Studies on Effects of External Electric Fields on Halobacteria with Fluorescence Intensity and Fluorescence Lifetime Imaging Microscopy

Takakazu Nakabayashi,[1] Hui-Ping Wang,[1,2] Kazuo Tsujimoto,[3] Seiji Miyauchi,[4] Naoki Kamo,[5] and Nobuhiro Ohta*[1]
[1]*Research Institute for Electronic Science (RIES), Hokkaido University, Sapporo 060-0812*
[2]*School of Medicine, Zhejiang University, Hangzhou 310031, P. R. China*
[3]*School of Materials Science, Japan Advanced Institute of Science and Technology (JAIST), 1-1 Asahidai, Nomi 923-1292*
[4]*Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812*
[5]*Faculty of Advanced Life Science, Hokkaido University, Sapporo 060-0810*

(Received February 4, 2008; CL-080132; E-mail: nohta@es.hokudai.ac.jp)

Effects of an external electric field have been measured for *Halobacterium salinarum* loaded with fluorescent dye BCECF. Aggregates of the halobacteria are effectively formed by an electric field. The fluorescence lifetime of BCECF remains unchanged after the formation of the aggregates, suggesting that intracellular environments are not affected significantly by an external electric field in spite of the aggregate formation.

Imaging of fluorescence intensity has been extensively used in biochemical research because it can be applied to cells and tissues under native and physiological conditions. Fluorescence intensity depends on a variety of biophysical and experimental factors such as concentration and optical conditions, whereas fluorescence lifetime is an inherent property of a chromophore and, therefore, independent of photobleaching, excitation power, and other factors that limit intensity measurements. Therefore, imaging of fluorescence lifetime may provide more information on the cellular microenvironment in live cells.[1–5]

We have recently constructed a system for fluorescence lifetime images and applied it to *Halobacterium salinarum* (*Hb. salinarum*) loaded with 2′,7′-bis-(carboxyethyl)-5(6)-carboxyfluorescein (BCECF).[5–7] *Hb. salinarum* belongs to the family of extreme halophilic archaebacteria and has been extensively studied in relation with the proton transport, phototaxis, or the adaptation of organism to extreme environments.[5,7–10] BCECF is one of the most used fluorescent dyes for evaluation of pH because of its highly pH-dependent fluorescence intensity.[11] It was shown that information on the intracellular property as well as the intracellular pH of the halobacteria can be obtained from the fluorescence lifetime of BCECF in cells.[5,7]

In the present study, we have measured the images both of the fluorescence intensity and of the fluorescence lifetime of *Hb. salinarum* after the application of an external electric field. BCECF was employed for a chromophore loaded in the halobacteria. Application of external electric fields to biological cells allows modulations of membrane permeabilization and intracellular environments. Application of electric fields to cells, therefore, has been widely used in chemical biology for gene transfection, drug delivery, and modulation of intracellular ion concentrations;[12–14] however, the detailed mechanisms of such electroperturbation processes remain unclear. We have applied an external electric field to *Hb. salinarum* loaded with BCECF and measured the shape and the intracellular environment of the halobacteria using the fluorescence of BCECF inside the cell.

The acetoxymethyl (AM) ester-loading technique was used for loading BCECF in *Hb. salinarum*.[5,7,8] The strains of *Hb. salinarum*, S9, were grown as previously reported.[15] A 10-cm^3 cell suspension was incubated with a 10 mm^3 of DMSO solution of BCECF/AM at 1.0×10^{-2} mol dm^{-3} in the dark at 18 °C for three days. The suspension of the halobacteria was washed out with the basal salt solution (4 mol dm^{-3} NaCl containing 2.5×10^{-2} mol dm^{-3} HEPES) at pH 6.8. Then, the dye-loaded halobacteria were resuspended in the basal salt solution at pH 7.0 on a 55-mm glass bottom dish.

Measurements of fluorescence lifetime images were carried out using a four-channel time-gated detection system.[1,5,6] The second harmonic of the output from a femtosecond mode-locked Ti:sapphire laser (450 nm) was used for excitation, and the fluorescence decay was measured in each pixel of the confocal microscope with a time-correlated single-photon counting method. To minimize the data generated, the time-gated detection system captures the fluorescence decay trace into four time-windows. Each fluorescence lifetime was evaluated by analyzing the four time-gated signals by assuming a single exponential decay, and the fluorescence lifetime image was obtained. We inserted two stainless steel electrodes into the cell suspension and applied a DC field between them. The halobacteria in the middle of the electrodes were observed. The distance between the electrodes was 3.2 cm.

Figure 1 shows the time course of the fluorescence intensity and the corresponding fluorescence lifetime images of *Hb. salinarum* loaded with BCECF. The applied field was 0.25 V cm^{-1}. Fluorescence in the 515–560-nm region was detected.[7] The distribution of the fluorescence lifetime over the whole cells in each image is also shown in the right of Figure 1. As shown in Figure 1A, the halobacteria were homogeneously dispersed in the basal salt solution, and the fluorescence lifetime of each bacterium could be identified in the lifetime image before application of an electric field. The halobacteria have the cylindrical shape with a long axis of 1–5 μm. The distribution of the fluorescence lifetime in Figure 1A exhibits a peak at ≈2.4 ns.

However, it is found that aggregates of the halobacteria are effectively formed after exposure to an electric field. As shown in panels A and B in Figure 1, the aggregate becomes larger as the passage of exposure time, and the aggregate larger than 100 μm is formed at 100 min after the exposure (see Figure 1C). The rate of the aggregate formation becomes much faster at higher external fields, e.g., at 0.8 V cm^{-1}. The halobacteria cannot clearly be seen at electric fields larger than 1.0 V cm^{-1}, which is probably due to destruction of the halobacteria in the presence of high electric fields. It is noted that not all of the halobacteria are the same in panels A–C in Figure 1 because of movement of the halobacteria, but the aggregates

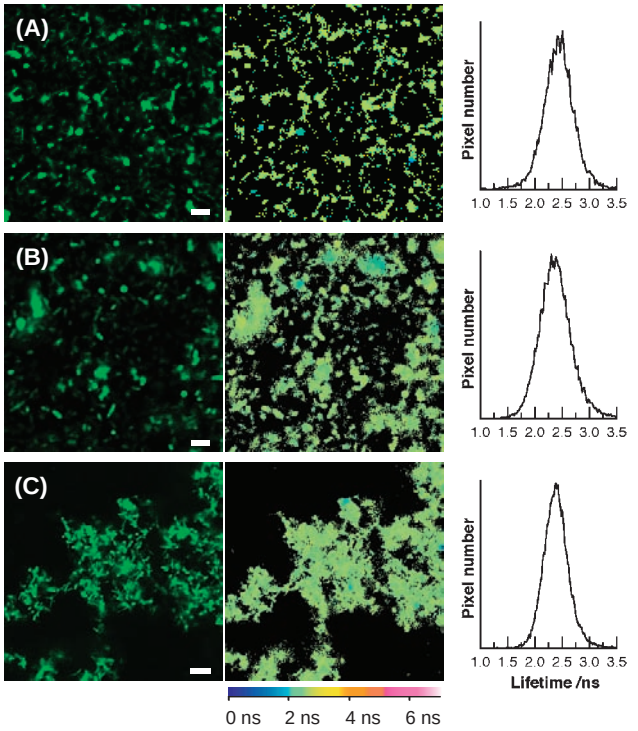

Figure 1. Fluorescence intensity images (left) of BCECF-loaded *Hb. salinarum* and the corresponding fluorescence lifetime images (middle) and the distributions of the fluorescence lifetime (right) at 0 (A), 60 (B), and 100 min (C) after exposure to an external electric field. The applied voltage was 0.8 V. The lifetime values are presented using the color scale in the bottom of the figure. Scale bar is 10 μm. Excitation wavelength was 450 nm. Fluorescence in the 515–560 nm was detected.

are observed in any regions around the middle of the electrodes. The cylindrical shape of the halobacteria seems to remain unchanged even after the formation of the aggregates.

The field-induced morphological change is clearly observed in the fluorescence intensity image, but the peak position of the fluorescence lifetime distribution remains constant at around 2.4 ns during 100 min after the exposure (see Figure 1). Intracellular pH is the important factor to understand states of cells because marked changes in pH inside a cell occur with many cellular functions.[16] The present result indicates that the halobacteria mostly exhibit the fluorescence lifetime of ≈2.4 ns regardless of exposure time; pH in the cell is essentially the same irrespective of the aggregate formation. It should be stressed that we analyze the fluorescence lifetime of the generated aggregate, not the lifetime change induced by the electric field of 0.25 V cm^{-1}.

We have previously shown the existence of the two halobacteria species exhibiting different fluorescence lifetimes of BCECF from each other: a small amount of the halobacteria exhibits the lifetime as short as 1.9 ns, while most of the halobacteria exhibit the lifetime at around 2.4 ns.[5] In the present study, the halobacteria having a short fluorescence lifetime of ≈1.9 ns are observed in the lifetime image at 0 min in Figure 1A, as presented by the blue color in the image, and still exist at 100 min after the exposure (see Figure 1C). Thus, it is concluded that the effect of the field-induced aggregate formation on the fluorescence lifetime of BCECF is very small both for the short and long fluorescence lifetimes.

The fluorescence lifetime of BCECF is influenced by various environmental factors such as pH, ion concentrations, and electric fields inside a cell.[5,7] The observation of two halobacteria species with short and long fluorescence lifetimes arises from the fact that the cells have different intracellular environments from each other. It is suggested from the electrofluorescence spectrum of BCECF that the observed difference in the fluorescence lifetime is ascribed to the difference in electric fields inside a cell.[5] Charged and polar groups of membranes and peptides of proteins cause electric fields that affect the photoexcitation dynamics of the chromophore.[5] The present negligible effect of the aggregate formation on the fluorescence lifetime, therefore, indicates that intracellular environments of the halobacteria remain unchanged after the formation of the aggregates.

In conclusion, we have measured external electric field effects on *Hb. salinarum* loaded with BCECF. The aggregates of the halobacteria are formed by an electric field, while intracellular environments remain unchanged for exposure to an electric field. Electroporation seems to be important for the aggregate formation; however, the generated pore should be small because of the preservation for the intracellular environments. We are now in progress to control morphological changes of the halobacteria with electric field pulses of short duration.

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