Biological Effects of Narrow Band Pulsed Electric Fields

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ABSTRACT

This paper describes the process of narrow band pulsed electric fields (NPEFs) and its effect on mammalian cells. The NPEF consists of a pulse modulated sinusoidal wave (PMSW), which allows delivery of well-defined electric fields in terms of frequency, field strength and deposition energy to the biological systems. 100 μs long sinusoidal electric fields with a frequency of 0.02, 2 or 50 MHz and field strengths of up to 2 kV/cm are applied to CHO cells with variation in the DNA density in the cells investigated by means of Acridine Orange assay. The experiments indicate that 50 MHz fields cause DNA degradation without cell membrane defects, while 0.02 MHz fields lead to an increase in membrane permeability which is similar to the effect known as electroporation. The intermediate frequency of 2 MHz influences both the membrane and DNA. It is demonstrated that the MHz range narrowband electric fields with the amplitude level of 1 kV/cm cause intracellular effects in mammalian cells.

Index Terms – **Narrow band pulsed electric field (NPEF), pulse modulated sinusoidal wave, CHO cell, intracellular effect, electroporation, DNA degradation, Acridine Orange assay**

1 INTRODUCTION

BIOLOGICAL effects of intense pulsed electric fields (PEFs) have been reported over the past three decades. Neumann firstly reported in 1972 permeability changes induced by PEFs in membrane [1]. Zimmermann explained the permeability changes as a pore formation of membranes due to its electrical breakdown, i.e. electroporation [2]. Since then there have been a number of studies on the mechanism of electroporation and its applications to gene transfection [3], cell fusion [4] and medical treatments [5]. PEFs with a pulse length of longer than 10 μs are generally used for electroporation because the cell membrane acts as a capacitor and has to be charged to a sufficient voltage to cause membrane defects. Schoenbach et al have started using nanosecond pulsed electric fields (nsPEFs) and reported their effects on various kinds of biological cells over the past decade [6-12]. Application of nsPEFs to biological cells results in intracellular effects with the intense electric field inside the cell seemingly adding a new stress to the internal biological system which will potentially be used for biotechnology and medical treatment. Also Vernier et al have reported the unique biological effects of nsPEF [13-14]. Recently, Schoenbach et al demonstrated that nsPEFs could cause melanomas in mice to self-destruct [12], which is a forerunner to practical medical application. The intracellular effects of the nsPEFs are a result of the high frequency components exceeding the MHz range which passes through cell membrane and directly stimulating the DNA and/or intracellular organelles. Based on the fact that cells are a complex of dielectric materials, the intracellular effects are expected to depend on frequency, field strength as well as input energy of the applied electric fields. Frequency components of a sub-hundred ns long rectangular pulse is widely distributed from zero to hundreds of MHz. Electric fields with narrowband frequency spectra enable us to discuss more clearly the frequency dependence of the biological effects.

There are numerous experimental and numerical studies on radio frequency (RF), electric fields irradiations on biological systems such as various kinds of cells, animals, and even on human bodies [15-16]. Most of these studies have used continuous RF fields and concluded that RF fields mainly result in thermal effects. There also are some studies on millimeter waves (> 30 GHz), which possibly show non-thermal effects on biological systems [17]. The electric field strengths of such continuous ac fields are as low as 1 kV/m, which might not give a significant moment

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to intracellular molecules [18] because the continuous intense ac electric fields heats up the cells and/or the suspending medium. Very few studies using pulsed high power microwaves were reported [19-20].

Here, we propose a narrowband pulsed electric field (NPEF) consisting of a pulse modulated sinusoidal wave (PMSW) to deliver well-defined electric fields to the biological cells with a minimum temperature increase. The PMSW allows to independently control physical parameters of electric fields to be given to the biological cells, such as frequency, strength and time duration. This paper describes the process of the NPEF and the responses of cultured biological cells to the NPEF with frequencies of 0.02, 2 or 50 MHz, and with field strengths of up to 2 kV/cm. The effects of the NPEF on biological cells in the suspending medium are discussed on the basis of Acridine Orange assay showing the temporal variation in DNA density distribution in the cells after the NPEF exposure.

2 MATERIALS AND METHODS

2.1 ELECTRICAL SETUP

Figure 1 shows the NPEF application system, which consists of a pulse generator, a signal generator, an amplifier, on-slide electrode set and fluorescent microscopy (Nikon ECLIPSE E600). Sinusoidal signals are generated by a signal generator (HP8116A, dc to 50 MHz) and the duration of the signals is determined by the pulse length from a pulse generator (Berkeley Nucleonics, BNC555). The amplified signals from an amplifier (EM Power model 2005, 10 kHz ~ 230 MHz, 50 dB) are delivered to the on-slide electrode through a 50 Ω coaxial cable. Duration of NPEF is fixed at 100 μs in order to minimize the temperature increase of the cell suspending medium. Figure 2 shows a time domain waveform (a) and the energy spectrum density (b) at a resistive load (50 Ω) for the burst sinusoidal electric field with 2 MHz, 100 μs duration and 1 kV/cm. The black box in Figure 2a contains 200 cycles of sinusoidal wave. In comparison, the energy density of NPEF at 2 MHz is two orders larger than that of a rectangular pulse with a 100 ns long rectangular pulse with 22.4 kV/cm amplitude, which is the same resistive energy as the above mentioned NPEF.

A 20 μm-thick silver layer is metal-plated on a slide glass forming a parallel electrode gap for microscopy. The electrode gap is approximately 120 μm, which allows application of average electric fields up to 35 kV/cm. Figure 3 shows the electric field distribution around the electrode gap, which is calculated using a two-dimensional finite element solver under ac electric fields (PAC/TriComp, Field Precision). The curves indicate equivalent potential surfaces and the differentiation between the curves is 0.83 V under the condition shown in Figure 3. The variation of the electric field strength in the conductive solution (ε_r = 80, ρ = 10 Ωcm) is 5%, while the

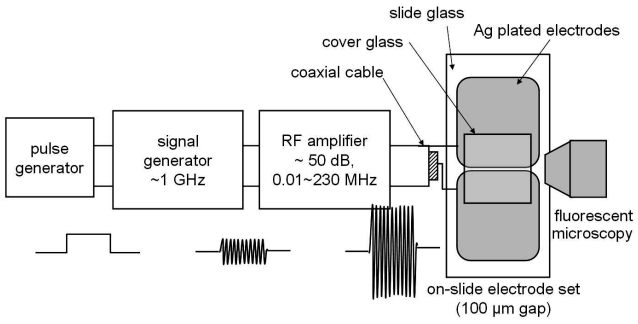

Figure 1. Schematic of the experimental setup. The system consists of an NPEF generator, on-slide electrodes, and a fluorescent microscopy.

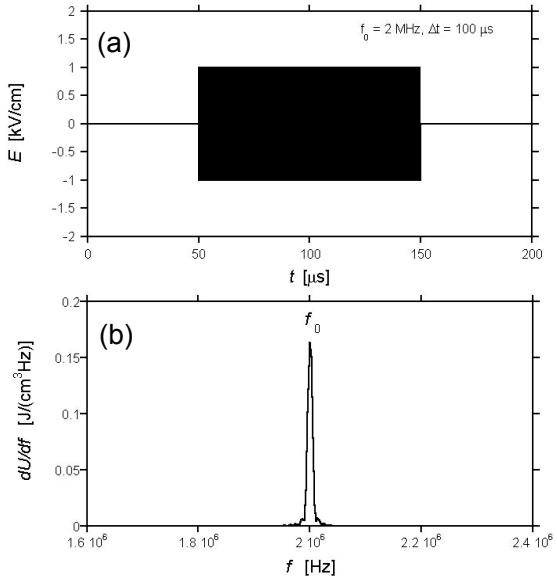

Figure 2. Waveforms of the NPEF with a frequency of 2 MHz, amplitude of 1 kV/cm and duration of 100 μs. (a) is the time domain waveforms and (b) is the frequency domain energy spectrum density of (a). Sampling time is 200 μs.

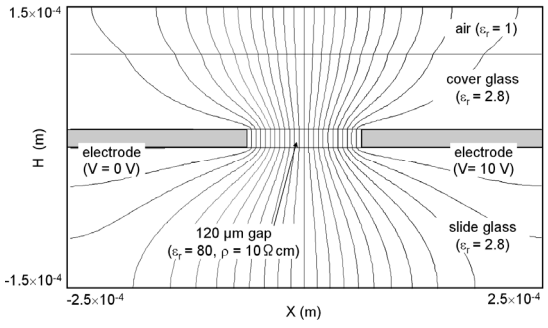

Figure 3. Calculated equivalent potential lines in the vertical plane perpendicular to the electrode surface. Potential difference between curves is 0.833 V.

field enhancement is limited near the electrode edges inside the glass plate. The field strength in the central region of the gap is 0.83 kV/cm that is almost the same as the applied voltage divided by the electrode separation. Note that the field strength near the electrode is probably larger than the

calculated value because the roughness of the electrode surface enhances the field strength in the medium. The electrode width is 10 mm with a resulting gap resistance, including cell suspension, of approximately 50 Ω. The capacitance is approximately 1.5 pF, which is negligible for the experimental frequency range. The voltage between the electrodes is monitored by a voltage probe (Tektronics, P5100, 250 MHz).

2.2 BIOLOGICAL PROCEDURE

The temporal change of DNA density in biological cells after the NPEF application is recorded by means of an Acridine Orange assay [21]. All experiments are conducted using Chinese Hamster Ovary (CHO) cells cultured with αMEM medium in a 5% CO_2 incubator. Cells are incubated routinely every two days before the cell culturing condition becomes 90% confluent. The cultured cells are washed twice using tris buffered saline (TBS), and re-suspended to 1×10^6 cell/ml with αMEM medium. Acridine Orange (AO) is used as a fluorescent nucleic acid dye that is permeable to living cells and stains the DNA and RNA. AO stained double strand DNA (dsDNA) and RNA fluoresce in green. AO also stains single strand DNA (ssDNA), which fluoresces in orange. The intensity of green fluoresces is dependent on the density of AO molecules bound to nucleic acid providing quantitative information of normal nucleic acid.

The experimental procedure is as follows. After adding AO solution to the electrode gap, a quantity of 10 μl of medium, which includes approximately 50 cells, is added to the electrode gap, and then a cover slip is placed on the medium. After the slide is fixed to the microscope stage, the electrodes are connected to the NPEF system via a coaxial cable. It takes approximately 3 minutes to prepare the slide. Both bright field and fluorescent images are taken in seven phases while the NPEF is applied to the cells. One is taken prior to the NPEF exposure and the other six are after exposure, each at 1, 2, 3, 5, 7, and 10 minutes. The microscopic observation is performed as quickly as possible (~ 20 seconds) to avoid damaging cells due to the excitation light (488 nm) of the fluorescent microscope.

3 RESULTS AND DISCUSSION

Figure 4 shows the fluorescent image of CHO cells placed between the electrodes on a slide glass. The observation focuses on cells more than 20 μm from the electrodes in order to avoid taking in account cells that receive higher electric fields than the average one, as discussed in Section 2.1. In the case of Fig. 4, we exclude three cells near the left hand side electrode from the observation. Figure 5 shows the temporal development of the fluorescent images of CHO cells during the 10 minutes after the NPEF exposure.

Figures 5a, 5b, 5c and 5d represent typical cell responses to the control and the NPEF exposures for three different frequencies of 0.02, 2 and 50 MHz, respectively. The NPEFs of 0.02, 2 and 50 MHz, respectively contain 2, 200 and 5000 sinusoidal wave periods during the 100 μs pulse. The DNA is mostly localized in the nucleus and mitochondria, and there is some in cytoplasm. Also there exists RNA in the cytoplasm, which has a similar structure to dsDNA. Therefore, the fluorescence comes not only from the nucleus but also the cytoplasm. No obvious change is observed during the pulse exposures. In the case of 0.02 MHz, as shown in Figure 5b, the fluorescent image at 5 minutes shows leakage of intracellular contents out of the cell. Also the fluorescent intensity in the cytoplasm gradually fades away, which is probably due to the leakage. In contrast, in the case of 50 MHz, the fluorescent intensity decreased quickly after exposure not only in the cytoplasm but also in the nucleus. As we described in Section 2.2, AO molecules interacts dsDNA by the intercalation and fluoresces in green. The decrease in the fluorescent intensity indicates dsDNAs degradation. Also the gradual

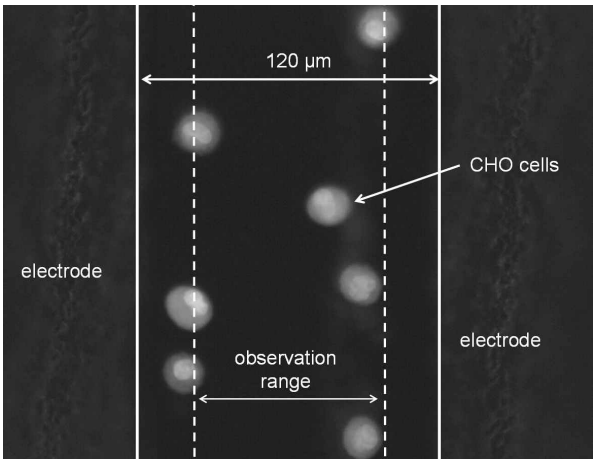

Figure 4. Fluorescent image of CHO cells between the on-slide electrodes. The electrode separation is approximately 120 μm. Two broken lines indicate the positions 20 μm from the electrodes. Cells between the broken lines were the targets used for observation.

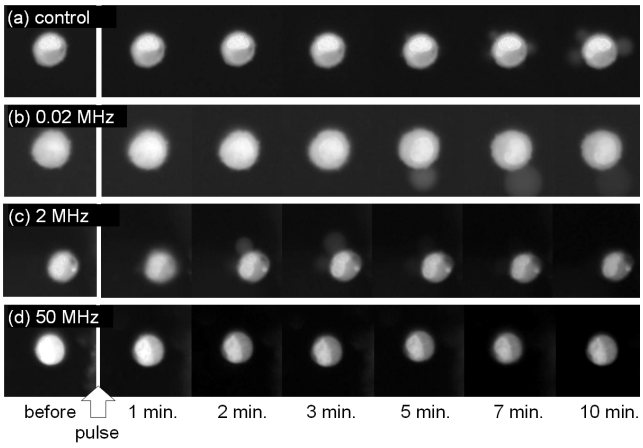

Figure 5. Temporal variation of fluorescent images of CHO cells before and after exposure to NPEF. (a) control, (b) 0.02 MHz, (c) 2 MHz and (d) 50 MHz. The electric field was fixed at 1.2 kV/cm.

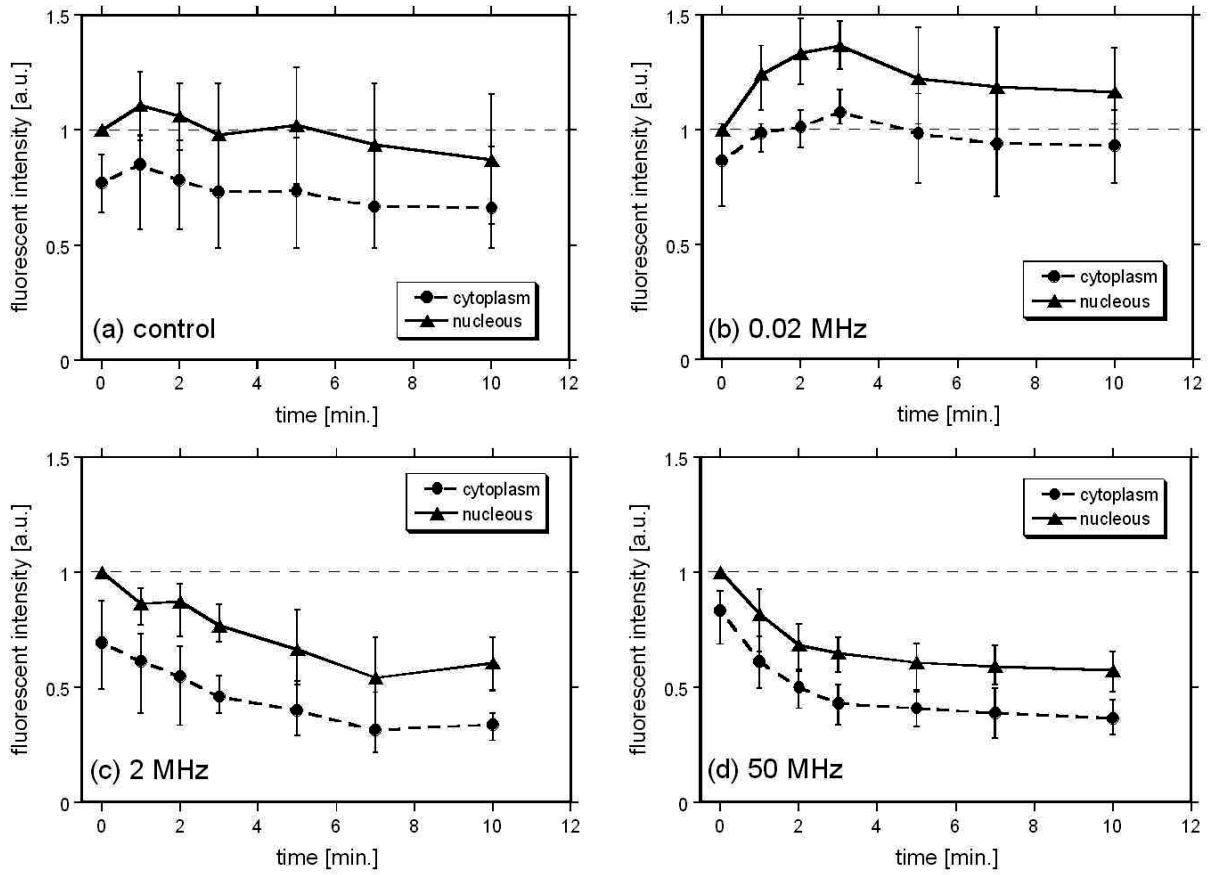

Figure 6. Temporal variation of the florescent intensity in both the nucleus and cytoplasm. (a) control, (b) 0.02 MHz, (c) 2 MHz and (d) 50 MHz. The electric field was fixed at 1.2 kV/cm.

decrease in the fluorescent implies NPEF might not break DNAs directly but might activate certain biological mechanisms to break DNA. Although we do not know what occurs in this process, we call in this paper the apparent decrease in the fluorescent intensity the DNA degradation.

Figure 6 shows the temporal variation of the fluorescent intensity for the control (a), NPEF with the frequency of 0.02 MHz (b), 2 MHz (c) and 50 MHz (d). Triangles and circles in each graph indicate the intensity averaged over the nucleus and cytoplasm, respectively. The intensity is normalized by the value of nucleus in the image taken prior to the electric field exposure. There are five trials for each condition. The control, cells without the exposure, shows a slight increase in fluorescent intensity during observation. This might be because the AO molecules are still penetrating into the cell during the observation. The cell responses for 0.02 and 50 MHz NPEF exposures appear quite different. The fluorescent intensity increases just after the 0.02 MHz NPEF exposure, then gradually decreases afterwards, especially in the cytoplasm. The momentary increase in fluorescent intensity after the 0.02 MHz exposure may be caused by the increase in density of AO molecules penetrating into the cell through the membrane with increased permeability due to the exposure. The subsequent decrease in intensity is possibly caused by the leakage of the intracellular contents back through the membrane. In the case of 50 MHz, the fluorescent intensities in both nucleus and cytoplasm decreased quickly. Since the characteristic time scale of the decrease in the fluorescent intensity is approximately 3 minutes that is much shorter than the time for the apoptotic DNA fragmentation process in the range of hours. The 50 MHz NPEF seems to activate the mechanisms of a rapid DNA degradation process. At the frequency of 2 MHz, the intermediate frequency between 0.02 and 50 MHz, the intensity fades more slowly than that in the case of 50 MHz. Leakage through the membrane is also often observed in this case.

Figure 7 shows the ratio of cells exhibiting leakage during the 10 minutes after the pulse exposure. Leakage occurrences are determined by the fluorescent images. Figure 7 indicates that 0.02 and 2 MHz NPEF causes membrane defects, while 50 MHz NPEF does not influence the membrane significantly. The increase in the permeability of the cell membrane by the 0.02 MHz NPEF exposure is assumed to be due to electroporation. For negligible membrane conductance, the charging time τ_c of the spherical cell membrane is given by.

$$\tau_c = \left(\frac{\rho_1}{2} + \rho_2 \right) C_m a \qquad (1)$$

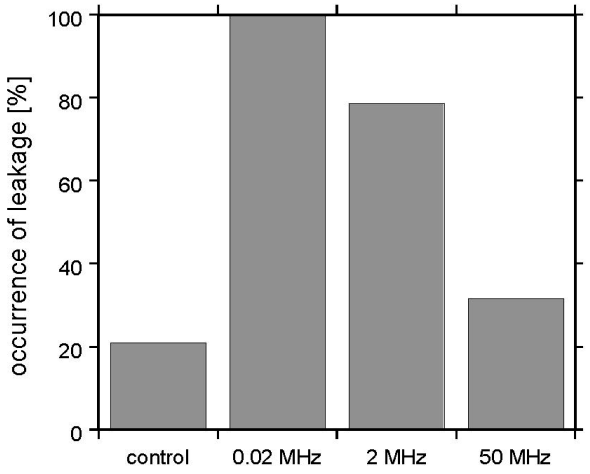

Figure 7. Ratio of the number of cells showing leakage of the cell contents for the control and three different frequencies of 0.02, 2 and 50 MHz. The electric field was fixed at 1.2 kV/cm.

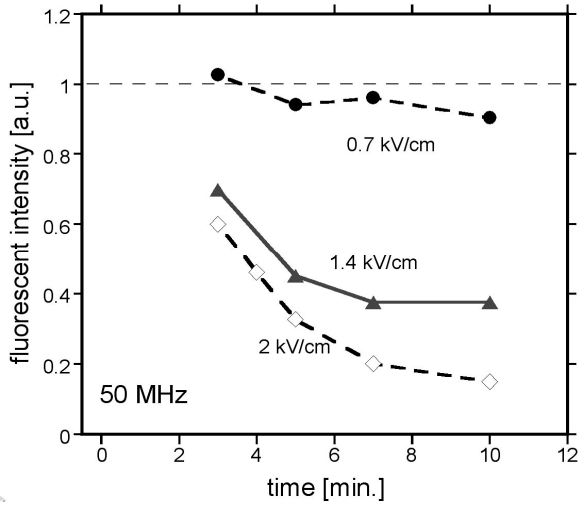

Figure 8. Temporal variations of the fluorescent images of CHO cells after NPEF exposure for different field strengths of 0.8, 1.4 and 2 kV/cm. The frequency was fixed at 50 MHz.

where ρ_1 and ρ_2 are the electrical resistivities of suspending medium and cytoplasm, respectively, C_m the capacitance of the membrane per unit area, and a the cell diameter [7]. τ_c for spherical cells with a diameter of tens of μm, such as CHO cells, is on the order of 100 ns which is much smaller than the characteristic time of the electric filed variation in the case of 0.02 MHz. Since the diameter of CHO cells is approximately 25 μm, a voltage of 2.5 V takes place across the cell under an electric field of 1 kV/cm. This voltage is fully built up across the membrane and sustained for a sufficiently long time to form pores in the membrane. Other parts of the cell are almost free of the electric field. In the case of 50 MHz, on the other hand, the membrane cannot be charged up because the electric field varies much faster than τ_c, and the electric field is distributed uniformly throughout the cell. In the case of 2 MHz, because τ_c is comparable to the electric field variation, cell membrane is charged up to some extent, resulting in the pore formation. The slower decrease in the fluorescent intensity in the case of 2 MHz can be the AO molecule penetration promoted by the membrane defect, which might be superposed on the intracellular effect shown in the case of 50 MHz. The AO assay is no longer sensitive to show the frequency dependence of the intracellular effect. DNA damage analysis using an electrophoresis is expected to show more detailed information on the frequency dependence.

The rapid temperature increase of the cell suspending medium due to a 1.2 kV/cm and 100 μs long PMSW is calculated to be 0.3 ºC. However, the higher conductivity of cytoplasm, which is approximately 100 Ωcm, might cause the higher temperature increase than that of the medium. This heat shock can be a stress to the cells in addition to the effect of electric fields.

The response of CHO cells exposed to 50 MHz and 1 kV/cm NPEF is quite similar to those of Leukemic cells exposed to nanosecond long rectangular PEFs [21]. However, the rectangular pulses need much higher field strength exceeding 10 kV/cm to cause the decrease in AO fluorescence. Because of the wideband spectra of the rectangular pulses, the electric field is scattered over the cell, while the energy of the NPEF might be localized on a certain organism or structure.

Figure 8 shows the response of CHO cells to the NPEFs for different electric field strengths. The frequency is fixed at 50 MHz. At an electric field of 0.7 kV/cm. No notable change in the fluorescent intensity was observed for at least 10 minutes after the exposure. Obviously electric fields exceeding 1.4 kV/cm cause the rapid decrease in the fluorescent intensity. With a higher electric field of 2 kV/cm, the fluorescent fades away more quickly. Since the electrical energy of the pulse penetrates into the cells in 50 MHz range, both the effects of the electric field and the heat shock are increased with increasing the field strength.

Thus we conclude that the rapid process of the DNA degradation is activated by the MHz range NPEF with the strength exceeding 1.2 kV/cm. This rapid process of the DNA degradation finally results in the cell death. Also it would be interesting to investigate biological reactions triggered by the field strength below 0.5 kV/cm, which does not cause the rapid process of the DNA degradation.

4 CONCLUSIONS

We experimentally showed the responses of mammalian cells to the narrowband pulsed electric fields consisting of pulse modulated sinusoidal waves. The experiments show that tens of kHz range electric fields affect cell membranes, while 50 MHz range fields cause the DNA degradation without the membrane defects. The field strength in the level of 1 kV/cm is sufficiently large to cause intracellular effects. The pulse modulated sinusoidal electric field is a favorable scheme to control the biological effects.

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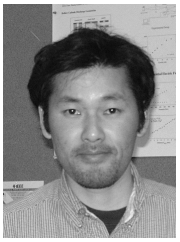

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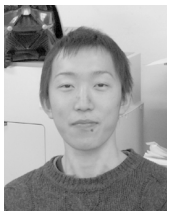

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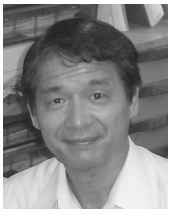

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