Original Article

Rapid tissue processing technique: A novel method using methyl salicylate

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Abstract

Introduction: Tissue processing involves transition of the biopsy tissue in graded concentration of various chemicals to make the tissue amiable for sectioning. The entire process takes 2–3 working days before a microscopic slide is ready for diagnosis. In order to shorten the turnaround time, rapid tissue processing method using methyl salicylate was developed.

Aim: The aim of this study is to develop a rapid tissue processing technique using methyl salicylate as a clearing agent and to compare it with routine tissue processing technique.

Materials and Methods: A total of 70 tissue specimens were cut into two equal halves. One each was processed by routine processing technique (RoPT) and rapid processing technique (RaPT). Tissue specimens were measured before and after processing. Quality of staining and cellular-level shrinkage were observed and scored for specimens. Statistical analysis using Welch's unequal variances *t*-test was performed. Costs of chemicals in both the techniques were compared to see the cost-effectiveness of RaPT.

Results and Conclusion: Outcomes of both the processing techniques were comparable with statistically not significant *P* values for all the parameters. Hence, the results of RaPT technique are satisfactory, and the use of this technique may prove beneficial to pathology laboratories.

Keywords: Methyl salicylate, rapid, routine, tissue processing, xylene

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INTRODUCTION

Tissue processing is an important histotechnique after biopsy procedure. It involves passing of the biopsy tissue in graded concentration of various chemicals in order to make the tissue amiable for sectioning. This includes dehydration, clearing and infiltration. Dehydrating solutions are usually alcohol-based solutions required to remove water and fixative such as formalin from the tissue specimen and replacing it with alcohol. The tissue is then placed in xylene, a clearing agent which makes the tissue receptive to wax impregnation by the removal of alcohol. This is followed by infiltration – to permeate the tissue with a support medium and embedding – to orient the tissue specimen in a support medium and allowing it to solidify. This entire process takes 2–3 working days before a microscopic slide is ready for diagnosis.[1]

The most commonly used means of tissue processing are routine manual method, rapid manual method and

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the microwave method. Routine manual method is a time-tested method used routinely in histopathology laboratories. Reliability and inexpensive nature are the positive factors for this method. The disadvantages of this method are turnaround time of 21–24 h and exposure to noxious chemicals such as xylene and formalin.[2,3]

Rapid manual tissue processing method and recent microwave methods have shortened the processing times but do not reduce high cost and exposure to noxious chemicals.[4]

In this study, we have used methyl salicylate in place of routinely used xylene for clearing step. Methyl salicylate (oil of wintergreen) is an organic ester and can be obtained either naturally (wintergreen plants – *Gaultheria procumbens* and *Betula lenta*) or commercially by esterifying salicylic acid with methanol.[5] It is an excellent clearing agent, used to study internal parts of embryo, insects and plants.[6] The nontoxic nature compared to xylene and rapid clearing property of methyl salicylate encouraged us to include it in our study.

The aim of this study is to shorten the turnaround time and use of alternate nontoxic chemical in tissue processing which is also cost-effective. This study was done to develop a rapid tissue processing method using methyl salicylate as clearing agent.

Aim

The aim of this study is to develop a rapid tissue processing technique using methyl salicylate as a clearing agent and to compare it with routine tissue processing technique with respect to quality of staining, shrinkage and cost.

MATERIALS AND METHODS

A total of 70 tissue specimens were considered for this study, out of which only 3 were hard tissue (2 – bone and 1 – tooth tissue) specimens. Each of the specimens was halved. One half of the tissue bit was processed by routine processing technique (RoPT) and the other half with rapid processing technique (RaPT).

Timings and chemicals used for both techniques are given in Tables 1 and 2.

Tissue bit dimensions were measured before and after processing by both techniques to check for shrinkage [Table 3 and Figures 1a, b and 2a, b].

All the sections were stained with hematoxylin and eosin stain to observe the histological details. Quality of staining of tissues was graded on a scale of 1–3 with scale 1 as excellent, 2 as good and 3 as satisfactory [Figure 3a and b]. This scoring system is a modified version from Panja *et al.*[2] Score 1 (excellent) was considered brilliant when both cellular and tissue structures were easily discernible with good staining contrast. Score 2 (good) indicated good staining and contrast and diagnosable tissue. Score 3 (satisfactory) is designated when tissue was not stained adequately or evenly and lacked details.

Nucleus and cytoplasm of cells of epithelium and connective tissue were observed for quality of staining and scored separately for specimens processed by both the techniques [Tables 4-8].

The sections were subjected to morphometric analysis to observe the difference in shrinkage at microscopic

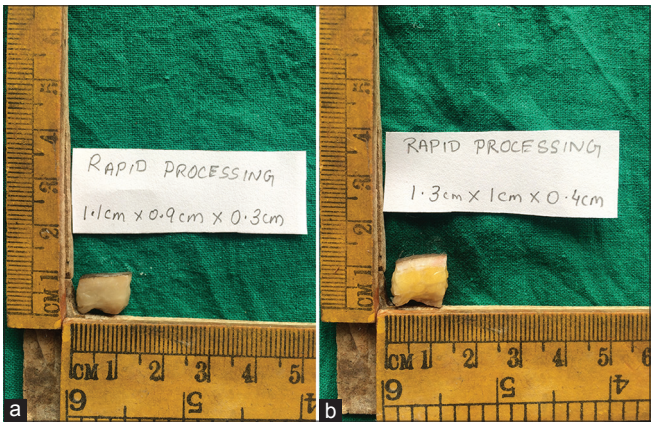


Figure 1: (a) The measurement of the tissue after processing with rapid processing technique. (b) The measurement of tissue before subjecting to rapid processing technique

Table 1: Steps involved in rapid tissue processing

Steps	Duration for different sizes (mm)		
	2-3	3-4	5-6
Fixation in neutral buffered formalin at 60°C	1 h	1 h (with agitation)	1 h (with agitation)
Water wash	15 min	15 min	15 min
70% alcohol	30 min	45 min	1 h
Absolute alcohol I	30 min	45 min	1 h
Absolute alcohol II	30 min	45 min	1 h
Methyl salicylate	20 min	30 min	45 min
Paraffin wax impregnation	1 h	1 h	1 h
Total time	4 h 5 min	5 h	6 h

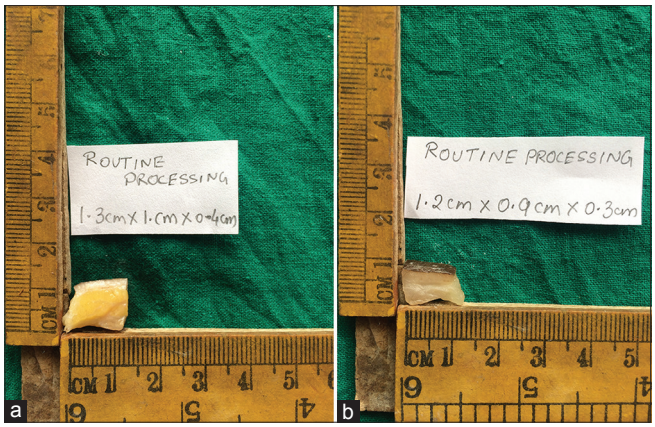


Figure 2: (a) The measurement of tissue before subjecting to routine processing technique. (b) The measurement of tissue after processing with routine processing technique

Table 2: Steps involved in routine tissue processing with xylene

Step	Duration
Fixation in 10% neutral buffered formalin	Overnight
Water wash	15 min
50% alcohol	1 h
70% alcohol	1 h
90% alcohol	1 h
100% alcohol	1 h
70% xylene	1 h
100% xylene	1 h
Molten wax impregnation	Overnight
Total time	2-3 working days

Table 3: Comparison of shrinkage of tissue at gross specimen level

Tissue processing method	Percentage of shrinkage (mean±SD)
Rapid	34.67±6.38
Routine	23.13±6.31
P=0.0000012*	

*Significant. SD: Standard deviation

Table 4: General quality of tissue with respect to artifacts, folds, and staining

Observer	Routine processing	Rapid processing
1	1.71	2.14
2	2.27	2.29
3	2.34	2.11
4	1.71	2.06
Mean	2.01	2.15
P=0.24*		

*Not significant

Table 5: Staining quality of nucleus of epithelial tissue

Observer	Routine processing	Rapid processing
1	1.77	2.23
2	2.3	2.29
3	2.21	2
4	1.76	1.9
Mean	2.01	2.11
P=0.30*		

*Not significant

level. Five most representative fields were chosen on each specimen from each group and images were captured.

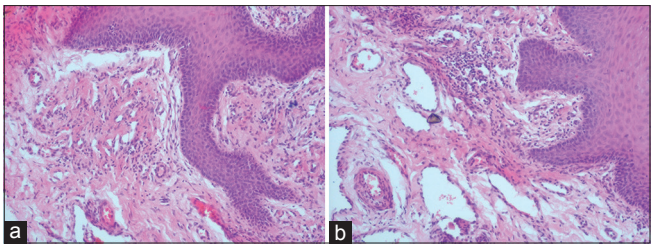


Figure 3: (a) Staining quality of tissue processed with routine processing technique. (b) Staining quality of tissue processed with rapid processing technique

Three-chip CCD camera attached to a trinocular research microscope was used for imaging with a ×40 objective and with a total magnification of ×400. The selected fields included representative cells with distinct cellular and nuclear outlines. A total of fifty cells (ten cells in each of five different high-power fields) were randomly selected and measured for mean cell area, mean nuclear area and nuclear-cytoplasmic ratio [Figures 4 and 5]. Measurements were made using a sophisticated, computer-assisted morphometry software. Measurements were transferred and stored in computer. The software automatically calculated cell and nuclear areas in square microns when the perimeter was traced. "Set scale" was done in software that converted default pixel measurements to micrometers [Table 9].

The scoring and assessment were carried out by four qualified observers to eliminate inter- and intra-observer bias, as most of the evaluative criteria were subjective.

The obtained data were tabulated and subjected to statistical analysis using Welch's unequal variances *t*-test, and the means were calculated using R 3.3.3 statistical package.

Costs (in Indian Rupees) of chemicals in both the techniques were compared to see which technique is cost-effective [Graph 1].

Steps in Tables 1 and 2 were followed by block preparation, sectioning, staining, mounting and reporting of the slide.

RESULTS

The slides were ready for staining in 6 h in rapid processing method as compared with 2 days in routine processing [Tables 1 and 2]. The study compared rapid and routine processing with respect to

1. Quality of staining [Table 4]
2. Clarity of nucleo-cytoplasmic differentiation of epithelial tissue [Tables 5 and 6]
3. Clarity of nucleo-cytoplasmic differentiation of fibrous tissue [Tables 7 and 8]

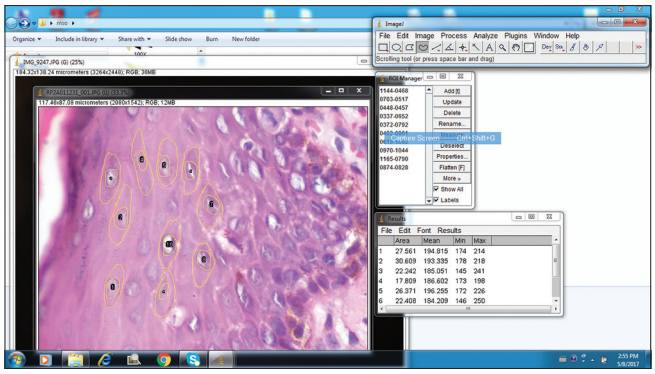


Figure 4: The marking and measuring using the software

Table 6: Staining quality of cytoplasm of epithelial tissue

Observer	Routine processing	Rapid processing
1	1.77	2.31
2	2.27	2.27
3	2.33	2.12
4	1.78	2.1
Mean	2.04	2.20
P=0.18*		

*Not significant

Table 7: Staining quality of nucleus of fibrous tissue (includes blood vessels, glandular tissue, inflammatory cells, osteoid components, muscles, and nerves)

Observer	Routine processing	Rapid processing
1	1.77	2.29
2	2.27	2.29
3	2.17	1.94
4	1.62	1.68
Mean	1.96	2.05
P=0.34*		

*Not significant

Table 8: Staining quality of cytoplasm of fibrous tissue (includes blood vessels, glandular tissue, inflammatory cells, osteoid components, muscles, and nerves)

Observer	Routine processing	Rapid processing
1	1.8	2.31
2	2.27	2.29
3	2.32	2.15
4	1.74	1.88
Mean	2.03	2.16
P=0.26*		

*Not significant

The *P* values were statistically not significant for all the parameters

4. The comparison of tissue shrinkage at gross level showed statistically significant difference between the two processing techniques [Table 3]; however, cellular-level shrinkage showed no significant difference [Table 9].

DISCUSSION

Routine (conventional) processing requires 2–3 working days before a diagnosis is delivered to the patient. The rapid processing schedules that are available currently require a minimum of 16–48 h for completion.[7-10]

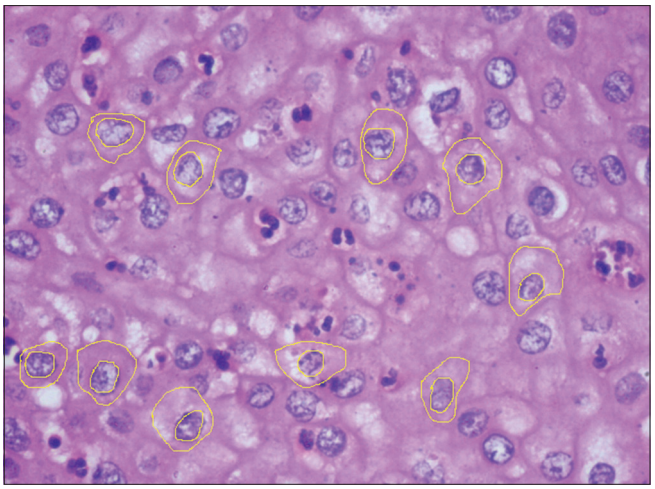


Figure 5: The cellular and nuclear measurement markings

Automation in tissue processing may hasten the process by continuing beyond the working hours of the laboratory, but it needs continuous power supply which cannot be ensured all the time. Automation is also an expensive affair.[11]

Diagnostic pathology is largely dependent on formalin-fixed paraffin-embedded tissue sections. This study presents a rapid processing technique (RaPT) [Table 1] and is compared with the routine processing technique (RoPT). The properties of tissues processed by rapid technique were comparable with routinely processed tissues, with reference to ease of sectioning, yield of good ribbons, good staining, permanency of stain (6–12 months observation) and satisfactory staining quality. These properties were observed in both soft tissues and hard tissues processed by these techniques. Furthermore, decalcified tissues were subjected to both the techniques to observe and compare. However, no statistical comparison was made for hard tissues as the sample size for hard tissues was less.

Rapidity of this technique is enhanced by the clearing agent methyl salicylate after dehydration step with alcohol. The property of making the root tissue transparent in a short time to study root canal morphology has been exploited in this study on tissue samples.[12,13]

Advantage of methyl salicylate over xylene is its low toxicity. However, their strong penetrating odors necessitate good laboratory ventilation. It causes minimal tissue shrinkage and hardening and tissues can remain in it indefinitely without damage.[14-16]

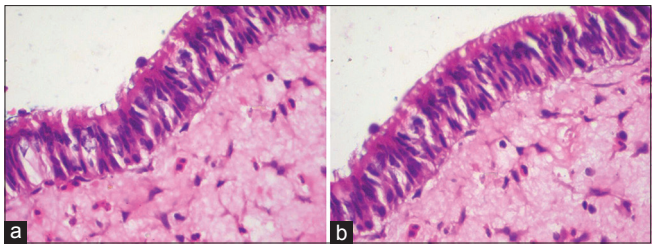


Figure 6: (a) Photomicrograph of stained specimen with rapid processing (immediately staining). (b) Photomicrograph of specimen in (a) after period of 1 year to show stability of staining

Table 9: Comparison of shrinkage of tissue at cellular level

Cytoplasm		Nucleus	
Routine processing	Rapid processing	Routine processing	Rapid processing
25.61±5.48	26.25±5.74	6.38±1.41	7.49±1.46
P=0.6289*		*P*=0.9849*	

*Not significant

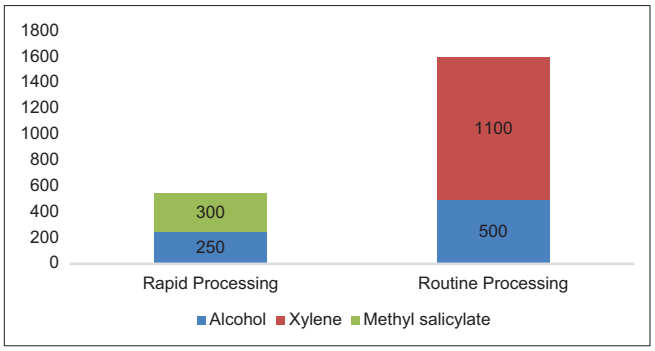
We compared the tissues and sections from RaPT with RoPT for general quality with respect to artifacts, folds and staining, and we arrived at a $P = 0.24$ which is statistically not significant [Table 5].

P value obtained for comparison of nuclear and cytoplasmic staining in epithelial and connective tissue was 0.30, 0.18, 0.34 and 0.26, respectively [Tables 5-8]. The result was considered statistically not significant for the same which indicates that the outcomes of both the processing techniques are comparable. Hence, the results of RaPT technique are satisfactory. To examine the stability of staining, the slides were observed periodically for a year and it was noticed that staining and its contrast were good and were comparable to RoPT [Figure 6a and b].

Processed tissue from RaPT showed relatively more shrinkage (34.67%) when compared to RoPT tissue (23.13%) with a statistically significant $P = 0.0000012$ [Table 3]. However, when cellular and nuclear areas were measured and compared for assessing shrinkage, there was not much difference between RaPT and RoPT with $P = 0.6289$ and 0.9849, respectively, which is statistically not significant [Table 9].

Authors of this study have also observed that RaPT was cost-effective with its minimal reagent requirement compared to RoPT with elaborate reagent setup [Graph 1].

There are no studies using methyl salicylate as clearing agent with which we can compare our observations. Our literature search did not reveal any other study using methyl salicylate for rapid processing. A study by Jali *et al.* claims a processing time of 8–9 h, but the study required a preliminary 24 h of



Graph 1: Cost (in Indian rupees) comparison between rapid and routine tissue processing (for 60 samples)

fixation step in neutral buffered formalin.[11] Our method completed the processing in 5–6 h depending on the size of the tissue, which includes fixation step as well and has yielded satisfactory results.

Limitations of our study

Tissue specimens with adipose component need longer time for processing. Standardization of timings in each solution is still in the process. Very few hard tissue samples were included in the study. Specimen thickness as mentioned in Table 1 is crucial as thicker specimens cannot be adequately processed within the time span mentioned.

Future objectives

The study will be further tested to check for the immunological viability of the RaPT tissue by performing immune-histochemical techniques and to apply the technique on variety of tissues to test the consistency of the procedure.

CONCLUSION

It should be possible to give a report on the same day of the arrival of the tissue specimen to the lab. The rapid processing technique proposed in this study helps in achieving just that with results comparable to routine processing technique at an affordable cost. Our study can prove to be beneficial to pathology laboratories by reducing the waiting period for the delivery of the report and definitely benefits the patients with early diagnosis and early treatment.

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Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Bancroft JD, Gamble M, editors. Tissue processing and microtomy. In: Theory and Practice of Histological Techniques. 5th ed. London: Harcourt Publishers; 2002. p. 85-92.
2. Panja P, Sriram G, Saraswathi TR, Sivapathasundharam B. Comparison of three different methods of tissue processing. J Oral Maxillofac Pathol 2007;11:15-7.
3. Rohr LR, Layfield LJ, Wallin D, Hardy D. A comparison of routine and rapid microwave tissue processing in a surgical pathology laboratory. Quality of histologic sections and advantages of microwave processing. Am J Clin Pathol 2001;115:703-8.
4. Culling CF. Handbook of Histopathological and Histochemical Techniques (Including Museum Techniques). 3rd ed. Butterworth and Co: (Publishers) Ltd.; 1974.
5. Available from: https://www.en.wikipedia.org/wiki/Methyl_salicylate. [Last accessed on 2017 Oct 25].
6. Niederegger S, Wartenberg N, Spiess R, Mall G. Simple clearing technique as species determination tool in blowfly larvae. Forensic Sci Int 2011;206:e96-8.
7. Godkar PB, Godkar PD. Text Book of Medical Laboratory Technology: Basic Histopathologic Techniques and the Laboratory Requirements. 2nd ed. Mumbai: Bhalani Publishing House; 2005. p. 1009-11.
8. Bancroft JD, Marilyn G. Theory and Practice of Histological Techniques: Tissue Processing and Microtomy. 5th ed. Philadelphia: Churchill Livingstone; 2002. p. 91-3.
9. Luna LG. Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology: Processing of Tissue. 3rd ed. New York: McGraw Hill Inc.; 1968. p. 16-8.
10. Sheehan DC, Barbara B. Theory and Practice of Histotechnology: Processing of Tissue. 2nd ed. Ohio: Battelle Press; 1980. p. 63-5.
11. Jali PK, Donoghue M, Gadiwan M. A rapid manual processing technique for resource-limited small laboratories. J Oral Maxillofac Pathol 2015;19:306-14.
12. Velmurugan N, Sandhya R. Root canal morphology of mandibular first premolars in an Indian population: A laboratory study. Int Endod J 2009;42:54-8.
13. Boruah LC, Bhuyan AC. Morphologic characteristics of root canal of mandibular incisors in North-East Indian population: An *in vitro* study. J Conserv Dent 2011;14:346-50.
14. Metgud R, Astekar MS, Soni A, Naik S, Vanishree M. Conventional xylene and xylene-free methods for routine histopathological preparation of tissue sections. Biotech Histochem 2013;88:235-41.
15. Kandyala R, Raghavendra SP, Rajasekharan ST. Xylene: An overview of its health hazards and preventive measures. J Oral Maxillofac Pathol 2010;14:1-5.
16. Tissue Sampling Processing and Staining. Available from: http://www.tissuesampling.weebly.com/processing.html. [Last accessed on 2017 Oct 20].