Transfer of R-Phycoerythrin into CHO-K1 cells using Fuse-It-P

This protocol describes the standard technique for handling Fuse-It-P, as well as provides instructions on how to design an approach to transiently transfer water-soluble proteins into human cells. A fusion experiment of CHO-K1 cells with Fuse-It-P delivering the protein R-Phycoerythrin is described as an example.

Keywords:
Fuse-It-P; Fuse-It; membrane fusion; protein transfection; proteofection; protein transfer; R-Phycoerythrin; R-PE; live cell imaging; single molecule imaging; molecular tracking

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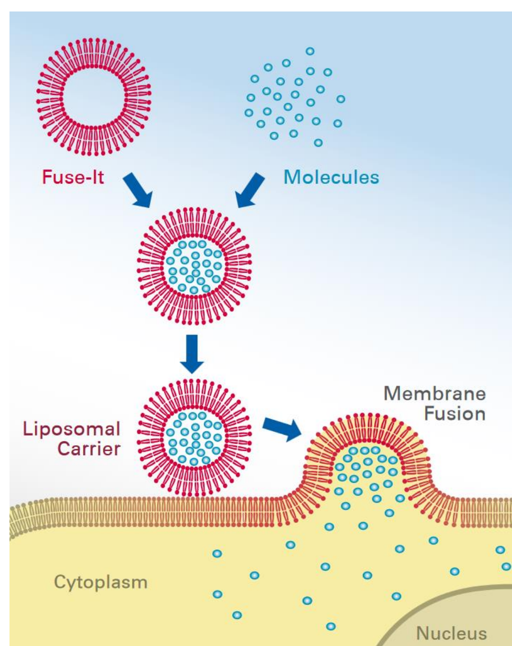
1. Background



Protein transfer using Fuse-It-P is based on fusogenic liposomes. As shown in Figure 1, the Fuse-It liposomal carrier, which includes the soluble protein, simply fuses with the cell membrane and then releases the protein directly into the cytoplasm. Unlike standard transfection protocols, membrane fusion is independent of endocytosis. This reduces cytotoxicity and time to protein expression. It also increases the efficiency of protein expression.

The electrostatic interaction between protein molecules and carrier liposomes is essential for successful transfer using Fuse-It-P. Fuse-It-P carriers are characterized by an overall positive charge, favoring the formation of proteoliposomes for negatively charged proteins.

Figure 1: Simplified illustration of the membrane fusion mechanism of Fuse-It-P.

Proteins describe a highly heterogeneous group of molecules with varying properties in terms of e.g. charge, hydrophobicity, size and structure due to specific amino acid compositions. Many proteins remain fully functional for a large pH range. Proteins are neutral at pH values close to their isoelectric point (pI). When the pH is higher than the isoelectric point, the protein has a negative net charge, and when it is lower, the protein has a positive net charge. Therefore, the net charges of proteins can be altered by

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changing the pH value of the buffer solution. This enables proteoliposome formation for neutral or positively charged proteins.

This Application Note focusses on the protein transfer of the well-known and easily available protein R-Phycoerythrin (R-PE), a fluorescent protein isolated from red algae.

2. Material and Equipment Required

For this protocol, the following materials are needed:

Table 1: Material and reagents needed for the fusion of CHO-K1 cells.

Name	Company	Order No.
Fuse-It-P	ibidi GmbH	60221
µ-Dish 35 mm, high, ibiTreat (3x)	ibidi GmbH	81156
CHO-K1 cells	DSMZ	ACC-110
DMEM-F12 supplemented with 10% FCS	Sigma-Aldrich Chemie GmbH Sigma-Aldrich Chemie GmbH	D8437 F0804
R-Phycoerythrin	ThermoFisher	46185
1 M HEPES (pH 7.0 - 7.6)	Sigma-Aldrich Chemie GmbH	H0887
1x PBS (pH 7.1 - 7.5)	Sigma-Aldrich Chemie GmbH	D8537
Fibronectin	Corning	354008

For this protocol, the following equipment and instruments are needed:

- Ultrasonic bath (power 50 W, frequency 35 kHz)
- Fluorescence microscope equipped with an appropriate filter set for R-PE (fluorescence properties: Ex_{max} 488 nm / Em_{max} 578 nm) and for the infrared fusion control dye (fluorescence properties: Ex_{max} 720 nm / Em_{max} 780 nm), and a stage top incubator (37°C, 5% CO_2)

3. Experimental Procedure

In this Application Note the transfer of R-Phycoerythrin into CHO-K1 cells using Fuse-It-P is optimized. Therefore, three different protein concentrations are tested.

3.1. Defining Protein Properties

The Fuse-It-P system cannot distinguish between proteins of interest and proteins used for stabilization, like BSA. Therefore, dilute the protein of interest in a protein free buffer system, e.g. HEPES. The net charge of the protein has to be determined to successfully transfer proteins by membrane fusion. This can be done by open source programs, e.g. http://pepcalc.com/.

R-Phycoerythrin is a large protein (240 kDa) that is not able to pass through mammalian cell membranes without molecular carriers. It has the following amino acid sequence:
MKSVITTTISAADAAGRYPSTSDLQSVQGNIQRAAARLEAAEKLGSNHEAVVKEAGD
ACFSKYGYNKNPGEAGENQEKINKCYRDIDHYMRLINYTLVVGGTGPLDEWGIAGA
REVYRTLNLPSAAYIAAFVFTRDRLCIPRDMSAQAGVEFCTALDYLINSLS.
It has a negative net charge at pH 7.0 – 8.0. Due to this, the pH values of the buffers (HEPES and PBS) do not have to be adjusted.

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Physiological pH is approximately 7.4 and not all cell types are viable at extreme pH values like pH 4 or 10. For this reason, it is necessary to check the cell viability at the calculated pH.

3.2. Optionally: Coating the µ-Dishes

1. Dilute 18 µl of the stock solution fibronectin (1 mg/ml) in 1182 µl of 1x PBS to get a final volume of 1200 µl. The final concentration of fibronectin is 15 µg/ml.
2. Mix by pipetting.
3. Add 400 µl of fibronectin dilution to each of three µ-Dishes.
4. Incubate 30-40 minutes at 37°C.
5. Remove the fibronectin solution from each µ-Dish.
6. Wash each µ-Dish once with 400 µl of 1x PBS.

3.3. Seeding Cells

1. Seed 60,000-80,000 CHO-K1 cells in each µ-Dish (growth area 3.5 cm²).
2. Incubate the cells for 24 hours at 37°C under standard cell culture conditions.
3. The optical cell confluence at the time of fusion should be 80%.

3.4. Fusion Protocol

The protocol described in this Application Note is already optimized for the transfer of R-Phycoerythrin into CHO-K1 cells. The standard protocol given in the "Fuse-It-P Instructions" should be followed for the first trial of Fuse-It-P.

1. Cool down the ultrasonic bath with an ice pack.
2. Dilute the 1 M HEPES solution 1:50 in sterile water to get a final concentration of 20 mM HEPES.
3. Vortex the R-Phycoerythrin stock solution (4 mg/ml) for 10 seconds and dilute it 1:30, 1:50 and 1:100 in 20 mM HEPES.
4. Add 25 µl of each protein dilution to one Fuse-It-P vial and mix by pipetting until the solution is homogeneous.
5. The lyophilized Fuse-It-P is not resuspended completely in the protein dilutions 1:30 and 1:50. Add 2 µl 1x PBS to the 1:50 dilution and 4 µl 1x PBS to the 1:30 dilution and mix by pipetting until the solution is homogeneous.

> **Important Note:** If the lyophilized Fuse-It-P cannot be resuspended completely, increase the concentration of ions by adding 1-5 µl of 1x PBS.
> Do not warm up the mixture for better solubility.

Figure 2: Resuspension of lyophilized Fuse-It-P vials.
The left images represent a Fuse-It-P solution that is not homogeneous and the right images represent a Fuse-It-P solution that is homogeneous.

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6. Sonicate the mixture in the cooled ultrasonic bath for 10 min.
7. Dilute 25 µl of the fusogenic mixture 1:20 in 1x PBS (pH 7.0) to make a final volume of 500 µl, and mix thoroughly.
8. Sonicate the dilution in the cooled ultrasonic bath for 5 min.
9. Remove the culture medium from the cells and wash once with 1x PBS.
10. Add the diluted fusogenic mixture drop-wise onto the cells.
11. Incubate for 10 min at 37°C.
12. Replace the fusogenic mixture with fresh culture medium to stop the fusion.

3.5. Live Cell Imaging

Image cells by phase contrast and fluorescence microscopy. Use a bandpass filter if possible, to visualize R-Phycoerythrin at its emission maximum of 578 nm. Liposomal fusion efficiency is best detectable by tracking the infrared control dye at 780 nm. Here, live cell images were taken on the Zeiss LSM710, a laser scanning confocal microscope.

Important Note: Keep in mind, that fusogenic liposomes are used to transfer low amounts of protein, preventing overexpression artifacts. Therefore, the fluorescence signals are also low, compared to overexpression of genes.

4. Results

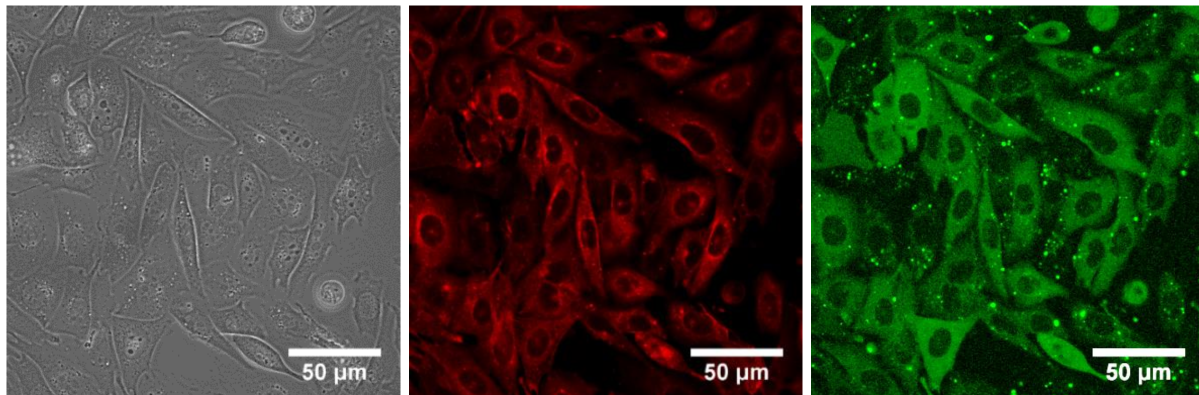

Figure 3: Images of CHO-K1 directly after fusion with Fuse-It-P and a 1:50 dilution of R-Phycoerythrin. The left image represents the phase contrast image. The middle image represents the infrared control dye of Fuse-It-P. The right image represents the fluorescence of R-PE.

The best result was achieved using the 1:50 dilution of R-Phycoerythrin, with a protein transfer efficiency of up to 90%. The intensity of the fluorescent signal of R-Phycoerythrin and transfer efficiency in the 1:100 and 1:30 dilutions were lower, compared to the 1:50 dilution.

These results emphasize the protein concentration dependency of the fusion process. The protein transfer efficiency cannot be increased by elevating the initial protein concentration. Higher concentrations impair fusion and therefore reduce protein transfer by covering the surface of the fusogenic vesicles. Likewise, it is also hard to resuspend the lyophilized Fuse-It-P with protein solutions that have higher concentrations.

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