Measurement of section thickness: are PAS prestained celloidin control cylinders the ultimate solution?

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The exact knowledge of the actual section thickness is a prerequisite for many quantitative analyses on histological sections [1]. Cabrini et al. [2] present an interesting contribution to this topic by modifying the method published by Gschwendtner and Mairinger [3]. The method is based on the staining of celloidin cylinders with PAS, thus resulting in a control material whose extinction is a function of the thickness of the sectioned material. This “thickness control cylinder” can be coembedded with the tissue to be analysed.

Such an approach would be of great advantage in quantitative pathology. Nevertheless, some important drawbacks of this method have to be discussed:

– The preparation of cylinders of celloidin 7% in equal parts of absolute alcohol and ethylic ether, hardened in 70% alcohol is not well standardized and hampered by the attributes of the substance (celloidin) which is inflammable and dangerous to process [4,5].
– The cylinders are stained with PAS and afterwards embedded in paraffin wax to be stored. We did not find any reference dealing with the stability of the stained celloidin cylinders embedded in paraffin wax over time. When used for quantitative methods, this point has to be clarified before relying on the method.
– The celloidin cylinders are coembedded together with the tissue under investigation in one paraffin block. This block is sectioned, thus containing a histological section of the tissue as well as the thickness control. The embedding of two different tissues in a single paraffin block is possible, though unusual. This is especially difficult when dealing with material like celloidin, as a well known disadvantage of celloidin is the fact that this medium cannot easily be sectioned even at a thickness of 5–10 μm [4,5]. If the cutting qualities of tissue and thickness control are different, it is questionable whether co-sectioning of both materials will lead to 2 pieces with equal thickness.
– The most important drawback, however is the usage thickness controls that have to be stained separately before the tissue under investigation is processed. Cabrini et al. propose to perform the Feulgen reaction just on the tumour section, avoiding overstaining of the control cylinder. In practice this means that, when performing a Feulgen reaction, the control has to be mounted on one end of the glass slide in a way that only the tissue section that has to be Feulgen stained is dipped in HCl for hydrolysis. The PAS-stained control cylinder, however is exposed to HCl steams during the hydrolysis process. It is a well known fact that PAS stain is highly susceptible to destaining by HCl. The HCl steams during one hour of hydrolysis may well affect the staining of the control cylinder in this setting.

Although the method proposed by Cabrini et al. would be of great importance for many quantitative analyses, these issues should be solved before this method can be widely applied.

References
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[3] A. Gschwendtner and T. Mairinger, How thick is your section? The influence of section thickness on DNA-cutometry on histological sections, Anal. Cell. Pathol. 9 (1995), 29–37.
[4] B. Romeis, Die Einbettung in Celloidin, in: Mikroskopische Technik, B. Romeis, ed., Vol. 16, R. Oldenbourg Verlag, München, 1968, p. 134.
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Comments on the Letter to the Editor by Tauber and Mikuz

Section thickness evaluation: PAS prestained celloidin control cylinders may become a practical solution

The issues raised by Tauber and Mikuz are interesting and contributory. We are grateful for the chance of enriching our work by addressing each of these points.
Letter to the Editor

– We have used celloidin for many years as an embedding medium for large samples of bone and teeth. We have taken the necessary precautions and have had no accidents whatsoever. Celloidin is only inflammable when it is pure and dehydrated. We use Pro-Celloidin Fluka that is stored in its metal container in the fridge. The mixture with ether–alcohol is made in small quantities to give a height of 1 cm in a Petri dish 5 cm in diameter. The partially covered Petri dish is placed overnight in a closed container alongside a glass containing 300 ml of 70% alcohol. The cylinders are obtained directly from the dish with a puncture biopsy needle.

– We have checked the stability of the staining of cylinders over a period of 1 year and found no variations. We usually use all the cylinders from a batch before that time. At that point we repeat the process and obtain a new calibration curve. Furthermore, the IOD values afforded by our equipment are checked regularly employing standards. These standards are constructed by coating glass slides with carbon and platinum to varying thickness.

– Paraffin-embedded PAS-celloidin cylinders are stored until use. They are sectioned easily both on their own or alongside tissue blocks employing routine disposable blades. We have sectioned them alongside blocks of liver, spleen and tumours of mucosa and skin and have found no section inhomogeneities.

– The fact that the sections are mounted such that the cylinder is on one end of the slide, allows for the Feulgen reaction, dewaxing included, to be performed solely on the tumour section. In this way the cylinder remains embedded and protected by paraffin during the acid hydrolysis. The dewaxing of the cylinder is performed with the xylene employed for clearing prior to mounting. The PAS reaction of the cylinder is remarkably strong due to the reactivity of cellulose. We have checked for possible destaining following the aforementioned procedure and the differences in values have always fallen within methodological error.

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Measurement of section thickness: bias and absolute versus comparative results

This commentary relates to the article by Cabrini et al. ("A technique for section thickness evaluation for microphotometry and image analysis of sectioned nuclei" in Anal. Cell. Pathol. 17, 125–130, 1998) and the resulting correspondence by Tauber and Mikuz ("Measurement of section thickness: are PAS prestained celloidin control cylinders the ultimate solution?"). Both refer to the practical difficulties of estimating section thickness in order to apply ‘corrections’ to DNA contents made using sectioned nuclei in tissue slices. I will not comment on the specific technical difficulties described by Tauber and Mikuz (and others before them) but, instead, make some general points about bias, its consequences and its handling.

Bias (sometimes called systematic error) is an important measurement error which affects accuracy or validity. It is independent of sample size and, consequently, cannot be eliminated by increasing the number of measurements, specimens or subjects (animals or patients). As the other correspondence implies, biases arise from several sources. They may be due to sampling (“selection bias”) or technical limitations (“technical bias”). The former is determined by how subjects, specimens and nuclei are selected and these matters are not always described. Technical biases include section thickness, staining reproducibility, tissue shrinkage, sectioning compression effects and so on. These biases may vary in magnitude and direction and, thereby, act antagonistically or synergistically to minimise or amplify overall biases.

Because of these considerations, a thorough systematic approach is required when dealing with bias correction and this means examining each and every aspect of the sampling protocol and of the other techniques which are employed. It does not make sense to focus on (and “correct”) only some of the biases and ignore others. This is pertinent since sectioning provides but one (albeit an important one) source of technical bias when determining DNA contents of sectioned nuclei.

A sensible preliminary question might be: "Do I need absolute data or will data having comparative worth suffice?" Clearly, seeking absolute data (e.g., wanting to know the exact DNA content of each nucleus) is a very demanding goal. If we are really seeking exact DNA contents, then we will need to know exact section thicknesses, be sure that different microtomes cut at the same notional thickness, be sure that control and tumour tissues have the same cutting and staining characteristics, etc. Less demanding, is the task of generating data which could be used to compare, say, the average nucleus in control tissue with that in a tumour. In this case, we can standardise relative
biases by various practical devices. For example, we might process and stain control and tumour tissue in tandem, cut all sections at the same notional thickness on the same microtome, etc.

Finally, some brief notes on aspects of the Cabrini et al. article. First, Figure 3 in the article is misleading as drawn since the section thickness axis (which starts at 1 $\mu$m rather than 0 $\mu$m) is different from the microtome setting axis (which starts at 0 $\mu$m). Both should have shared the same zero origin. Consequently, although the linear relationship between the measured thickness and the microtome setting appears to be very good (a nice straight line), in fact this microtome was cutting at a thickness of 1 $\mu$m when the notional thickness was zero and cutting at about 9 $\mu$m when the setting was 10 $\mu$m. In other words, the microtome bias varied with section thickness! Second, the method proposed (based on section recutting) provides a thickness estimate which may not represent the actual thickness on the microslide because of section compression and other artifacts. Third, any relationships established apply only to that microtome. However, in our hands, we have found that microtomes by the same manufacturer may vary in section thickness bias and precision (see Simpson et al., Placenta 13, 501–512, 1992).

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