Short Communication

Technical Note: Measuring the thickness of histological sections by detecting fluorescence intensity of embedding foam

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ARTICLE INFO

ABSTRACT

Fluorescence intensity of embedding foam in paraffin blocks can be used to measure the thickness of histological microsections. We embedded samples of embedding foam and produced several microsections of varying thicknesses using routine processing and staining. Fluorescence intensity in the blue area of the embedding foam detected with a slide scanner was compared to absolute thickness as measured using confocal microscopy. Correlation analysis displayed a clear linear correlation with convincingly low prediction interval. The concept of measuring thickness of histological microsections by detecting fluorescence intensity of embedding foam is suggested as an approach to high-throughput measuring of histological sections applicable for a fully digitized pathology department. No acquisition of dedicated equipment is required and the method can be applied as a fully automated technique requiring no time consumption.

Introduction

Variation in thickness of histological tissue sections is a source of uncertainty, which should raise concern in the wake of digital image analysis. Pathologists evaluating histological sections by eyesight are well familiar with this variation, which originate in the inescapable imprecisions of planning a macrostructure in micrometer slices. Certainly, pathologists include this variation honorably in their subjective histopathological evaluations. However, digital image analysis systems relying on algorithms assuming uniform microsection thicknesses may not fare so breezy.[1,2] Among preanalytical factors impacting intensity of both hematoxylin & eosin (HE) and immunohistochemical (IHC) staining, microsection thickness was considered a critical parameter comparable to that of staining protocol and IHC biomarker being detected.[3–5]

Estimating the thickness of histological sections has been the subject of few previous studies. Practical approaches involving measuring the drop in thickness of the paraffin block or vertical reembedding[6–8] of microsections are considered inaccurate[9] and are not likely to become part of routine. Methods involving dedicated equipment such as spectral reflectance measurement as tested on plastic embedding material[9] and absolute gradient focus as tested on tissue sections[10] provide impressive estimates of microsection thickness. Techniques used in the thin film industry, e.g., ellipsometry and interferometry, and advanced microscopy techniques, e.g., confocal microscopy, can probably be appropriated for high throughput microsection thickness measurement of high precision.

Here is presented a simple method for estimating the thickness of histological sections with a precision in the range of $\pm 0.5\,\mu m$ requiring no dedicated equipment. The method is based on measuring the fluorescence intensity of sections of polymeric foam embedded and microsectioned along with the tissue. Foam material is routinely embedded along with tissue and the method can be readily implemented in a pathology department with a slide scanner.

Methods

Three samples of embedding foam (Sakura FineTek Denmark APS, Brøndby, DK) (Fig 1a + b) were put in formalin and embedded in paraffin following routine alcohol dehydration. From each paraffin block 2 microsections were cut at settings 2, 4, 6, 8, 10, and 12 μm producing in total 36 microsections with various thicknesses. Sections were deparaffinized with (TissueTek, Sakura FineTek Denmark APS, Brøndby, DK) and HE-stained using routine method. A beam of the embedding foam was imaged with a Zeiss Axioscan Z1 slidescanner mounted with a Colibri 2 LED fluorescence light source (Carl Zeiss A/S - Microscopy, Birkerød, DK). Ring aperture contrast was used for autofocus and imaging was done in the blue area (excitation/emission wavelengths 353/465 nm) with no gain using 30 ms exposure time and 40x objective. An example image is shown in Fig. 2a. To measure the thickness, the same foam beam was imaged with a Zeiss LSM 700 confocal microscope (Carl Zeiss A/S - Microscopy, Birkerød, DK), with excitation/emission wavelength 488/512 nm

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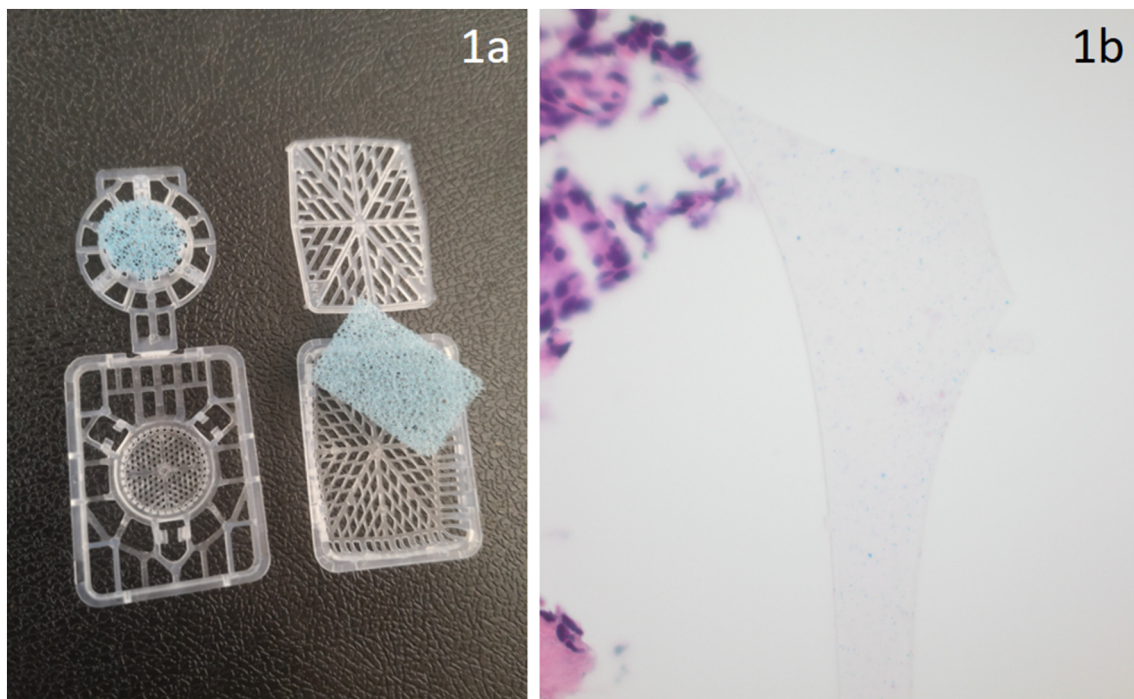*E-mail address:* thomas.hartvig.lindkaer.jensen@regionh.dk (T.H.L. Jensen).

Fig. 1. 1a) In our laboratory, 2 different versions of embedding foam are available. 1b) A foam beam as seen with a conventional light microscope.

using a 63x Plan-Apochromat oil immersion objective producing Z-stacks with thickness 0.35 μm. Two example images are shown in Fig. 2b and 2c.

The fluorescence intensity of beams imaged with the slide scanner was measured by adding a thin rectangular ROI and reading the modus intensity of the corresponding histogram, Fig. 3a + b. The thickness of the confocally imaged beams is measured using the "Plot Z-axis Profile" in ImageJ on the Z-stack of the same area using a line-shaped ROI of approximately same length and location, Fig. 3d. The thickness was defined as the distance between Z-stacks bordering the Z-stacks with highest drop in mean fluorescence intensity, Fig. 3d.

Measured fluorescence intensities and thicknesses were correlated using linear regression (Origin 2019, OriginLab Corporation, Northampton, USA).

In addition, a random and anonymized immunoperoxidase-stained microsection from routine embedded with foam was imaged with confocal microscope using same settings as described above. The measurements

were done to confirm that thickness of embedding foam beams is representative of the thickness of adjacent tissue microsections.

Results

The microscopic imaging produced applicable images in focus. Regression analysis between microsection fluorescence intensities and measured thicknesses are presented in Fig. 4. A clear linear relationship is observed. The regression line is inserted, adjusted R^2 is 0.9904. The red band is the 95% confidence interval and the light red band is the 95% prediction interval. The vertical height of the prediction interval is ± 0.5 μm.

Confocal images of a beam of embedding foam and an immunostained tissue section from the same microsection is presented in Fig. 5a and 5b, respectively. Intense autofluorescence is seen in the upper part of the tissue section (5b). This decreases in the deeper sections due to lower penetrance.
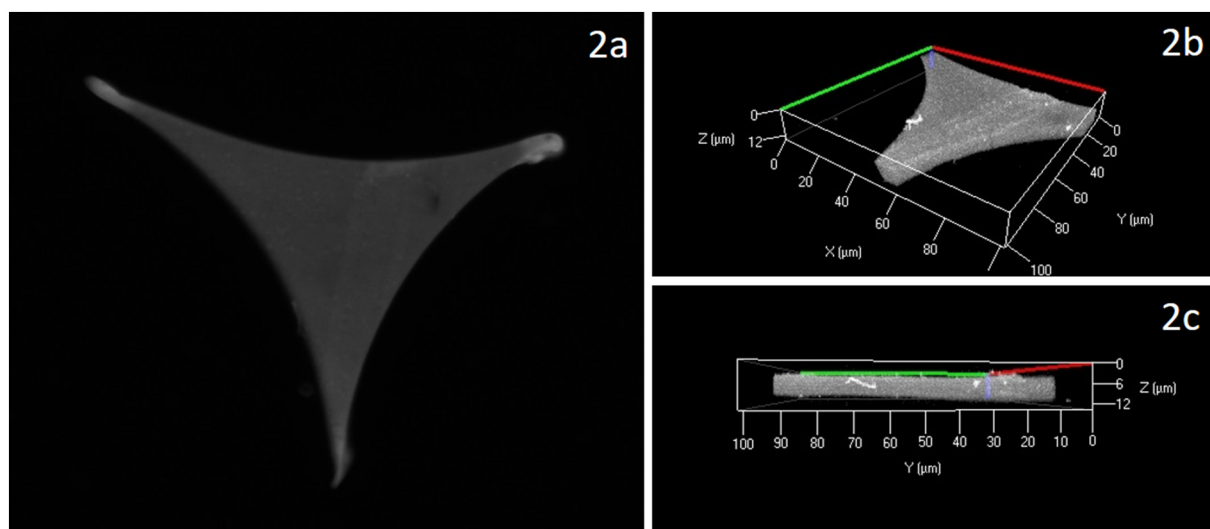

Fig. 2. 2a) Foam beam imaged with fluorescence light source in the blue area. 2b) The same foam beam imaged with a confocal microscope, b) oblique view and c) side view displaying the thickness.
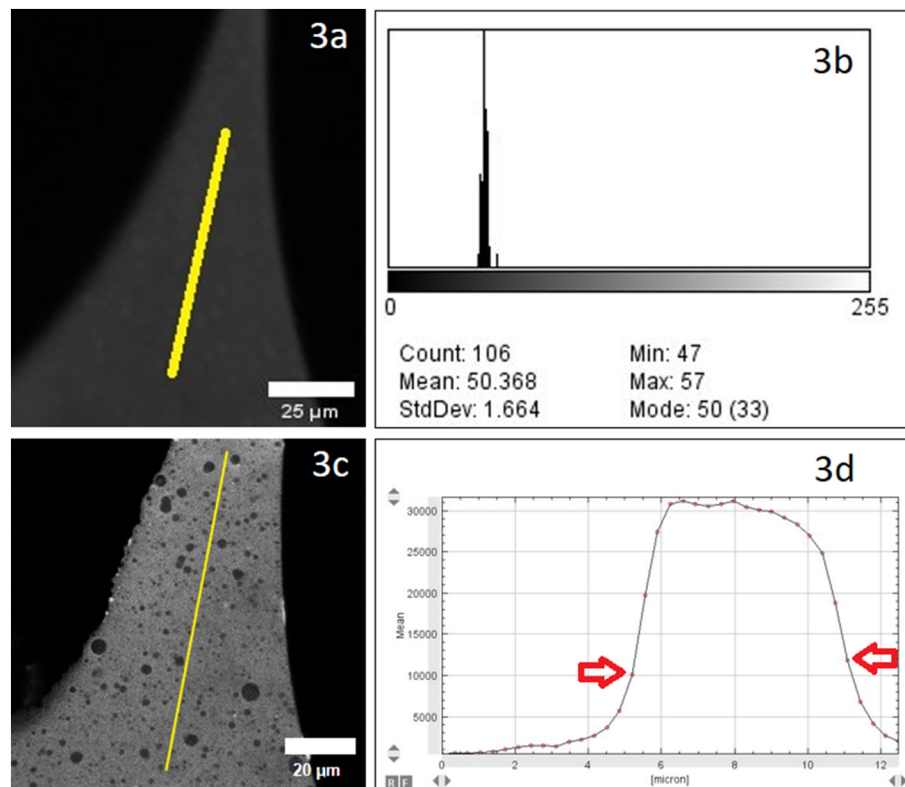
Fig. 3. 3a) Thin rectangular ROI on foam beam observed in the slide scanner and 3b), the corresponding intensity histogram. 3c) Line-shaped ROI on foam beam observed with confocal microscope. 3d) Measuring the thickness of the foam as the distance of the highest rise and drop in mean fluorescence intensity as indicated by red arrows.
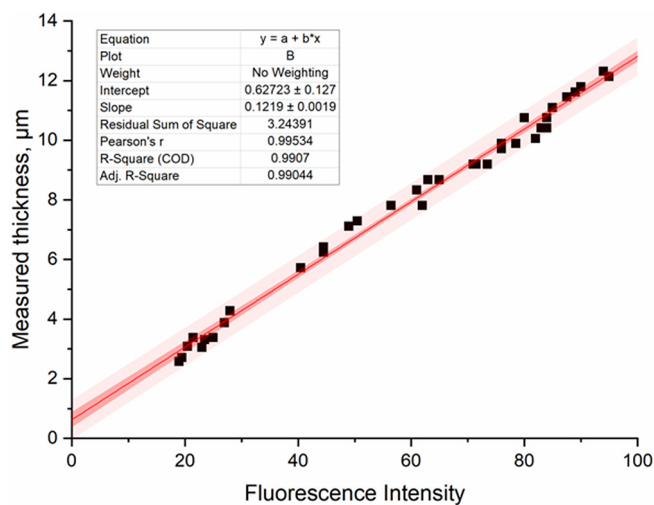

Fig. 4. Linear correlation between measured thickness and fluorescence intensities.

When aligned and merged, the tissue and foam beam sections appear equal in height (5c).

Discussion

We have shown that the fluorescence intensity of embedding foam in histological microsections is linearly correlated to the microsection thickness with convincingly low residuals. This relationship can be applied as an approach for estimating microsection thickness. In addition, it is observed that the thickness of embedding foam is equal to adjacently embedded tissue. The setup is based on routine histological preparation methods and a commercially available slide scanner. The 95% prediction interval for the microsection thickness is ± 0.5 μm. More precise methods involving dedicated equipment have been reported.[1,9,10] The presented method, however, holds gravity as a readily available approach in laboratories with a slide scanner mounted with a fluorescence illumination module. It should be noted that only one type of embedding foam has been tested on one type of slide scanner. Laboratories with alternative equipment are encouraged to identify a fluorescence intensity corresponding to the optimal microsection thickness or establish their specific linear correlation.

Traditionally, thickness of histological microsections is not considered as a source of uncertainty in histological research. Controlling microsection thickness is expected to have a wide impact. *Impact on digitization:* Uniform thickness would provide optimal foundation for identifying the focus level during scanning. *Impact on routine stains:* Digital image analysis will likely expand the quantitative detail in basic histology far beyond the capabilities of eyeballing.[11] The tremendous potential of this development could be lowered by variation in microsection thickness. In particular, nuclear details must be reproducibly presented. *Impact on immunostaining:* It seems reasonable to assume that staining intensity as perceived by a pathologist correlates with thickness in a linear fashion. In light of the importance of immunostain based diagnostic and predictive parameters (e.g., Ki67-, PD-L1-, and HER2-positivity), where variation in microsection thickness may have a direct impact on the result, this lack of consideration is worrisome. A recent study has documented effect of microsection thickness on HER2-scoring of breast cancer.[1] With the otherwise eager attention devoted to reproducibility in histological laboratories and the current introduction of digital image analysis, it seems appropriate to suggest routine measuring of histological microsections.

Integration of the presented method in to laboratory work flow at minimal cost and time consumption could be obtained by embedding a suitable piece of foam in a specified area or have it preinstalled in the tissue capsules. In the following digitization, the slide scanner programming includes a step dedicated to detecting the fluorescence intensity of the area in question, which could be automatically documented. The method may prove useful in scientific work, but the approach is probably not suitable for
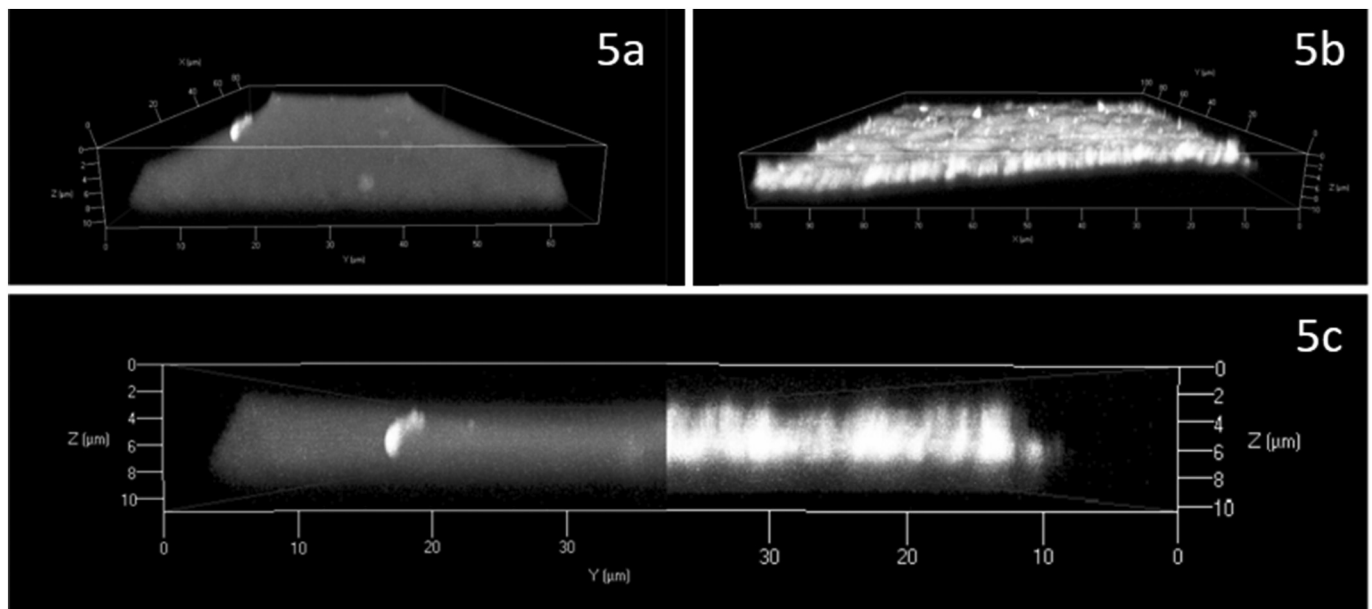
Fig. 5. 5abc) Aligning immunostained tissue and adjacent foam beam sections.

routine, where equipment not involving a microscopy step is essential. The optimal solution for routine is a device mounted on the microtome measuring the thickness of the paraffin part of the section immediately after cutting as opposed to being part of the microscopy. This pre-miscroscopy approach allows for discarding microsections with undesired thickness before further processing.

Conclusions

Thickness of histological microsections can be estimated by measuring the fluorescence intensity of embedding foam included in the paraffin block. The method can be applied by use of a slide scanner mounted with a fluorescence illumination module. We suggest directing attention to precision in microsection thickness as part of quality assurance in digital image analysis in particular and histopathology in general.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

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