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Detachment of methacrylate-embedded sections from microscope slides can be prevented by heating on hotplate

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Abstract

Glycol methacrylate (GMA, a resin also called 2-hydroxyethyl methacrylate) embedded sections are useful for quantitative (morphometric or stereological) study of histological structures, but detachment of sections (especially thick ones) from microscope slides is common during staining. We found that heating of the sections at a high temperature could prevent the section loss. To confirm our finding, we designed and conducted a series of experiments, comparing the numbers of sections detached after different treatments such as different temperatures (90-290°C) of heating (on hotplate for 30 minutes), different slides (conventional and adhesive), and different thicknesses (10 and 20 μm) of sections. We showed that heating at 115-140°C prevented the GMA sections from detaching, with the light microscopic image unaffected.

Keywords: Detachment of sections, glycol methacrylate, histological structures, thickness of sections

Introduction

Glycol methacrylate (GMA), a resin or plastic also called 2-hydroxyethyl methacrylate, has been used for embedding and then sectioning of biological specimens for light microscopy since 1970s, yielding semi-thin sections morphologically superior to paraffin embedded sections [1,2]. Besides, GMA embedded sections have negligible or much less distortion or shrinkage of the tissue embedded, especially useful for quantitative (morphometric or stereological) study of histological structures [3]. With the necessity of thick sections for sampling or counting particles (e.g., nuclei or nucleoli) according to the method of optical disector in stereology [4], thick (e.g., 20-25 μm in thickness) GMA-embedded sections have become an essential part of contemporary stereological tools [3]. However, thick GMA sections were liable to detaching from microscope slides during staining. In managing to solve problems arising from our recent work with thick GMA sections [5], we found, to our surprise, that heating of the sections at a high temperature could prevent the detachment. A series of experiments were therefore designed and conducted in this study, using GMA-embedded testicular sections available in our laboratory, to confirm the finding and determine a heating temperature that would not only prevent section detachment but also maintain section quality.

Materials and methods

10 testicular tissue blocks of similar sizes, obtained from normal male Sprague-Dawley rats (age 8 weeks), were embedded in GMA (Historesin by Leica Microsystems Nussloch GmbH, Germany); 10–30 sections were cut from each block at a set thickness of 10 or 20 μm using a microtome (RM2235, Leica Biosystems Nussloch GmbH), with the average areas of the tissue sections and the entire sections (including the tissue and the GMA around the tissue) being approximately 24 and 64 mm^2, respectively. The sections were randomly assigned to different experiments with different treatments such as different temperatures (90-290°C) of heating of sections, different slides (conventional slides, and adhesive slides with positively charged surface produced by Yifan Experiment, China), and different procedures of staining, see **Tables 1** and **2**.

Our protocol of sections staining was as follows: 30 minutes in 1% periodic acid, 1×3 minutes (i.e., 1 minute for 3 times) in distilled water, 50 minutes in Schiff's reagent, 10 minutes in running tap water, 1 minute in distilled water, 30 minutes in hematoxylin, a few seconds in running tap water, 10 minutes in tap water, 1 minute in distilled water, 2 minutes in 70% ethanol (this step of dehydration was omitted in 1 experiment, see **Table 1**), 2 minutes in 95% ethanol, 2×2 minutes in absolute ethanol,

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Table 1. Number of sections detached after heating at different temperatures.

	Experiment 1				Experiment 2	
	Staining without 70% ethanol		Staining with 70% ethanol		Staining with 70% ethanol	
	Not detached	Detached	Not detached	Detached	Not detached	Detached
90°C	5[a]	5[a]	0[a]	10[a]	-	-
140°C	10[b]	0[b]	6[b]	4[b]	14	6
190°C	5	0	5	0	6	0
240°C	5	0	5	0	6	0
290°C	-	-	-	-	6	0

In Experiments 1 and 2, glycol methacrylate embedded testicular sections (cut at a set thickness of 20 µm and mounted onto conventional slides) were placed on a hotplate at 90-290°C for 30 minutes before staining (periodic acid-Schiff's reagent and hematoxylin), which was not or was followed by dehydration in 70% ethanol for 2 minutes. P≤0.05 for the contingency table data a or b (Fisher exact test).

Table 2. Number of sections detached in experiment 3.

	Not detached	Detached
90°C, conventional slides, 10 µm [a]	13	7
90°C, conventional slides, 20 µm[b]	2	18
90°C, adhesive slides, 20 µm[c]	4	16
115°C, conventional slides, 10 µm[d]	20	0
115°C, conventional slides, 20 µm[e]	15	5
115°C, adhesive slides, 20 µm[f]	14	6

In Experiment 3, conventional or adhesive glass slides were used to mount the glycol methacrylate embedded testicular sections, which were cut at 10 µm or 20 µm (thickness) and heated on a hotplate at 90°C or 115°C for 30 minutes before staining with periodic acid-Schiff's reagent and hematoxylin followed by dehydration in 70% ethanol for 2 minutes. x^2 test: P<0.001 for the contingency table data of a, b and c; P≤0.05 for d, e and f. x^2 test (Yates correction for continuity used): P>0.05 for the contingency table data of b and c, d and e, or e and f; P≤0.05 for a and b, a and d, b and e, or c and f.

and 2×2 minutes in xylene. Before staining, sections were heated on a hotplate (H17.5D, Ingenieurbüro, M. Zipperer GmbH, Germany) for 30 minutes. Care was taken to ensure that the bottom surface of the glass (slide), not the upper surface on which the section was mounted, was put on the surface of the hotplate. After staining, they were mounted with a neutral balsam.

Detachment of a section was defined in this study as fully detaching of the section from the glass slide on which the section was mounted, i.e., the section fell off (or became separated from) the slide during staining.

Thickness of some sections (**Tables 1** and **3**) were measured after staining. As we previously described [**6**], the section was observed on a computer screen (final magnification×2240), using a×100 oil lens (UPlanSApo, NA 1.40, Olympus, Japan) on an Olympus BX51 microscope equipped with a stereology image system (Visiopharm, Denmark); 10 fields (distance between fields 1000 µm) were sampled per section in a systematic random manner; a frame (35 µm×26 µm) was superimposed at the center of each field; the upper surface of the section was first brought into focus within the frame area, and then the bottom surface was focused within the frame area; the distance between the 2 focal planes, which was measured by a microcater (Dr. Johannes Heidenhain GmbH, Germany) and shown on the screen, was a thickness of the section at the frame area. For thickness measurement of the thinner stained section (**Table 3**), the bottom surface in focus was the bottom surface of the stained structures (especially nuclei) whose shapes could be clearly seen, not the bottom surface of the embedding medium.

Table 3. Thickness (µm,$\bar{X}$±SD, median, range) of sections after heating at different temperatures.

	GMA sections	Thinner stained sections
90°C	19.8±0.6, 19.6, 18.7–20.9 (n=10)	-(n'=0)
140°C	19.8±1.1, 19.7, 18.4–22.6 (n=10)	18.2±1.5, 17.8, 16.3–21.4 (n'=10)[*]
190°C	19.0±0.7, 19.0, 17.4–20.1 (n=11)	13.2±2.0, 12.7, 10.9–17.1 (n'=11)[#*]
240°C	18.6±0.7, 18.7, 17.3–19.4 (n=11)[#]	5.4±6.0, 4.8, 0.0–17.2 (n'=11)[#*]
290°C	12.9±4.5, 12.1, 8.7–18.5 (n=6)[#]	0.1±0.4, 0.0, 0.0–0.9 (n'=6)[#*]

n is the number of glycol methacrylate embedded testicular sections cut at a set thickness of 20 µm. A thinner stained section is part of the entire GMA section with stained histological structures, whose thickness is thinner than that of the entire section; n' is the number of thinner stained sections. [#]*P*≤0.05 for comparison with the thickness at 140°C in the "GMA sections" or "Thinner stained sections" group. [As normality test failed (P<0.01), Kruskal-Wallis one way analysis of variance on ranks was used, in combination with the Dunn's method for comparison of the "140°C" subgroup with each of the other subgroups.] *P<0.001 for thickness comparison between "GMA sections" and "Thinner stained sections" (one-tail paired t-test).

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Results

With heating of sections (thickness 20 µm) at 90°C, half (without dehydration in 70% ethanol after staining) or all (with dehydration in 70% ethanol) of the sections detached from slides before mounting (**Table 1**). When the heating temperature increased to 115°C or 140°C, no sections (without dehydration in 70% ethanol) or less than half of the sections (with dehydration in 70% ethanol) detached (**Tables 1** and **2**). At 190°C or higher heating temperatures, detachment of sections was completely prevented (**Table 1**).

Detachment occurred in fewer thinner sections (thickness 10 µm) compared to thicker ones (thickness 20 µm), and none of the thinner sections detached after heating at temperature 115°C (**Table 2**). In contrast, the use of adhesive slides did not show significant effect on the prevention of sections detachment (**Table 2**).

Detachment of sections occurred after staining with periodic acid-Schiff's reagent (PAS) and hematoxylin. For more than 80% of the sections detached, it occurred at the step of dehydration in 70% ethanol (if there was this step in the staining) or in 95% ethanol (if there was not the step of dehydration in 70% ethanol).

In regard to the microscopic effect or quality, staining followed by dehydration in 70% ethanol, in comparison with staining without this step of dehydration, appeared to offer a slightly clearer section image. With increasing heating temperatures, the sections were stained increasingly lighter (**Figure 1**); at 290°C, only the shade of the structures was seen. In parallel, the embedding medium and, in particular, the tissue section embedded in the medium became thinner and thinner beyond 140°C; at 290°C, almost no structures were stained (**Table 3**). Poor staining of the tissue section started from its bottom surface on the slide. That is, the burning effect on the upper part of the section happened later. However, heating of sections up to even 240°C did not appear to induce appreciable changes in the size or shape of structures (nuclei or tissues) that were stained and seen.

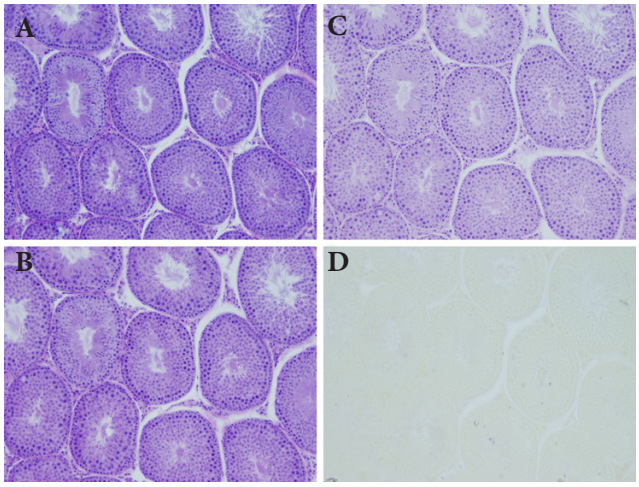

Figure 1. Testicular micrographs (each with width 870 µm) taken from the focal plane at the upper surface of 20 µm thick GMA sections, which were heated, before staining with periodic acid-Schiff's reagent and hematoxylin followed by dehydration in 70% ethanol, at 90°C (**A**), 140°C (**B**), 190°C (**C**) and 240°C (**D**) for 30 minutes, respectively.

Discussion

GMA sections, often obtained with semi-thin thickness (e.g., 1-2 µm), were usually left to dry up, e.g., at room temperature overnight [**7**] or at 50-70°C for 1-4 hours [**8**,**9**], to ensure their adhesion to slides. But for thick GMA sections, their detachment from slides is a common problem. We have found in this study that this problem can be effectively solved by heating them on a hotplate at higher temperatures.

Based on the limited experiments in the current study, we recommend a heating temperature of 115-140°C (for 30 minutes) for detachment prevention of thick (10-20 µm) GMA sections (**Tables 1** and **2**). For sections less than 10 µm in thickness, 90°C (30 minutes) may be enough. Importantly, the morphology of the tissue structures or the thickness of the tissue sections (**Table 3**) was essentially unaffected by heating at these temperatures, suggesting that the embedding medium GMA, which was not dissolved or removed during staining, served as a good protection for the tissue embedded.

Apart from GMA-embedded testicular sections used in this study, as shown in our recent experience, the heating method also applies to GMA-embedded sections of the rat eye, seminal vesicle and spinal cord for effective prevention of section detachment. Whether the immunohistochemical staining will be affected by the heating, however, remains to be further studied. The study of paraffin sections (see below) suggests that it may be affected.

The experimental designs in the current study did not demonstrate a significant effect of adhesive slides on the affixing of GMA sections to slides (see **Table 2**). Although this does not necessarily mean that adhesive slides have no use for GMA sections, we can rest assured that the use of adhesive slides is not as effective as the heating of slides for the prevention of GMA sections detachment.

According to our years of experience, as also shown in the current study, detachment of GMA sections occurs frequently at the step of dehydration in 70% ethanol after staining. This procedure was designed in this study in order to obtain contrasting results. As expected, we obtained efficient results with statistical significance using limited numbers of sections and experiments (**Tables 1** and **2**).

With respect to paraffin sections, according to our additional study (data not reported), heating (after dewaxing) of paraffin embedded renal and spinal cord sections at 90-140°C prevented detachment of sections, but resulted in not only marked reduction of the section thickness but also marked damage to the sections, including disappearance or poor staining of some nuclei, and more non-specific immunohistochemical staining and fewer immuno-positive structures. Similarly, dec-

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reased intensity of immunostaining was previously reported after heating at 80°C for 16 hours [**10**]. Thus the implication is that the high temperature heating method does not apply to paraffin sections.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	YX	ZWY
Research concept and design	--	✓
Collection and/or assembly of data	✓	--
Data analysis and interpretation	--	✓
Writing the article	--	✓
Critical revision of the article	--	✓
Final approval of article	--	✓
Statistical analysis	--	✓

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