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Continuous specimen cooling during slicing and thickness measurement contributes to improved accuracy in image analysis of pathologic specimens

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Summary. We measured section thickness (ST) after slicing using a film thickness meter and investigated the relationship between ST and the percent area of positive staining using computer-assisted image analysis. Methods: Sections were prepared from a paraffin-only block and formalin-fixed paraffin-embedded (FFPE) blocks containing fish sausage and human liver specimens. The ST was compared between the sections prepared with cooling using an ice pack (IP) or a continuous cooling device (CCD) paired with a sliding microtome set at an ST of 4 μm. The sections were stained with eosin or aniline blue, and the association between the percent area of positive staining and ST was determined using computer-aided analysis of images captured with a whole slide scanner. Results: The average STs of the paraffin-only block sections measured by four practitioners were 5.01-5.41 and 4.09-4.33 μm in samples prepared using an IP and a CCD, respectively. Therefore, subsequent analyses included sections prepared using the CCD. The ST of the tissue surface was significantly thinner than that of the paraffin surrounding the tissue section. Furthermore, the percent areas of positive staining for eosin and aniline blue were significantly correlated with ST in both the fish sausage and liver sections. The analysis of the ST and percent area of positive staining in 60 sections of the same block, which were categorized into quantiles based on ST, revealed a significant difference in the percent area of positive staining between the thicker and thinner sections. Discussion: Specimen sectioning should be performed with a CCD, ST should be measured before the staining of pathologic specimens prepared for quantitative analysis, and histologic examination should be performed using specimens with uniform ST.

Key words: Formalin-fixed paraffin-embedded block, Continuous cooling during slicing, Slicing accuracy, Specimen thickness measurement, Computer-assisted image analysis

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Introduction

Various microtomes are used for thin sectioning of specimens, including rotary, base sledge, rotary rocking, sliding microtomes, and ultramicrotomes (Spencer, 2019). Among these, the rotary microtome is primarily used for formalin-fixed/paraffin-embedded (FFPE) specimens (Sy and Ang, 2019); the base sledge microtome is primarily used for large blocks, hard tissues, and whole-mount blocks, among others (Spencer, 2019); the rotary rocking microtome is used for cryosectioning (Spencer, 2019); and the ultramicrotome is used to prepare samples for electron microscopy. Furthermore, the sliding microtome, originally developed for celloidin-embedded specimens (Spencer, 2019), is used for FFPE specimens (Randall et al., 1988). However, no study to date has examined the exact specimen thickness or the accuracy of sectioning performed with specific microtomes.

Conversely, automatic immunostaining equipment is increasingly used to determine the therapeutic utility of molecular targeted drugs through the routine pathologic evaluation of specimens immunostained for HER2 (Thorat et al., 2021), programmed death 1 (Huan et al., 2023), or programmed death ligand 1 (Jing et al., 2023).

In the present study, we aimed to investigate the optimal section thickness (ST) and the stainability of FFPE specimens by measuring the ST of unstained specimens after drying and sectioning with a microtome. In addition, we compared the slicing conditions between

FFPE blocks precooled with an ice pack (IP) and those precooled with a continuous cooling device (CCD).

Materials and methods

Samples

In the present study, samples from fish sausage (Toyo Suisan) and human liver were used. Human samples were prepared from resected liver tissues collected between April 1, 2019 and March 31, 2021 in Gunma University Hospital from patients aged above 20 years diagnosed with hepatocellular carcinoma and hepatitis C virus infection. The human liver samples were prepared from residual tissues preserved with formalin fixation after the final pathologic diagnosis. A parapet (Muto Pure Chemicals) was used to measure ST after slicing the paraffin-only-embedded specimen.

The present study was conducted under the approval of the Gunma University Ethical Review Board for Medical Research Involving Human Subjects (HS2021-018).

Sectioning

To prepare sections, a microtome blade was attached to the knife. Fish sausage was sliced to prepare sections with dimensions of 1.5×1.5×4 mm. The liver sample was cut into pieces to prepare sections with dimensions of 1.5×1.5×4 mm.

The cassettes containing the fish sausage and liver samples were immersed in phosphate-buffered 10% formalin for 28h before embedding.

Tissue embedding

The tissue embedding equipment embedding Center (Sakura Finetek Japan, Chuo-ku, Japan) was used for tissue embedding. First, a 2×2 cm embedding dish was placed in the center of the embedding equipment and filled with melted paraffin. To prepare the paraffin-only block, an embedding cassette was placed on the dish, the melted paraffin was poured over the cassette until the cassette cavity was filled with paraffin, and the dish was placed on a cold plate maintained at 4°C for cooling. To prepare the fish sausage and liver sample blocks, the tissue piece was removed from the embedding cassette and placed in the center of the embedding dish, forceps were used to press the sample to the bottom of the dish and to fix the sample position in paraffin while simultaneously cooling paraffin by placing the dish on the cold plate. After confirming that the sample position was fixed, the embedding cassette used during tissue processing was placed on the dish, and the melted paraffin was poured over the cassette until the cassette cavity was filled with paraffin. Next, the dish was placed on a cold plate maintained at 4°C for cooling.

After confirming paraffin solidification, the paraffin-only and the fish sausage and liver samples were removed from the embedding dish and placed on the embedding cassette by flipping the paraffin blocks.

Slicing

Slicing was performed using a Retratome REM-710 sliding microtome (Yamatokoki Kogyo). Before slicing, the paraffin blocks were cooled on an AS ONE cool plate (ZCP-15150). The paraffin block stand on the sliding microtome (Fig. 1A-C) was replaced with a paracooler (PC-110; Yamatokoki Kogyo) (Fig. 1A) (Fig. 1B) to cool the paraffin block throughout the slicing at 9-11°C. A water bath maintained at 42°C attached to a paraffin stretcher (PS-110WH; Sakura Finetek Japan) was used to stretch and place the paraffin-embedded sections on glass slides. A low-plate type T-76 stretcher (42°C) (Takashima Shoten) was used to stretch the sections before drying. For samples that were not cooled during slicing, the paraffin blocks were placed on the paraffin block stand and cooled by placing an IP on the paraffin block (Fig. 1C). A denture adhesive (New Liodent Pink; Lion) was used to fill the air gap between the back of the paraffin block and the CCD or the paraffin block stand so that the CCD's temperature could be transferred, and the sample was cooled for 30 min on the CCD before slicing. The room temperature was maintained at 25°C by air conditioning.

First, the block was placed on the CCD or the stage and the level was adjusted so that the block surface was horizontal. After fixing the block, a microtome blade (Feather Safety Razor) was set; the blade was replaced after approximately 50 slices. The lights and humidifier were also turned on, and the steam from the humidifier hit the surface of the paraffin block to prevent curling of the sliced sections before rough slicing to remove the surface paraffin covering the sample. Next, the steam was turned off, and the IP was placed on the block for approximately 2 min to cool the block and bring the initial block temperature without the CCD closer to the block temperature achieved with the CCD.

Before slicing, the date of slicing, person performing the slicing, ST set on the microtome, room temperature, humidity, paraffin block temperature before the start of slicing, and CCD temperature (or stage temperature if CCD was not used) were recorded. The surface temperatures of the paraffin block and the CCD were measured using an infrared radiation thermometer (A Life) at approximately 12 cm from the measurement surface. For blocks containing the fish sausage and liver samples, the exposed tissue surface on the paraffin block was used to measure block temperature.

For slicing, the power supply for the humidifier was turned back on and the sliced sections were picked up using a piece of paper made from a business card and floated in the water tank for continuous sectioning. At this time, the duration of slicing of the paraffin block and the time interval between the end of slicing and the touching of the blade to the paraffin block to resume slicing were recorded. For the paraffin-only block, the slicing interval was set to 15 sec. For blocks containing

the fish sausage and liver samples, the interval was also fixed to 15 sec.

For the examination of sections prepared from the paraffin-only block, two sets of six slices were prepared. For the examination of the fish sausage and liver sections, two sets of 60 slices were prepared. After the completion of slicing, the humidifier power was turned off and the surface temperatures of the paraffin block and the CCD at the end of the slicing were recorded as described above.

Next, the sections were placed on glass slides (Star Frost Edge Polished, Muto Pure Chemicals) so that the start part of the sliced section was placed on the upper left of the glass slide. Next, the sliced sections were stretched in hot water using a hot water bath-type paraffin stretcher at 42°C. After wiping off excess moisture with Kimwipe® (Nippon Paper Crecia), the sections were stretched by placing them on a paraffin stretching plate for at least 30 min. Subsequently, the sections were dried overnight in a paraffin melter (Sakura Seiki) at 37°C. For detection condition setting for computer-assisted image analysis of eosin-stained

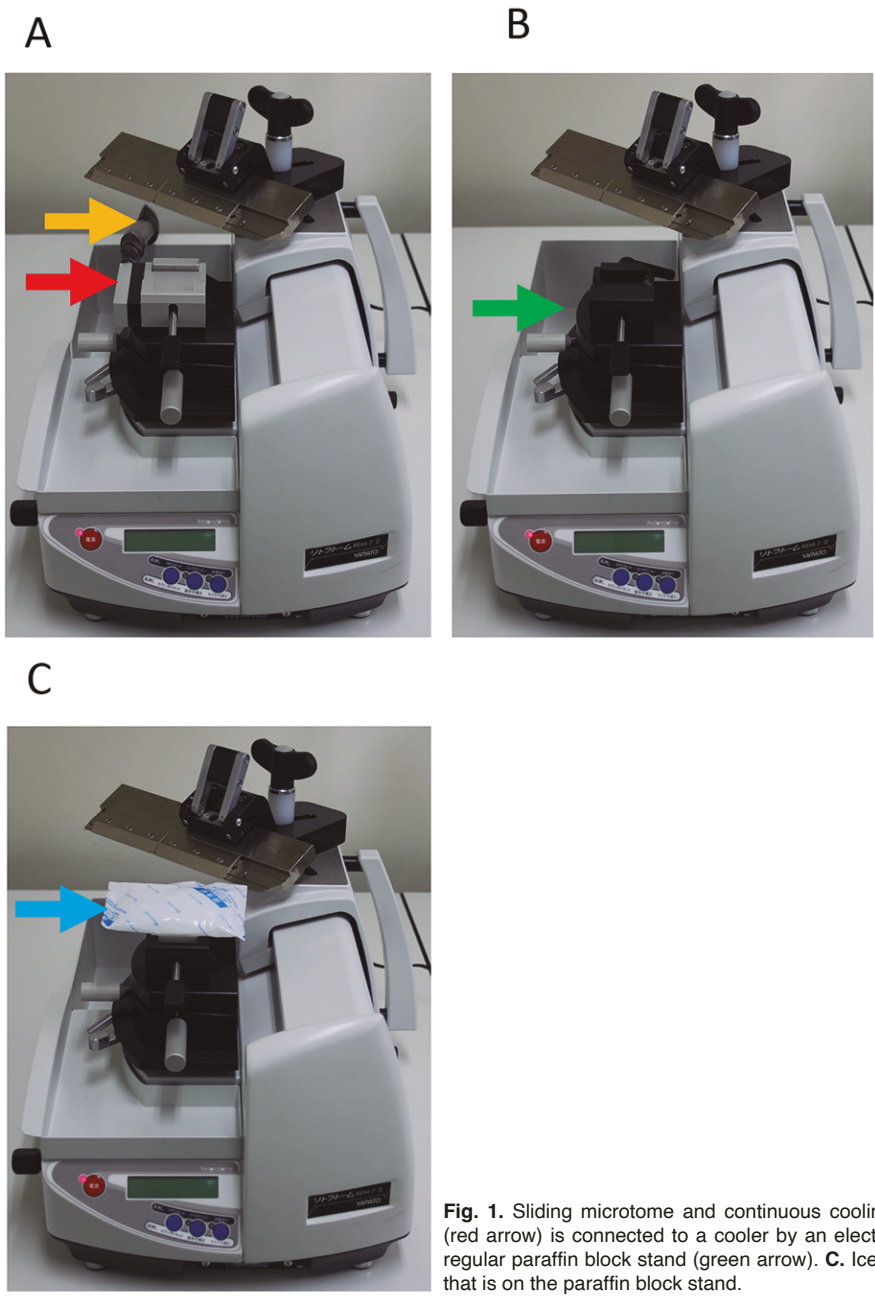

Fig. 1. Sliding microtome and continuous cooling device (CCD). **A.** Paraffin block stand-type CCD (red arrow) is connected to a cooler by an electric wire (orange arrow). **B.** Sliding microtome with a regular paraffin block stand (green arrow). **C.** Ice pack (IP, blue arrow) is placed on the paraffin block that is on the paraffin block stand.

specimens, the microtome was set at 4 and 8 µm. In another experiment, the microtome was set at 4 µm.

Measurement of the temperature of the paraffin block surface

Alterations in the temperature of the paraffin blocks were measured under CCD or cooling by IP conditions.

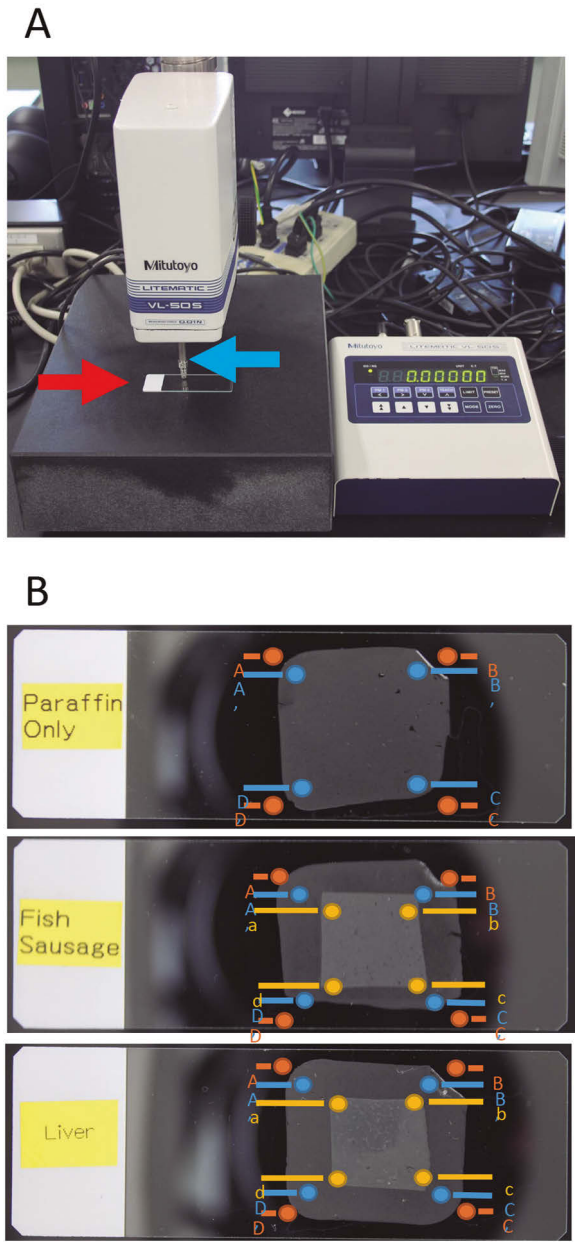

Fig. 2. Measurement of section thickness using a film thickness meter. **A.** The glass slide (red arrow) is placed on the stand of the film thickness meter, and the measuring needle (blue arrow) is placed in contact with the glass slide. **B.** Paraffin-only (upper panel), fish sausage (middle), and liver tissue (lower) sections on glass slides are shown. Red dots indicate the glass surface, blue dots indicate the paraffin surface, and yellow dots indicate the tissue surface.

To measure temperature with CCD, the temperature of the block was measured every 10 sec for a total of 180 sec. To measure temperature with cooling by IP, the IP was placed on the paraffin block to cool before temperature measurement. Two minutes later, the IP was removed and the temperature was immediately measured. The temperature was measured every 10 sec for a total of 180 sec. The measurements were saved in a Microsoft Excel file.

Measurement of the thickness of the sliced sections

The thickness of the sliced sections was measured using a film thickness meter (Lightmatic VL-50S; Mitutoyo Corporation, Tokyo, Japan) (Fig. 2A). Figure 2B shows an example of thickness measurement of the paraffin-only sections. Measurements were performed on four corners of the sections. First, the glass slide thickness was measured at four corners (red dots, A to D). Next, the thickness of the paraffin section plus the thickness of the glass slide were measured near the locations where the glass slide thickness was measured (blue dots, A' to D'). Measurements were performed in the order of A → A' → B → B' → C → C' → D → D', with the first measurement performed at A. Measurements were performed three times for each location, and average values were used for analyses. In addition, in sections containing the fish sausage and liver specimens, points on the tissue surface were defined as a, b, c, and d (yellow dots), as shown in Figure 2B. Measurements were performed in the order of A → A' → a → B → B' → b → C → C' → c → D → D' → d, and each point was consecutively measured three times. The measurements were saved in a Microsoft Excel file together with the data recorded during sectioning. After thickness measurement, the surface of the sections was coated with paraffin by Para-Mate (Kaken Genex, Tokyo, Japan).

Eosin staining

The sections containing fish sausage were stained with eosin. First, the sections were deparaffinized by immersion in xylene three times for 5 min each, followed by immersion in absolute alcohol for 1 min, 99% alcohol for 1 min, 95% alcohol for 1 min, and 70% alcohol for 1 min. The sections were washed with running water. Next, the sections were immersed in New Eosin Type M solution (Muto Pure Chemicals) for 5 min, rinsed with distilled water, and immersed consecutively in 70% alcohol for 1 min, 95% alcohol for 1 min, absolute alcohol twice for 1 min each, and xylene three times for 5 min each. The sections were then mounted using a mounting medium.

Aniline blue staining

Aniline blue staining was performed on liver-containing sections using a modified Azan staining

method. First, the sections were immersed in xylene three times for 5 min each, followed by immersion in absolute alcohol for 1 min, 99% alcohol for 1 min, 95% alcohol for 1 min, and 70% alcohol for 1 min. After washing with running water and immersion in distilled water for 1 min, the sections were immersed in dichromic acid for 10 min, washed with running water, and immersed in distilled water for 1 min. Next, the sections were immersed in 2.5% phosphotungstic acid for 10 min, 1% acetic acid for 1 min, aniline blue solution (Muto Pure Chemicals) for 5 min, and 1% acetic acid for 1 min. Subsequently, the specimens were washed with running water and immersed in 70% alcohol for 1 min, 95% alcohol for 1 min, anhydrous alcohol twice for 1 min each, and xylene three times for 5 min each before mounting.

Image capture using a whole slide scanner

After staining, images of the fish sausage and liver sections were captured using a whole slide scanner (Nano Zoomer; Hamamatsu Photonics) in manual mode. Briefly, the glass slides were placed in the scanner, the file name and profile were entered, and the magnification was set to 20×. The slides were scanned after a left drag was performed on the tissue section, and the region for image capture and the section to be focused were manually selected.

Computer-assisted image analysis

For computer-assisted image analysis, the captured images were converted using the Slide Converter program (3D HISTECH). The area of the converted image for analysis was determined using the annotation function of Panoramic Viewer (version 1.15.4; 3D HISTECH), and the software settings for image analysis were created using the Quant Center HistoQuant Module (3D HISTECH) of the Panoramic Viewer. Fat and fish meat are mixed in fish sausage; therefore, the settings for the fish sausage sections were adjusted by adjusting the color tone of the eosin staining the fish meat at 40% under the condition of 8 µm thickness. The settings were as follows: hue, 312-348; sat, 12-52; noise reduction: blur (Gauss), 1 without using "separation" or "fill holes" functions or the size filter. The settings for the liver sections were as follows: hue, 204-234; sat, 34-100; noise reduction: blur (Gauss), 1 without using "separation" or "fill holes" functions or the size filter. Additionally, adjustments were performed to maintain a positive staining area of approximately 30% in liver sections at 4 µm thickness staining with aniline blue. The data obtained using these settings were saved in a Microsoft Excel file.

Statistical analysis

JMP Pro version 15.2.0 (SAS Institute Japan, Tokyo, Japan) was used for all statistical analysis. The Steel-Dwass test was used for nonparametric comparisons of three or more groups, and the Wilcoxon/Kruskal-Wallis test was used for nonparametric comparisons between two groups. A P value of ≤ 0.05 was considered to indicate statistically significant differences.

Results

Continuous cooling provides a stable temperature for the FFPE block

To examine whether temperature changes before and after slicing paraffin blocks affect ST, we first evaluated the variations in the surface temperature of the paraffin block processed using a CDD or an IP. As shown in Figure 3, a minimal change in the surface temperature of the block was observed when CDD was used for cooling. However, the surface temperature ranged between 15°C and 16°C at the start of the temperature measurements and rose to 20 to 22°C over 3 min when an IP was used for cooling. These results indicated that a constant surface temperature could be maintained using a CCD.

Continuous cooling during slicing facilitates section thickness that matches the slicing setting

We investigated the difference in ST between the sections prepared from paraffin blocks cooled with CCD and IP during sectioning. In our analyses of the paraffin-only block (Fig. 4), the sections were significantly thinner with CCD than with IP cooling in specimens prepared by four different practitioners (Practitioner A: CCD 4.12±0.53 µm, IP 5.01±1.05 µm, P=0.0004; Practitioner B: CCD 4.33±0.60 µm, IP 5.34±0.83 µm, P<0.0001; Practitioner C: CCD 4.12±0.34 µm, IP 5.07±0.84 µm, P<0.0001; and Practitioner D: CCD

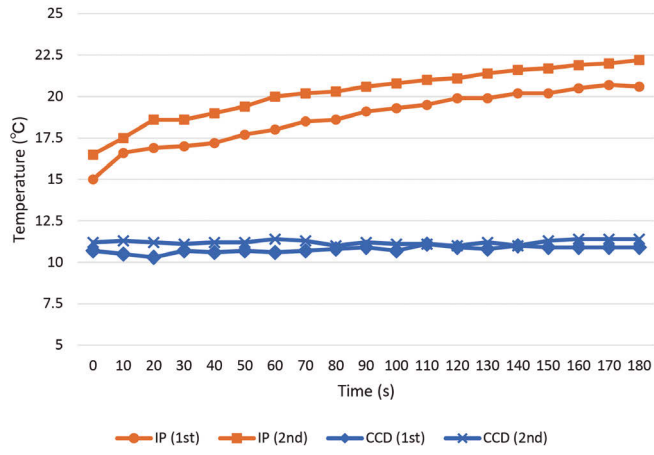

Fig. 3. Changes in temperature on the paraffin block surface over time while slicing using a CCD or an IP. Orange symbols indicate the surface temperature of the paraffin-only block with an IP, and blue dots indicate the surface temperature of the paraffin-only block with a CCD.

4.09±0.24 µm, IP 5.41±0.90 µm, P<0.0001). The average ST was very close to the set slice thickness of 4 µm in sections prepared using CCD, however, the thickness varied across the slides. These results indicated that the ST varied with each stroke although cooling with a CCD allowed the preparation of thin sections close to the set value.

The tissue surface is significantly thinner than the paraffin surface

Next, we compared the thickness of the tissue and paraffin surfaces after thin sectioning. The mean paraffin and tissue surface thicknesses were 4.68±0.59 and 3.14±0.59 µm, respectively, in the fish sausage sections (Fig. 5A) and 4.16±0.63 and 3.10±0.53 µm, respectively, in the liver sections (Fig. 5B). These results indicated that the tissue surface was significantly thinner than the paraffin surface in both the fish sausage and liver sections and that the tissue in the center of the section was more depressed than the paraffin surface based on the measurement of the dried thin sections prepared from FFPE blocks.

Section thickness correlates with the area of positive staining

Next, we evaluated whether ST was associated with the percent area of positive staining using the fish sausage and liver sections stained for only eosin or aniline blue. As shown in Figures 6A and B, the analysis of the fish sausage sections revealed that the percent area of positive staining positively correlated with paraffin surface thickness (r=0.667209) and tissue surface thickness (r=0.721652). Similarly, the analysis of the liver sections revealed that the percent area of positive staining positively correlated with paraffin surface thickness (r=0.405681) and tissue surface thickness (r=0.651926) (Fig. 6C,D). These results indicated that specimen stainability was associated with ST.

Variations in section thickness led to significant differences in the positive staining area in image analysis

To determine whether ST impacted the percent area of positive staining in image analysis, we prepared a set of 60 sections from the same specimen and placed them in descending order of thickness on the paraffin surface (section 1 being the thinnest and section 60 being the thickest). Next, we compared the percent area of positive staining in sections categorized into quantiles based on thickness. As shown in Figure 7, the percent area of positive staining was significantly different between the thickest and the first or second thinnest groups for both the fish sausage and liver sections. In detail, the percentages of areas with eosin positivity were 17.06±3.05%, 19.47±3.30%, 18.63±3.67%, 22.23±3.74%, and 25.11±5.98% in fish sausage sections 1-12, 13-24, 25-36, 37-48, and 49-60, respectively,

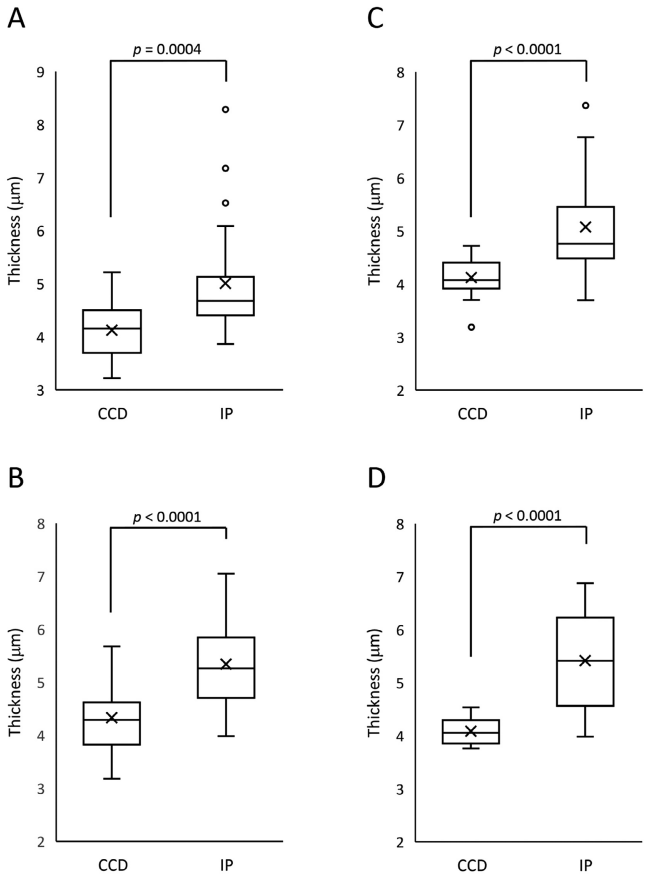

Fig. 4. Comparison of the thicknesses of sections prepared using a CCD or an IP. **A-D.** Each graph shows the measurements obtained by an individual practitioner. In box plots, × indicates the mean value, the line in the box indicates the median value, and the upper and lower sides of the box indicate quartiles.

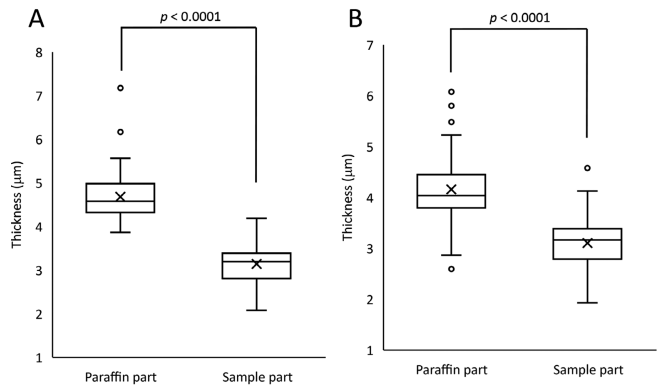

Fig. 5. Comparison of the section thickness between the paraffin and tissue surfaces. **A.** Thickness of the fish sausage sections. **B.** Thickness of the liver sections. In box plots, × indicates the mean value, the line in the box indicates the median value, and the upper and lower sides of the box indicate quartiles.

categorized according to paraffin surface thickness. Additionally, the percentages of areas of eosin positivity were 17.01±3.40%, 17.84±3.15%, 20.75±3.10%, 20.77±2.65%, and 26.13±5.90% in fish sausage sections 1-12, 13-24, 25-36, 37-48, and 49-60, respectively, categorized according to tissue surface thickness. Conversely, the percentages of areas of aniline blue positivity were 21.23±11.11%, 29.5±9.34%, 30.60±6.39%, 34.63±7.17%, and 441.85±9.67% in liver sections 1-12, 13-24, 25-36, 37-48, and 49-60, respectively, categorized according to paraffin surface thickness. Additionally, the percentages of areas of aniline blue positivity were 20.85±11.47%, 29.03±8.11%, 33.19±7.17%, 34.28±8.95%, and 40.49±9.15% in liver sections 1-12, 13-24, 25-36, 37-48, and 49-60, respectively, categorized according to tissue surface thickness. These results indicated that the percent area of positive staining was significantly associated with ST regardless of the analysis using the thickness of the paraffin surface or the tissue surface.

Discussion

In recent years, results of immunostaining of tissue specimens have been utilized worldwide as criteria for choosing treatment methods for patients; one representative example is the use of HER2 immunohistochemistry in breast cancer (Thorat et al., 2021). Among these, staining density is used as an evaluation method (Thorat et al., 2021). In the present study, we presented three main observations or aspects. First, the ST varied with each stroke during a session of 60 strokes using an optimally operating slicing microtome with a preset ST. Second, the staining density varied depending on the ST, and the percent area of positive staining determined by image analysis significantly correlated with the actual measurement value of the slice. Finally, in sections prepared from FFPE specimens, the tissue surface was depressed compared with the paraffin surface. Therefore, the evaluation of immunostaining for markers such as HER2 requires standardization of the

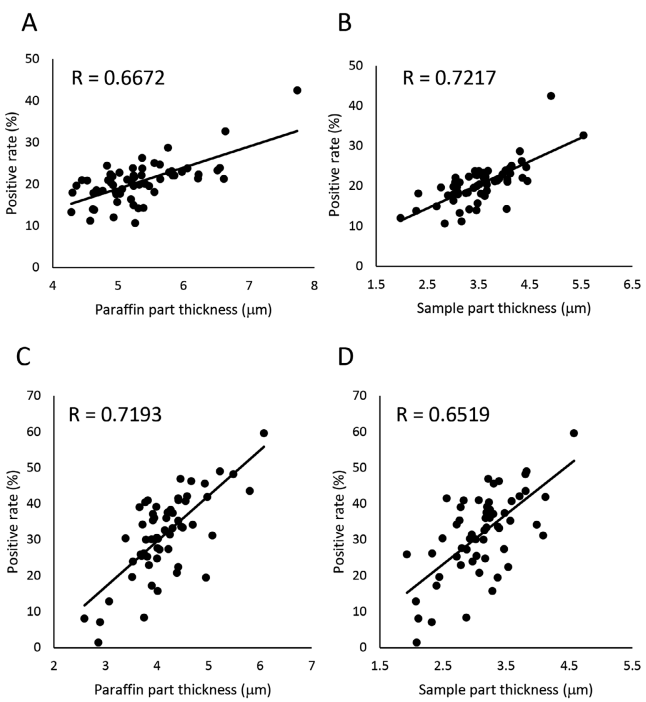

Fig. 6. Correlation between section thickness and percentage area with positive staining in fish sausage and liver sections. **A, B.** Correlation between percentage areas with eosin positivity and paraffin surface thickness **(A)** and tissue surface thickness in fish sausage sections **(B)**. **C, D.** Correlation between percentage areas with aniline blue positivity and paraffin surface thickness **(C)** and tissue surface thickness in liver tissue sections **(D)**. In all graphs, lines indicate the regression line. Correlation coefficients were calculated using linear regression analysis.

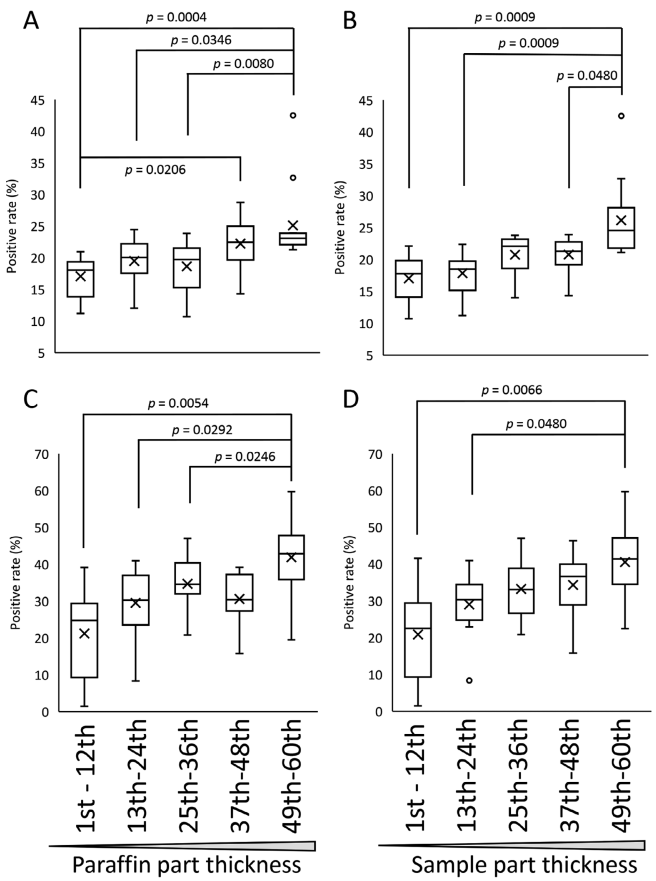

Fig. 7. Rates of positivity for specific stains in liver and fish sausage sections categorized into quantiles based on section thickness. **A, B.** Percentages of areas with dye positivity in fish sausage sections categorized according to paraffin surface thickness **(A)** and section thickness **(B)**. **C, D.** Percentages of areas with dye positivity in liver sections categorized according to paraffin surface thickness **(C)** and section thickness **(D)**. In box plots, × indicates the mean value, the line in the box indicates the median value, and the upper and lower sides of the box indicate quartiles.

ST to obtain samples with uniform thickness. In the present study, we used a relatively inexpensive film thickness meter due to budget constraints. However, specimen surface thickness can be measured in a shorter time using laser equipment and manufacturers should develop devices dedicated to easily measuring the surface thickness of sliced specimens on glass slides; such devices would be easily implemented in pathology laboratories and hospital pathology departments around the world. We do not advocate that specimens with inappropriate STs should be discarded. Nonetheless, quantitative data should be obtained from sections of identical thicknesses, whereas sections with differing thicknesses should be used for qualitative evaluation to avoid wasting valuable patient specimens.

Regarding the depression found in the tissue surface in the present study, water in the tissue was replaced first with ethanol, followed by xylol and paraffin, during tissue processing and embedding in the present study. Therefore, paraffin in the tissue section should not contain xylol. However, paraffin may not completely replace clearing agents (Kiernan, 2015). Conversely, a modular embedding center is generally used during paraffin embedding and new paraffin is poured from the dispenser. In other words, the paraffin in the tissue might contain a small amount of xylol during tissue processing, whereas the paraffin around the tissue does not; in such cases, the remaining xylene in the tissue evaporates during the drying step, leading to a reduction in tissue volume, which does not occur in the surrounding area that contains paraffin and not xylene. This difference might explain the depression observed only on the tissue surface. Even in the gross appearance of preserved paraffin blocks, we have observed the same depression phenomenon of tissue parts (unpublished observation). In summary, this phenomenon is considered to be caused by the difference between the paraffin containing residual xylene in the tissue and the new paraffin added around the tissue.

This is the first study examining the impact of specimen cooling on ST and the accuracy of image analysis of stained samples, highlighting the need for the standardization of ST in the future. As researchers and medical personnel (pathologist and laboratory technician), we hope that pathology and histology research will develop quantitative data analysis methods based on proper ST.

Conflict of interest statement. The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author contributions. MS conducted this study as the principal investigator and created the experimental design; prepared paraffin blocks; performed image analysis, specimen evaluation, statistical analysis, and figure preparation, and wrote the manuscript. RK and YK prepared paraffin blocks and performed slicing, specimen thickness measurements, specimen staining, image analysis, statistical analysis, and figure preparation. AI and YA prepared paraffin blocks and performed slicing, specimen thickness measurements, specimen staining, image analysis, and statistical analysis. SK and YN prepared paraffin blocks, and instructed 4 practitioners on how to slice specimens, and stain tissues as laboratory technologists because the practitioners were all students and thus beginners in slicing and staining.

References

Huan X., Zou K., Zhang P., Ding H., Luo C., Xiang C., Xu S., Zhuang Y., Wu C., Wang Y., Wu X., Chen C., Zhang J., Yao X., Liu F., Liu S. and Wu Z. (2023). Neoadjuvant chemotherapy is linked to an amended anti-tumorigenic microenvironment in gastric cancer. Int. Immunopharmacol. 127, 111352.

Jing X., Luo Z., Wu J., Ye F., Li J., Song Z., Zhang Y., Shi M., Sun H., Fang Y., Jiang Y. and Ji X. (2023). The genomic and immune landscapes of gastric cancer and their correlations with HER2 amplification and PD-L1 expression. Cancer Med. 12, 21905-21919.

Kiernan J.A. (2015). Dehydration, clearing, and paraffin embedding. In: Histological and histochemical methods. Scion publishing. Banbury. pp 53-54.

Randall H.W., Monticello T.M. and Morgan K.T. (1988). Large area sectioning for morphologic studies of nonhuman primate nasal cavities. Stain Technol. 63, 355-362.

Spencer L. (2019). Microtomy for paraffin and frozen sections. In: Bancroft's theory and practice of histological techniques, 8 ed. Suvarna S., Layton C. and Bancroft J. (eds). Elsevier. Amsterdam. pp 84-95.

Sy J. and Ang L.C. (2019). Microtomy: Cutting formalin-fixed, paraffin-embedded sections. Methods Mol. Biol. 1897, 269-278.

Thorat M.A., Levey P.M., Jones J.L., Pinder S.E., Bundred N.J., Fentiman I.S. and Cuzick J. (2021). Prognostic and predictive value of HER2 expression in ductal carcinoma *in situ*: Results from the UK/ANZ DCIS randomized trial. Clin. Cancer Res. 27, 5317-5324.

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