30 hPa. On the contrary, the cells exhibited a strong fluorescence signal when the injection pressure was increased to 50 or 70 hPa.

The results gathered in the current study have demonstrated the importance of concentration optimization for cell injection. Protein with a low concentration (less than 1 mg/ml) could not be tracked in the cells as the protein had been diluted in the intracellular liquid or extracellular culture medium. Therefore, the findings of this study indicated a slightly higher protein concentration at a range of 1.5-2.5 mg/ml. Nevertheless, too high protein concentration might block the microcapillary or induce osmosis, which could subsequently lead to cell burst.

In summary, the findings have demonstrated the importance of injection pressure in delivering sufficient protein into the cells. MBP protein was visible at high injection pressures (50 and 70 hPa), but it could not be tracked at a reduced injection pressure (30 hPa). The findings have indicated that higher concentration of protein, along with a sufficient injecting pressure, is a prerequisite for successful delivery. However, not all successful injections have shown fluorescence at higher protein concentration, and this is due to protein leakage to culture medium. Therefore, variable factors can pose influence on determination of success rate of cell microinjection at various protein concentrations (0.8 to 2.5 mg/ml).

CONCLUSIONS

This study has demonstrated that the protein concentration ranging from 1.5 - 2.5 mg/ml, at the injection pressures of 50 to 70 hPa, as the most favourable parameters in protein microinjection into human breast cancer cells. These recommended pressure and protein concentration can be starting platforms for the optimization of recombinant MBP-fused protein delivery into adherent cells for cell functional studies.

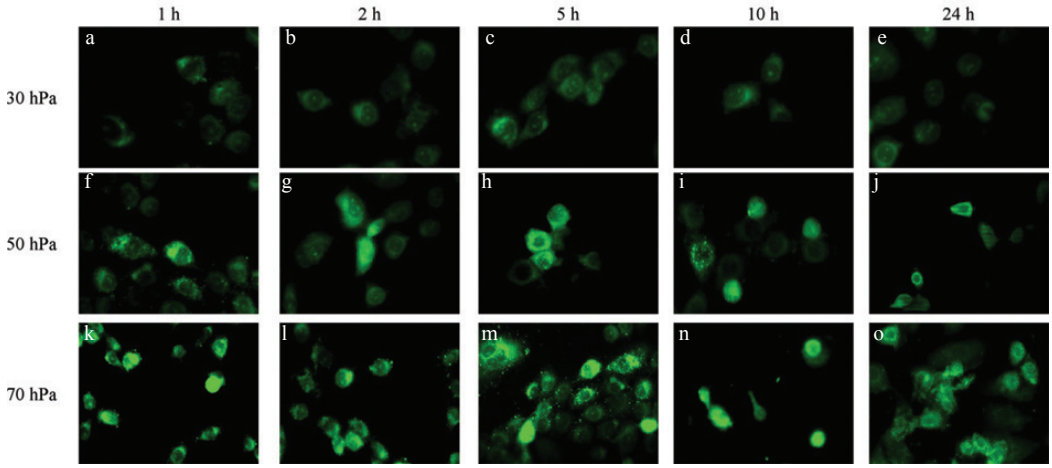

Fig. 5: Indirect immunofluorescence staining of the injected MCF7 cells with 2 mg/ml of the purified MBP protein. The cells which had been injected at 30 hPa (Figs. a-e) showed very little or mild fluorescence signal, whereas 50 hPa (Figs. f-j) and 70 hPa (Figs. k-o) showed strong fluorescence signals

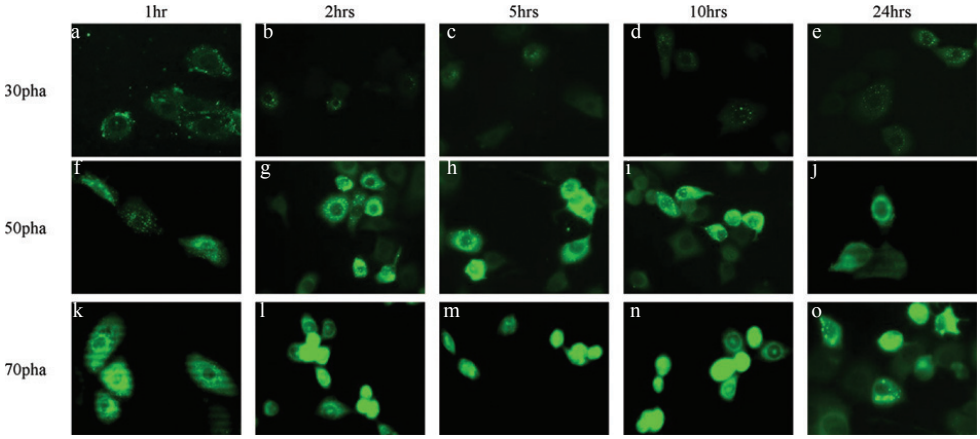

Fig. 6: Indirect immunofluorescence staining of the injected MCF7 cells injected with 2.5 mg/ml of purified MBP protein. The cells that had been injected at 30 Hpa (Figs. a-e) showed very little or mild fluorescence signal, 50 hPa (Figs. f-j) and 70 hPa (Figs. k-o) showed strong fluorescence signal

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REFERENCES

Derouazi, M., Flaction, R., Girard, P., de Jesus, M., Jordan, M., & Wurm, F.M. (2006). Generation of recombinant Chinese hamster ovary cell lines by microinjection. *Biotechnology Letters*, *28*, 373-383.

Feramisco, J., Perona, K., & Lacal, J.C. (1999). Needle microinjection: A brief story. In J.C. Lacal, R. Perosa & J. Feramisco (Eds.), *Microinjection* (pp. 9-15). Switzerland: Library of Congress.

Viigipuu, K., & Kallio, P. (2004). Microinjection of living adherent cells by using a semi-automatic microinjection system. *Alternatives to Laboratory Animals (ATLA)*, *32*, 417–423.

Komarova, Y., Peloquin, J., & Borisy, G. (2007). *Microinjection of protein samples*. Cold Spring Harbour Protocol. Retrieved May 11, 2000 from http://cshprotocols.cshlp.org/cgi/content/full/2007/1/pdb.prot4657

King, R. (2003). Gene delivery to mammalian cells by microinjection. Nonviral gene transfer techniques. *Methods in Molecular Biology*, *245*, 167-173.

Smaili, S.S., Rosenstock, T.R., & Hsu, Y.T. (2008). Evaluation of some cell death features by real time real space microscopy. *Methods in Enzymology*, *442*, 27-50.