

# Agar-Paraffin Double Embedding Over Conventional Embedding for Minute Oral Biopsies- Cohort Study

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## Abstract

Agar-Paraffin double embedding technique is a simple technique that combines the advantages of both the embedding material. It preserves the minute tissue biopsies in orientation and holds them together from getting lost. Oral biopsies, being most commonly small incisional tissues, have to be preserved all through the processing and embedding to ensure optimal visualization of all the mucosal layers without compromise. In the present study, samples were divided into two groups: Group A: Agar-paraffin double embedding (APE) and Group B: conventional paraffin embedding (PE). Tissue samples collected were sectioned into two approximately equal sized bits. Both the bits were simultaneously processed, embedded in two different techniques (APE and PE method). Sections obtained were scored by an observer and analyzed using independent sample t-test (SPSS software version 21) to evaluate the efficacy of agar-paraffin double embedding technique in comparison to the conventional paraffin embedding technique. Agar paraffin embedded tissue was found to be well processed, firm and well preserved. Orientation was comparatively easier and the blocks yielded sections of good quality. They showed no interference with staining and cell morphology was of good clarity. Thus Agar-paraffin embedding technique represents a simple, reliable method that can greatly improve the quality of diagnostic information.

**Key words:** Agar-Paraffin, double embedding, oral biopsies

## Introduction

Agar-Paraffin double embedding refers to the technique whereby the tissue is first impregnated with agar and subsequently blocked in Paraffin to combine the advantages of both the embedding media [1]. Use of Agar-Paraffin embedding for small specimens in routine surgical pathology was first reported in 1959, which was developed further by Herbert Z Lund in 1961 [2]. Agar-formalin solution to aggregate fragments of tissues prior to paraffin embedding was described by Cook and Hotchkiss in 1977 [3].

Oral mucosal biopsies, being the small incisional ones, in common instances poses frequent problem in appropriate orientation. Proper visualization of mucosal layers is most necessary to accurate diagnosis. Although Agar-Paraffin double embedding holds numerous advantages, it remains little used technique in oral pathology laboratory.

The present study aims to assess the efficacy of Agar-paraffin double embedding technique in comparison with conventional paraffin technique to emphasize the usefulness of the technique in handling small oral specimens.

## Materials and Method

Ethical approval was obtained from the institution standard review board prior to the commencement of the study and oral mucosal biopsies were prospectively collected during gingivectomy and operculectomy procedures (Prospective experimental study). Each

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specimen obtained was sectioned into two approximately equal sized bits (Figure 1b). One bit was given to Group A- agar-paraffin embedding group, APE and the other bit to Group B - conventional paraffin embedding group PE. Group A and B specimens were processed together and subsequently embedded in paraffin. Except for the difference that Group A specimens (eight samples) were embedded in 3% agar (type 1 bacteriological agar) prior to processing. Both the tissue sections were scored and analyzed by an observer who is blind to the choice of the procedure. All the data obtained were tabulated and statistically analyzed using independent sample t-test (software: IBM SPSS Statistics version 21).

**Preparation of Agar-Paraffin (APE) blocks**

3% Agar solution was prepared by adding 3gms of Type I agar (extra pure bacteriological grade Type I agar, GRM666 - HIMEDIA research lab, Mumbai, India) to 100 ml of distilled water in a conical flask and bringing to a boil on continuous stirring. Stirring was kept until the solution appeared clarified and no particle of agar was visible (Figure 1). The agar solution was slowly cooled to room temperature. Then using a dropper/ micropipette the solution was added slowly in the L-mold containing the fixed/unfixed minute oral specimen in correct orientation to cover the entire specimen. After cooling and solidifying the agar block (Figure 2) was (put for fixation if unfixed) processed in the usual way and subsequently embedded with paraffin. It was made sure at least 3mm of agar surrounds the tissue in all directions. The remaining agar are stored in a beaker and maintained in the laboratory refrigerator. As needed, sufficient quantities are re-liquefied by heating.

**Fixation, Processing, Sectioning & Staining**

Both the Group A and Group B tissue samples were fixed by leaving the tissues in 10% neutral buffered formalin overnight.

These samples were simultaneously processed without any difference in dehydrant (isopropyl alcohol),

clearing (2 changes of xylene), infiltrated in molten paraffin wax and subsequently embedded in paraffin. 2-3 um thick section of each block were taken, stained with routine hematoxylin and eosin and cover slipped with DPX mounting medium.

**Results**

Conventional oral biopsies as small as 0.2 to 0.5 cm were processed by this technique. These biopsies are further reduced in tissue volume and surface area to nearly half during formalin fixation and paraffin processing making it difficult to identify embedding surface. Agar paraffin Embedding technique has helped to overcome this problem as the fresh tissue has already been embedded in agar before processing. The colorless Agar block was easily viewed in liquid and gel forms, orientation of the biopsy was easily monitored. Agar had not interfered with the penetration of fixative or processing chemical reagents, thus all the APE tissues were well processed.

There were eight sections in each group [Group A-Agar paraffin embedded sections (APE) and Group B- Paraffin embedded sections (PE)]. The sections obtained from each group (Figure 4) were scored in observer score sheet based on quality of section, staining properties, optimal orientation and cell morphology. The results are summarized in the Table 1.

The mean score for section quality of APE sections was 1.63±.744 whereas those for PE sections was 1.50±.535 (p= 0.228). The mean score for staining clarity of APE sections was 1.88±.354 whereas those for PE sections was 1.38±.744 (p= 0.052). Both the APE & PE sections showed a mean score of 0.88±.354 (p= 0.052) for orientation. The clarity of the cell morphological characteristics of APE and PE sections were with average score of 1.5±0.535 and 1.38±0.518 respectively (p= 0.744).

**Table 1: T-test for parameters comparing agar-paraffin double embedding and Paraffin embedding methods.**

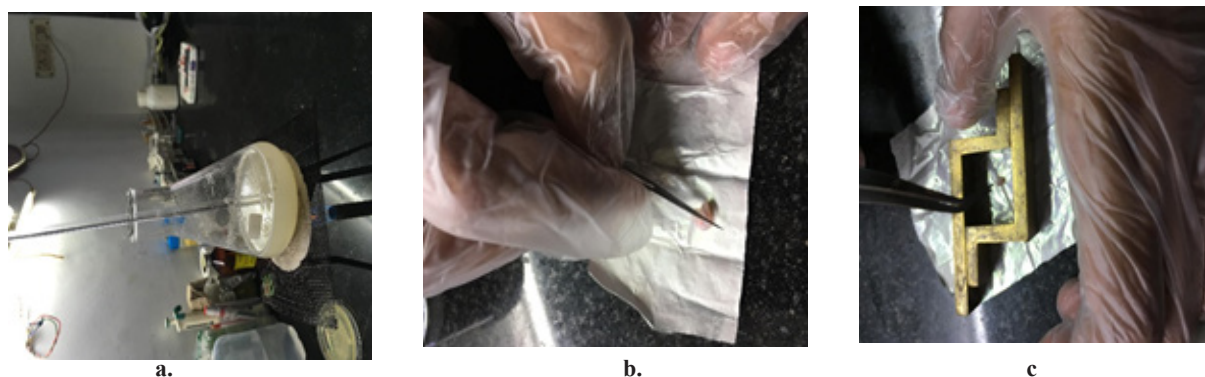
Criteria	Sub-criteria	Agar-paraffin section (total 8)	Paraffin sections (total 8)	P value
Quality of sections	Torn	1 (12.5%)	0	.228
	Wrinkled	1(12.5%)	4 (50%)	
	Good	6 (75%)	4 (50%)	

**Cont... Table 1: T-test for parameters comparing agar-paraffin double embedding and Paraffin embedding methods.**

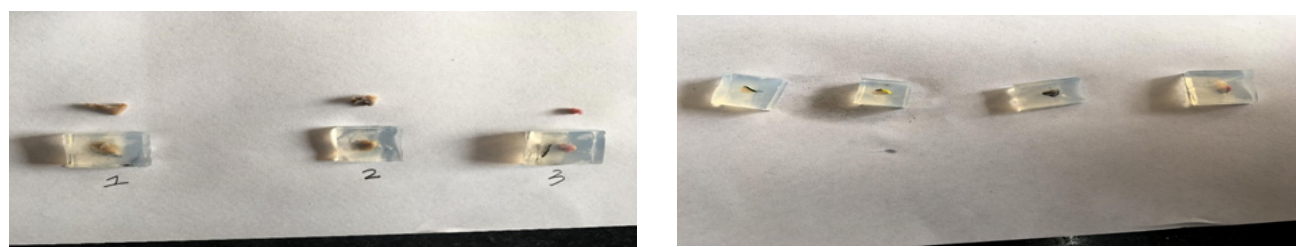
Clarity of stain	Weak staining	0	1 (12.5%)	0.052
	Patchy staining	1(12.5%)	3 (37.5%)	
	Optimal staining	7(87.5%)	4 (50%)	
Orientation	Wrong orientation	1(12.5%)	1(12.5%)	0
	Optimal orientation	7(87.5%)	7(87.5%)	
Cell morphology	Acceptable	4(50%)	5 (62.5%)	.744
	Good clarity	4(50%)	3 (37.5%)	

**Table 2: T-test for Agar embedding with unfixed and fixed specimens**

Criteria	Agar embedding with Unfixed specimen (total 4)	Agar embedding with Formalin fixed specimen (total 4)	P value
Quality of sections	2.00	1.25±.957	0.080
Clarity of stain	2.00	1.75±.500	0.006
Orientation	.75±.5	1.00	0.058
Cell morphology	1.25±.5	1.75±.5	0.006



**Figure 1: a. Preparation of 3% agar solution, b. sectioning of oral biopsy specimen to equal sized bits, c. orientation of specimen bit held in L-mold around which molten agar poured.**



**Figure 2: Prepared Agar pre-embedded block along with the corresponding bit for conventional embedding.**

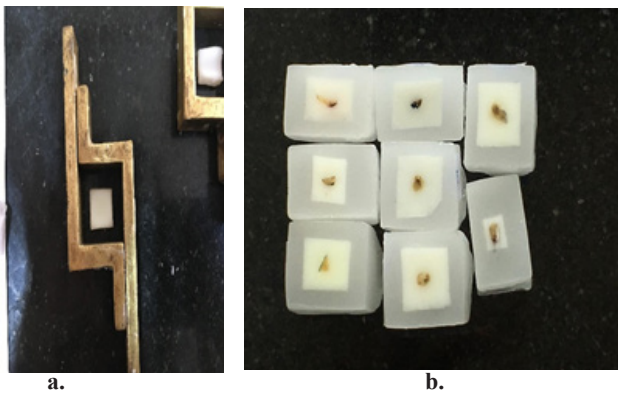


Figure 3: a. Orientation of processed agar block in L-mold for paraffin embedding, b. Prepared Agar-paraffin embedded block (APE).

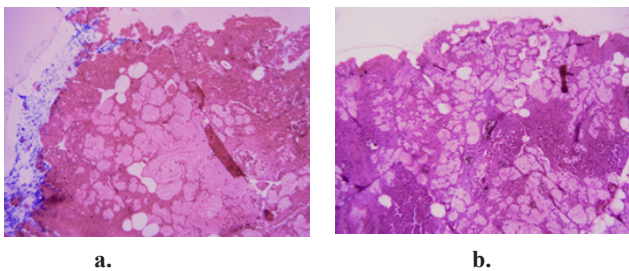


Figure 4: Comparison of APE sections (a) and PE sections (b)

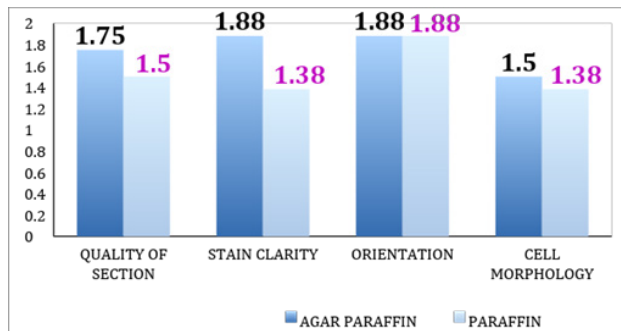


Figure 5: Graphical representation of Comparison - APE and PE Sections on various parameters.

### Discussion

Embedding involves enclosing of properly processed, correctly oriented specimens in a support medium that provides external support during microscopy [4]. Agar stabilizes the specimen by penetrating and replacing the intercellular fluid between the cells of the mucosa. It forms the cheapest source of embedding medium.

Agar is good choice for tissue embedding because of its property of hysteresis as it remains solid at  $36^{\circ}\text{C} \pm 1.5^{\circ}\text{C}$ , which remains firm even at  $60-65^{\circ}\text{C}$  thus holding the tissue firm and oriented in molten paraffin wax. Agar melts only at  $87^{\circ}\text{C} \pm 1.5^{\circ}\text{C}$ , a temperature range which tissue processing never reaches [5]. Being a product of cell wall polysaccharides, Agarose and Agarpectin has an easy melting and good gel stability

properties even at high temperatures. It is a common gelling agent used in various industries [6].

Improper choice of embedding medium can cause difficulty in section cutting resulting in poor quality of tissue sections. Among the faults in section cutting, crumbling of sections into fragments, wrinkling or folds are common [4]. We found that Agar-Paraffin sections showed 25% more good quality sections compared to paraffin sections. This could be attributed to the strong penetration of tissue by dual materials, agar followed by paraffin, forming a homogeneous block from which sections could be cut easily. Improved cohesion of tissue layers and plasticity given by agar with facility of better ribboning characteristics offered by paraffin are combined to produce sections without inclusion of much of artifacts.

Staining processes are used to give tissue contrast and color before they are examined with the microscope. Presence and absence of embedding medium during staining not only manipulate sections but also result in coloration of the embedding media [7]. The staining characteristics of APE were better than the PE sections by 37.5%. This data proves that the agar was better cleared away from the tissues unaffected the senility and specificity of the stain used. Moreover agar does not generally stain with hematoxylin and eosin unlike media like gelatin.

In our study, we had used acrylic color paints (Faber-Castell 6 Fabric colors Acrylic paint PC: 1410501, Mumbai, India) to mark the margins of the specimens and found that inking on the specimens were well preserved all through out the processing by agar embedding whereas the inking materials over the specimen margins was lost during conventional processing as the specimen is passed through successive processing solutions. Thus it adds on the credit of using even the cheapest and easiest mode of inking, which can be preserved by the Agar- Paraffin embedding technique.

Improper embedding due to change in shape of the tissue during the processing can result in tangential sectioning which may interfere with reading or cause incorrect critical measurement such as thickness of the tumor. Often difficulty of identifying embedding surface on the minute oral specimens ( $\sim 0.2-0.4\text{cm}$ ) is produced due to shrinkage produced by formalin fixation and paraffin embedding [8]. In the study, Agar held the specimen intact through the processing and since the orientation is also maintained which is accordance with previous studies [9,10,11], it yielded better quality of sections apart from saving the time for the pathology

technicians. Although, we found no significant difference between the tissue orientation achieved by Agar – Paraffin and conventional paraffin embedding, ease of orientation had been better with pre-embedding in agar.

The tissue processing procedure should preserve the underlying tissue architecture giving more comprehensive view of disease and its effect on tissues [4]. APE sections produced sections with well-preserved, discernable cell features- 12.5% more than that achieved by PE sections. Mechanical processing can distort the shape of the specimen due to action of the processing chemicals, which was prevented by agar block.

In the study, we had subdivided group A specimens (8 nos.) into two subgroups A<sub>1</sub>, A<sub>2</sub> (4 no each). A<sub>1</sub> group specimens were first embedded in agar followed which they were fixed in 10% neutral buffered formalin for a period of 24 hours whereas A<sub>2</sub> group specimens were fixed (24 hours) prior to agar embedding. While the A<sub>1</sub> group produced good quality sections with better staining characteristics, orientation and cellular morphology was better with A<sub>2</sub> group [Table 2]. It was evident from the A<sub>1</sub> sections that formalin had penetrated and fixed the tissue through the pores present in the agar. Cumulative intercellular tissue stabilization, removal with ease during de-waxing procedure and other physical characteristics of agar was responsible for section quality and staining character. The cellular morphology could have been compromised in A<sub>1</sub> compared to A<sub>2</sub> group due to probable imbibition of water from aqueous formalin solution or due to autolytic changes produced by the delay in the fixation during the process of agar embedding.

Certain slides of Agar-Paraffin showed edematous appearance of the cells showing the imbibition of water by agar. Agar blocks tend to hold more water even at high concentrations (due to imbibing action of agar). Increased duration in the dehydrating and infiltrating medium while tissue processing rectified the problem.

### Conclusion

The Agar-paraffin embedding technique represents a simple, reliable user-friendly method that can greatly improve the quality of diagnostic information one can obtain from minute biopsies by improving tissue orientation, quality of sections and shortened turn around time. This study is the first comparative study to prove the efficacy of Agar-Paraffin double embedding over conventional Paraffin embedding.

**Conflicts of Interest** – Nil

**Source of Funding** – Self-funded

**Ethical Clearance**- Taken from Institutional standard review board (Approval no: SRB /SDMDS16 OMP/02).

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