Original Article

Rapid Coverslip Removal and Immunohistochemical Analysis: A Comparative Study

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Abstract

Introduction: Rapid restaining and reuse of archival slides for diagnostic and research purposes is impossible without decreasing the time spent on coverslip removal. Old archival slides can also be used in research, project, dissertation study, IHC, special staining or molecular study.

Aim: The aim of the study is to analyse various methods of coverslip removal from the stored H&E stained slides and utilisation of the same for subsequent diagnosis of tumour subtypes using immunohistochemical analysis.

Materials and methods: The archival slides were retrieved from the Department of Oral Pathology, Saveetha Dental College and Hospital. Samples were taken for 5 groups to analyse the duration for coverslip removal using xylene (RT), xylene (56°C), acetone, refrigerator -18°C, Endo-frost. 10 H&E stained archival slides were taken in each group. Destaining and Immunohistochemical analysis was performed for a basic panel of 9 markers. Stained slides were graded by two independent observers. Good: Equal or superior intensity compared to positive control; Fair: Intensity graded lower compared to positive control; Poor: No adequate intensity and staining. SPSS v25 software was used for Statistical analysis. Descriptive analysis was done for various reagents time duration taken for coverslip removal. One way ANOVA was done to compare statistical significance between old archival H&E stained slides and newly sectioned slides.

Results: In conventional xylene method (Room temperature), the mean time for removal of coverslip was 48 hour 30 minutes. Mean duration in which coverslip came-off was 5 minutes by Endo-frost minutes, 22 minutes by freezer -18°C method, 25 hours 50 minutes with xylene at 56°C and 56 hours with acetone method. One way ANOVA, post hoc Tukey HSD test showed significant results between groups ($p<0.05$). In our study, Pan CK, vimentin, SMA, S100, CD34 and CD45 showed good intensity; CD3 and CD20 showed fair intensity in old archival slides; Desmin showed poor intensity in old archival compared to newly sectioned slides

Conclusion: Alternative methods especially freezing using Endofrost and refrigerator, xylene at high temperature and acetone method can be used for removal of coverslip. These methods are reliable within available resources for immunohistochemical analysis for research purposes.

Keywords: archival slides; coverslip removal; destaining, immunohistochemistry

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INTRODUCTION

Formalin-fixed and paraffin-embedded tissues are a valuable resource in research. Hematoxylin and eosin (H&E) histopathology slides are preserved in archives for training and research purposes. Formalin Fixed Paraffin Embedded (FFPE) can be used in future diagnostic and retrospective research studies. However, some retrospective investigations are limited due to depleted tissue samples or a lack of material in stored blocks for serial sections. The benefit of reusing and destaining the archived H&E-slides eliminates the requirement of repeat sectioning. At times when specimens of interest are not available for further sectioning, it is possible to repeat the sectioning and staining of archival slides (1).

The mounting media is the solution used to cover the sectioned specimens over the glass slides using cover slip. To preserve the specimen from the environment, resinous (natural/artificial) and aqueous mounting mediums can be utilised. DPX (Distrene 80) refractive index of 1.52 is the most often used mounting material. For coverslip removal in H&E slides, chemicals such as

xylene, petrol, diesel, freezing technique, and acids are utilised (2). Rapid restaining of archival slides is impossible without decreasing the time spent on coverslip removal. Xylene was most popular and widely used for coverslip removal despite its duration taken coverslip removal (3,4).

The existing literature evidence on whether old archival destained slides could be used for Immunohistochemistry (IHC) staining is still debatable (5). Heat-induced antigen retrieval method is the most popular method for antigen retrieval used in immunohistochemistry. It will be unnecessary to utilise additional progressively sectioned slides, which is a major benefit of a method that permits the reuse of the H&E-stained slide (6). Recently whole slide imaging has gained popularity, restaining archival slides can be used to prepare virtual libraries (7,8). The rationale of this study is to find a novel method for rapid coverslip removal and destaining to use for immunohistochemistry purposes.

The aim of the study is to analyse various methods of coverslip removal from the stored H&E stained slides and utilisation of the same for subsequent diagnosis of tumour subtypes using immunohistochemical analysis.

The objectives are to evaluate the removal of coverslip using various methods and to analyse whether destained H&E slides used for Immunohistochemical analysis.

Materials And Methods

Reagents and materials

Xylene, acetone, grades of alcohol, Distilled water, Tris buffer (7.6 ± 0.2 pH), H&E stained archival slides, FFPE tissues, Microscopic slides, Slide coverslips, Mounting medium.

The archival slides were retrieved from the Department of Oral Pathology, Saveetha Dental College and Hospital. 10 samples were taken for 5 groups to analyse the duration for coverslip removal using xylene (RT), xylene (56°C), acetone, refrigerator -18°C, Endofrost. 10 H&E stained archival slides were taken in each group. Destaining and Immunohistochemical analysis was performed for a basic panel of 9 markers. The antibodies used for further immunostaining of 10 de-stained archived H&E stained slides for analysis and comparison with 10 newly sectioned slides. The antibodies used in this study are Pan CK, Vimentin, Desmin, S100, SMA, CD34, CD45, CD3 and CD20.

Destaining Procedure for the archived H&E slides: Duration of procedure: ~4 to 5 hours

Retrieve H&E archived slides and rinse in distilled water

Placement of slides Freezer -18°C/ Spray Endo Frost over the coverslip and remove the cover slip ~5 to 10 mins.

To avoid tissue injury, carefully remove the coverslip using forceps, Remove the coverslip.

Rinse slides three times in a xylene wash to remove any leftover adhesive, then leave slides in the water to remove any mountant ~10 to 15 minutes

Rinse slides (3-4 times) in grades of alcohol (90% propanol to 50% propanol) for 10 minutes, with 3 minute hold intervals between rinses to remove eosin.

Rinse slides in distilled water to remove excess.

Rinse slides in Acid alcohol 1 min to remove Hematoxylin.

Rinse in Tap water to remove excess hematoxylin and allow it to dry.

TRIS buffer rinse for 10 minutes

Antigen retrieval using electrical cooker at pre-set temperature and pressure for 30 minutes

Rinse the slides in distilled water and bring to room temperature

Peroxidase blocking for 30 minutes

Rinse the slides twice in Wash buffer for 5 minutes each

Primary antibody for 1 hour

Rinse the slides twice in Wash buffer for 5 minutes each

Secondary antibody for 30 mins

Rinse the slides twice in Wash buffer for 5 mins each

DAB chromogen for 3 mins

Alum Hematoxylin staining for 2 mins

Rinse in tap water

Dehydrate slides with grades of alcohol and xylene, coverslip using mounting medium DPX

Stained slides were graded by two independent observers. Good: Equal or superior intensity compared to positive control; Fair: Intensity graded lower compared to positive control; Poor: No adequate intensity and staining.

Statistical analysis: SPSS v25 software was used for Statistical analysis. Descriptive analysis was done for various reagents time duration taken for coverslip removal. One way ANOVA was done to compare statistical significance between old archival H&E stained slides and newly sectioned slides.

Results

In conventional xylene method (Room temperature), the mean duration for removal of coverslip was 48 hour 30 minutes. Mean duration in which coverslip came-off was 5 minutes by Endofrost, 22 minutes by freezer -18°C method, 25 hours 50 minutes with xylene at 56°C and 56 hours with acetone method.

One way ANOVA, post hoc Tukey HSD test showed significant results between groups ($p<0.05$).

In our study, Pan CK, vimentin, SMA, S100, CD34 and CD45 showed good intensity in both old archival and newly sectioned slides; CD3 and CD20 showed fair intensity in old archival slides; Desmin showed poor intensity in old archival slides compared to newly sectioned slides. Apart from Desmin, all other markers showed good Immunohistochemical staining when compared to newly sectioned slides.

Discussion

There is a scarcity of literature on unconventional coverslip removal procedures. Methods that are easily accessible and viable were employed in this investigation. This is the first comprehensive research that has detailed the use of several ways for removing coverslip. Few studies have also detailed the use of liquid nitrogen, ultrasonic vibrations, and scratching coverslips, as well as the use of an ice block to remove coverslips. The difficulty with these approaches is that they are either not easily available at the setup, such as liquid nitrogen, or they may cause tissue damage, such as ultrasonic or scratching methods (9).

Xylene was most popular and widely used for coverslip removal despite its duration taken coverslip removal (3). With xylene, the turnaround time is longer; coverslip removal might take ranging from 72 to 94 hours (10). Freezing Liquid Nitrogen, however not significant, has also shown good results in coverslip removal (3).

Moore et al studied and successfully removed epoxy resin mounted glass coverslips using hydrofluoric acid (11). Mechanism of action of Diesel, Petrol and Xylene at room temperature is same i.e., they all are solvents and dissolve the DPX. Heating aids in penetration as well as fastens melting of DPX.

Our study applied up-to-date coverslip removal techniques, in H&E stained archival slides and heat induced antigen retrieval method for highly sensitive detection of antibodies. The introduction of Endofrost technique has further been emphasised. In the present study, it was found out that the most efficient and fastest method for coverslip removal was the freezing method. Freezing the DPX mounted slides crystallises the DPX and dissolves the DPX when placed in xylene.

The most typical reason for removing a coverslip is to collect DNA for molecular analysis in research. In the age of molecular testing, when target therapy is in high demand, even a little specimen can be beneficial. Identification of mutations in the EGF receptor, BRAF, and KRAS genes can be used for target treatment. Archival slides are sometimes the only source for such studies. Other research areas where needle removal is used (12)(2).

Grillo et al conducted an immunohistochemistry study on old archival paraffin blocks and concluded that antigenicity for cytoplasmic markers for 60 years or more, whereas nuclear antigens, Ki67 and CD31 markers showed poor antigenic intensity in archival slides (13). In our study, Pan CK, vimentin, SMA, S100, CD34 and CD45 showed good intensity; CD3 and CD20 showed fair intensity in old archival slides; Desmin showed poor intensity in old archival compared to newly sectioned slides.

Blind et al. proposed paraffin coating as a strategy for reducing slide oxidation and increases the longevity of the antigenicity (14). DiVito et al. (15) previously reported this, as well as other approaches, as a methodology for overcoming storage-induced loss in antigenicity. The effects of storage temperature, inert environment (nitric oxide storage), and slide paraffin coating were

investigated. The greatest results were obtained when stored at 4°C, although paraffin coating provided no further protection against deterioration over time. On the contrary, a modest negative effect was identified. Most antigens in FFPE blocks are stable throughout time. Antigen degradation is especially common in membrane and nuclear antigens (particularly Ki67).

The limitations of the study are small sample size, only the basic panel of IHC markers were studied. The future scope of research includes Immunohistochemical studies subjected to archived specimen samples of less than 5 years and further studies on archival tissue blocks comparing several decades to find the loss of antigenicity. IHC Studies on nuclear markers like Ki67 need to be studied. Multiplex Immunohistochemical staining should also be studied to reduce wastage of slides.

Conclusion

To conclude, freezing using Endofrost and refrigerator showed superior results for coverslip removal followed by xylene at high temperature and acetone method. Further studies on studying antigenic potential between decades and several immunochemical markers can be studied. Old archival slides can be used as resources for immunohistochemical analysis for diagnostic and research purposes.

Fig 1: Retrieval of old H&E stained archival slide.

Fig 2: Coverslip removal using Endofrost

Fig 3: Archival slide stained with immunohistochemistry after coverslip removal

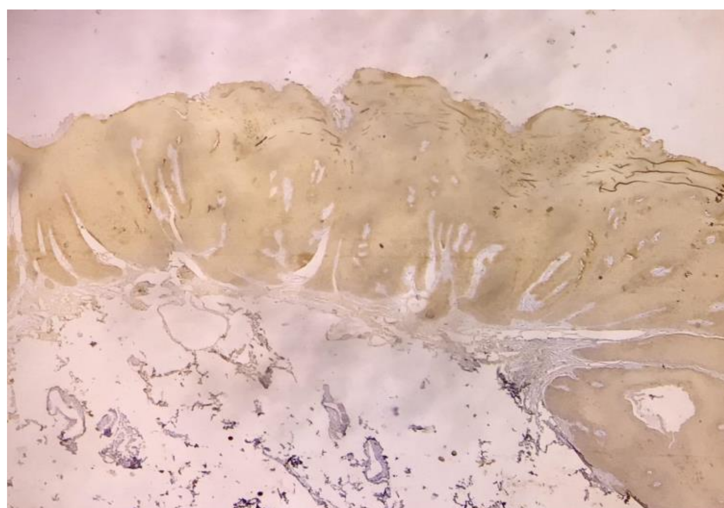

Fig 4: Immunohistochemistry staining done on old destained archival slides (PANCK, 10x)

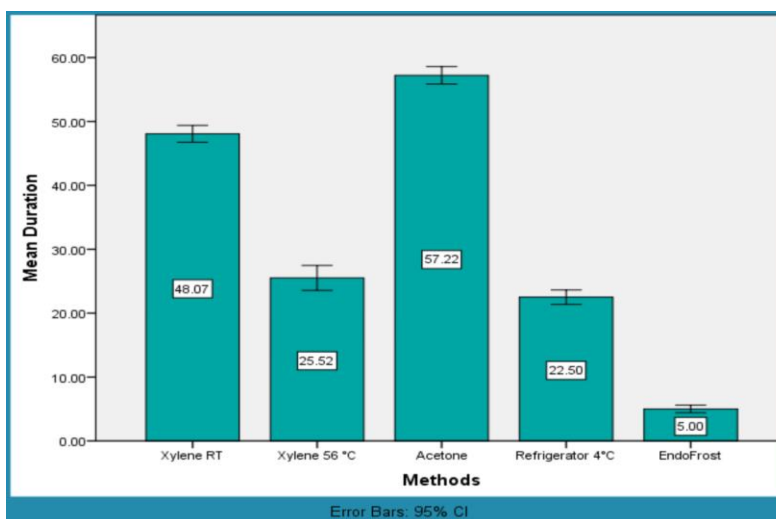

Fig 5: bar graph represents mean duration taken for coverslip removal using 5 different methods like Xylene room temperature, Xylene at 56°C, Acetone, Refrigeration at 4°C, Endofrost.

Table 1: Descriptive analysis for duration of coverslip removal using 5 different methods

Descriptives								
Duration								
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Xylene RT	10	48.0700	1.85176	.58558	46.7453	49.3947	45.40	51.30
Xylene 56 °C	10	25.5200	2.71980	.86008	23.5744	27.4656	21.60	30.30
Acetone	10	57.2200	1.91532	.60568	55.8499	58.5901	54.10	59.70
Refrigerator 4°C	10	22.5000	1.58605	.50155	21.3654	23.6346	20.50	25.20
EndoFrost	10	5.0000	.83267	.26331	4.4043	5.5957	3.50	6.10
Total	50	31.6620	19.01072	2.68852	26.2592	37.0648	3.50	59.70

Table 2: One way ANOVA analysis significant results between groups (p<0.05).

ANOVA					
Duration					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	17549.625	4	4387.406	1239.124	.000
Within Groups	159.333	45	3.541		
Total	17708.958	49			

Table 3: Post hoc analysis One way ANOVA- Turkey HSD showing significant results (p<0.05).

Multiple Comparisons						
Dependent Variable: Duration						
Tukey HSD						
(I) Methods	(J) Methods	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Xylene RT	Xylene 56 °C	22.55000*	.84151	.000	20.1589	24.9411
	Acetone	-9.15000*	.84151	.000	-11.5411	-6.7589
	Refrigerator 4°C	25.57000*	.84151	.000	23.1789	27.9611
	EndoFrost	43.07000*	.84151	.000	40.6789	45.4611
Xylene 56 °C	Xylene RT	-22.55000*	.84151	.000	-24.9411	-20.1589
	Acetone	-31.70000*	.84151	.000	-34.0911	-29.3089
	Refrigerator 4°C	3.02000*	.84151	.007	.6289	5.4111
	EndoFrost	20.52000*	.84151	.000	18.1289	22.9111
Acetone	Xylene RT	9.15000*	.84151	.000	6.7589	11.5411
	Xylene 56 °C	31.70000*	.84151	.000	29.3089	34.0911
	Refrigerator 4°C	34.72000*	.84151	.000	32.3289	37.1111
	EndoFrost	52.22000*	.84151	.000	49.8289	54.6111
Refrigerator 4°C	Xylene RT	-25.57000*	.84151	.000	-27.9611	-23.1789
	Xylene 56 °C	-3.02000*	.84151	.007	-5.4111	-.6289
	Acetone	-34.72000*	.84151	.000	-37.1111	-32.3289
	EndoFrost	17.50000*	.84151	.000	15.1089	19.8911
EndoFrost	Xylene RT	-43.07000*	.84151	.000	-45.4611	-40.6789
	Xylene 56 °C	-20.52000*	.84151	.000	-22.9111	-18.1289
	Acetone	-52.22000*	.84151	.000	-54.6111	-49.8289
	Refrigerator 4°C	-17.50000*	.84151	.000	-19.8911	-15.1089
*. The mean difference is significant at the 0.05 level.						

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