



In situ observation of a cell adhesion and metabolism using surface infrared spectroscopy

著者	Miyamoto Ko-ichiro, Yamada Parida, Yamaguchi Ryo-taro, Muto Takami, Hirano Ayumi, Kimura Yasuo, Niwano Michio, Isoda Hiroko
journal or publication title	Cytotechnology
volume	55
number	2-3
page range	143-149
year	2007-11
権利	(C) Springer Science + Business Media B.V.
URL	http://hdl.handle.net/2241/98148

doi: 10.1007/s10616-007-9111-2

Running head *In-situ* infrared spectroscopic observation of a cell adhesion and metabolism

Article type general paper

Title *In-situ* observation of a cell adhesion and metabolism using surface infrared spectroscopy

Authors Ko-ichiro MIYAMOTO¹, Parida YAMADA², Ryo-taro YAMAGUCHI³, Takami MUTO³, Ayumi HIRANO³, Yasuo KIMURA³, Michio NIWANO³ and Hiroko ISODA²

Affiliations

¹ Department of Electronic Engineering, Graduate School of Engineering, Tohoku University, 6-6-05 Aza-Aoba, Aramaki, Aoba-ku, Sendai, Miyagi, 980-8579, Japan

² Alliance for Research on North Africa, Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1Tennodai, Tsukuba, Ibaraki, 305-8572, Japan

³ Research Institute of Electrical Communication, Tohoku University, 2-1-1 Katahira, Aoba-ku, Sendai, Miyagi, 980-8577, Japan

Address for correspondence

Hiroko ISODA, Alliance for Research on North Africa, Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1Tennodai, Tsukuba, Ibaraki

305-8572, Japan; (Telephone:+81-29-853-5775, Fax:+81-29-853-5776, E-mail:

isoda@sakura.cc.tsukuba.ac.jp)

Abstract

In this study, we report on an *in-situ* monitoring system of living cultured cells using infrared absorption spectroscopy in the geometry of multiple internal reflections (MIR-IRAS). **In order to observe living cultured cells**, the temperature in **the** sample chamber of a FT-IR spectrometer was maintained at 37 °C and a humidified gas mixture containing 5% CO₂ was introduced into the sample chamber. Human breast cell line MCF-7 cultured on Si MIR prisms were placed in the sample chamber and infrared spectra of MCF-7 cells were collected for 5 h. It was found that **the adhesion and metabolism** of MCF-7 cells can be monitored by the absorption intensity of amide-II protein band (1545 cm⁻¹) and also by the absorption intensities of CH_x bands (2700-3100 cm⁻¹). These results suggest that our system is useful for a nondestructive and non-label monitoring of cell viability. **Our method based on infrared absorption spectroscopy has a potential for bioscreening application.**

Keywords

Human breast cell line MCF-7, Si prisms, infrared absorption spectroscopy, cell metabolism, in-situ observation

Introduction

Bioassay is the search for new chemicals in living matter and an effective method to discover a new drug. Many kinds of new biological active substances have been found from a biological resource. It is necessary to shorten the screening time because most of bioprospecting research activity is a race against time. The bioassay using cultured cell has been developed to screen for kinds of the biological active substances and their influences (H. Isoda *et al.* 2002, J. Han *et al.* 2002, T. Talorete *et al.* 2002).

In our previous report, we have successfully determined genetically modified maize, and distinguished between genotypes of rice varieties using infrared absorption spectroscopy (IRAS) (K. Emura *et al.* 2006). And, recently we proposed an *in-situ* monitoring of biomolecular interactions using IRAS (K. Miyamoto *et al.* 2005 and 2006). High surface sensitivity was achieved using the multiple internal reflections IRAS (MIR-IRAS) geometry using a small Si prism, with MIR-IRAS particularly, which is well-suited for observing interactions or conformational changes of such biomolecules in aqueous environment. Thus MIR-IRAS can be a powerful tool for the primary screening of bioassay. Infrared absorption spectrum provides various information on conformation or structure of biomolecules and it may attend to the needs of multifactorial screening.

On the other hand, there is a large body of literatures about IRAS measurement of biomolecules: DNA, protein, tissue sample, bacteria sample and cultured cells (H.H. Mantsch and D. Chapman 1996, H.U. Gremlich and B. Yan 2001, T.B. Hutson *et al.* 1988, T. Miyazawa and E.R. Blout 1961). T.B. Hutson *et al.* (1988) have reported on a technique for monitoring cell growth of Chinese hamster ovary cells using attenuated total reflection (ATR) FT-IR measurement, however, general ATR method needs expensive ATR crystal made of germanium or zinc selenide and a size of the crystal is much larger than our MIR-IRAS prism. Then MIR-IRAS prism made of Si has advantages in terms of cost and size. And a surface stability of Si prism covered with chemical oxide layer should be another advantage.

In this study, we report on a method for *in-situ* observation of cell adhesion and metabolism using MIR-IRAS. We maintained the environment of a sample chamber of an infrared spectrometer to be the same as a general cell culture and succeeded in *in-situ* observation of the cell adhesion and metabolism on the Si prism. The result of the MIR-IRAS measurement shows spectral enhancements at amide-I, amide-II and CH_x absorption bands due to the cell adhesion and metabolism.

Materials and Methods

Cell and cell culture

The Human Breast Tumor Cells (MCF-7, RCB No.1904) were purchased from the Riken cell bank, Japan. The cells were cultured in RPMI 1640 medium, supplemented with 10% heatinactivated FBS, penicillin (50 I.U./ml) and streptomycin (50 µg/ml). The cells were maintained at 37°C in a humidified 5% CO₂ atmosphere in an incubator.

Materials

RPMI 1640 medium (Gibco), FBS (Sigma), streptomycin-penicillin solution (MP Biomedicals Inc.), 25% glutaraldehyde solution (Wako), paraformaldehyde (Wako), 4% osmium tetroxide solution (Heraeus), 1M HEPES buffer (pH 7.0-7.6, Sigma).

Infrared spectroscopy

Figure 1(a) illustrates the equipment made of Teflon® we used in this study for MIR-IRAS measurements. This equipment was also used in our past studies (K. Miyamoto *et al.* 2005 and 2006). The volume of the sample solution was 100-200 µl. A homemade Si prism was 0.5 x 10 x 30 mm³ with 45° bevels on each of the short edges, and contacts with the sample solution. An infrared light beam from an interferometer

(BOMEM MB-100) was focused at normal incidence onto one of the two bevels of the Si prism, and penetrated through the Si prism, internally reflecting about 60 times. The light that propagated through the Si prism through the other bevel was focused onto a liquid-nitrogen cooled Mercury-Cadmium-Telluride (MCT) detector. The **internal reflections excited** an evanescent field on the Si prism surface (**Figure 1(b)**), and the chemical species of the biomolecules inside the evanescent field **influenced** the propagated light.

To observe the living cultured cell, we constructed an environment control system. A schematic view of the environment control system is shown in **Figure 2(a)**. First we mounted a homemade temperature controller (a heater, a small fan and thermo controller 1) to the infrared spectrometer described above. Second, a mixed gas containing 5% of CO₂ gas was introduced into MIR-IRAS equipment via a bubbler bottle. To humidify the mixed gas, the bubbler bottle was maintained at about 40°C using thermal controller 2 and an attached heater. The temperature of the sample chamber of the spectrometer is maintained at 37°C and the MCF-7 cell sample can be cultured under enough humidity and CO₂ 5% as same as a typical cell culture environment. A time course of the temperature monitored by thermo couple 1 is shown in **Figure 2(b)**. The temperature of the sample chamber is well maintained at 37±0.2°C

for 30 min.

Microscopic observation

Optical images of the MCF-7 cells on the Si prism were observed using Digital microscope (VHX-200, Keyence Co., Japan). And a cross-sectional image of MCF-7 on a silicon wafer was observed using scanning electron microscope (VE-7800, Keyence Co., Japan). To obtain the cross-sectional image, the MCF-7 cells on the silicon were pre-fixed over night. The pre-fix solution was composed of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1M HEPES buffer. The sample was post-fixed for 1 h in 1% osmium tetroxide in 0.1M HEPES buffer. The sample was then dehydrated through graded ethanol and a thin gold layer was deposited. The fixed sample and the Si wafer were cleaved before the observation.

Result and discussion

As a preliminary experiment, we have estimated MIR-prism dependence on the cell **adhesion** of MCF-7 cells. Two kinds of MIR prisms, Si and GaAs, were compared. We found that MCF-7 did not **adhere** on the GaAs surface as shown in **Figure 3(a)**. It may be due to a toxicity of arsenide. On the other hand, when MCF-7 cells (2×10^5 cells/ml, 200 μ l) were introduced into the MIR-IRAS equipment with a Si prism in **Figure 1(a)**, and incubated for 24 h in the CO₂ incubator, the MCF-7 cells adhered on the Si prism surface, and its surface **area** coverage was about 70%, indicating a stable cell **adhesion** on the Si prism (**Figure 3(b)**). Considering that less toxic SiO₂ layer was formed on the surface of the Si prism during washing with Piranha solution (H₂SO₄:H₂O₂ = 1:1), the SiO₂ layer enabled cell growth of MCF-7 cells on the Si prism. Thus we used the prisms made of Si in this study.

We next examined the cell **adhesion** of MCF-7 cells in the MIR-IRAS equipment in the FT-IR sample chamber and infrared spectra of the cultured MCF-7 cells on the Si prism were collected for 300 min at an interval of 30 min. A reference spectrum was collected at 60 min after the temperature in the sample chamber became stable. The collected spectra of MCF-7 on the Si prism are shown in **Figure 4**. **Figure 4(a)** and **(b)** show different region in 1450-1750 cm⁻¹ and 2700-3100 cm⁻¹, respectively. In **Figure**

4(a), it is clearly visible that two absorbance bands are centered around 1640 cm^{-1} and 1545 cm^{-1} . The absorption band around 1640 cm^{-1} corresponds to amide-I protein absorption band and is mainly attributed to C=O stretching mode, and the other absorption band around 1545 cm^{-1} corresponds to amide-II protein absorption band mainly attributed to N-H bending mode and C-N stretching mode (G. Socrates 2001). In Figure 4(a), these amide-I and II absorption bands were enhanced as incubation time is increased.

In another region in $2700\text{-}3100\text{ cm}^{-1}$, some CH_x absorption peaks are observed. In Figure 4(b), three absorption peaks at 2854 , 2925 and 2960 cm^{-1} increased in elapsed time. These absorption peaks are known as stretching modes of C-H stretching modes. According to G. Socrates (2001), the peak at 2854 and 2925 cm^{-1} can be assigned to a symmetry stretching mode and an asymmetry stretching mode of CH_2 group, respectively. Another peak at 2960 cm^{-1} can be assigned to an asymmetry stretching mode of CH_3 group. These absorption peaks are also enhanced as incubation time is increased as in the case of amide-I and II absorption bands. These spectral changes shown in Figure 4 may be due to the metabolic activity of the MCF-7 on the Si prism.

On the other hand, the surface coverage of the MCF-7 cells, which was estimated through the microscopic observation, was about 70% before MIR-IRAS measurements

and almost 100% after 300 min as shown in Figure 3(b) and (c). It suggests that the environment of the spectrometer was maintained appropriately for cell culture and the spectral changes described above are due to not only the metabolism but also the adhesion of the MCF-7 cells. The surface coverage on the Si prism surface should directly affect on the infrared absorption spectrum.

We also performed a control experiment without MCF-7 cells. Absorption spectra obtained by the control experiment are shown in Figure 5. In Figure 5(a), any significant spectral changes are not observed in amide-II absorption band. On the other hand, small spectral changes were observed in amide-I absorption band, which is probably due to a strong absorption of water overlapping with the amide-I absorption band. In the higher wavenumber region, enhancements of the absorption peaks of CH_x groups were observed, as shown in Figure 5(b). However, the intensities of the observed peaks are smaller than those in Figure 4(b), and the relative intensities of the peaks are different from those in Figure 4(b). Although the strongest peak is at 2925 cm^{-1} in Figure 4(b), the strongest peak is at 2960 cm^{-1} in Figure 5(b). The result of control experiment therefore suggests that the background signal from medium is relatively small and the spectral changes in Figure 5 are attributed to other species different from the case of the experiment with MCF-7 cells.

The time course of the peak intensities show another evidences. In Figure 6(a), the intensities of the strongest absorption peak at 1545 cm^{-1} in Figure 4(a) and 5(a) as a function of time. In the case of MCF-7, the intensity of the peak increased and saturated after 210 min. And there is no absorption peak as shown in Figure 5(a) in the control experiment. In Figure 6(b), the three CH_x absorption peaks in Figure 4(b) and 5(b) are plotted against the time. These intensities in the absorption spectra of MCF-7 also saturated after 210 min as same as the peak intensity at 1545cm^{-1} as shown in Figure 6(a). And the intensities in the control experiment increased continuously as incubation time increased. A clear difference can be seen between the time courses of two experiments. The continuous increasing of CH_x peaks in the control experiment may be due to the adsorption of medium components. These intensities of CH_x peaks in the control experiment are smaller than those in the experiment with MCF-7. And besides, MCF-7 cells adhered on the Si prism and covered the Si prism surface, as shown in Figure 3(b) and (c), suppressing the effect of the background signal from the medium. Thus the obtained signal should be mainly due to the adhesion and the metabolism of MCF-7 cells.

Figure 7 is a cross-sectional scanning electron microscopy (SEM) image of the MCF-7 cells on a Si wafer. We estimated the thickness of the MCF-7 cells is about 1-2 μm , and

the thickness of the excited evanescent field on the Si prism was estimated about 500 nm from the surface as shown in Figure 1(b). The thickness of the evanescent field covers 25-50 % of that of the MCF-7 cells, thus the obtained absorption spectra should contain many absorption bands of lipid membranes, membrane proteins, part of nuclear and also organelle proteins of the MCF-7 cells. T. Miyazawa and E.R. Blout (1961) have assigned absorption bands of typical secondary structures of protein; α -helix, β -sheet and random coil. If these absorption bands in Figure 4(a) were mainly attributed to the kinds of protein, the absorption band at 1545 cm^{-1} can be assigned to the α -helix structure. However, it is probable that the amide-II absorption band contains other absorption bands attributed to the β -sheet and random coil structure at 1530 and 1535 cm^{-1} , respectively. According to their study, although the α -helix structure also has another strong absorption band at 1650 cm^{-1} in amide-I band, it is difficult to identify the peak from Figure 4(a). This is because of an overlapping of H_2O strong absorption band at about 1640 cm^{-1} to amide-I band. And it is also difficult to identify the bands attributed to the β -sheet and the random coil structured proteins for the same reason.

On the other hand, it is most likely that the absorption bands of other cell components such as the nucleic acid (K. Miyamoto *et al.* 2005 and 2006) or the lipids (G. Socrates 2001) complicate the obtained spectra. For example; the absorption bands attributed to

the asymmetric stretching mode of COO^- group overlaps to the amide-II region, the bending vibrational mode of the N-H group also overlaps to the amide-I and amide-II region, and the base residues of the nucleic acid also have kinds of absorption bands in the amide-I region. And it is also difficult to assign the CH_x absorption peaks in $2700\text{-}3100\text{ cm}^{-1}$. There must be large number of CH_x groups on the Si prism surface. As described above, although the assignment of the absorption peaks is very complicated, these absorption bands should be good index to monitor the **cell adhesion and metabolism** by MIR-IRAS measurement.

Conclusion

We developed the monitoring system of living cultured cell using infrared spectroscopy and demonstrated that MIR-IRAS is capable of *in-situ* observation of living culture cell in this study, specially the cell **adhesion and metabolism** without any reagents. The sample chamber of the spectrometer was maintained **for to optimum the condition of the cell culture**, and the confluence of MCF-7 can be monitored by the absorption intensity at 1545 cm^{-1} of amide-II absorption band and also by the absorption intensities of CH_x absorption bands in $2700\text{-}3100\text{ cm}^{-1}$. To screen various biological active substances, one of the important information is cell viability or toxicity, which

affects the cell **metabolism**. Thus, this novel method can be used for **the** screening of many biological active substances. We have tried to observe the other cell types and other cellular activities such as apoptosis or necrosis using MIR-IRAS and these results will be discussed **in the next article**.

Acknowledgement

Part of this work was supported by Grants-in-Aid for Scientific Research (Grant No. 17206004) from the Japan Society for the Promotion of Science (JSPS).

References

- H. Isoda, T. P. N. Talorete, M. Kimura, T. Maekawa, Y. Inamori, N. Nakajima and H. Seki (2002) Phytoestrogens genistein and daidzin enhance the acetylcholinesterase activity of the rat pheochromocytoma cell line PC12 by binding to the estrogen receptor. *Cytotechnology* 40:117-123.
- J. Han, H. Isoda and T. Maekawa (2002) Analysis of the mechanism of the tight-junctional permeability increase by capsaicin treatment on the intestinal Caco-2 cells. *Cytotechnology* 40:93-98.
- T. Talorete, H. Isoda and T. Maekawa (2002) *Agaricus blazei* (Class Basidiomycotina) aqueous extract enhances the expression of c-jun protein in MCF-7 cells. *J Agric Food Chem* 50:5162-5166
- K. Emura, S. Yamanaka, H. Isoda and K. N. Watanabe (2006) Estimation for different genotypes of plants based on DNA analysis using near-infrared (NIR) and fourier-transform infrared (FT-IR) spectroscopy. *Breeding science* 56:399-403
- K. Miyamoto, K. Ishibashi, K. Hiroi, Y. Kimura, H. Ishii, M. Niwano (2005) Label-free detection and classification of DNA by surface vibration spectroscopy in conjugation with electrophoresis. *Appl Phys Let* 86:053902.

- K. Miyamoto, K. Ishibashi, R. Yamaguchi, Y. Kimura, H. Ishii and M. Niwano
(2006) *In-situ* observation of DNA hybridization and denaturation by surface
infrared spectroscopy. J Appl Phys 99:094702.
- H. H. Mantsch, D. Chapman (eds) (1996) Infrared spectroscopy of biomolecules.
Eiley-Liss, Inc, New York
- Hans-Ulrich Gremlich, Bing Yan (eds) (2001) Infrared and Raman Spectroscopy of
Biological Materials. Marcel Dekker, Inc, New York
- T. B. Hutson, M. L. Mitchell, J. T. Keller, D. J. Long and M. J. W. Chang (1988)
Mammalian Cell Growth Studied In Situ by Fourier Transform Infrared
Spectroscopy. Anal Biochem 174:415-422.
- T. Miyazawa and E. R. Blout (1961) The Infrared Spectra of Polypeptides in Various
Conformations: Amide I and II Bands. J Am Chem Soc 83:712-719
- G. Socrates (2001) Infrared and Raman Characteristic Group Frequencies. John Wiley
& Sons Ltd, pp50-67 and pp333-338

Figure legends

Figure 1. (a) Experimental setup of MIR-IRAS measurement and (b) schematic illustration of evanescent field on the Si prism surface. Evanescent field of infrared light penetrates about 500 nm deep into the medium solution.

Figure 2. (a) Schematic view of a FT-IR sample chamber. A mixed gas containing 5% of CO₂ gas was introduced into MIR-IRAS equipment via a bubbler bottle, and temperature of the sample chamber was maintained at 37±0.2 °C. (b) A Temperature vs. time plot obtained for the FT-IR sample chamber.

Figure 3. The micrographs of the MCF-7 cell on (a) GaAs substrate and (b), (c) on a Si prism surface. (b) The cell were added in the MIR-IRAS equipment and incubated for 24 h in a CO₂ incubator. (c) The cells after (b) were placed in the FT-IRA sample chamber for 300min of MIR-IRAS measurement.

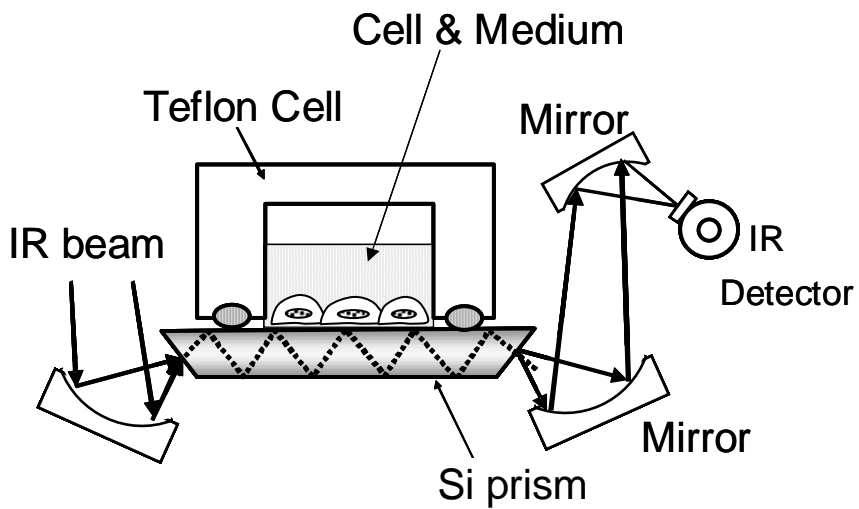
Figure 4. MIR-IRAS spectra of MCF-7 in the wavenumber region of (a) 1450-1750 cm⁻¹ and (b) 2700-3100 cm⁻¹.

Figure 5. MIR-IRAS spectra obtained from a control experiment without MCF-7 cells in the wavenumber region of (a) 1450-1750 cm⁻¹ and (b) 2700-3100 cm⁻¹. The spectra were measured at every 30 min.

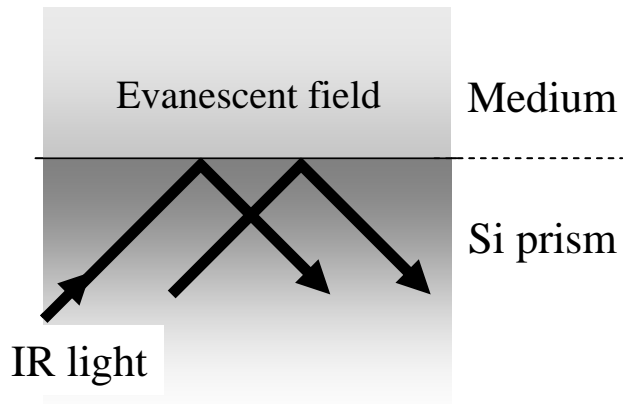
Figure 6. Time course of the peak intensities at (a) 1545 cm⁻¹, (b) 2925 cm⁻¹ (■), 2960

cm^{-1} (\square) and 2854 cm^{-1} (\blacktriangle). Solid lines indicate the peak intensities of MCF-7 cells (Figure 4), and dashed lines indicate the peak intensities of control experiment (Figure 5).

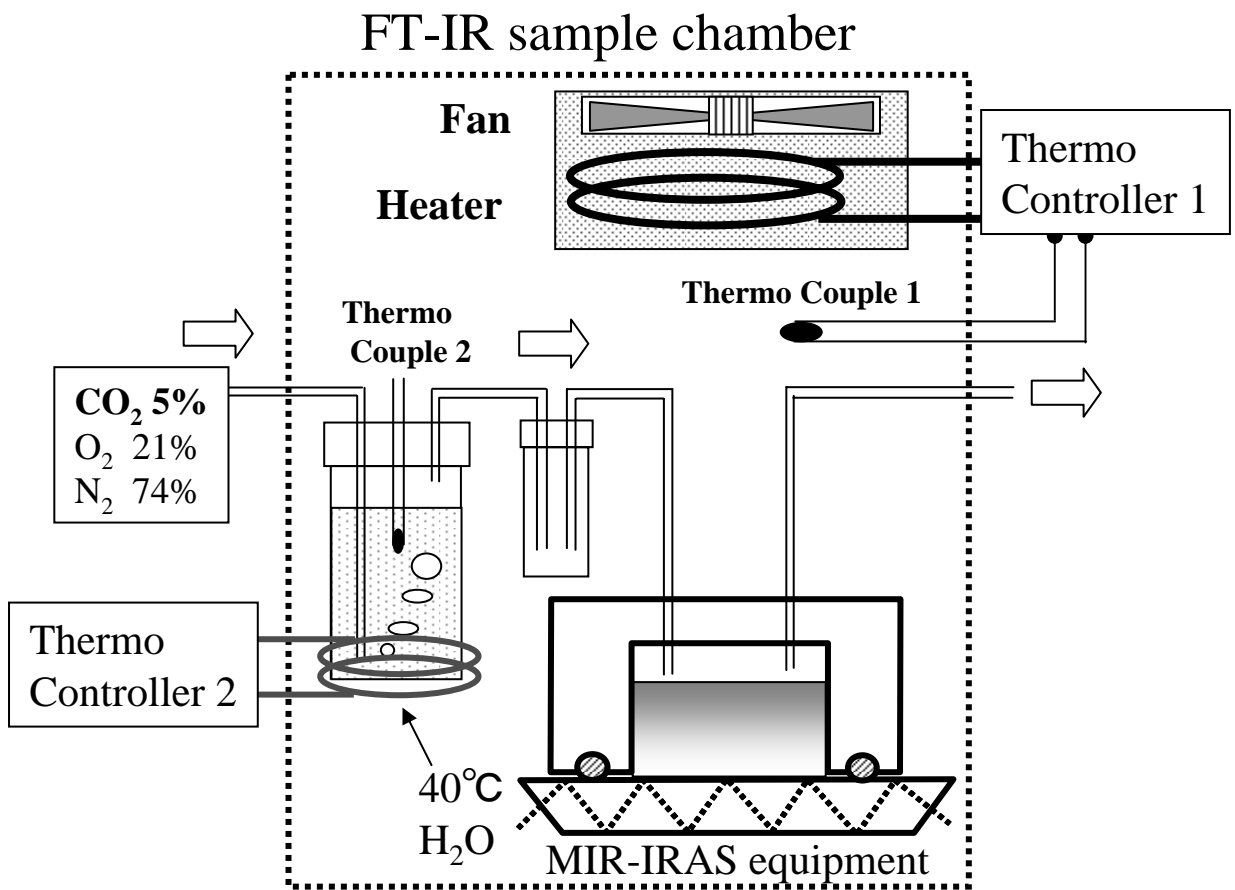
Figure 7. The cross sectional SEM image of MCF-7 cells on the Si prism



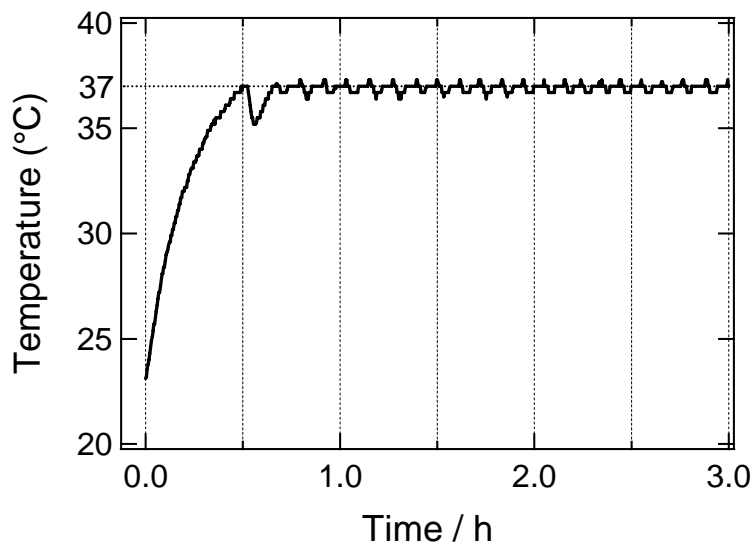
(a)



(b)



(a)



(b)

