Lecture 7

Elemental Images of Single Neurons by Using SR-XRF

7.1. Introduction

The interactions and responses of neurons to transition metal elements are widely investigated because of the fact that transition metals can accept or donate single electrons to promote the free radical formation [1]. Iron or other transition metals such as copper are believed to induce neuronal injury by converting super-oxide (O_2 •) and hydrogen peroxide (H_2O_2) into highly reactive, toxic hydroxyl radicals (OH •) in a sequence of reactions that is referred to cumulatively as the Haber-Weiss reaction [2]. It is suggested that neuro-degenerative diseases are caused by these reactive oxygen species, and their excessive amount is generated through the reactions with transition metal elements, such as iron, zinc and copper. For example, aluminum is considered to be one of the most important sources related to Alzheimer's disease. Abreo et al. [3] displayed that the accumulation of aluminum in Neuro2a cells can result in the increased uptake of iron, inhibition of cell growth, and expression of NFT (Neurofibrillary Tangle) protein, partially mimicking the pathological hallmarks of Alzheimer's disease (AD), namely, loss of neurons and formation of NFTs [4]. On the other hand, copper is considered to possibly have a central role in several neurodegenerative disorders, including AD, CJD (Creutzfeldt Jakob disease), and ALS (amyotrophic lateral sclerosis) [3]. Furthermore, in case of ALS, it is suggested that the observed accumulation of calcium in motor neurons is closely related to the internalization of zinc into the cell [5,6]. Increased iron levels within neurons have been observed in several neurological disorders, e.g. Parkinson's disease and Huntington's disease [7-10]. The storage of iron within ferritin may act as a protective mechanism, but heavily loaded ferritin may still produce free radicals. It is possible that the increased loading of ferritin in Parkinson's disease provides an environment that encourages free radical generation and hence neuronal damage [11].

It is probable that trace elements within neurons may display significant fluctuations when internalization and accumulation of certain transition metals are induced. In an experiment described in the following, the interactions between metals and neurons in-vitro was investigated. A sample neuron, Neuro2a, was cultured in chromium oxide and vanadium chloride solution environments. After the cell culture, the cells were studied by SRXRF and EPMA-EDX analysis. It is anticipated that the ability to measure elements at ultra trace levels may clarify their role within neurons

affected by dosing metal elements. Especially, intracellular iron and calcium are considered to play important roles in order to control the fate of the cells against foreign metal elements. Iron is essential to tissue metabolism in brain and other organs. However, iron catalyzes lipid peroxidation and free radical production with results that could be especially destructive to a lipid-rich structure, such as the brain [12]. Normal brain iron may be toxic if it is released from its tightly controlled compartments and excessive accumulation of iron is induced within neurons [13].

Here, interactions between single neuron cells and metal elements are investigated via the measurement of the density fluctuation of the elements in the matrix and also of the localization of those elements in single cells, using samples that have been cultured in a metallic solution environment.

7.2. Procedures of Cell Culture and Morphological Observation

7.2.1. Neuron

The human brain – the control center that stores, computes, integrates, and transmits information – contains about 10^{12} neurons, each forming as many as a thousand connections with other neurons. The function of a neuron is to communicate information, which it does by two methods: electric signals processing and transmitting information within a cell. Chemical signals transmit information between cells, utilizing processes similar to those employed by other types of cells to signal each other. Information from the environment creates special problems because of the diverse types of signals that must be sensed – light, touch, pressure, sound, odorants, the stretching of muscles. Sensory neurons have specialized receptors that convert these stimuli into electric signals. These electric signals are then converted into chemical signals that are passed on to other cells – called interneurons – that convert the information back into electric signals.

7.2.2. Procedures of Cell Culture

Neuro2a was employed in this experiment. These cells were provided in American Type Culture Collections (ATCC). The culture medium was Eagle's Minimal Essential medium that is modified by ATCC to contain: 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids and 1.5 g/L sodium bicarbonate. 10% FBS (Fetal Bovine Serum) was added into the culture medium. The cells in DMSO (Dimethyl Sulfoxide) and culture medium which were frozen in liquid nitrogen were defrosted in warm water (37 °C). After defrosting, the cells were pipetted into a sterilized tube with the culture medium (5mL). After the centrifugation of the tube, the cells were distributed into 550

mm diameter dishes with 5mL culture medium. The cells were cultured in an incubator fixed at 37 °C and 5 % of concentration of carbon dioxide. When the number of cells increased and the dish was covered with a lot of cells, a part of the cells were moved into the new dish with the fresh culture medium.

After the cell division, axon and dendrites of each neuron spread in all directions and make networks with other neurons. The scanning electron microscope image of neurons making networks is shown in figure 7.1. In their cell bodies, there are many sub-cell structures. In order to make them clear, Giemsa's solution, Methyl Green solution, Mayer's Hematoxylin solution and Eosin Y Ethanol solution (0.5 %) were employed. The light microscopic photographs of neurons stained by these procedures are shown in figure 7.2, 7.3 and 7.4. The nucleus, the nucleoli in the nucleus, dendrites and axons are visible clearly in each figure.

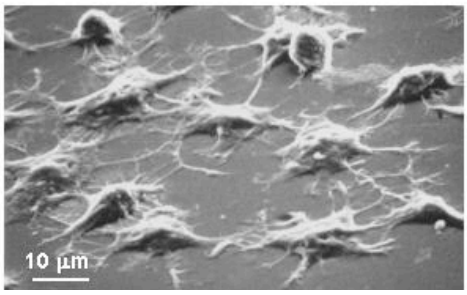


Figure 7.1

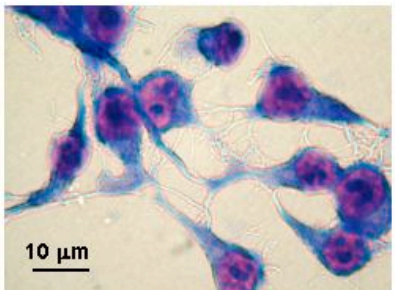


Figure 7.2

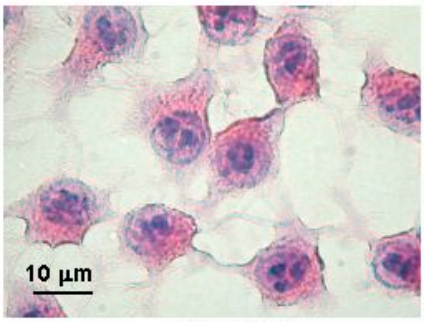


Figure 7.3

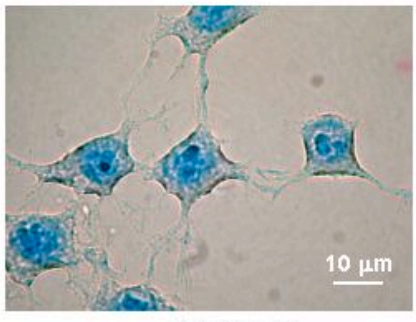


Figure 7.4

Figure 7.1, 7.2, 7.3 and 7.4. The scanning microscope images of neurons making networks with other neurons. In order to make sub-cell structures clear, Giemsa, Hematoxylin-Eosin and Methyl Green staining procedures are employed and shown in figure 7.2, 7.3 and 7.4, respectively.

The procedures for preparing the metallic solutions were as follows. The metal

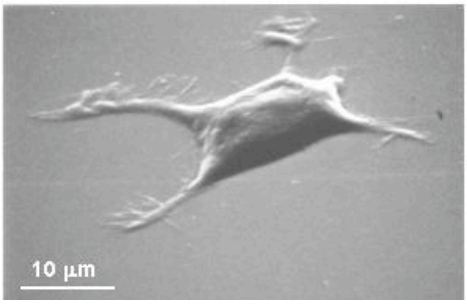
compounds utilized in this experiment were CrO_3 and VCl_3. These metal powders were enclosed in the bottle and were sterilized in an autoclave for 30 min. The metal powders were dissolved by the sterilized PBS solutions. The metallic solutions were further sterilized through the filter with 0.45 μm holes. If the cells were cultured in a metallic solution environment, the ratio of the culture medium and metallic solution was 100 to 1.

7.2.3. Morphologic Observation With Scanning Electron Microscope

Neurons have many dendrites and axons spreading from their bodies to create the networks with other neurons. The axons and thick dendrites can be seen by light microscope, but fine threads of dendrites cannot be observed by this method. In order to visualize the structures of the axons, dendrites and cell bodies after exposing cells to a metallic solution environment, the SEM (scanning electron microscope) analysis was employed.

For SEM, silicon plates were employed as the substrate because of the good electro-conductivity. The silicon plates were immersed in ethanol solution and sterilized by the ultraviolet ray for 24 h. Neuro2a were cultured on these silicon plates and fixed by a formalin solution for 24 h. After fixing, carbon was deposited on the silicon plate. Selection of the fixation solution is important to make the fine structures of the cells clear. In order to compare formalin fixation technique with other ones, the macrophage cells were fixed by ethanol, methanol and formalin solution, shown in figure 7.5, 7.6 and 7.7 respectively. When the cells were fixed by methanol or ethanol solution, the fine ruffle structures on the cell surfaces and fine threads of dendrites were not visible. With formalin, the fine structure of the cell bodies, dendrites and axons of the cells were visible distinctly as can be seen in figure 7.7.

The cell cultured under normal condition is shown in figure 7.8. The cell cultured in a vanadium chloride solution (0.04 g/L) for 1.5 h. are shown in figure 7.9. The fine structures of the surface of the cell body and the many fine threads of dendrites are clearly visualized in figure 7.8. However, the fine threads of dendrites are lost because of V solution environment, shown in figure 7.9. The lost of dendrites because of the V toxic effect is considered to be reasonable because the injured neuron with no dendrites can not accept the signals from other cells and therefore the incorrect information can not spread around the injured cell. The question remains whether the V solution directly injures the fine threads of dendrites. There are many factors related to this phenomenon, but it is plausible that the effect of V solution may appear within the cell body first and a certain degenerative reaction may induce the degeneration of dendrites afterwards.



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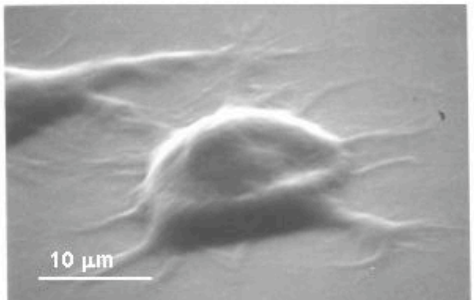

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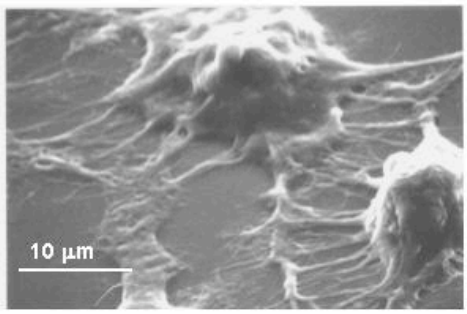

Figure 7.7

Figure 7.5, 7.6 and 7.7. Macrophages fixed with ethanol, methanol and formalin solutions are shown in figure 7.5, 7.6 and 7.7, respectively.

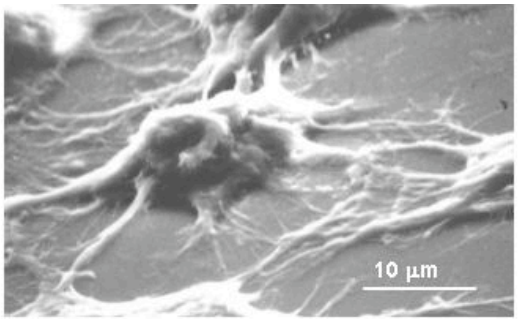

Figure 7.8

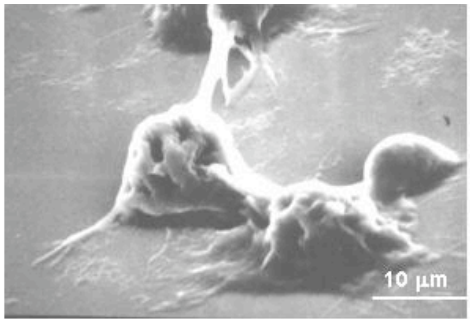

Figure 7.9

Figure 7.8 and 7.9. Cells cultured under normal conditions are shown in figure 7.8. Cells cultured in a 0.04 g/L V chloride solution for 1.5 h. are shown in figure 7.9.

7.3. Sample Preparation and Experimental Methods

The x-ray fluorescence spectra and elemental images were mainly obtained at Beam Line 4A in High Energy Accelerate Institute using the X-ray beam emitted by 2.5 GeV storage ring "Photon Factory". The final beam size was 6 μm in diameter in this study. Fluorescent x-ray was detected by a SSD (Si(Li)) in the air. The incident beam energy was 14.2 keV.

The x-ray absorption spectra and some fluorescence spectra were measured at the Beam Line 39XU in Japan Synchrotron Radiation Research Institute using the X-ray beam emitted by the 8 GeV storage ring "SPring-8".

The cells (treated neurons) were cultured while being exposed to solutions containing chromium oxide, vanadium chloride and also cells without metal uptake (untreated neurons) were cultured under normal conditions. The Neuro2a mouse neurons were provided by the American Type Culture Collections. These neurons were cultured on the sterilized PET films in Eagle minimum essential medium supplemented with 10 % Fetal Bovine Serum. After exposure to metallic solutions, the cells were washed with culture medium and fixed in formalin solutions for 24 h.

7.4. Challenge for In-Vivo and In-Situ Measurement of Living Single Neurons

7.4.1. Objective

For the elemental analysis, it is possible to measure samples in the air and solutions by employing the SR source. For investigations into the cell functions, in-vivo and in-situ measurement are an ideal procedure. When the biological samples are measured by SR-XRF imaging technique, the fixed and thin sections of tissues or cells are normally employed. Here, the elemental distribution patterns are observed in-vivo and in-situ by SR-XRF imaging technique in order to obtain the real figure of the cell.

7.4.2. Procedure

In order realize in-vivo and in-situ measurement, it is necessary to perform the cell culture near the beam line. In the biological imaging center in SPring-8, it is possible to do cell culture for the measurement sample. At Kyoto University, neurons (Neuro2a) were cultured and frozen by liquid nitrogen. The frozen cells cooled in the dry ices were brought into the biological imaging center in SPring-8. These cells were defrosted and cultured on a thick PET film. The films were first sterilized using ethanol solution, washed with sterilized water and dried under ultraviolet rays. Two kinds of PET films, normal and special ones, were prepared for the cell culture. Special PET films were processed by the excimer laser irradiation in order to form the

microstructures on their surfaces. The surface structures of the PET film after irradiation are shown in figure 7.10. It can be observed that there are many ruffles on the surface of the PET films. The SEM photograph of the neurons cultured on the special PET film is shown in figure 7.11. These microstructures on the surface of the PET film are designed to improve the adhesion power of the cells.

About a week later, the neurons on the surface of the PET films formed a tightly composed network with each other. Three kinds of samples were prepared for the measurement. They are the control neurons sample, and two treated neurons samples that were cultured in a chromium oxide solution (0.04 g/L) for 4 h. on the surface of the normal PET film and on the surface of the special PET film. Before the measurement, the PET films were put on a thick acrylic container poured with the culture medium. The acrylic container has a hole at the bottom. The PET film is stretched over the hole and glued by adhesive agent. When the PET film with the cultured neurons is put on the acryl container, the surface with neurons must be faced on the culture medium. After putting the film on the acryl container, a thick acryl cover with a big hole was pushed on the film. At the end, the neurons were confined between the two PET films filled with the culture medium. The thickness of the liquid phase is about 0.1 to 0.5 mm. After making the samples, they were brought into the beam line soon. The schematic drawing of procedures of making samples is shown in figure 7.12.

SR-XRF analysis was performed in the air. The strong adhesion power between the cells and film is required during the measurement because the film with the cultured neurons must be mounted vertically.

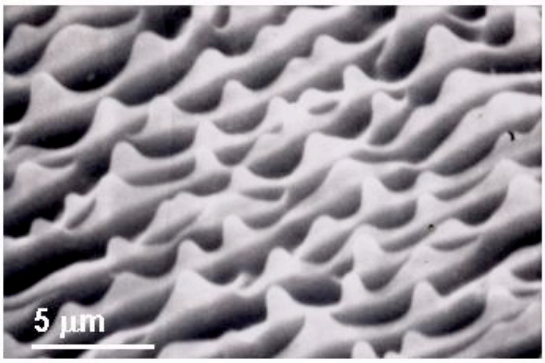

Figure 7.10

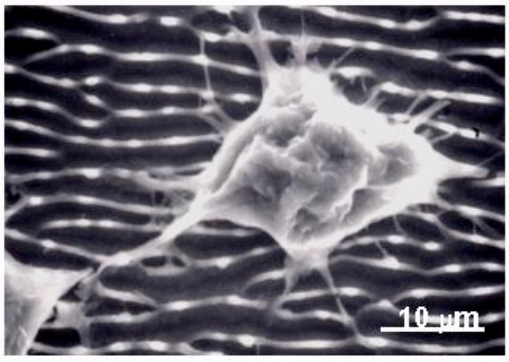

Figure 7.11

Figure 7.10 and 7.11. Two kinds of PET films, normal and special ones, were prepared for the cell culture. Special PET films were processed by the excimer laser irradiation in order to form the microstructures on the surfaces of them. The surface structures of the PET film after irradiation are shown in figure 7.10. The SEM photograph of the neuron cultured on the special PET film is shown in figure 7.11.

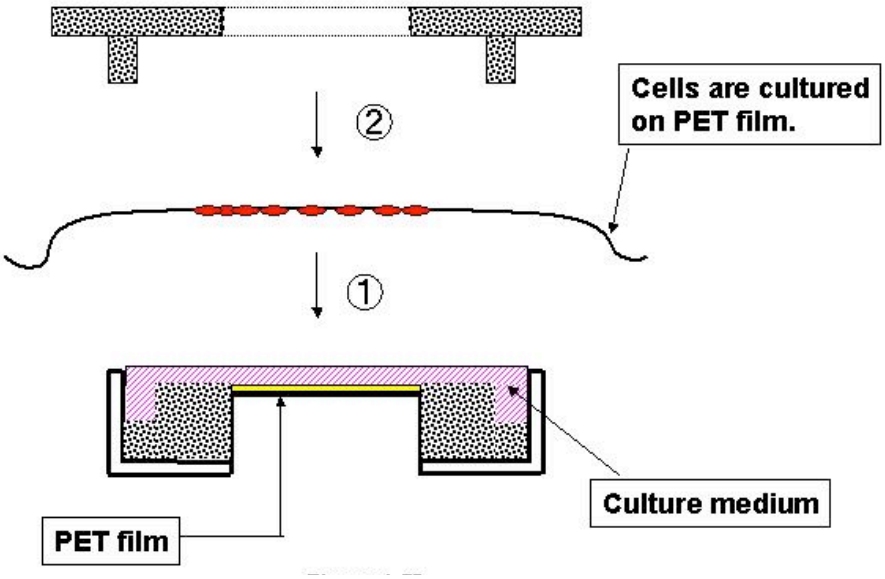

Figure 7.12. Before in-vivo and in-situ measurement, the PET with living cells were put on the acryl container poured with the culture medium. The acryl container has a hole at the bottom of it. The PET film is stretched over the hole and glued by adhesive agent. When the PET film with the culture neurons is put on the acryl container, the surface with neurons must be faced on the culture medium. After putting the film on the acryl container, a thick acryl cover with a big hole was pushed on the film.

7.4.3. Results

In order to obtain the elemental distribution pattern of the control cells cultured on the normal PET film, in-vivo and in-situ measurement was employed and the image is shown in figure 7.13a-d. When SR-XRF imaging technique was employed to the fixed cells, the distribution patterns of iron, calcium, potassium, phosphorus and sulfur were clearly observed within neurons. However, the distribution patterns shown in figure 7.13 are obscure. It is considered that the elements included in the culture medium shade off the gradation of the elemental distribution patterns within the cells.

The cells cultured in a chromium solution environment for 4 h. on the normal and special PET films are shown in figure 7.13c and 7.13d, respectively. These images are also obscure.

Normally, SR-XRF imaging technique can not detect the elemental distributions during watching the area under scanning. Therefore, the elemental distribution patterns obtained by SR-XRF must be corresponded to the photograph of the sample. In this system, if the elemental distributions do not display the distinct patterns, it is impossible to correspond the scanned images to the photograph of the sample. In order to realize

the in-vivo and in-situ measurement, it is necessary to employ the real-time observation of the scanned area precisely. Furthermore, it must be considered that the contents of the culture medium are controlled to obtain the clear images of the cells.

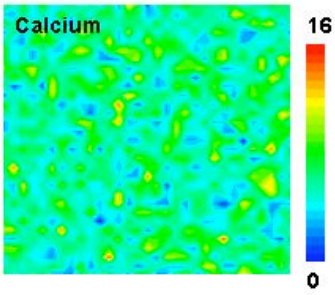

Figure 7.13a

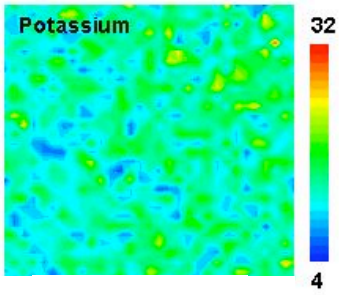

Figure 7.13b

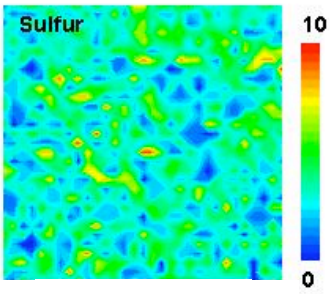

Figure 7.13c

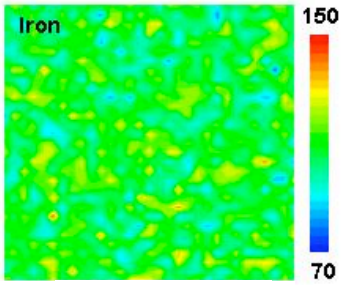

Figure 7.13d

Figure 7.13a-d. In order to obtain the elemental distribution pattern of the control cells cultured on the normal PET films, in-vivo and in-situ measurement were employed. The elemental images of Ca, K, S and Fe are shown in figure 7.13a-d, respectively

4.4. Summary

By using synchrotron radiation source, in-vivo and in-situ measurement of the elemental images of the cell may be realized. It is considered that there are no methods except SR-XRF to obtain the various elemental images within the living cell simultaneously. There are many phenomena that we have never observed within the cell. Especially, the instant and temporary reactions involved in the transfer of the many elements in the cell are considered to be important for the investigations into the cell functions, but it is impossible to obtain the real-time images of the intracellular elements by using the current procedures. It is probable that in-vivo and in-situ measurement of the elemental images of the cells by using SR-XRF will be the essential method in order to approach the cell functions in spite of the incomplete technique to be improved and developed.