SPECTROSCOPIC DIAGNOSTIC IMAGING OF CANCER RAW TISSUE BY FT-IR MICROSCOPY IN THE MIDDLE INFRARED AND BY NEAR-FIELD SPECTROSCOPY IN THE SUB-THZ REGIONS

Norio Miyoshi[1, 4], Kazuhiro Karaya, Yoshiaki Imamura, Ichiro Shimada, Akira Yamaguchi, Toshiharu Takahashi[2], Shigeki Nishina[3], Motoki Imamura[3], Yukihiro Fukunaga[1], Andriana B. Bibin[1], Kunihiko Tajima[4]

[1]Faculty of Medical Sciences, University of Fukui, Japan
[2]Research Reactor Institute, Kyoto University (RRI-KU), Osaka, Japan
[3]ADVANTEST, Co., Sendai, Japan
[4]Department Molecular Chemistry, Kyoto Institute of Technology, Kyoto, Japan

Abstract

The usage of H&E staining of morphological images for a pathological diagnosis has a long history. Even nowadays it is still used for cancer diagnosis by collecting biopsy samples from a surgical operation. Such images have only few quantitative parameters and sometimes this leads to ambiguous and subjective judgments. In fact, during the staining processes, some amount of the lipid and/or aqueous components and thereby the secondary conformation components of the protein are being lost as the cancer raw tissue is treated with methanol, xylene, heat, etc. It is possible, however, to use molecular vibrational spectroscopic imaging of raw tissue without such treatments in the operation room by utilizing Fourier-Transform Infrared (FT-IR) microscopy as a rapid screening method instead of the conventional H&E staining.

We have developed a special sample holder for a FT-IR microscope, which keeps the biopsy sample and preserves its freshness allowing such instrument to be used as a rapid but more perfect screener of cancer grades. For this analysis, we have used a calibration curve and an algorithm based on the principal component regression (PCR) implemented in the software that has been used for many pathological cancer (adenocarcinoma) case studies.

It has been found that the specialized sample holder, which is designed for keeping the frozen-section slice of the biopsy tissue fresh, prevents the destruction of the ATP components in the raw tissue and preserves 60% of its quantity for 1 hour (Observation #1). It has been observed that the lipid (-CH_2- and -CH_3 rich macromolecules) and phosphoric acid (PO_4) components in the raw cancer region were distributed more uniformly than those around the normal one (Observation #2). The α-helix component fraction of the secondary protein construction of the viable cancer region was increased compared to the normal one, which was estimated by the PCR analysis. The β-sheet component fraction was increased in the necrotic cancer area when compared with that of the viable one (Observation #3). Finally, it can be concluded that the developed FT-IR microscopic imaging of cancer markers, using the PCR calibration curve in 4 different colors, may prove itself an appropriate tool for a rapid screening in the operation room in the near future.

Key words: *spectroscopic diagnostic imaging, cancer raw tissue, ft-ir microscopy*

1. INTRODUCTION

Over the last 140 years, the pathological diagnosis and the judgment concerning the grade of cancer were based mainly on observing the hematoxylin-eosin (H&E) staining images. Nowadays, however, more important for the surgical pathology is the faster diagnosis that is based on the analysis of biopsy samples collected during the surgical operation. Recently, we have reported on the molecular analysis and imaging of cancer raw tissue using Fourier transform infrared (FT-IR) microscope, which may allow one to make distinction between the necrotic and the viable areas of the cancerous tissue by assessing the changes in the protein molecules [1]. It should be noted that such distinctive changes can't be observed from H & E staining images.

The history of frozen-sectioned samples for the rapid screening histology is also very long. It can be traced back to the pioneering work of L.B. Wilson [2], published in 1905. The articles by S. P. Hermanek [3] as well as by A. A. Gal and P. T. Cagle [4], that have been dedicated to the anniversaries of their seminal contribution, can also be quoted in this regard. Despite its advantages, however, this technique has also well-known drawbacks. For example, J. E. Fisher et al. [5] have drawn the attention to the fact that the sample is very fragile and can be used only on the day of its preparation. G. Keeney and K. Lesito have also reviewed the preparation of fresh tissues for microscopic studies [6]. Recently, a frozen-sectioned slice (7 μm) was used for the FT-IR micro-spectroscopy in the field of pathology by M. A. Cohenford, S. Lim, C. Brawn., et al. [7]. They, however, have not provided information how the freshness of the slice was preserved during their lengthy measurements at a room temperature. Here we underline the importance of this circumstance because it is well known that the conditions of the frozen-sectioned slices inside the tissue change very quickly and thereby a large difference between the changes inside the normal and the cancerous samples could occur even during the first 15 mins.

2. EXPERIMENTS AND METHODS

2.1. Experimental Tumor Model

The glial tumor cell line (C6; ATCC CCL-107, Sumitomo Cell Bank Co., Tokyo, Japan) of rat was cultivated with RPMI1640 medium (Katayama Chemical Co. Ltd., Osaka, Japan) containing 5%-BSA in the CO_2 incubator under the conditions of 37°C and 5% CO_2 gas. Around $2x10^5$ cells were harvested and gathered to inject 0.1 ml of the new medium solution under the skin on the thigh of the Balb-c-nu/nu strain mouse (CLEA Japan Inc., Tokyo, Japan), and finally after waiting for 3 weeks a tumor model tissue was developed on the thigh of the mouse.

2.2. Preparation of the Samples of the Frozen-section

The frozen-sectioned samples with a thickness of 8-10 μm were prepared using the cryostat instrument (Type-CM-1950-OUVVM, Leica Biosystems Co, Ltd., Germany). The frozen-sectioned samples were stored in a plastic flask (dish) and then transported to the SR center in dry-ice.

2.3. Biochemical analysis of the ATP contents in the frozen-sectioned slices of C6 tumor and normal brain tissues

The frozen-sectioned samples were homogenized in a PBS solution using a disposable homogenizer (Biomasher II of Nippi, Inc. Tokyo, Japan) followed by a centrifugation at 1,000 x g at 4°C for 5 minutes to remove the cellular debris. The supernatant was separated and then used for the measurement of the ATP contents and protein concentration. The level of ATP was determined using Tissue ATP assay kit (Toyo B-net Co., LTD, Tokyo, Japan). ATP was extracted from the supernatant and luciferin-luciferase mixture was added according to the manufacturer's instruction. The bioluminescence of the sample was evaluated using the Luminescence-PSN AB- 2200 luminometer (ATTO Co. LTD, Tokyo, Japan) and compared to that of the standard amounts of ATP used in calibration curve prepared by standard ATP in the kit. The protein concentration of the sample supernatant was determined using the BCA protein assay kit (Pierce, Rockford, IL USA).

2.4. Measurement of the secondary conformations content in the protein components of cancer tissue by the FT-IR microscope

The frozen-sectioned samples were put on the BaF_2 circular plate (with a diameter of 10 mm and a thickness of 1 mm) and set into the holder fixed on the stage of the FT-IR spectrophotometer (Spectrometer: FT/IR-6100-ST Type, Microscope: IRT-3000M Type, JASCO Co, Tokyo, Japan). The IR spectra of the raw cancer samples were measured using an aperture size of 2.5x2.5 μm^2. All these spectra (more than 7,000 altogether) were analyzed within 10 min by a curve-fitting method (Voigt-function) at Amide-I peak in the region 1,700-1,600 cm^{-1} using a dedicated software (IR-SSE type, JASCO Co., Tokyo, Japan) to estimate 4 different secondary conformations (α-helix, β-sheet, β-turn, and the one that includes random coil) of the protein content. The total measuring and imaging time of less than 15 min is appropriate for the envisaged prototype of a surgical cancer screener that uses the developed sample holder.

3. RESULTS AND DISCUSSION

3.1. Prototype of the FT-IR microscope system as a Surgical Cancer Screener

The prototype of the sample holder includes 4 parts: (i) temperature controller; (ii) generator and compressor of dried air; (iii) moisture generator; and (iv) humidity controller as shown on the upper left side (a) in Figure 1. The humidity is supplied by the moisture generator (d) in a rate that depends on the readouts of the detector of the dew point inside the holder (c). The surface windows of the sample holder are dried for preventing dew formation using the dried air generator (b).

3.2. Other parts of the developed sample holder

The experimental setup (see Fig.2) includes also: (i) frozen sliced sample (thickness 10 μm); (ii) heat bridge structure (diameter 1 mm, height 3 mm); (iii) humidity controlled gas generator; (iv) barium fluoride windows (diameter 30 mm, thickness 2 mm); (v) constant temperature plate; and an internal BaF_2 plate (diameter 1 mm, thickness 1 mm). This structure provides a 1.5 mm narrow space with a controlled humidity at a constant temperature. It includes a heating bridge, which prevents the condensation of dew on the top cover. It should be underlined that the overall thickness of the holder is only 9 mm.

3.3. Actual generation of the FT-IR spectra of a cryo-sectioned slice of tumor model tissue inside the developed holder on the stage of the micro-spectroscope

The claer FT-IR spectra was generated without noises of vapor ($O\text{-}H_{sv}$: stretching vibrational components between 3,100-4,000 cm^{-1} and $N\text{-}H_{sv}$: stretching vibrational one at 3,400 +/- 50 cm^{-1}) for the cryo-sectioned slice tumor tissue measured by the microscope inside the developed cell holder chamber at 6°C and 60 % humidity as shown in Figure 3. The upper and lower spectra (expanded parts) show the anti-symmetrical P=O stretching vibrational ($P{=}O_{asv}$) and the symmetrical ($P{=}O_{sv}$) components, respectively. It was considered that the noise was excluded by controlled shortening (1.5 mm) of the distance between the upper window and the sample surface.

3.4. Measurement of ATP contents and an estimation of secondary conformation contents of the proteins in the sliced tumor tissue

The ATP contents in the cancerous region were analyzed by the fluorescent detection using an ATP assay kit. They were decreased exponentially in time when the sample was outside of the holder at room temperature (19°C) as shown in Figure 3 (see the red curve). On the other hand, the ratio of β-sheet/α-helix of cancerous region in the secondary conformation was increased linearly with time (<60 min) as shown by the blue curve. Therefore, there were reciprocal and correlated ATP losses and protein conformation changes.

Inside the sample holder with a controlled humidity (66% at 5°C) the value of ATP contents of the protein were preserved (preventing 60% of the losses that take place outside the holder) for both the C6 glial tumor model and for the normal brain tissues as shown in Figure 4. All these demonstrate that the ATP losses are a sensitive indicator of the freshness losses of the raw tissue. Therefore, we had been able to carry out FT-IR spectroscopic analysis and mapping of the images inside the sample holder within 15 minutes without changing the protein conformation.

3.5. The content ratio (β/α) of α-helix and β-sheet conformation changing the temperature in the sample holder

Even in the sample holder, the changes of temperature induced large changes in the conformations of the normal tissue as shown in Figure 5 for the case of the normal brain tissue. A significant difference in the sensitive marker of the protein 2ndary conformation damages for the ratio of β-sheet and α-helix, which had been developed by our team previously [1] in the normal brain tissue and for the tumor model, can be observed. Fig. 6 shows the temperature dependency of α-helix and β-sheet contents (%) of the tumor and normal tissues against the increasing (each 2 min steps) of the temperature controlled inside the sample holder. The upper pairs of the graph are α-helix contents (%) of C6 glial tumor (Blue) and normal (Red) tissues. The lower pairs of the graphs are β-sheet contents (%) of C6 glial tumor (Blue)

and normal (Red) tissues. The right panel of the figure shows the temperature dependency of protein stability for the secondary conformation ratio in the frozen-sectioned slices of normal muscle (Black) and tumor (Blue) tissues using the left hand-side data.

From a surgical screener, it is expected to better measure the contrast of the mapping images at lower temperature. The newly developed sample holder for the FT-IR microscope completely satisfies the above formulated requirements and provides the necessary functionality.

4. CONCLUSION

In this paper, we presented a novel versatile sample holder for FT-IR microscope, which preserves the freshness of the sliced tissue by controlling and maintaining the humidity to 60% at 6°C. In such a way the noise in the spectrum due to the moisture inside the holder can be greatly reduced. Moreover, it has been proved that the fresh tissue inside the holder preserves 60% of the ATP content at least for 60 min.

We believe that the sample holder presented here can be considered as a prototype for the development of an optimized and more advanced instrument that could be used as a surgical cancer screener in the operation room. Moreover, another prospective application of this holder is as an additional plug-in to the existing fluorescence microscopes.

Disclaimer

Any opinions, findings, conclusions or recommendations expressed in this material are those of the authors and contributors and do not necessarily reflect those of the editors or the publisher. The editors and the publisher cannot accept liability for damages arising from any errors or omissions in this material. Please inform the editors of any errors.

ACKNOWLEDGEMENTS

This project has been supported by the JST (Japan Science and Technology Agency) foundation and funded by the Japanese Government as a 3.5 years project. During this research the machine time cost and the travel expenses were supported by a fund of the National Collaboration Research Foundation in the Research Reactor Institute at Kyoto University. One of the authors (N. M.) wishes to express many thanks to Emeritus Prof. Haruo Hisazumi of Kanazawa University and Dr. Kazumori Ishiguro for their academic supports. The study was supported also by a Grant-in-Aid for Scientific Research (C-2: No.11672293; B-2: 14370793, B-2: 11557116 and Special study Area: Photo-functional Surface: Grant No. 17029024) of the Japan Society for the Promotion of Science (JSPS).

REFERENCES

1. T. Yamada, N. Miyoshi, T. Ogawa, K. Akao, M. Fukuda, T. Ogasawara, Y. Kitagawa, and K. Sano: Observation of molecular changes of a necrotic tissue from a murine carcinoma by Fourier-transform infrared microscopy. *Clinical Cancer Research,* **8 (6):** 2010-2014 (2002).
2. L.B. Wilson: A method for the rapid preparation of fresh tissues for the microscope. *JAMA,* **45:**1737-1740 (1905).
3. P. Hermanek: 90 years of rapid-section histology, *Pathology*, **3(1):** 53-54 (1981).
4. A. A. Gal, P. T. Cagle: The 100-years anniversary of the description of the frozen section procedure. *JAMA*, **294 (24):** 3135-3137 (2005).
5. J. E. Fisher, P. C. Burger, E. J. Portman: The frozen section yesterday and today: Pediatric solid tumour. *Pediatr. Dev. Pathol.*, **4(3):** 252-266 (2001).
6. G. Keeney and K. Lesito: Preparing fresh tissues for the microscope. *JAMA* **300(9):** 1074-1076 (2008).
7. M. A. Cohenford, S. Lim, C. Brown, et al.: FT-IR microspectroscopy of mouse colon tissues: insight into the chemistry of carcinogenesis and diagnostic potential. *Am. J. Pathol.*, 2012, **181(6):** 1961-1968.

FIGURES AND LEGENDS

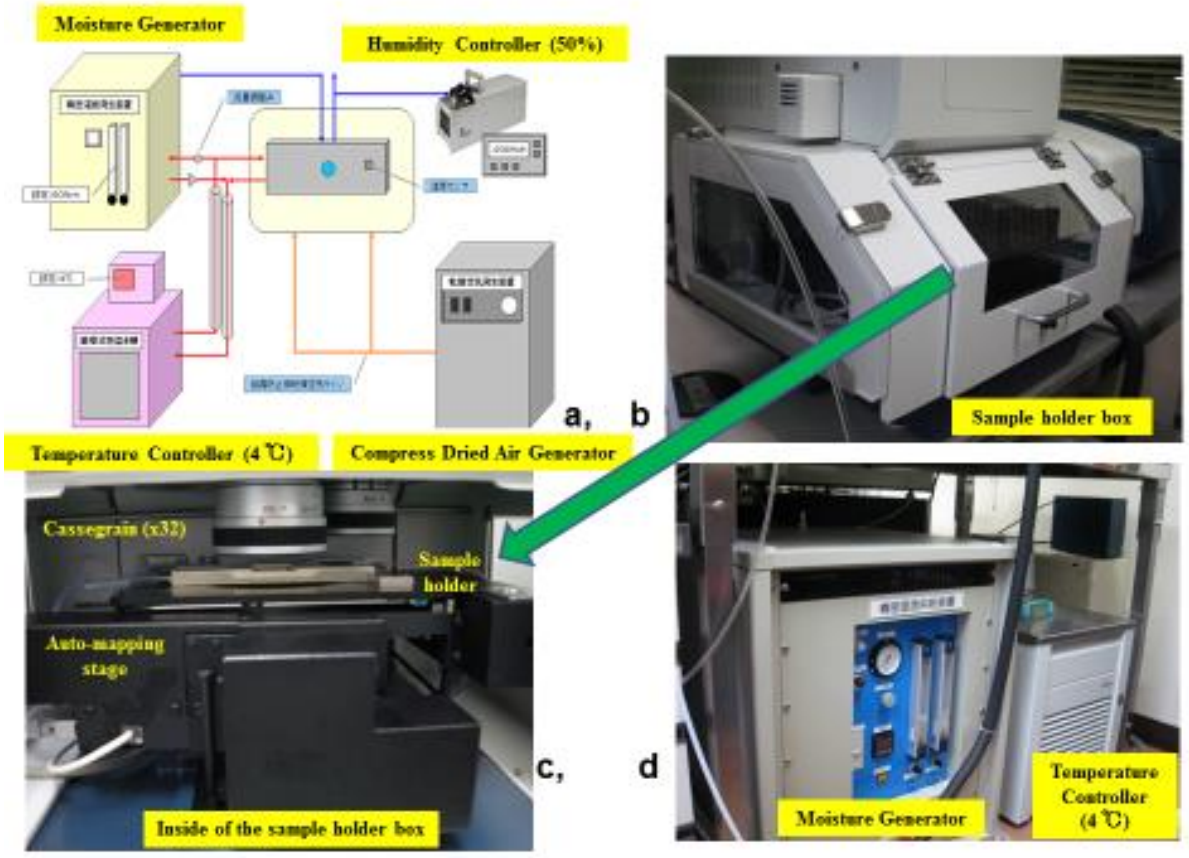


Figure 1. Control system of the sample holder (a); sample holder box and the stage of the FT-IR microscope (b); expanded interior of the holder box (c); moisture generator and temperature controller of the water bath (d).

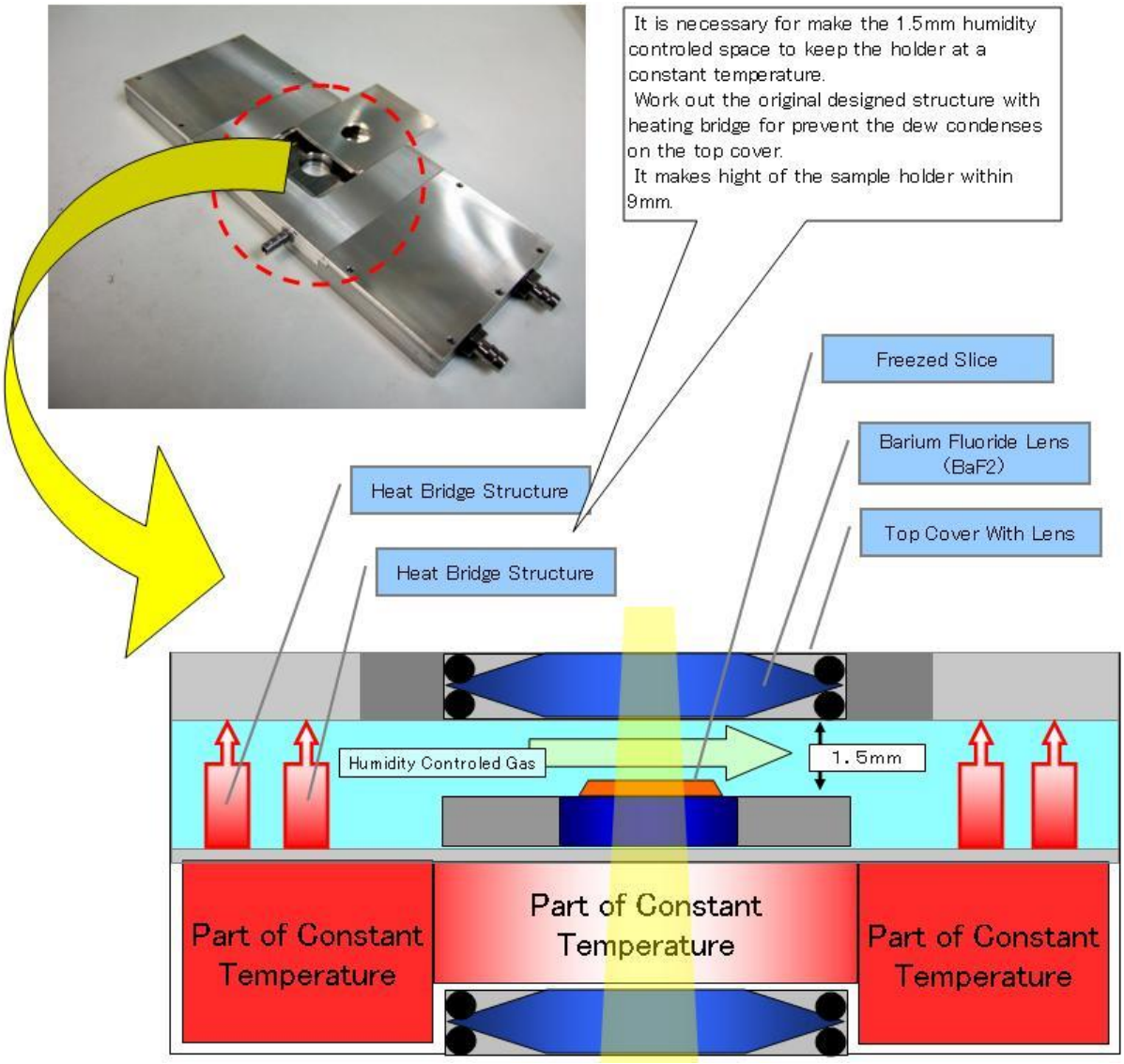


Figure 2. A photo and a schematic layout of the developed sample holder.

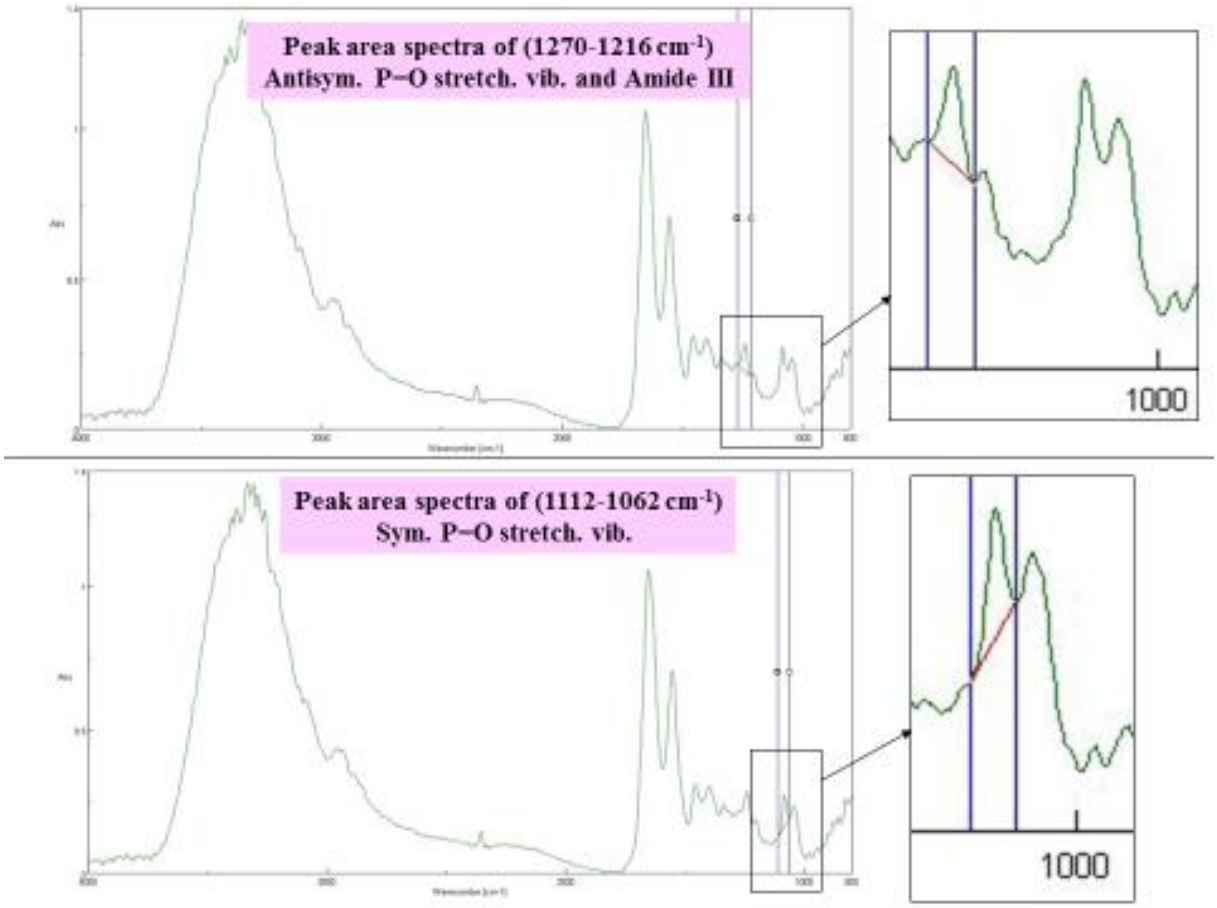


Figure 3. Actual FT-IR spectra of the cyo-sectioned slice tumor tissue measured by the microscope inside of the developed cell holder chamber at 6 degree C and 60 % humidity. The upper and lower spectra (expanded parts) show the anti-symmetrical of P=O stretching vibrational and the symmetrical components, respectively.

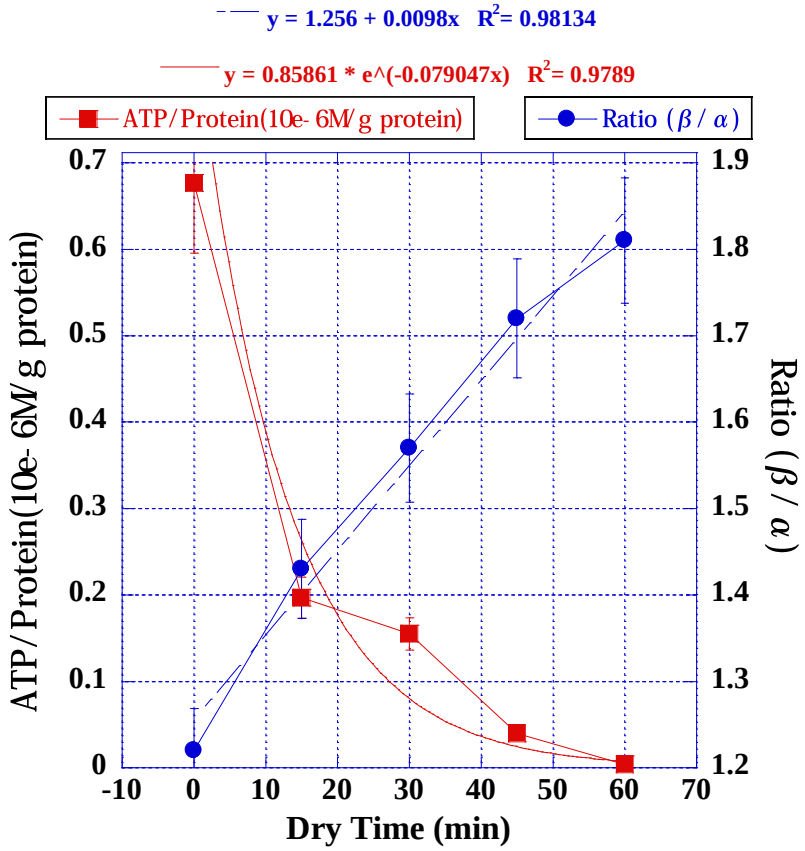


Figure 4. Changes of the ratio of the concentration of ATP against a unit (g) of protein (10E-6 M/ g protein) and the ratio (b-sheet/a-helix) estimated from FT-IR spectral analysis of Amide-I against the drying time (min) outside of the holder.

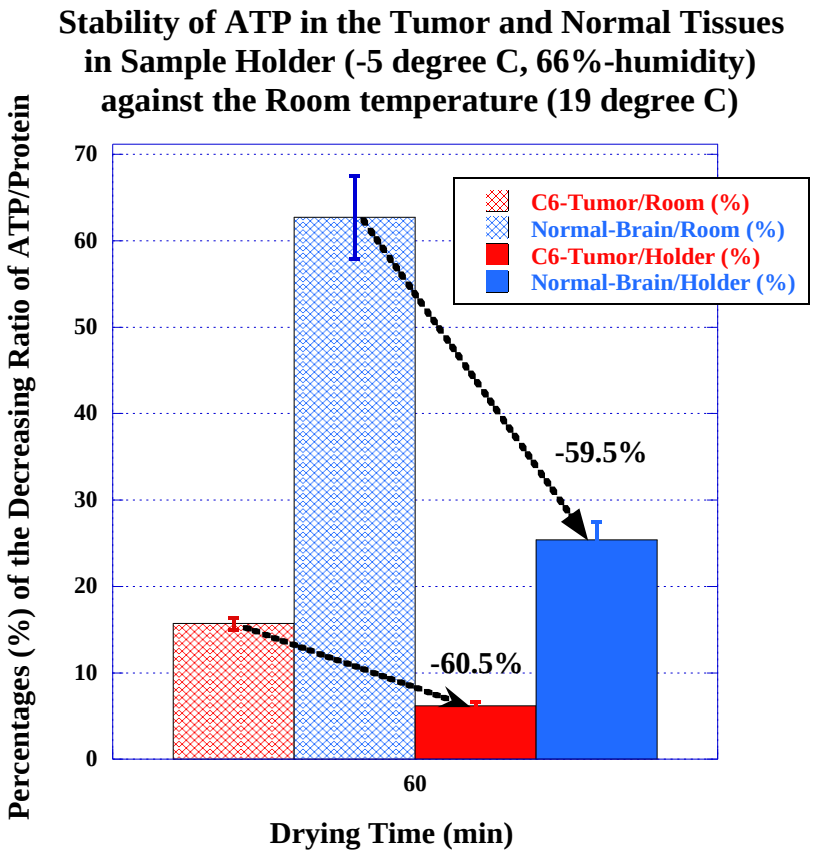


Figure 5. Stability of ATP in the tumour and normal brain tissues in the sample holder at room temperature (19°C).

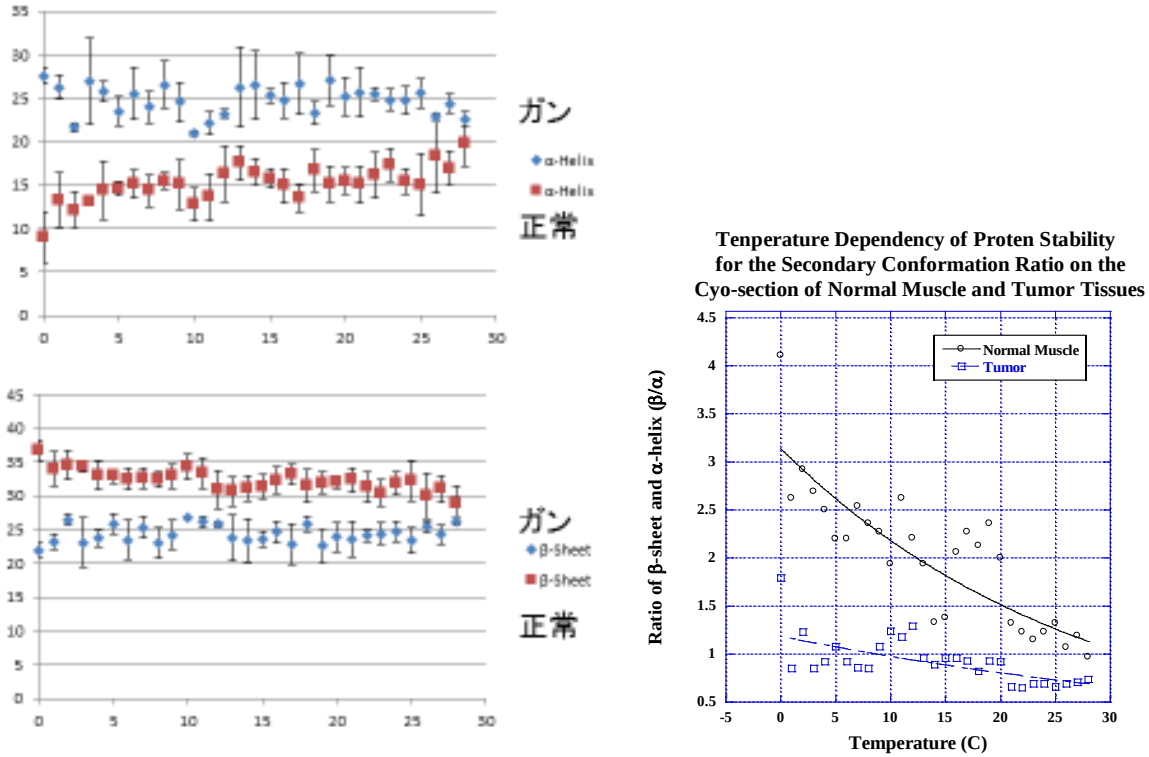


Figure 6. Temperature dependency of α-helix and β-sheet contents (%) of tumour and normal tissues against the increasing (each 2 min steps) temperature controlled inside of sample holder.