Study of Biological Effect of MC3T3-E1 *in vitro* by A Novel 2.4GHz Radiofrequency Electromagnetic Field Exposure System

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Abstract — A 2.4GHz mobile-phone/blue-tooth radiofrequency electromagnetic field (RF-EMF) exposure system for cellular experiment MC3T3-E1 *in vitro* was designed to study the biological effects and demonstrated in this report. Experimental results have illustrated the observation of signal at 14Hz through algorithm analysis of the measured near field magnetic fluctuation and the modulation of gap junction intercellular communication (GJIC) within mouse osteoblast cells (MC3T3-E1) under RF-EMF power density at 0.3mWatt/cm^2. [Life Science Journal. 2009; 6(4): 41 – 49] (ISSN: 1097 – 8135).

Keywords: GJIC, Near Field, RF-EMF

1. Introduction

Mobile telephones, television and radio transmitters and radar produce RF fields. These fields can be used to transmit information over long distances as well as radio and television broadcasting all over the world. Microwaves are RF fields at high frequencies in the range of GHz range. At radio frequencies, electric and magnetic fields are closely interrelated and typically we measure their levels as power densities in watts per square centimeter (W/cm2). Bluetooth provides a way to connect and exchange information between devices such as mobile phones, telephones, laptops, personal computers, printers, Global Positioning System (GPS) receivers, digital cameras, and video game consoles through a secure, globally unlicensed Industrial, Scientific and Medical (ISM) 2.4 GHz short-range radio frequency bandwidth. The Bluetooth specifications are developed and licensed by the Bluetooth Special Interest Group (SIG). The Bluetooth SIG consists of companies in the areas of telecommunication, computing, networking, and consumer electronics [1,2]. Bluetooth devices are divided into three power classes with a maximum output power of 100 mW (class 1), 2.5. mW (class 2), and 1 mW (class 3) and transmit distances ranging from 10 to 100 meters [3]. Both scientific and public concerns have been raised about the potential health impacts from regular use of microwave mobilphone [4]. Accordingly, even through numerous studies have reported no significant relationship between mobile phone use and health, a lot of scientists still have been dedicated research in the years on the possible and real effects of RF-EMF on biological system [6,7,8,9]. From our previously experiments cellular signal may be induced from the exposure of RF-EMF due to the outcome that we found a small power signal embedded in the ambient near field magnetic fluctuation [10]. Essentially, biological signal embedded in ambient fluctuation being found is not a surprising, such as cell membrane current is 10^{-15} ampere [11]. This signal is comparatively very much even less than the amount of sharp noise current from operation of the switch of electrical equipment when measuring the current [12]. As we know, ambient EMF fluctuation originates from a self-propagating wave in space, such as earth or through active devices. Not only EMF has an electric and magnetic field components, which oscillate in phase perpendicular to each other and to the direction of energy propagation, but also can be classified into types according to the frequency of the waves; these types

include radio waves, microwaves, terahertz radiation, infrared radiation, visible light, ultraviolet radiation, X-rays and gamma rays. Our previously experiments revealed that cells in vitro should be affected by electromagnetic field and the GJIC being modulated. Basically, GJIC is an indicator to express the cell's response affiliated with many pathological endpoints [13], such as cell aging, apoptosis, proliferation and differentiation, are associated with the modulation of GJIC. To measure GJIC modulation, scrape loading dye transfer of Lucifer yellow [13] was used in this report. The degree of modulation of GJIC can show the degree of cellular response to the EMF exposure. Therefore, in this report, we assured the impact of GJIC caused by RF-EMF by using a novel exposure system for the experiments. Previously, a proposed microstrip patch antenna providing a RF-EMF at 2.4GHz to the cultured rat liver epithelial cells with observable modulated GJIC was published in reference [14]. In this work, a more customized microstrip patch antenna providing a RF-EMF at 2.4GHz to the cultured mouse osteoblast cells with modulated GJIC was proposed for further discussion of the EMF impact to the cells *in vitro*. An inexpensive FR4 plate, a print circuit board (PCB) was used in convenience [15] for mounting the cell co-culture dish to the antenna. A customized designed check-shape radiator was constructed for measuring gain and justifying the transmitting power. The scanning probed near field magnetic fluctuation was recorded to correlate the GJIC modulation within the cells in GJIC assay by the exposure RF-EMF at 2.4GHz [16]. Usually, possible EMF sources that may cause the biological effects include the ambient field [17, 18], cells internally resonant signals, and the RF-EMF originated near field cross polarization fluctuation etc. are equally important to the health impact study of the EMF related biological effect concerns. In this work, we adjusted the size of the ground of the antenna to reduce the perturbation of the cross-polarization [19] and the power as well as the gain. Also, since scientists have suggested a lot of possible interaction models to explain the experimental results and the biological effects caused by RF-EMF as well as ELF interaction [20], we proposed a new model combining design of a customized microstrip antenna with special algorithm and the cellular assay of GJIC for assuring the biological effect being found. Even through in still, controversial outcome remains the conclusion in ambiguity.

2. Configuration of the Customized 2.4GHz RF-EMF Transmitter

The customized 2.4GHz transmitter (a microstrip antenna) mounted with a co-culture dish depicted in Fig.1. In the design, a V-shaped radiator provides RF-EMF at 2.4GHz. Cultured cell dish can be mounted on the slotted patch between the end point of V-shape radiator and the FR4 plate by two plastic clips to avoid the perturbation of the signals. A T-type patch ground was printed on 18.56mm×2.9mm×0.4mm FR4 plate and a small microstrip is linked to the SMA connector using as transmission line. The optimization length of the radiator is 20 mm.

3. Materials and methods

3.1 Cell culture:

The mouse osteoblastic MC3T3-E1 cell line was donated from Dr. C.C. Chang, Department of pediatrics, School of Medicine, Michigan State University, originally was obtained from D.T. Yamaguchi, Research Service and Geriatrics Research, Education, and Clinical Center, VAMC, West Los Angeles, California, USA and has been maintained in D-medium same as the cell line in Dr. C.C. Chang's Lab in Michigan State University, East Lansing, Michigan, USA. The cells were incubated at 37˚C in a humidified atmosphere containing 5% CO2 and 95% air and were fed or trypsinized every two to three days. Cells in culture for seven days can be used for experiments.

3.2 GJIC Assay:

The scrape load/dye transfer (SL/DT) technique was

used to measure the GJIC within cells [21]. After the treatment, the cells were rinsed with phosphate buffered saline (PBS), and a PBS solution containing 4% concentration lucifer yellow fluorescence dye is injected into the cells by a scrape using a scalpel blade. Afterwards the cells were incubated for 3 minutes and extra cellular dye was rinsed off and fixed with 5% formalin. We then measured the area of the dye migrated from the scrape line using digital images taken by an epifluorescent microscope and quantitated with Nucleotech image analysis software for the GJIC images.

3.3 Measurements of the near filed fluctuation in exposure of RF-EMF and the ambient filed:

The probe is the accessory magnetometer of the instruement F.W. Bell 9550 provided by National Instruement, Austin Texas, USA. The scanning probe is fixed at 1mm above perpendicularly to keep the phase at 90 degree with the plan of the cell layer. The RF-EMF at 2.4GHz was transmitted by the antenna proposed as in Fig. 1. The experimental data was recorded and transferred to the 54621A oscilloscope, made by Agilent Compony, Santa Clara, California, USA and saving as the file of Microsoft

3.4 Math Analysis:

The signal s_{ij} (jth element in ith ensample) is used as input to a background n_{ij}, the output $\{x_{ij}\}$ can be transformed to electrical voltages shown on oscilloscope. By using HP Benchlink, we collected the output data and transformed it to Microsoft Excel as text files. Matlab and FORTRAN programs were performed to analyze the data and get power density spectrum. We proposed a simple one dimensional filter algorithm used to amplify the difference between the input signal amplitude and the output power to an observable level so that we can exam any biological responding signal embedded in noise by power density spectrum calculation [22]. Consider the data output sequence $\{x_{ij}\}= \{s_{ij}\}+\{n_{ij}\}$. Set six values from v_1 to v_6 by taking v_1 the maximum amplitude of ambient geomagnetic fluctuation and then take $v_2 = 0.95v_1$, $v_3 = 0.85v_1$, $v_4 = 0.75v_1$, $v_5 = 0.65v_1$, $v_6= 0.55v_1$ for six levels, and therefore, $v_1>v_2>\ldots..>v_6$. If more values being selected, more calculation should be involved. And then, compute $\bar{x}_{ij}$ (the average value of x_{ij}), If $x_{ij} > \bar{x}_{ij}$, define m_h so that a high threshold value m_h is defined as $m_h = x_{ij}$. If $x_{ij} < \bar{x}_{ij}$, define a low threshold value m_1 so that $m_1 = \bar{x}_{ij}$. If $x_{ij} > m_h$, set $m_{hh} = x_{ij}$, then, a second high threshold m_{hh} should be defined. We first defined m level values. Select m so that m is more than $\bar{x}_{ij}$ but less than m_h and set m_{h1} being equal to $\bar{x}_{ij}$ and compare that If $m_1 < \bar{x}_{ij} < m$, $\bar{x}_{ij} = m_{1h}$, otherwise If $\bar{x}_{ij} < m_1$, set $\bar{x}_{ij} = m_{11}$, the $m_{hh} > m_h > m_{h1} > m > m_{1h} > m_1 > m_{11}$ should be kept. If $x_{ij} > m_{hh}$, set $\bar{x}_{ij} = v_1$, If $m_{1h} < x_{ij} < m_{hh}$, set $x_{ij} = v_2$, If $m_{h1} < x_{ij} < m_h$, set $\bar{x}_{ij} = v_3$, If $m < x_{ij} < m_{h1}$, set $x_{ij} = v_4$, If $m_{1h} < x_{ij} < m$, set $x_{ij} = v_5$, If $m_1 < x_{ij} < m_{1h}$, set $x_{ij} = v_6$. The noise is defined as all unpredictable signals in power density spectrum. We also must define ASNR = $\frac{A_s}{A_n}$ where A_s is the amplitude of the signal and A_n is the noise of the background. SNR = $\frac{P_s}{P_n}$ is defined where P_s is the power of the signal and P_n is the noise power of the background can be calculated, the plot of ASNR versus SNR produces a function curve showing the relation between the input signal amplitude and responding output power. Accordingly, even through noise may have its own characteristic; we can calibrate the function curve with the help of adjusting input signal's amplitude to amplify the power difference between the noise and the signal. The application of this function curve can be considered for the frequency component in power density spectrum of the noise depends upon the characteristic of the signal. When the exposure RF-EMF source is absent, we can measure the signal dependent noise, which is the combination of the ambient field with the cell, induced signal fluctuations. Meanwhile, if

the switch of the RF-EMF is open, we can measure the responding fluctuation from the cells under the reaction of RF-EMF if we can filter out the noise. Through the formula of the correlation,

$$R_q = \left(\frac{1}{N}\right)\sum_{p}^{N} V_p V_{p+q}, \quad S_k = \sum_{q=1}^{N} R_q e^{\frac{i2\pi Kq}{N}}$$

3.5 Algorithm to identify the intrinsic signal and it's SNR:

Let {1Hz, 2Hz, ...20Hz, 21Hz, 22Hz…58Hz,59 Hz} denote the given sequence of frequencies $\{\omega_i\}$, ω_1=1Hz, ω_2 = 2Hz, ω_3 = 3Hz,.....ω_{50} = 50Hz,….ω_{59} = 90Hz, respectively.

1. Take the maximum Vmax from ambient geomagnetic fluctuation {V (t)}
2. Mathematically add sin(ωit) signal for a ωi with amplitude Vmax to { V(t) } and name the result as {Vg(t)}.
3. Perform autocorrelation and Fourier transform to get the power density spectrum (PDS) of {Vg (t)}
4. Calculate the SNR at maximum of {SK} and represented as $S_{max}(\omega_i)$ for checking every possible ω_i in PDS. Notably, (a) the power of the signal is defined as the area under the frequency at ω_i, and (b) the power of the noise is defined as the total power of the all possible ω_i in PDS, $\{S_k, \text{noise}(\omega_i)\}$ of the {Vg(t)}, and (c) the SNR is defined as the power of the source signal divided by the power of the noise of the system, therefore, SNR can be rewritten as $SNR = \frac{S_{max}(\omega_i)}{S_{k,\,noise}(\omega_i)}$.

5. Repeat step 2, step 3 and step 4 with the amplitudes 0.7Vmax at ω_i to calculate Smax (ω_i) at ASNR=0.7 by assuming $S_{max,0.7}(\omega_i) = X_{0.7}$.

0.4Vmax at ω_i to calculate Smax (ω_i) at ASNR = 0.4, by assuming $S_{max,0.4}(\omega_i) = X_{0.4}$.

0.03Vmax at ωi to calculate Smax (ω_i) at ASNR = 0.03 by assuming $S_{max,0.03}(\omega_i) = X_{0.03}$

0.003Vmax at ωi to calculate Smax (ω_i) at ASNR = 0.003 by assuming $S_{max,\,0.003}(\omega_i) = X_{0.003}$ and so on.

6. Thus, if $pX2 + qX + r_{ASNR}(\omega_i) = 0$, using square curve fitting, we can easily get the corresponding p,q and $r_{ASNR}(\omega_i)$ values at each ω_i .

$$pX_{0.7}^2 + qX_{0.7}^2 + r_{0.7}(\omega_i) = 0$$
$$pX_{0.4}^2 + qX_{0.4}^2 + r_{0.4}(\omega_i) = 0$$
$$pX_{0.03}^2 + qX_{0.03}^2 + r_{0.03}(\omega_i) = 0$$
$$pX_{0.003}^2 + qX_{0.003}^2 + r_{0.003}(\omega_i) = 0$$

Consequently, p, q and r_{ASNR} can be calculated. The most interesting result is that by extrapolation method, r_0 (ω_i) value is not equal to zero. Notably, the $r_0(\omega_i)$ spectrum is characterized by the possible given intrinsic frequency ω_i buried in {V(t)} [16]. By analyzing the measured {V(t)} of osteoblast cells in the exposure of RF-EMF and {Vg (t)} of ambient geomagnetic field, we found $r_{=0}(\omega_i)$=0 at 14Hz for osteoblast cells is about 50 times more than that in the ambient geomagnetic field. The filter algorithm that we previously proposed in reference [12] can improve the amplification of the extremely low frequency to 10^4 times more than the noise. The comparison of calculation results is listed in table I which shows that we can recognize easily if the ELF signal being kept 104 times lower than the ambient noise. Measurements of return loss (RL) means a measure of power reflected from imperfections in an electrical link being defined as the ratio PR / PT, representing the power of the wave reflected from the imperfection (PR) to that of the transmitted, wave (PT) [23]. For best performance, the reflected signal should be as small as possible, meaning the ratio PR / PT should be as small as possible. Return loss is expressed in decibels. The return loss value describes the reduction in the amplitude of the reflected energy, as compared to the forward energy. It will always be a loss and therefore a negative dB. However, we can write, for instance, -3 dB as simply 3 dB of loss, dropping the negative sign and adding loss, which means the reflected energy from the device, PR, is always 3dB lower than the transmitted energy PT. When expressed in dB, larger (in magnitude)

negative numbers represent larger return losses and thus smaller reflected power PR.

Measurements of Antenna Gain: It is defined as the ratio of the radiation intensity of an antenna in a given direction to the intensity of the same antenna as it radiates in all directions (isotropically). Since the radiation intensity of an isotropically radiated power is equal to the power into the antenna divided by 360 degrees, we can express the following equation: Gain = $4\pi\times\frac{\text{Radiation Intensity}}{\text{Input Power}}$ The near field magnetic fluctuation is defined for the electromagnetic induction and electric charge effects on the EMF which effects decreasingly with increasing distance from the antenna. Typically, near field magnetic fluctuation would not be important farther away than a few wavelengths of the operation frequency of the antenna [24]. These near field effects involve energy transfer coupling directly to receivers near the antenna and affecting the power output of the transmitter. In a sense, the near field offers the information of energy transfer which is available to a receiver but sensed by the transmitter. The source of 2.4GHz RF-EMF was provided by HP/AGILENT 8722A/C (50MHz to 40GHz) Microwave Vector Network Analyzer (MVNA) [25]. The MVNA can be used for various kinds of applications, both for the classical type microwave engineering circuit characterization and many novel ones in research. Because of its unique capabilities, we used MVNA to transmit the RF-EMF to the cells with the power density of 0.3mWatt/cm2 at 2.4GHz. It becomes a useful tool in our research to explore cell responding signal in RF-EMF exposure. The 1-1000 GHz frequency domain covered by the MVNA bridges the interval between ordinary microwaves and infrared techniques. In particular, in energy units, the Microwave Vector Network Analyzer covers the range 0.03~4 meV for each drifting electron. This range contains characteristic energies of many elementary excitations.

4. Results and Discussion

In Fig. 2, it depicts the measurements of the return loss and the antenna gain of the proposed antenna. The operation frequency is at 2.4 GHz. Both with cells and without cells in the mounted culture dish have no effect to the measurements of the return loss, antenna gain as well as the operation frequency. There is also no influence of all antenna measurements to the proposed antenna mounted culture dish in comparison to the antenna only. However, the GJIC of the osteoblast cells reveals the biological effect for the exposure of RF electromagnetic wave at 2.4GHz. Meanwhile, the new method we previously developed for the calculation of signal to noise ratio verified the experimental result which shows the absorption frequency at 14 Hz. The measure magnetic fluctuation and mathematical calculation results are shown in Fig. 3. Meanwhile, we found the GJIC modulation within osteoblast cells under 2.4 GHz exposure of electromagnetic wave is observed at 20% which is shown in Fig. 4. Basically, all cancers have been generally viewed as the result of a disruption of the homeostatic regulation of a cell's ability to respond appropriately to extra cellular signals of the body which trigger intracellular or intercellular signal transducting mechanisms by modulating gap junctional intercellular communication between the cells within a tissue. In short, carcinogenesis means mutation of the genetic material of normal cells to interrupt the normal balance between proliferation and cell death. From Fig. 4., we can see the modulation of the GJIC within the mouse osteoblast cells by RF-EMF. The consequence of the GJIC modulation by RF-EMF may cause the cell being possibly in mutant, quickly aging, stop proliferation and even in instantaneous death [26]. The mechanism of releasing of 14Hz signal may through the models referred to calcium oscillation, cyclotron resonance, stochastic resonance and cell growth oscillation. However, due to the consideration of

potential health impact, RF-EMF may be an odd factor to human health we should be careful to deal with. Additionally, not only RF-EMF can be characterized by the amplitudes of electric and magnetic field which oscillate in phase perpendicular to each other and to the direction of energy propagation, but also can be classified into types of energy and power mathematically according to the values of multiplication of frequency and amplitude [27]. Thus, the total absorbed power by the mouse osteoblast cell system exposed into RF-EMF is about 3×10-16 Watt which is very small and supposedly has no hurt to the cell for our design. However, experimental result has shown significant modulation of the GJIC . In this report, we only proposed a new process algorithm to show the existence of 14Hz signal by measuring near field fluctuation and reveal this signal is a power signal that may caused the modulation of GJIC within mouse osteoblast cells. No mechanism model we would suggest due to the complexity of the signal transduction on the surface of the cell membrane and the lack of molecular biology experimental evidence.

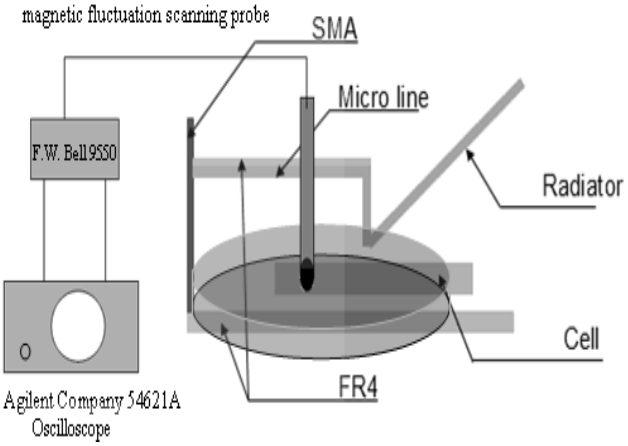


Fig 1: Schematic drawing of the cellular exposure

(a)

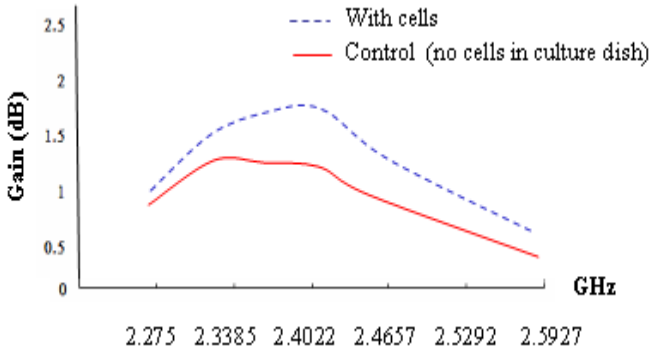
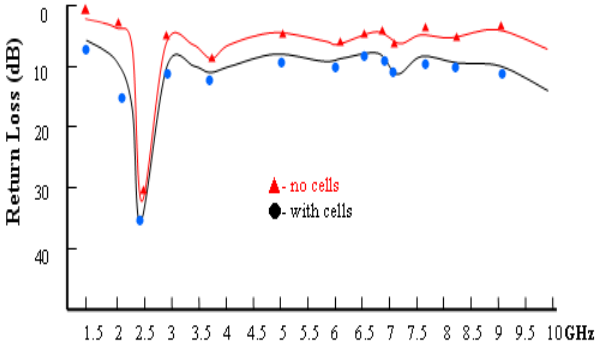


Fig 2: The measured (a) Return Loss
(b) antenna gain



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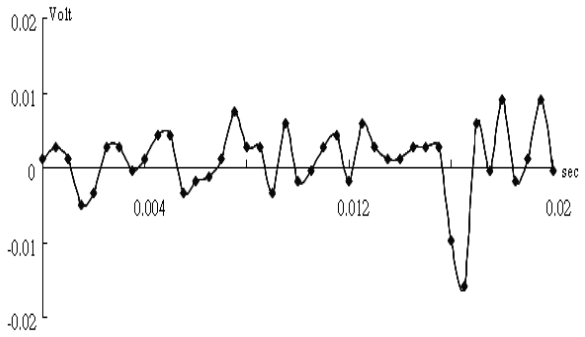
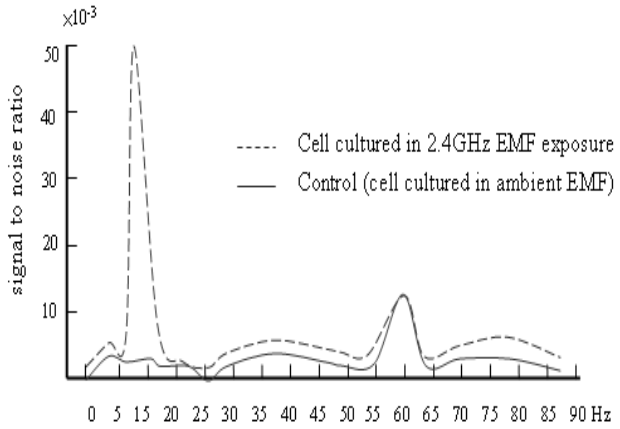


(a)



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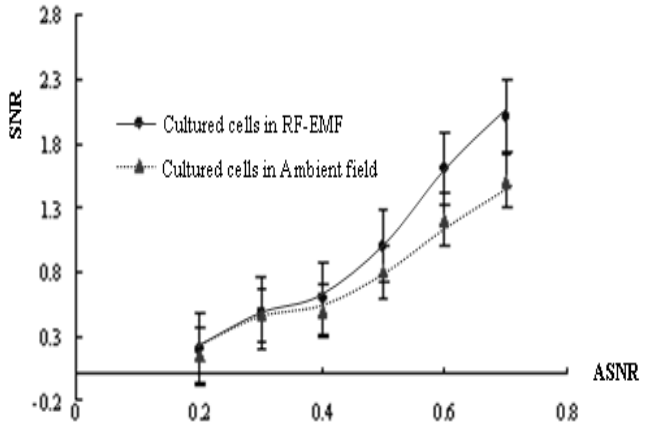
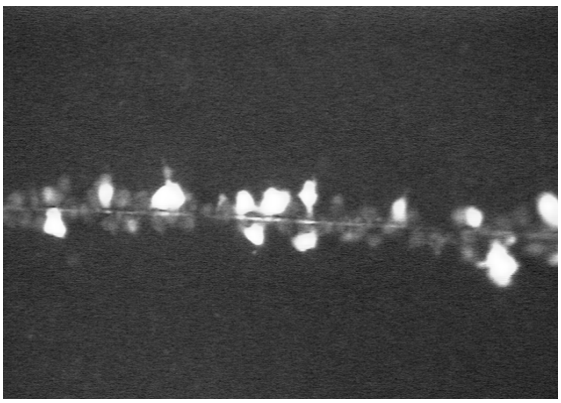
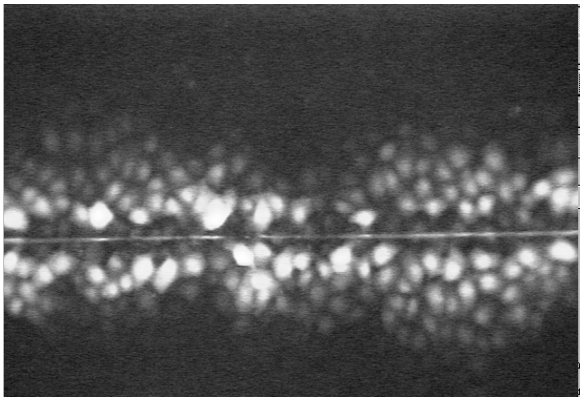


(c)

Fig 3. (a)Measurement of near field magnetic fluctuation of the mouse osteoblast cells by the exposure of RF-EMF at 2.4GHz (b) $r_0(\omega_i)$ spectrum of the frequency range from 0 to 90 Hz (c) SNR vs. ASNR fitting curve of exposure mouse osteoblast cells, which respond signal frequency at 14 Hz, in 2.4GHz RF-EMF Exposure.



(a)



(b)

Fig 4: (a) GJIC modulation within mouse osteoblast cells by RF-EMF at 2.4GHz (b) original GJIC within mouse osteoblast cells in ambient geomagnetic field

Table1.
Comparison of the power density spectrum calculation
-: not able to identify ELF at 15Hz embedded in ambient field
+: able to identify ELF at 15Hz embedded in ambient field

Input ASNR	PDS	PDS with adding filter algorithm	Output S/N Ratio
1.0	+	+	1.5
0.5	+	+	0.37
0.1	-	+	0.015
0.05	-	+	0.004
0.01	-	+	0.0001

5. Conclusion

According to the measurements, the proposed antenna mounted culture dish can provide an exposure of RF-EMF at 2.4GHz which can significantly affect GJIC within the mouse osteoblast cells cultured *in vitro*. The RF -EMF may stimulate the cultured mouse osteoblast cells to respond an extremely low frequency at 14Hz. Mathematical calculation can reveal the existence of 14 Hz signal in the power density spectrum of the probed near field magnetic fluctuation when the GJIC modulation being initiated significantly within the cells.

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