Facile Preparation of a Fluorescence-Labeled Plasmid

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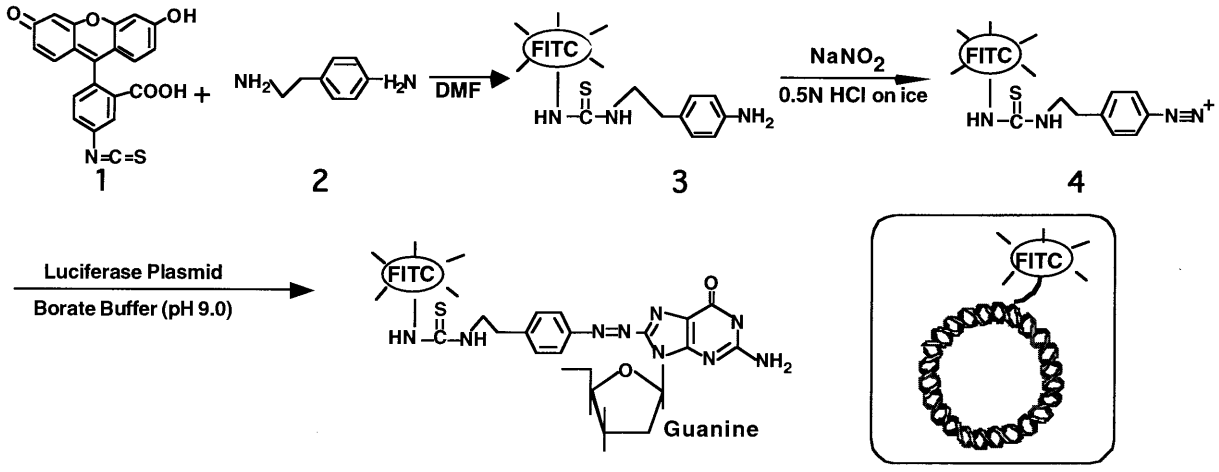
We developed a facile method for labeling plasmid DNA with a fluorescent probe (FITC). The obtained FITC-labeled plasmid kept luciferase activity, and had enough fluorescence intensity to monitor with flow cytometer and confocal laser scanning microscope.

Gene therapy is focused on medical treatment of disease such as cancer, cystic fibrosis and so on.[1] There are mainly two gene delivery systems such as viral and non-viral vector. As non-viral vector, cationic lipids, liposomes, and polymers have been developed to deliver plasmid into mammalian cells.[2] Though their transfection efficiencies have been investigated in many papers, the transfection mechanisms have not been clear yet. For making sure of the transfection mechanism, quantification of cell uptake of plasmid and analysis of intracellular trafficking will be indispensable information. One of the reasons why such experiments are backward is the lack of facile synthesis for a circular plasmid labeled with a fluorescence probe. A Linear DNA has been labeled with FITC at 5'-end.[3] Since circular plasmids do not have reactive 5'-end, a fluorescent probe having azide has been introduced to open chain aldehydes of ribose under UV illumination.[4] In this paper, we developed a more convenient labeling method by using diazonium reaction. The advantage of diazo coupling is that the reaction between 8-position of guanine base and diazonium salt proceeds at mild condition (pH 9.0). It has been reported that diazonium reaction is useful for labeling guanine with biotin[5] and saccharides.[6]

Scheme 1 showed the synthetic procedures for FITC-labeled plasmid. At first, FITC-benzylamine **3** was prepared by reacting fluorescein isothiocyanate **1** (FITC, 0.257 mmol, Dojindo Laboratory, Japan) and 2-(4-aminophenyl)-ethylamine **2** (0.257 mmol, Aldrich) in 1.5 ml of DMF. FITC was used without further purification. This reaction was carried out overnight at 25 °C under stirring. The proceeding of the reaction was monitored by silica gel chromatography. Elution solvent was mixture of hexane and ethyl acetate (2:8, v/v). Spots of compounds **1** (R_f=0.74) and **2** (R_f=0.1) completely disappeared and the new spot of **3** (R_f=0.26) appeared. The structure of compound **3** was analyzed by IR (disappearance of $\nu_{N=C=S}$ (2021 cm^{-1}) and appearance of $\nu_{C=S}$ (1754 cm^{-1})) and MALDI-TOF-MS ([M+H$^+$]=526, Voyager-DE, PerSeptive Biosystems). FITC-diazonium salt **4** was prepared by reacting FITC-aniline **3** (25.7 μmol in 150 μl of DMF) with sodium nitrite (110 μmol) in 2 ml of 0.5 M HCl for 5 min at 0 °C under stirring. The reaction was stopped by adding 1 mL of 1 M NaOH. Next, the solution of FITC-diazonium salt **4** (25 μmol) was mixed with the solution of luciferase plasmid (2 mg) in 15 mL of 0.1 M borate buffer (pH 9.0). The reaction was carried out for 15 min at 25 °C under stirring. The FITC-labeled plasmid was isolated by ethanol precipitation and gel-exclusion chromatography (Sephacryl S-200, Amersham Pharmacia Biotech). The average number of FITC linked to one plasmid was determined by absorbance at 260 nm and fluorescence intensity (Ex=495 nm, Em=520 nm), and was found to be 1.6**.** The yield of FITC-labeled plasmid was 1.6 mg (80%). The higher order structure of the FITC-labeled plasmid was analyzed by 1% agarose gel electrophoresis. As shown in Figure 1, the FITC-labeled plasmid showed both relaxed and super-helix structure of plasmid as same as non-labeled plasmid**.**

Hela cells were transfected with the FITC-plasmid by using cationic liposome (lipofectin, GIBCO) which is commercially available. Figure 2 showed no significant differences in the transfection efficiency between the FITC-labeled and the



Scheme 1. Synthetic procedure of FITC-labeled plasmid.

non-labeled plasmid. Uptake of the FITC-labeled plasmid by Hela cells could be monitored with flow cytometer (EPICS XL, Coulter). As shown in Figure 3, the shift of fluorescent intensity due to the cell uptake of the FITC-plasmid/lipofectin complex was clearly observed. Furthermore, the sub-cellular distribution of the FITC-labeled plasmid in Hela cells was observed with confocal laser scanning microscope (DMIRBE, Leica). Figure 4 showed the clear fluorescence image due to the FITC-plasmid.

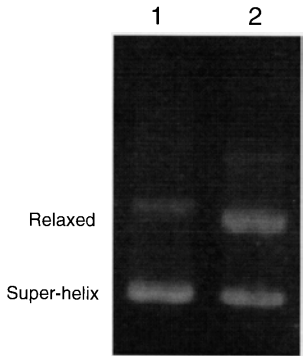


Figure 1. Agarose gel electrophoresis (1%) of non-labeled plasmid (lane 1) and FITC-labeled plasmid (lane 2).

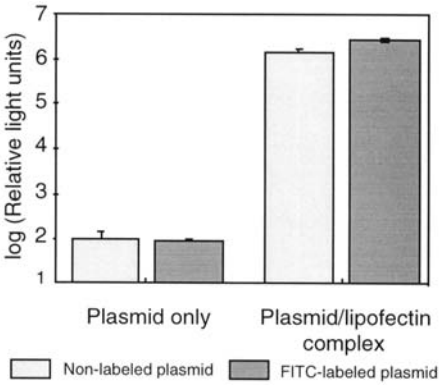


Figure 2. Luciferase activity of FITC-labeled and non-labeled plasmid for Hela cells. Hela cells were transfected with plamid only or plasmid/lipofectin complex (plasmid:lipofectin=1:2.5, by weight, [plasmid]=10 μg ml^{-1}) in serum-free medium (ASF-104, Ajinomoto Co., Inc, Japan)

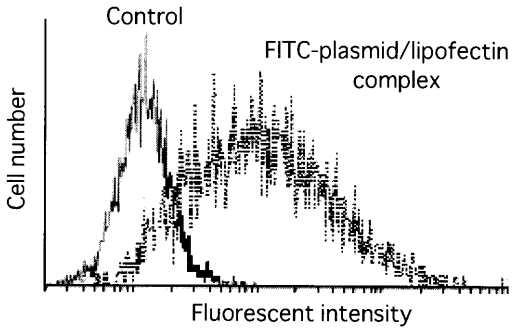


Figure 3. Flow cytometric analysis for uptake of FITC-plasmid by Hela cells. Hela cells were incubated with plasmid/lipofectin complex in eagle MEM containing 10% FBS for 2h (plasmid:lipofectin=1:2.5 by weight, [plasmid]=10 μg ml^{-1}).

Figure 4. Fluorescence images of FITC-labeled plasmid in Hela cells. Experimental condition was the same as that of Figure 3. The outline of Hela cell is drawn with white line.

In conclusion, we developed a facile method for coupling plasmid DNA with FITC by using diazonium reaction. The obtained FITC-labeled plasmid kept the original conformation and the trasfection activity. Furthermore, it was found that the FITC-plasmid synthesized in this paper had enough fluorescence intensity to monitor cell uptake efficiency with flow cytometer and sub-cellular distribution with confocal laser scanning microscope. Thus the FITC-labeled plasmid will be expected to be useful for analyzing the transfection mechanism.

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