



**Addis Ababa University**  
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*Performance evaluation of conventional formalin fixation and rapid microwave, formalin free fixation of large biopsy specimens*

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A research Thesis Submitted to Addis Ababa University, College of Health Sciences, School of Medicine Department of Pathology, As Part of Partial Fulfillment of Masters of Science in Histotechnology

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# **Performance evaluation of conventional formalin fixation and rapid microwave, formalin free fixation of large biopsy specimens**

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## **List of abbreviations**

**°C:** Degree Centigrade

**CLSI:** clinical and laboratory standards institute

**DEC:** Department Ethical Clearance committee

**DPX:** Diethyl Plasticized Xylem

**FNAC:** Fine Needle Aspiration Cytology

**NaCl:** Sodium Chloride

**P1:** Pathologist One

**P2:** pathologist Two

**PBS:** Phosphate Buffered Saline

**PPM:** Parts per Million

**TASH:** Tikur Anbessa Specialized Hospital

## **Abstract**

**Background:** Fixation is the first most important preparative technique. Formaldehyde causes a burning sensation to the eyes, nose, and lungs at high concentrations and also has possible associations with cancers of various organs in occupationally exposed populations. It takes about 24 hours for complete fixation. Microwave fixation method is an alternative tissue fixation technique which can decrease formalin exposure and fixation time.

**Objective:** The objective of this study was to compare the quality of slides prepared by formalin and microwave fixation methods and to compare the time duration of the two methods.

**Materials and Methods:** A prospective cross sectional study was conducted in Tikur Anbessa Specialized Hospital (TASH) from January 2017 to August 2018. A total of 120 paired tissue samples were collected by convenient type of sampling method. Domestic type of microwave and ten percent formalin were used for microwave and conventional fixation respectively. Chi square and kappa statistics were computed for association and agreement tests respectively.

**Results:** Microwave fixation method reduced the time of fixation to 11 minutes while it was 24 hours in formalin fixation. According to observer I, 89.2% and 45% of slides prepared by formalin and microwave fixation methods respectively, gave at least satisfactory result. While as per observer II, 97.5% and 91.2% of the slides prepared by the two fixation methods respectively, gave the same result. The agreement between the two fixation methods on slide qualities was positive except for some parameters. The slide grade of 33 % and 17% of all types of tissue was significantly associated ( $p < 0.05$ ) with fixation method according to observers I and II respectively. While the slide grades of the rest (67% and 83%) of slides were not dependent on methods of fixation according to these observers respectively..

**Conclusion:** Microwave irradiation under controlled conditions can reduce fixation time to 11 minutes without significant loss of specimen quality. It can also reduce staffs exposure to formalin. Further assessment need to be done before implementing these methods in various settings.

**Key words:** *Fixation, Formalin, Microwave, Slide quality, Duration of tissue fixation*

# **1. Background**

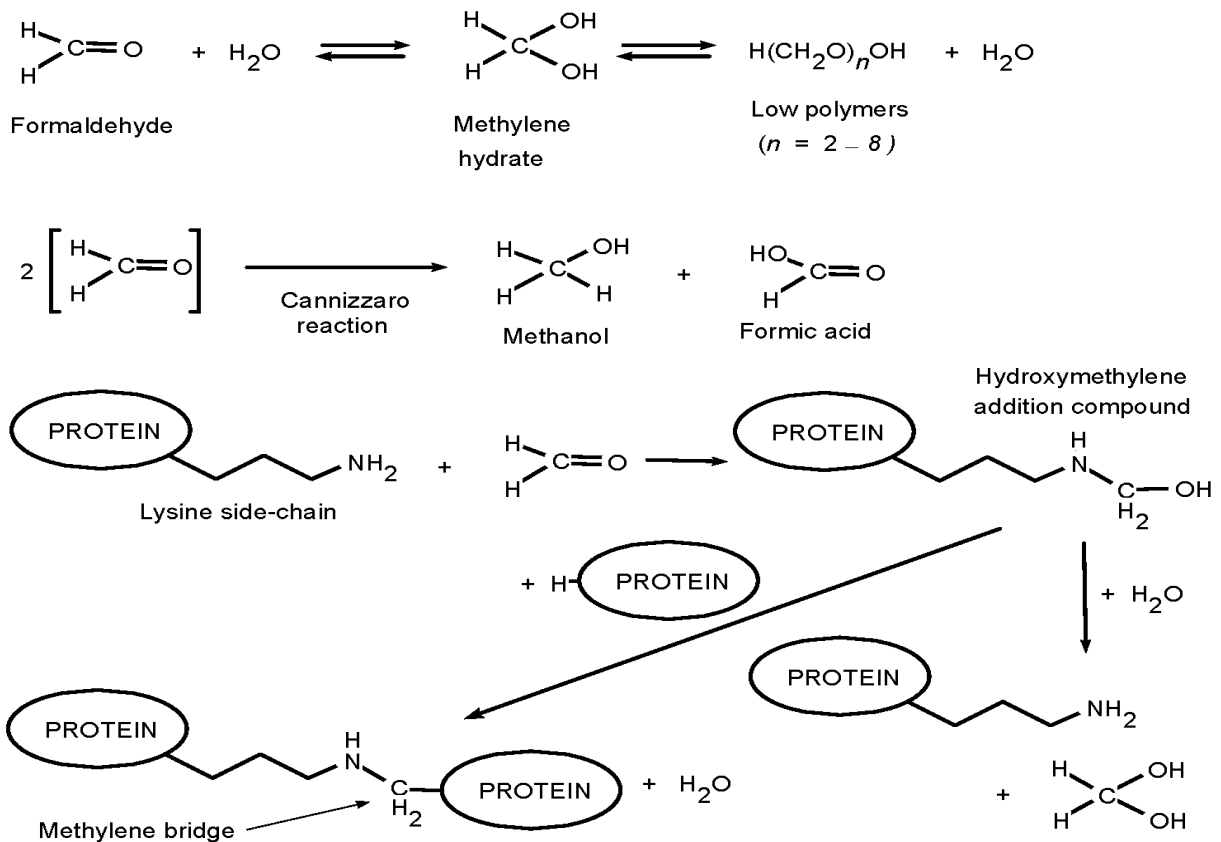
## **1.1.Introduction**

Fixation is the first most important preparative histopathological technique. It is a process by which the cells or tissues are stayed in chemical and partly physical state so that they can withstand continuous treatment with various reagents, with limited distortion of morphology and with no decomposition. It targets to preserve the biopsies as close to their living state as possible and to prevent autolysis and bacterial attack. It also aims to prevent tissues from changing their shape and size during processing. It helps to harden the tissues, to allow clear staining of sections subsequently and to improve the optical differentiation of cells & tissues. Fixation results in tissues protein denaturation and coagulation. Some fixatives have a property of forming cross links between proteins, thereby forming a gel, keeping everything in their in vivo relation to each other. Such methods which are currently used to manipulate tissues for microscopic examinations were developed in the early 1900s [1-4].

Different types of fixatives and fixation methods are known in routine activity of histopathology laboratory. The properties of these fixatives depend on both the physicochemical nature of the fixing agent and the vehicles used in the fixative solution. Fixation schedules for a given histopathology laboratory are often a matter of trial and error. Apart from the nature of the fixing agent itself, other requirements must be followed to develop these schedules. Those are maintenance of appropriate pH in the solution during the fixation process, choice of suitable vehicles and appropriate osmolality to avoid swelling or shrinkage as much as possible and control of ionic and micro environmental constitution within intracellular and extracellular spaces. This is to minimize extraction and precipitation of macromolecules or cellular organelles [1, 5].

The methods of tissue fixation in histopathology laboratory were evolved largely after the establishment of formalin as a fixative that could rapidly and permanently preserve fairly large tissue specimens. After the properties and potential use of formaldehyde were intensively studied, it became attractive as an alternative reagent to the old established histological fixatives. It penetrates tissue quickly but fixes slowly, which is the rate limiting step in formaldehyde fixation. Formaldehyde fixation is the formation of cross-links by the reactive aldehyde group (-

CHO) between proteins, proteins and nucleic acids as well as formation of coordinate bonds with calcium ions. The cross-links and the co-ordinate bonds may be responsible for the masking of epitopes by altering the three-dimensional structure of proteins. In an aqueous solution, formaldehyde forms ethylene hydrate, a highly reactive methylene glycol or methylols with uncharged amino groups. Each methylene bridge cross links an amino group to another functional group. By blocking the amino groups, formaldehyde renders the tissue more acidic thus increasing the negative charge. Only loosely bound formaldehyde is removed by washing for several hours but any bridging that has already occurred may remain in the tissue. The residual formaldehyde is gradually removed when tissues are left in water for a number of years (**figure 1**) [5, 6].



**Figure 1: Process of fixative reactions of formaldehyde with water, with itself, and with protein [6]**

Microwave fixation method is a relatively recent tissue fixation technique first used by Boon and Kok in 1985. Microwaves were invented by Percy Spencer in 1945 and soon became an integral part of our daily lives. Microwave energy is part of the electro-magnetic spectrum of similar frequency to radar beam energy, and at a frequency of 2,450 megacycles per second penetrates several centimeters into biological material. This energy is absorbed and converted into heat within the tissue. In effect microwave energy is an alternating electromagnetic field, the direction of which changes 2,450,000,000 times every second. Dipolar molecules, such as water, present in the field, are forced to oscillate at this frequency and this increases their thermal agitation and generates heat. The amount of heat produced within the tissue can be controlled by adjustment of the energy level and the duration of exposure [7-11].

## 1.2. Statement of the problem

Formaldehyde is a colorless, flammable gas at room temperature which has a pungent, distinct odor and may cause a burning sensation to the eyes, nose, and lungs at high concentrations. It can enter our body after we breathe it in, drink or eat it, or when it comes in contact with our skin. It is quickly absorbed from the nose and the upper part of our lungs. When it is eaten and drunk, it is also very quickly absorbed. It can react with many other chemicals, and it will break down into methanol (wood alcohol) and carbon monoxide at very high temperature. It irritates tissue when it comes into direct contact with them. Some people are more sensitive to the effects of formalin than others. Eg. People with asthma may be more sensitive to the effects of inhaled formalin than other people. Severe pain, vomiting, coma, and possible death can occur after drinking large amounts of formaldehyde. Skin can become irritated if it comes into contact with a strong solution of formaldehyde [12-16]

Repeated exposure to formaldehyde vapors at 40 parts per million (ppm), for 6 hours/day, 5 days/week for up to 13 weeks produced 80% mortality in B6C3F1 mice. Deaths occurred predominately in the fifth and sixth week of exposure and were associated with ataxia, severe body weight and depression. It caused inflammation and metaplasia in the nasal cavity, larynx, trachea, and lungs. These were attributed to occlusive tracheal lesions and/or prominent sero-purulent rhinitis [14, 17].

It is also noted that there are possible associations between formaldehyde and cancers of various organs in occupationally exposed populations. Chronic exposure to air concentrations in the range of 6–15 ppm [15] causes malignancy in respiratory tracts like nasal cavity and, lung cancers. Results of toxic kinetic and metabolic studies indicate that most inhaled formaldehyde is deposited within the upper respiratory tract. It also causes different types of malignancy in non respiratory tract including multiple myeloma, non-Hodgkin's lymphoma, ocular melanoma, and malignancy of brain. Cancer of connective, pancreatic, leukemic, lymphoid, hematopoietic and colon tissues are rarely resulted in formalin exposure [18].

On the other hand the effect of exposure to microwave irradiation on the biological systems of the body is uncertain. It is speculated that microwaves, unlike X-rays, and gamma rays, are not sufficiently energetic and too small an energy level to effect ionization in matter. Likewise, it

cannot be said whether microwave irradiation has an effect on biological molecules of proteins with dielectric properties. The exposure to microwave irradiation decreases rapidly with increasing distance from the oven. The operating manual recommends a location or standing distance of one meter from the oven, when it is in use [19-21].

The foundation of all good tissue specimen preparations is complete fixation. But the conventional method of tissue fixation is time consuming, taking not less than twenty four hours. It depends on relatively slow infiltration of the solution from the outer surfaces. This results in personal exposure to hazardous chemicals, formaldehyde [14, 22].

Search for alternate solution of formalin fixation in the histopathology laboratory to preserve the macromolecules has led to development of various substitutes with substantial technical advantages. The changing situation caused by modernization in the field of medical technology, has led to the placement of traditional techniques by newer ones. As the microwave is gaining more acceptances in histopathology, there is a need therefore to investigate the effect of this innovation on tissue morphology. Time and reagent consumption as well as decreasing exposure to hazardous consumables are also issues [9, 23- 24].

In Ethiopia especially in TASH pathology department, even though it is not measured quantitatively, there is high exposure of professionals to formaldehyde vapor. The turnaround time of tissue diagnosis is also longer due to different reasons including the lengthy formalin fixation time. But enough study was not conducted on the possibility of replacing formalin fixation method by other alternative fixation method which can resolve these problems in the country.

Therefore, this work was aimed at investigating the possibility of reducing the total time required for formalin fixation and minimizing the exposure to hazardous chemicals without compromising morphological quality by replacing it with microwave fixation. It also targets to assess the reliability of microwave fixation in histopathology and to investigate the effect of microwave fixation on tissue morphology and dye uptake.

### **1.3. Significance of the study**

If conventional formalin fixation is replaced by rapid microwave fixation, it can decrease the turnaround time of the tissue diagnosis. It can also decrease the hazardous effect of formalin on health professionals. Therefore this research can be used as a base line for other studies to investigate the possibility of adopting the rapid microwave fixation method in the routine histopathology diagnosis procedure without compromising morphological quality.

## 2. Literature review

A study was conducted by Login GR in 1978 on Microwave fixation versus formalin fixation of surgical and autopsy tissues. In the microwave irradiation, normal saline, ten percent phosphate buffered formalin, and distilled water were used as fluid media. Tissue sections were fixed by both microwave irradiation and 10 % phosphate buffered formalin. The slides were stained by hematoxylin and eosin stains. The result showed that Saline microwave fixation was comparable and superior to formalin fixation [25].

A prospective cross sectional study was conducted by Leong ASY *et al* in 1986 on a method of rapid fixation of large biopsy specimens using microwave irradiation with a total of 212 large and fresh specimens. All specimens were subjected to non-ionizing irradiation in a domestic microwave oven for 4-9 minutes. After fixation tissues were rendered sufficiently firm to be dissected and sampled immediately without the need for any formalin fixation. These were hardened and remained pliable, retained much of their natural color and were free of noxious fumes. Lymph nodes tissues became opaque and firm and their pink-tan color contrasted with the bright yellow of adipose tissue, making them easily dissectible [26].

A cross sectional comparative morpho-metric study was conducted by Kayser K *et al* in 1988 on rapid microwave fixation by taking tissue from primary bronchial carcinoma of lung and mediastinal and hilar lymph nodes. Solutions of tris buffer, buffered formalin, sodium chloride,; distilled water, acetone, methanol and gluteraldehyde were used in the procedure. The result revealed that histo-morphological sections fixed with tris buffer in a domestic microwave oven appeared best morphological results showing more contrast in chromatin distribution of nuclei in comparison to conventional formalin-fixed specimens. The research concluded as microwave fixation using tris buffer was a time-saving fixation method compared to conventional formalin fixation. It avoids hazards to the environment which is not possible in formalin fixation [27].

Similar study was conducted by Chaudhary K *et al* in 2000 on microwave technique in histopathology and its comparison with the conventional technique taking 25 formalin fixed human tissues from different organs and 50 fresh animal tissues. In this study recording of temperature could not be done in domestic microwave irradiated tissue fixation. Among the fresh tissues, 34 pieces were fixed in 10% formalin and 16 were stabilized in normal saline, with

microwave irradiation. The result showed that 80% cases of formalin fixation gave satisfactory result, while only 30% cases of microwave irradiation tissue fixation were satisfactory [28].

A prospective cross sectional study was conducted by Tripathi M *et al* in 2013 in Meerut, India on the comparison of routine fixation of tissues with rapid tissue fixation. The study constituted 140 paired tissue specimens which were taken randomly excluding very tiny biopsies. By this study tissue samples were fixed by microwave irradiation and conventional method to compare the two methods by the quality of histological section and turnaround time. This study demonstrates that microwave irradiation under controlled conditions can reduce fixation time to about 5 minutes and thus reduce the overall processing time in surgical pathology without significant loss of specimen quality. The p-values of all parameters were not significant except for the overall morphology owing to loss of tissue in some cases [29]. There was no similar study conducted on fixation methods in Ethiopia.

### **3. Objectives**

#### **3.1.General objective**

The objective of this study was to compare the quality of slides prepared by formalin and microwave fixation methods and to compare the time duration of the two methods.

#### **3.2.Specific objectives**

- This study compares the quality of tissue slides prepared by formalin and microwave fixation methods.
- To determine statistical agreement between the two fixation methods on the slide quality.
- To compare the time duration of fixation by microwave irradiation and fixation by conventional method (formalin fixation)

## **4. Materials and Methods**

### **4.1. Study design**

A prospective cross sectional comparative study was conducted.

### **4.2. Study area**

The study was conducted in Addis Ababa University Tikur Anbessa Specialized Hospital (TASH) which is located in Addis Ababa, Lideta sub city, Woreda 10. It is the largest of the public teaching hospitals having more than 26 departments including pathology department.

TASH pathology department has academic and no academic staffs. Academic staffs include 7 senior pathologists at the level of assistant professors and above, one molecular and cancer biologist at the level of assistant professor, 5 candidates of pathology speciality, 8 histotechnologists at a level of lecturer and assistant lectures. Non academic staffs including laboratory assistants, reception and result despatching personnel, secretaries and cleaners play great role in achieving the goal of the department routine clinical and teaching activities. The department also has post graduate students of pathology resident ship and masters of histotechnology who came from other health and teaching institutions of the country.

It has different working areas including reception room, tissue grossing room, tissue processing and special stain room, tissue sectioning room, fine needle aspiration room, immune histochemistry room and slide examination room. About 10,000 biopsy and post mortem samples come to the department reception room every year to be diagnosed and to despatch the result to the respective requesting department. It also works on cytology (FNAC), body fluid cytology, and haematology, with a total of 12,000 patient specimens annually.

### **4.3. Study period**

From the starting to complete the study, it took more than one and half years (January 2017 to August 2018).

#### **4.4. Sample size, sampling method and sampling procedure**

A total of 120 paired tissue samples were included in this study. This was determined according to clinical and laboratory standards institute (CLSI) guide line. According to this guide line, the number of sample for method comparison study should be 40 and above [31]. Convenient type of sampling method was used to collect tissue specimens. In the TASH major operation room, larger tissue specimens were selected and part of it was put into a container having normal saline (0.9% sodium chloride (NaCl) solution prepared at home) and transported to department of pathology. This continued until the number of specimen reach to 60. In the grossing room of pathology department, two samples were taken from each specimen for microwave fixation (a total of 120) and other two samples were prepared for the conventional formalin fixation (a total of 120).

#### **4.5. Inclusion and Exclusions criteria**

##### **4.5.1. Inclusion criteria**

Larger and soft biopsies (at least it can be cut in to 4 slices of tissue with a size of 3mm X 5mm X 2mm each) were included in this study.

##### **4.5.2. Exclusion Criteria**

Tissues lasted more than an hour without fixatives after surgical operation were rejected.

#### **4.6. Study variable**

##### **4.6.1. Dependent variable**

- Quality of slides
- Agreement between the two fixation methods
- Length of time for complete fixation

##### **4.6.2 Independent variable**

- Type of method of tissue fixation
- Difference in nature of specimen
- Inter observer personal difference in grading of slides

## 4.7 Laboratory methods and data collection

### 4.7.1 Pilot study

A pilot study was done on 10 slides of 3 tissue specimens using a domestic microwave, which has power level adjustment starting from power level 10 (warm) to power level 0 (high) and also has a time adjustment key.



#### **Samsung domestic microwave (kitchen microwave) used in the research**

According to the user manual, if a key of microwave is adjusted on power level 0, the machine irradiates microwaves at frequency of 2.45 megacycles per second with a power being converted to heat measuring high temperature; if it is adjusted at power level 20, it measures low temperature; then continues to power level 90 with temperature of sute and power level 0 (high). The following table shows the power level and the corresponding qualitative temperature of the microwave as indicated in the user manual of the microwave used (table 1).

**Table1: Domestic microwave qualitative temperature with respect to power level**

No.	Power Level	Qualitative Temperature	No.	Power Level	Qualitative Temperature
1	10	Warm	6	60	Simmer
2	20	Low	7	70	Medium high
3	30	Defrost	8	80	Reheat
4	40	Medium Low	9	90	Sute
5	50	Medium	10	0	High

Since this kitchen type microwave oven does not show us the exact temperature at a given power level is adjusted, the temperature of one litre phosphate buffered saline (PBS) was measured at different power level with different duration of time using high temperature thermometer. This was aimed to optimise the temperature at which the tissue fixed well within the minimum possible time without morphological distortion.

When sample was fixed at lower temperature for small time, it was not well done and tissues remain flashy. To the contrary when it was fixed with high temperature and for much more time tissues were burned. The best temperature was found to be 68 °c. This can be detected by first putting tissue cassettes to one litter PBS solution of glass container, next place it in microwave machine and finally irradiate it at low power level for 40 minutes. Or, following the same procedure but finally irradiating the solution at high power level for 6 minutes and then low power level irradiation for 5 minutes. The minimum possible time with optimum fixation temperature was found to be 11minutes which was used for this study this was found by try and error (after repeated times of testing) (table 2).

**Table 2: Level of tissue fixation in one litre PBS against power level and time duration**

No.	Power Level	Time duration (minutes)	T <sup>0</sup> of 1 litter PBS (°c)	Level of tissue fixation
1	20 /low power level	30	57	Not fixed well/flashy
2	20 /low power level	38	67	Not fixed well/flashy
3	20 /low power level	40	68	Well fixed/Optimum
4	30 /defrost	10	45	Not fixed well/flashy
5	30 /defrost	20	65	Not fixed well/flashy
6	30 /defrost	30	80	Tissue burned out
7	<b>0 (high power level)</b>	<b>6</b>	<b>68</b>	<b>Well fixed/ optimum</b>
	<b>20(low power level)</b>	<b>5</b>		
8	0/high power level	10	90	Tissue burned out

After this adjustment was done, the fixations of all the 120 tissue samples were done with this microwave. This tissue fixation was done with heat and microwave resistant glass container.



**Microwave resistant glass container**

#### **4.7.2. Tissue grossing**

After the specimens were transported to pathology department grossing room, tissues were transferred to the fume cabinet and observed for any macroscopic abnormality. Then four samples were taken from each specimen with the sizes of 3mm X 5mm X 2 mm using a sharp scalpel blade. Each of the individual tissue samples were placed in tissue cassettes and labeled with a three digit number starting from '001' followed by the first four English capital letters as '001A' or '001B' or '001C' or '001D' etc. Two of them were placed to the container of 5% PBS at a P<sup>H</sup> of 7.2. Then it was immediately fixed using a domestic microwave at a temperature of 68 °c with a power level and time duration indicated above. The other two were put in to a container of 10% formalin which was kept to be fixed for the next 24 hours [22]

### **4.7.3. Microwave tissue fixation method**

One liter of 5% new PBS was prepared by adding 50 milliliter of concentrated PBS solution to 950 milliliter of distilled water. Tissue cassettes in the PBS solution were transported to the Immune-histo-chemistry room. The 5% PBS solution were drowned to the microwave safe container and put into the microwave oven. Then power level was adjusted as 0 (high temperature) and kept for 6 minutes then the temperature was measured to be about 68 °c. The cassettes were added then microwave oven power level was adjusted to 20 (low temperature) and kept for five minutes. Then the heated PBS solution with cassettes was drowned to the original plastic container. This container was transported to tissue processing room and kept there until its temperature decreased to room temperature. The tissue kept there to be processed together with the routine patient tissue cassettes.

### **4.7.4. Tissue processing**

All samples were processed with the conventional tissue processing method with TISSE 'TEK II rotary tissue fill fully automated tissue processor. Both types of tissue cassettes fixed with formalin and microwave irradiation were first dehydrated by passing through exchange of graded alcohol starting with 70% ethanol followed by 80% alcohol then 96% ethanol and finally three jars of absolute alcohol. Two jars of xylene were used as clearing agent and as a bridging chemical between absolute alcohol and the next infiltrating/impregnating agent. The final two jars were filled with molten paraffin wax for tissue infiltration to give internal support.

### **4.7.5. Tissue embedding and sectioning**

At the end of tissue processing, tissues were embedded using embedding cassettes on which the processing was performed by Tissue Tek embedding Centre. Tissues were removed from tissue cassettes and placed on the embedding chamber. Molten paraffin wax was dispensed to full capacity into Tissue Tek embedding mold by pressing the tap backward. Using a toothed forceps, each tissue was picked and orientated in the molten paraffin wax in the mold. The cover of the labeled cassette was removed and the reverse side of the cassette was placed on the mold containing the tissue embedded in paraffin wax. Then embedded mold was placed on the cooling chamber and allowed to cool and

solidify. This was repeated for individual tissues until all tissues were embedded. After a period of 10 minutes, the block was detached from the mold (now containing the tissue embedded in the solidified wax). Then tissue blocks were sectioned by a rotary microtome at a thickness of three micro meters.

#### **4.7.6. Tissue / slide staining and mounting**

The prepared slides were stained by a Leica Auto-Steiner XL slide Steiner and mounted with diethyl plasticized xylene (DPX) mounting medium. First of all tissue sections were deparaffinized in a dry oven at 70 °c for one hour, then cleared with two turn of xylene. Tissue slides passed through exchanges of graded alcohol (Absolute alcohol, 96 % alcohol, 80% alcohol and 70% alcohol in its descending order) then immersed in tap water for tissue rehydration. Mayer's hematoxylin was used as primary stain; tap water was used as bluing medium and eosin as counter stain. The stained slides were then dehydrated again using graded alcohol (70%, 80%, 96% and Absolute alcohols in ascending order). Then finally the tissue slides were cleared with other two turns of xylene and then mounted with DPX.

#### **4.7.7. Microscopic examination of the slides and recording of the data**

All slides (a total of 240 for both fixation methods) were examined and rated for the adequacy of fixation by two pathologists in a blinded fashion. Seven parameters including cellular outline, cytoplasmic detail, nuclear detail, erythrocyte integrity, lymphocyte appearance, overall morphology and overall staining, were used as measuring tools in rating of slides. First each parameter was rated as '**Poor**' if it was assumed to be below satisfactory (a minimum quality that can enable to diagnose with some difficulties), '**Fair**' if slide quality was assumed to be satisfactory '**Good**' if the slide was assumed to be at a quality that enable to diagnose easily '**Excellent**' if the slide was assumed to be at a perfect quality of enabling diagnosis and '**Outstanding**' if the slide was assumed to include some special qualities in addition to enabling diagnosis). These rates were valued as a score of 1, if it was poor, 2, if it was fair, 3, if it was good, 4 if it was excellent and 5, if it was outstanding. Finally, overall grading of the slide was calculated by adding the scores of each parameter and it was again rated as '**Poor**' if the sum of scores of all parameters fall within 7-13, '**Fair**' if it fall between 14-20, '**Good**' if 21-27,

*'Excellent'* if it was 28-34 and *'Outstanding'* if it was above 34 as described by previous study [29].

An evaluation data sheet was prepared with spaces for recording the slide code, pathologist code, scores of each parameter, nature of the specimen, and diagnostic result of the tissues (annex I).

Slides were given to each observer in 6 rounds (40 slides for each round). The first 40 slides (001A-010D) were arranged in ascending order of numeric code in a slide box. The code of each slides were recorded on the 'slide code' portion; and code of observer was recorded as 'p1' at the 'pathologist code' portion of each evaluation data sheet. A slide box containing 40 slides and the corresponding 40 data sheets were given for observer I. He was expected to write the score of the slides on each parameter according to his judgment based on the above criteria. The second round was given to observer II in the same fashion recording the code of observer as 'P2'. The expected activities of observer II was similar with observer I for all slides and data sheet. After collection of the slides and evaluation data sheet of the first round from observer I and second round from observer II it was continued interchangeably until the last round evaluation datasheet was collected with the corresponding slides from the last observer (observer I in this case).

#### **4.7.8. Data quality assurance**

Every data was collected carefully and samples were investigated with appropriate laboratory procedure. Senior professionals (senior pathologists and technologists) were participated in the supervision and technical aspect as well. The pre analytical, analytical and post analytical quality assurance procedures are explained below independently.

##### **a. Pre analytical quality assurance**

Normal saline was prepared following appropriate measurement of sodium chloride (NaCl) (using digital balance which increased its accuracy) and using distilled water. Specimen collection, transportation and microwave fixation were done within 60 minutes after each surgical operation was done. This was aimed to prevent tissue petrification [7, 30]. Enough size of each specimen (the size of specimen at least for 4 cassettes) was taken from operation room.

## **b. Analytical quality assurance**

In the grossing room tissues with similar macroscopic appearance; including their size were taken in to each of the four cassettes from one specimen. The labeling of samples from one specimen were not in ordered fashion (E.g. if sample numbers 001A and 001B were fixed with microwave irradiation, sample numbers 002B and 002D would be fixed with formalin). This was aimed to avoid the pre anticipation of the scale of the next slide depending on the present one during slide examination. The code of the sample and the corresponding fixation method to which it was going to be done were recorded in a log book. In the microwave fixation, temperature adjustment and duration of fixation were similar for all specimen types. The ph of PBS was strictly measured to be 7.2 for each specimen. Both microwave and formalin fixed tissues were processed together with the routine tissue cassettes in the same condition. The same was true for tissue embedding, slide sectioning, staining and mounting procedures.

During slide examination the two slide observers were blinded. This means observer I did not know who was observer II and vice versa. They also did not know the score of the slide given by one another. They were not informed as by which method of fixation a given slide was done.

## **c. Post analytic quality assurance**

On each evaluation data sheet all the data including the method of fixation of the corresponding slides were carefully recorded. Overall grade of the slide were calculated carefully by adding the scale of each parameters. SPSS data entry was also done with a great care.

## **4.8 Data processing and analyses**

All the data were entered to SPSS version 20, to be analyzed.  $\chi^2$  test was done to each parameter of the quality of slides to check if dependent variables have statistically significant association with the independent variables. Ninety five percent confidence level and 'P' values less than 0.05 were considered as statistically significant in the chi-square statistics.

Kappa statistics was done to assess the agreement between the two observers on the slide assessment results and to determine if there was an agreement of the slide qualities of the two techniques (fixation methods). Since the sample size of this study was large enough (above 100)

[31], all kappa values above zero were considered as significant. The *P* values in this kappa statistics was not used to test the strength of agreement; it tested whether or not the estimated kappa was due to chance [32].

The time taken for microwave tissue fixation were measured by a stop watch and recoded each time. The mean value of each turn was calculated manually.

#### **4.9 Ethical consideration**

Ethical clearance was obtained from the department of pathology ethical clearance committee (DEC), college of health sciences, school of medicine, Addis Ababa university. A letter was written for the surgical department and got permission to collect the tissue specimens.

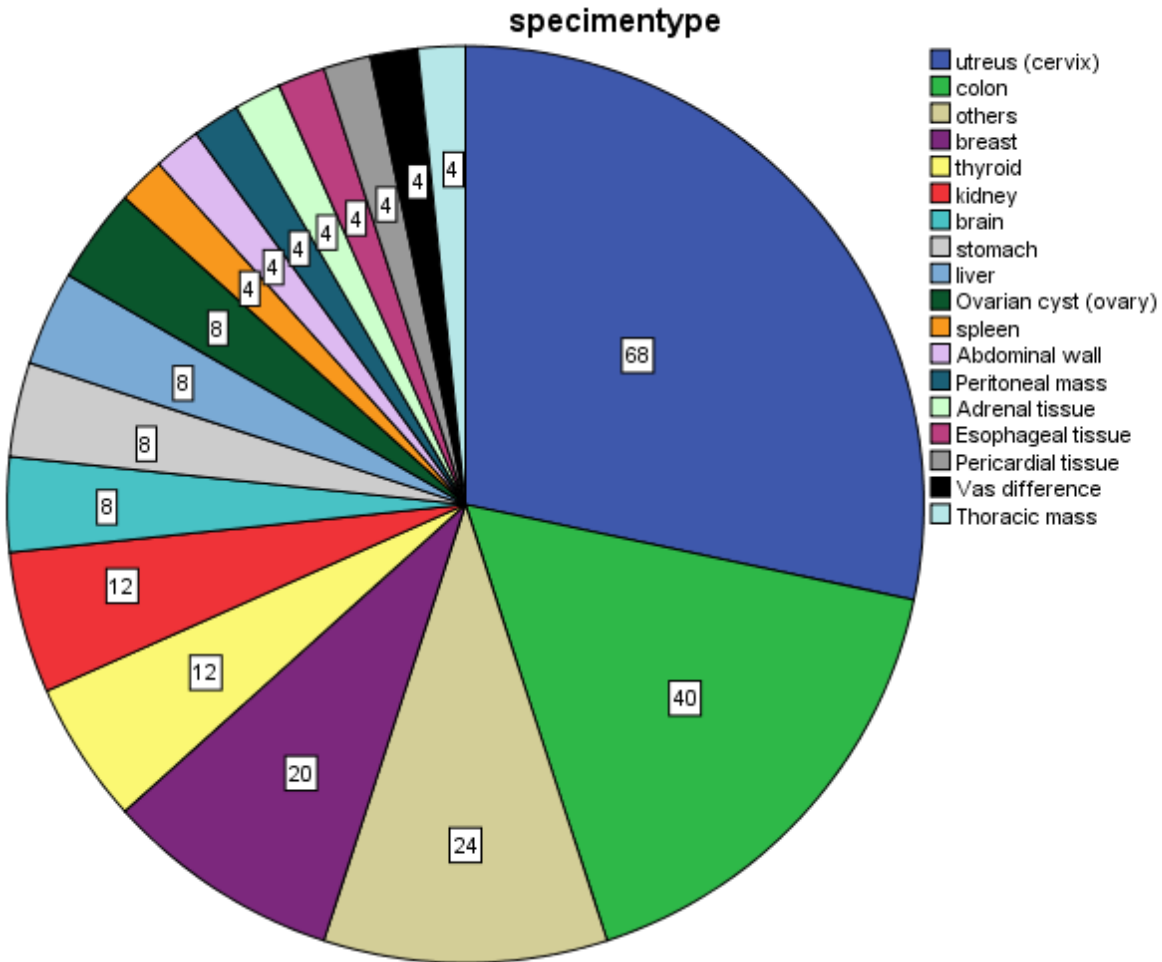
#### **4.10. Operational definitions**

- ***Observer I:*** the first pathologist who examined the research slides.
- ***Observer II:*** the second pathologist who examined the research slides.
- ***Tissue processing:*** passing the tissue through steps in exchange of chemicals from tissue dehydration to tissue impregnation
- ***Domestic microwave:*** the type of microwave used for kitchen purpose (for domestic activity)
- ***Kappa value:*** a quantitative measure of the magnitude of precision (agreement) between observers or, among laboratory methods. It ranges between -1 and 1 with interpretation as: kappa value less than zero means less than chance agreement; 0.01–0.20 means slight agreement, 0.21– 0.40 means fair agreement; 0.41–0.60 means moderate agreement; 0.61–0.80 means substantial agreement and 0.81–0.99 means almost perfect agreement. But with a large enough sample size (100 and above) [31]; any kappa above 0 will become statistically significant. The *P* value in kappa statistics tests whether the estimated kappa is not due to chance; it does not test the strength of agreement [32]

## 5. Results

### 5.1. General description

A total of 120 pairs of samples from about 18 tissue types were fixed by rapid microwave and conventional formalin fixation methods. The dominant tissue type was uterus, followed by colon biopsy. Figure 2 below depicted the type of biopsies used for this study.



**Figure 2: Quantitative distribution of different specimen types processed by both microwave and conventional fixation methods**

In this study comparison of turnaround time and quality of histological sections was assessed. The duration of time for tissue fixation by microwave irradiation was 11 minutes. But it takes about 24 hours (1440 minutes) for formalin fixation; which is more than 130 fold of the time taken by microwave method.

After fixation by microwave irradiation, tissue sections were firm and shrunken by one third. But after formalin fixation tissue sections were darker and shrunk by one fourth from the original biopsy. Some tissues like uterus, after fixed with microwave irradiation became harder in tissue microtome. While other soft tissues like brain, after fixed with microwave were more comfortable to cut than tissues fixed with formalin.

## 5.2. Inter observer agreement on quality of slides

On microscopic examination by the two pathologists, inter-observer agreement was found to be positive within the range of slight agreement except on **cellular outline** and **grade of the slide**. The value of agreement ranges from -0.028 to 0.102 with p value ranging from 0.003 to 0.537 in the measure of the seven parameters (table 3).

**Table 3: Inter observer agreement (Kappa) on the scale of each parameter for tissue slides**

No.	Parameter	Kappa value	P-value
1	Cellular outline	-0.028	0.368
2	Cytoplasmic detail	0.023	0.472
3	Nuclear detail	0.102	0.003
4	Erythrocyte integrity	0.056	0.067
5	Lymphocyte appearance	0.023	0.537
6	Overall morphology	0.066	0.054
7	Over all staining	0.030	0.392
8	Slide grade	-0.022	0.392

## 5.3. Association of slide quality with tissue type

The association between the scale of each parameter and the type of the specimen were computed using chi-square test for each observer independently. The result shows that as examined by observer I, scales of all parameters for the tissue fixed by formalin have no significant association with the tissue nature; while only the scales of **cellular outline**, **lymphocyte appearance** and **over all staining** of the tissue fixed by microwave method were significantly associated with specimen nature ( $p < 0.05$ ). On the other hand, the scales of all parameters except **over all staining** and **grade of the slide** for the tissue fixed by microwave method and examined by observer II were significantly associated with nature of the tissue. From the tissues fixed by formalin and examined by observer II, scales of **cytoplasmic detail**,

**nuclear detail and over all staining** have statistically significant association(  $p < 0.05$ ) with the nature of the tissue (table 4).

**Table 4: Chi-square analysis of each parameters of tissue slides against tissue type**

No.	Parameter	p. value			
		Observer I		Observer II	
		Formalin fixed slides	Microwave fixed slides	Formalin fixed slides	Microwave fixed slides
1	Cellular outline	0.126	0.034	0.180	0.008
2	Cytoplasmic detail	0.526	0.242	0.001	0.002
3	Nuclear detail	0.275	0.098	0.033	<0.0005
4	Erythrocyte integrity	0.265	0.283	0.525	0.008
5	Lymphocyte appearance	0.168	0.020	0.165	0.001
6	Overall morphology	0.074	0.245	0.324	<0.0005
7	Over all staining and	0.300	0.040	0.004	0.055
8	Slide grade	0.605	0.110	0.670	0.152

#### **5.4. Comparison of the two fixation methods based on slide quality**

From 120 tissue slides prepared by conventional fixation method and as examined by observer I, 13 (10.8%) were graded as poor, 39 (32.5%) fair, 56 (46.7 %) good, 10 (8.3%) excellent and 2 (1.7%) slides had outstanding quality. Another 120 tissue slides prepared by microwave irradiation fixation method and examined by the same observer were graded as 66 (55%) poor, 34 (28.3%) fair, 15 (12.5%) good, 3 (2.5%) excellent and 2 (1.7%) outstanding. This indicates that according to observer I, 89.2 % and 45 % of slides prepared by formalin and microwave fixation methods respectively, fulfill the minimum quality which enables tissue diagnosis.(table 5).

**Table 5: Scales of slides based on the seven parameters as examined by observer I**

		Scale of the slides examined by observer I																			
		Conventional fixation method										Microwave fixation method									
		Poor		Fair		Good		Excellent		Outstanding		Poor		Fair		good		Excellent		Outstanding	
		Fr eq	%	Fr eq	%	fre q	%	Fr eq	%	Fr eq	%	fr eq	%	fr eq	%	Fr eq	%	fr eq	%	Fr eq	%
Parameter	Cellular outline	15	12.5	34	28.3	46	38.5	23	19.2	2	1.7	57	47.5	26	21.5	23	19.2	12	10	2	1.7
	Cytoplasmic detail	10	8.3	34	28.3	49	40.8	25	20.8	2	1.7	58	48.3	38	31.7	15	12.5	7	5.8	2	1.7
	Nuclear detail	9	7.5	19	15.6	41	34.2	47	39.2	4	3.3	36	30	45	37.5	24	20	13	10.8	2	1.7
	Erythrocyte integrity	6	5	24	20	43	35.8	42	35	5	4.2	69	57.5	30	25	12	10	7	5.8	2	1.7
	Lymphocyte appearance	3	2.5	22	18.3	52	43.3	41	34.2	2	1.7	27	22.5	50	41.7	35	29.2	6	5	2	1.7
	Overall morphology	5	4.2	26	21.7	53	44.2	34	28.3	2	1.7	41	34.2	45	37.5	28	23.3	4	3.3	2	1.7
	Overall staining	5	4.2	25	20.8	54	25	34	28.3	2	1.7	45	37.5	39	32.5	30	25	4	3.3	2	1.7
	Slide grade	13	10.8	39	32.5	56	46.7	10	8.3	2	1.7	66	55	34	28.3	15	12.5	3	2.5	2	1.7

Each of the slides given for observer I was also seen by observer II. From 120 tissue slides prepared by conventional fixation method, observer II graded as 3 (2.5%) poor, 33 (27.5%) fair, 44 (36.7%) good 38 (31.7%) excellent and 2 (1.7%) outstanding. Whereas another 120 slides prepared by microwave method were graded by this observer as 10 (8.3%) poor, 54 (45%) fair, 42 (35%) good, 13 (10.8%) excellent and 1 (0.8 %) outstanding. This indicates that according to observer II, 97.5 % and 91.2 % of slides prepared by formalin and microwave fixation methods respectively, fulfill the minimum quality which enables tissue diagnosis (table 6).

**Table 6: Scales of slides based on the seven parameters as examined by observer II**

		Scale of the slides examined by observer II																			
		Conventional fixation method										Microwave fixation method									
		Poor		Fair		Good		Excellent		Outstanding		Poor		Fair		Good		Excellent		Outstanding	
		Fr eq	%	Fr eq	%	fr eq	%	Fr eq	%	Fr eq	%	fr eq	%	fr eq	%	fr eq	%	fr eq	%	fr eq	%
Parameter	Cellular outline	1	0.8	14	11.7	50	41.7	44	36.7	11	9.2	8	6.7	31	25.8	56	46.7	22	18.3	6	5
	Cytoplasmic detail	0	0	23	19.2	41	34.2	48	40	8	6.7	7	5.8	37	30.8	56	46.7	17	14.2	3	2.5
	Nuclear detail	0	0	15	12.5	48	40	45	37.5	12	10	6	5	36	30	50	41.7	22	18.3	6	5
	Erythrocyte integrity	1	0.8	15	12.5	52	43.3	43	35.8	9	7.5	7	5.8	33	27.5	60	50	16	13.3	4	3.3
	Lymphocyte appearance	1	0.8	10	8.3	55	45.8	48	40	6	5	2	1.7	31	25.8	64	53.3	19	15.8	4	3.3
	Overall morphology	1	0.8	13	10.8	48	40	48	40	10	8.3	7	5.8	31	25.8	57	47.5	21	17.5	4	3.3
	Over all staining	4	3.3	17	14.2	45	37.5	44	36.7	10	8.3	7	5.8	31	25.8	59	49.2	19	15.8	4	3.3
	Slide grade	3	2.5	33	27.5	44	36.7	38	31.7	2	1.7	10	8.3	54	45	42	35	13	10.8	1	0.8

### 5.5. Agreement between the two fixation methods based on slide quality

The agreement between the two fixation methods on the scales of the slides was positive except for **cytoplasmic detail, nuclear detail and over all morphology** with the p value ranging from 0.001 to 0.931 as examined by observer I. The agreement of the two methods on the slide grade was 0.037 with p-value of 0.396. This agreement was considered as significant (table 7).

As examined by observer II, the agreement between the two fixation methods on the scales of the slides was also positive except for only **nuclear detail** with the p value ranging from 0.024 to 0.937. The agreement of the two methods on the slide grade was 0.121 with p-value of 0.024. This agreement was considered as significant (table 8).

**Table 7: kappa and p-values of two fixation methods on scales of each parameter within specimen type as examined by observer I**

parameter		Tissue type																	Total	
		Uterus (cervix)	Thyroid	Brain	Colon	Breast	Kidney	Stomach	Spleen	Liver	Abdominal wall	Peritoneal mass	Adrenal tissue	Esophageal tissue	Pericardial tissue	Vas deference	Thoracic mass	Ovarian cyst (ovary)		Others
cellular outline	Kappa	-	-	.20	.465	.242	-	-	-	.00	.000	-	.00	.00	.00	.00	.000	-	.0	.05
	-v	.021	.440	.0			.241	.231	.333	.00		-	.00	.00	.00	.00	.000	.143	.16	.07
	p-v	.770	.075	.505	.000	.214	.063	.230	.157	-	--	-	-	-	-	-	-	.248	.846	.199
cytoplasmic detail	Kappa	-	.50	.09	.025	-	.06	-143	-	-	.000	-	.00	.00	.00	.000	.000	.00	-	-
	-v	.115	.0	.01		.029	.03		.1000	.231		-	.00	.00	.00	.000	.000	.082	.0	.064
	p-v	.100	.070	.728	.813	.863	.439	.248	.157	.046	-	-	-	-	-	-	-	-	.332	.107
nuclear detail	Kappa	-	-	.20	.016	-	.09	-	.33	.00	.000	.00	.00	.00	-	.000	.00	.1	-	
	-v	.056	.200	.0		.282	.01	.333	.33	.00		.00	.00	.00	.000	.000	.050	.012		
	p-v	.417	.418	.248	.885	.054	.273	.248	.157	-	-	-	-	-	-	-	1.000	.060	.766	
erythrocyte integrity	Kappa	.07	.35	.33	-	-	.06	-	-	.00	.000	.00	.00	.00	-	.000	-	-	.01	
	-v	.070	.357	.333	.068	.111	.03	.333	.333	.00		.00	.00	.00	.333		.007	.059	.017	
	p-v	.167	.036	.046	.419	.408	.439	.206	.157	-	-	-	-	-	.157	-	.505	.299	.599	
lymphocyte appearance	Kappa	-	-	1.0	.116	.104	.25	-	.000	.00	.000	-	.00	.00	.00	.000	-	-	.02	
	-v	.028	.333	.00		.104	.250	.333		.00		-	.00	.00	.00	.000	.143	.158	.021	
	p-v	.696	.273	.046	.864	.578	.025	.157	-	-	-	-	-	-	-	-	.248	.007	.031	
overall morphology	Kappa	-	.000	.000	.105	.081	.25	.157	-	.00	.000	.00	.00	.00	.00	.000	-	-	-	
	-v	.107					.250	.157		.00		.00	.00	.00	.00	.000	.333	.164	.074	
	p-v	.113	1.000	1.000	.765	.380	.625	.206	-	-	-	-	-	-	-	-	.046	.001	.072	
over all staining	Kappa	-	.14	-	.114	.130	.06	-	-	.00	.000	.00	.00	.00	.00	.000	.07	.1	.00	
	-v	.086	.03	.200		.130	.03	.333		.00		.00	.00	.00	.00	.000	.077	.158	.003	
	p-v	.190	.327	.248	.252	.332	.439	.206	-	-	-	-	-	-	-	-	.505	.070	.931	
slide grade	Kappa	.10	-	.000	.116	-	.08	-	1.0	.1	.000	-	.00	.00	.00	.000	.00	.0	.03	
	-v	.104	.250		.116	.111	.081	.600	.00	.143		-	.00	.00	.00	.000	.000	.048	.037	
	p-v	.108	.289	-	.288	.525	.273	.070	.157	.248	-	-	-	-	-	-	1.000	.542	.369	

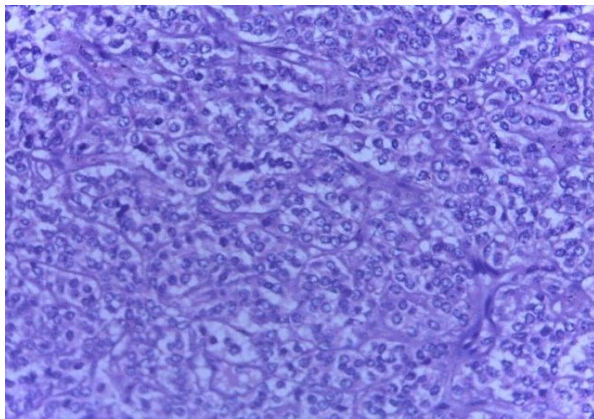
**Table 8: kappa and p-values of two fixation methods on scales of each parameter within specimen type as examined by observer II**

		Tissue type																		
parameter		Uterus (cervix)	Thyroid	Brain	Colon	Breast	Kidney	Stomach	Spleen	Liver	Abdominal wall	Peritoneal mass	Adrenal tissue	Esophageal tissue	Pericardial tissue	Vas deference	Thorcic mas	Ovarian cyst (ovary)	Others	Total
cellular outline	Kappa-v	.196	-.111	-.333	-.121	.333	.063	-.333	.000	-.333	.000	-.000	.000	.000	.000	-.333	.333	.077	-.071	.090
	p-v	.056	.604	.046	.394	.072	.439	.046	-.000	.102	-.000	-.000	-.000	-.000	-.000	-.000	.157	.689	.565	.082
cytoplasmic detail	Kappa-v	-.045	-.071	-.333	.189	.286	-.071	-.333	.000	.500	.333	.000	.000	.000	-.000	-.000	1.000	.000	-.025	.009
	p-v	.684	.574	.046	.126	.096	.540	.102	-.000	.248	.157	-.000	-.000	-.000	-.000	-.000	.157	-.000	.065	.861
nuclear detail	Kappa-v	-.094	.357	-.143	.103	-.229	.000	-.333	.000	-.333	.000	.000	.000	.000	.000	.000	1.000	-.231	.103	-.009
	p-v	.382	.036	.248	.363	.236	-.000	.102	-.000	.157	-.000	-.000	-.000	-.000	-.000	-.000	.154	.046	.492	.937
erythrocyte integrity	Kappa-v	.175	-.071	-.333	.064	.286	-.071	-.500	.000	.000	1.000	.000	.000	.000	.000	.000	1.000	-.143	.068	.093
	p-v	.053	.709	.043	.422	.205	.624	.248	-.000	1.000	.157	-.000	-.000	-.000	-.000	-.000	.157	.248	.557	.069
lymphocyte appearance	Kappa-v	.007	-.241	-.333	.111	-.163	-.125	-.500	-.000	.000	.007	.000	.000	.000	.000	-.000	1.000	.000	.333	.053
	p-v	.390	.137	.046	.414	.412	.121	.248	-.000	-.000	.390	-.000	-.000	-.000	-.000	-.000	.157	-.000	.029	.323
overall morphology	Kappa-v	-.013	-.071	-.333	.058	-.163	.000	-.455	.000	-.333	.000	.000	.000	.000	.000	-.000	1.000	-.231	.192	.012
	p-v	.894	.674	.046	.675	.336	-.000	.082	-.000	.102	-.000	-.000	-.000	-.000	-.000	-.000	.157	.046	.171	.810
overall staining	Kappa-v	-.136	.142	.143	.236	.283	.143	-.333	.000	-.200	.000	.000	.000	.000	.000	.000	1.000	.143	-.091	.046
	p-v	.139	.473	.248	.051	.153	.327	.102	-.000	.248	-.000	-.000	-.000	-.000	-.000	-.000	.157	.248	.557	.366
slide grade	Kappa-v	.082	.111	.077	-.099	.516	.000	-.333	-.000	.500	.000	.000	.000	.000	-.000	1.000	1.000	-.067	-.006	.121
	p-v	.450	.581	.689	.480	.020	1.000	.102	-.000	.248	-.000	-.000	-.000	-.000	-.000	.157	.157	.505	.942	.024

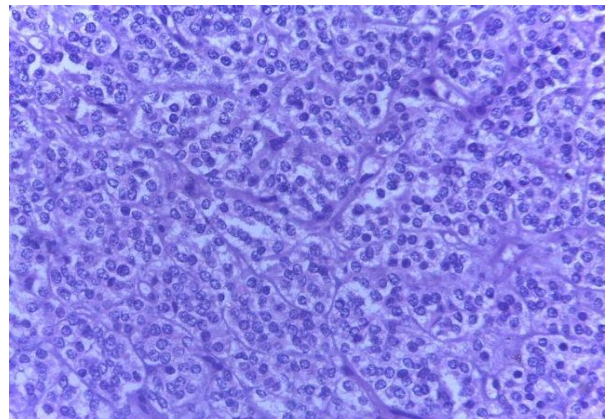
## 5.6. Association between scale of slide grade and method of fixation

The association of the scale of slide grade and the method of fixation were computed with chi-square test for each specimen type as examined by each observer independently. According to observer I 12 of 18 tissue types (68%) of slide grade was not associated with the type of fixation method. While 6 of 18 (33%) tissue types (**uterus (cervix), brain, colon, kidney, abdominal wall and esophageal tissue**) have slide grade which was significantly associated with method of fixation ( $p$ -value  $< 0.005$ ). On the other hand, the slide grade of 15 of 18 types of tissue (83%) was not associated with fixation method and only the slide grade of 3 tissue types (17%) (**Adrenal tissue, esophageal tissue and kidney**) have significant association with method type ( $p$ -value  $< 0.05$ ); as examined by observer II.

Microscopic photographs of a few selected slides of different tissue types were taken to show the comparability of the two fixation methods based on the quality of slides according to each seven parameters. The following figures are typical microscopic photographs of slides prepared by both conventional (formalin) and microwave fixation methods showing comparable slide qualities (figure 3-9).

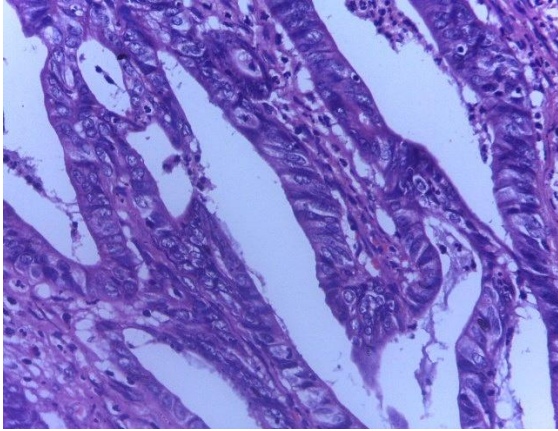


3a: Tissue fixed by formalin (H&E 400X)

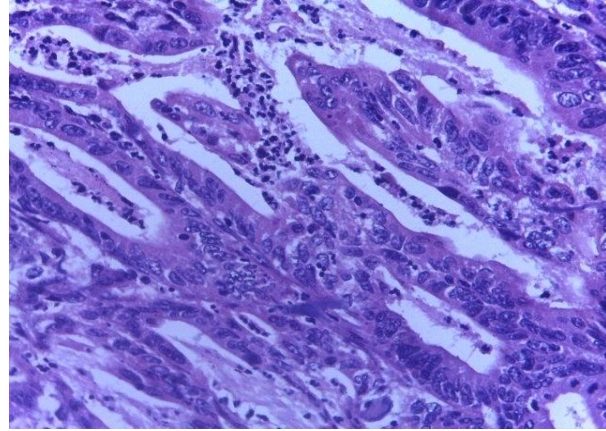


3b: Tissue fixed by microwave (H&E 400X)

**Figure 3: Cellular outlines of carcinoid tumor**

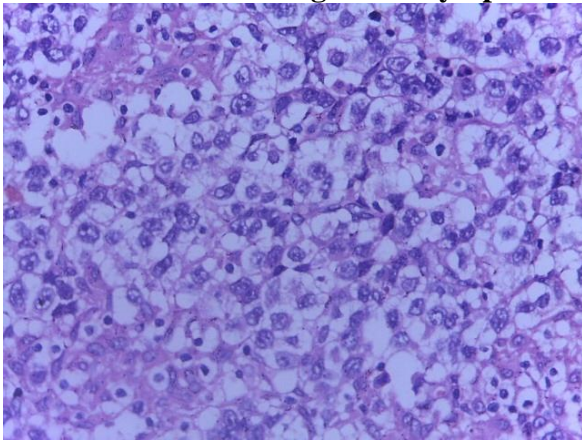


4a: Tissue fixed by formalin (H&E 400X)

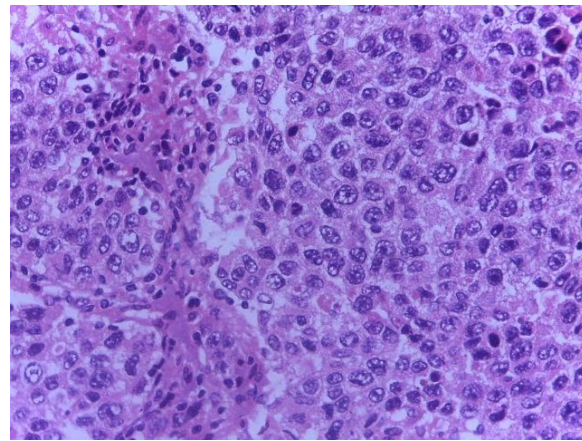


4b: Tissue fixed by microwave (H&E 400X)

**Figure 4: Cytoplasmic details of adeno-carcinoma**

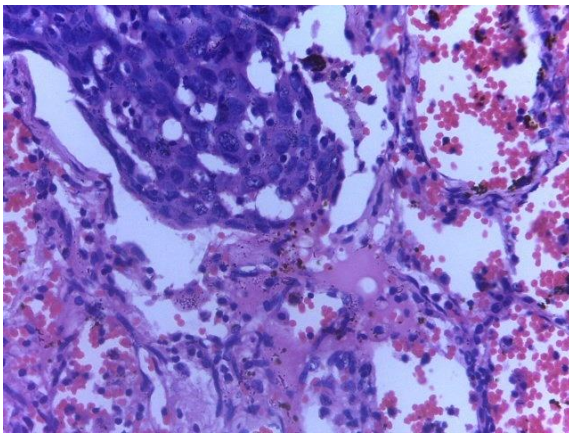


5a: Tissue fixed by formalin (H&E 400X)

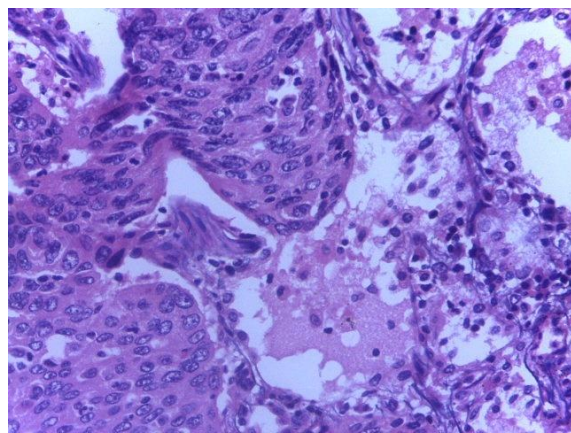


5b: Tissue fixed by microwave (H&E 400X)

**Figure 5: Nuclear details of leiomyoma**

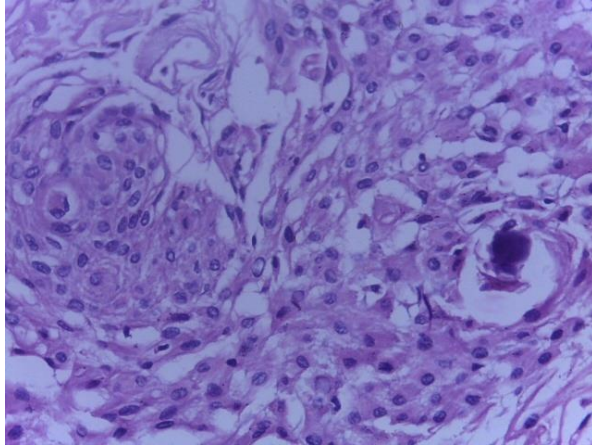


6a: Tissue fixed by formalin (H&E 400X)

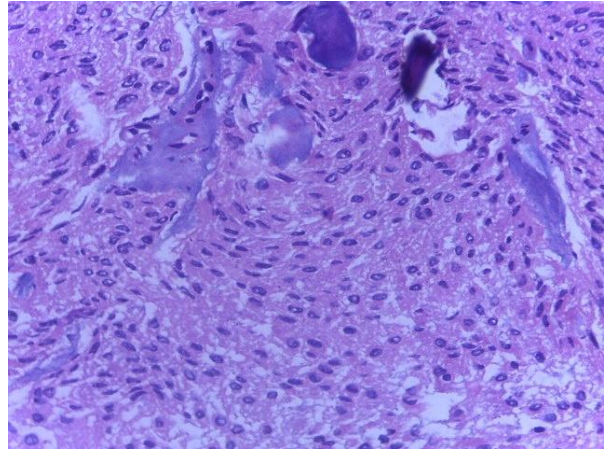


6b: Tissue fixed by microwave (H&E 400X)

**Figure 6: Erythrocyte integrity of lung carcinoma**

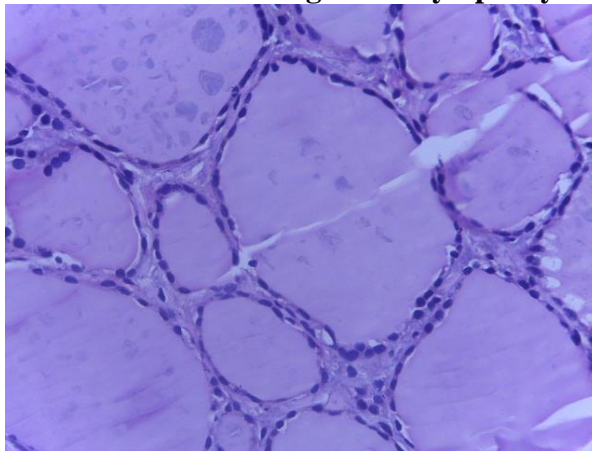


7a: Tissue fixed by formalin (H&E 400X)

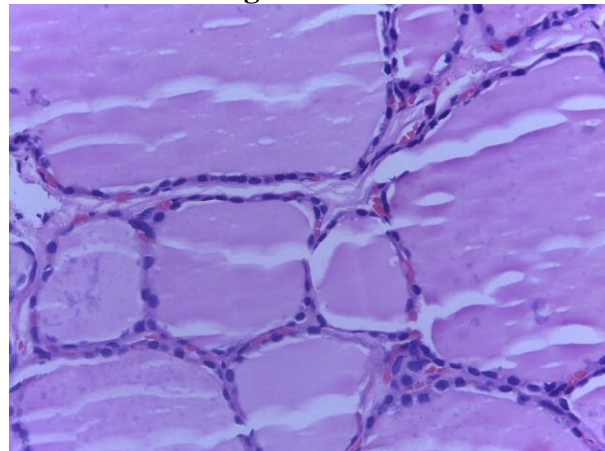


7b: Tissue fixed by microwave (H&E 400X)

**Figure 7: Lymphocyte appearances of meningeoma**

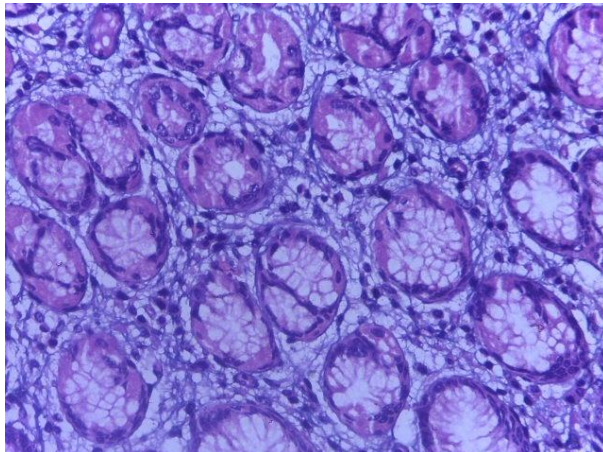


8a: Tissue fixed by formalin (H&E 400X)

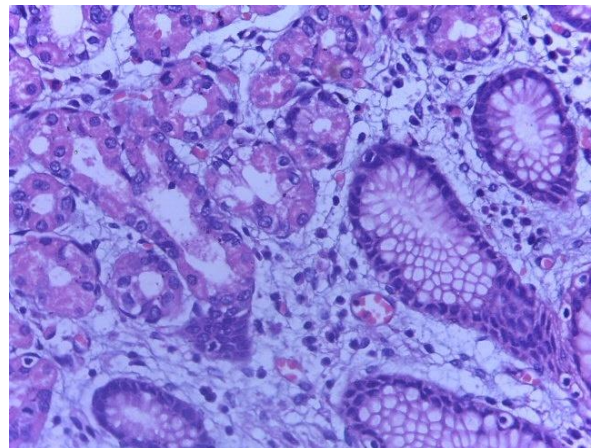


8b: Tissue fixed by microwave (H&E 400X)

**Figure 8: Overall morphologies of thyroid tissue**



9a: Tissue fixed by formalin (H&E 400X)



9b: Tissue fixed by microwave (H&E 400X)

**Figure 9: Overall staining of colon tissue**

## 6. Discussion

This research was aimed to investigate the possibility of adopting the rapid microwave fixation method in the routine histopathology diagnosis procedure without compromising morphological quality. Since it is the first study by its type in Ethiopia, it can be used as a base line for other studies. Even there are only few studies done worldwide especially in microwave fixation method.

In the present study tissues were received in normal saline as a transport medium. This was because one of the aims of this study was to decrease the potential exposure of professionals from hazardous effect of formalin chemical. This was similar with the researches done by Login GR [25], Leong ASY *et al* [26] and Kayser K *et al* [27]. In the present study four slices were taken from each specimen received which helps to decrease the probability of missing the appropriate tissue sample from the specimen. But in the study of Tripathi M *et al* [29] tissues were received in 10% formalin as a transport medium and two slices were taken from each gross specimen received.

In the present study domestic microwave was used for tissue fixation which does not display the exact temperature at which the fixation by microwave irradiation is done. It was used for both the pilot and the main part of the study with the adjustment of approximate fixative temperature using thermometer. Like this study Tripathi M *et al* [29] used the same microwave for the pilot study; but unlike the present study they used an enzyme retriever microwave, equipped with temperature probe accurate to  $\pm 1^{\circ}\text{C}$ . Due to this difference the duration of time for microwave fixation lasts about 11 minutes in the present study but only 5 minutes in the other one. The time duration for formalin fixation was consistent with each other which last about 24 hours. The type of microwaves used for tissue fixation in the studies done by Leong ASY *et al* [26], Kayser K *et al* [27] and Chaudhary K *et al* [28] were similar with that of the present study.

In the present study microwave irradiation has been used only for fixation of tissues which was similar with all studies indicated above [25-29]. Phosphate buffered saline was used as a medium for microwave irradiation in both the present study and the study done by Tripathi M *et al* [29]. But Tris buffer was used in the study of Kayser K *et al* [27] and normal saline was in the study of Chaudhary K *et al* [28] in microwave fixation as a media.

The tissue sections after microwave fixation were stained darker than fixed by formalin in all studies [25-29]. This may be because: in the process of formalin fixation the charge of protein slightly changes [5, 6]. This may have its own effect on the dye uptake of the tissue. But this is not the case of microwave fixation: nothing changes the charge of the tissue fixed by microwave irradiation [7-11] so that the tissue took up the stain with its maximum potential resulting darker stain.

All of the studies [25-29] including the present study showed that the time for microwave fixation was significantly decreased when compared with formalin fixation. This is because the high frequency of microwave irradiation facilitated the tissue protein coagulation. While in the formalin fixation the protein cross-linkage was done spontaneously taking its own time [5-11].

In the present study, the kappa statistics for inter observer agreement was within the range of slight agreement. This may be because less experienced pathologists (final year pathology residents) examined the slides. This can internally result in inter observer variability on rating slide quality compared with experienced pathologists [33]. To avoid the effect of the inter observer personal difference and nature of the tissue on the comparative result of the two fixation methods, statistics for each observer and for each specimen type was computed independently.

In the present study according to observer I about 89 % of the tissue slides prepared by formalin fixation were satisfactory and only 45% of microwave fixed slides got fair and above. This was comparable with the study of Chaudhary K *et al* [28] in which 80% cases of formalin fixation gave satisfactory result, while only 30% cases of microwave irradiation tissue fixation were satisfactory. But according to observer II, about 97% and 91% of the slides which were fixed by formalin and microwave respectively were satisfactory and above. This internally was in agreement with the study of Login GR [25] which showed that microwave fixation was comparable and superior to formalin fixation.

In the present study both of the observers agreed on the positive agreement of the two fixative methods on the scale of the slides of **cellular outline, erythrocyte integrity, lymphocyte appearance, over all staining and slide grade**. This comes into consistent with the study of Tripathi M *et al* [29]. On the other hand the two methods disagree (negative kappa value) on the scale of **nuclear detail** of the slides in which both observers agree on it and consistent with the

study of Tripathi M *et al*. This may be because of some loss of nuclear details and heat artifacts due to high fixative temperature in microwave irradiation [7-11, 34].

The study of Tripathi M *et al* [29] showed that the overall morphology of microwave fixed slides was slightly inferior to that of conventionally formalin fixed tissues. This is in agreement with observer I of the present study; but for observer II there was no significant difference between the two methods on overall morphology. To the contrary of these, the study of Kayser K *et al* [27] gave that histo-morphological sections fixed with tris buffer in a domestic microwave oven gave best morphological results showing more contrast in chromatin distribution of nuclei in comparison to conventional formalin-fixed specimens. This may be because Tris buffer decrease the effect of high fixative temperature in microwave irradiation on some loss of nuclear details.

## **7. Strength and limitation of the study**

The total number of samples (120) was large enough when compared to the minimum sample size for method comparative studies which was 40[31]. Specimen collection without formalin and doing microwave fixation within 30 to 60 minutes was also the positive aspect of this work. But specimen transportation with normal saline from out of TASH (farther places) cannot be realized within this limited time.

Since the scale of slide quality had an association with the nature of tissue, we were enforced to compare the two fixation methods for each type of the specimen. But the number of samples for some tissue types was not representative to come to conclusion. Microwave fixation was done by an available domestic microwave which would be better if it could have been done with standardized laboratory microwave. In addition to this the slide observers were examining the slides as an additional work besides their routine pathology diagnosis activity. This resulted in taking long time to complete the work.

## **8. Conclusion and recommendation**

This study demonstrates in microwave irradiation nuclear detail was poor due to heat artifact. But under controlled conditions it can reduce fixation time to about 11 minutes without significant loss of specimen quality. This can allow tissue processing on the same-day of surgical operation. Especially it is essential for the diagnosis of specimens when urgent reporting is required. It can also decrease the use of formalin which is potentially hazardous and irritating to the health professionals.

To incorporate the new fixation method in to the routine histopathology procedure, further assessment need to be done taking the corrective measure of the above limitations.

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## 10. Annexes

### 10.1. Annex I: Evaluation data sheet

Nature of the specimen: \_\_\_\_\_

Sample No \_\_\_\_\_ pathologist cod \_\_\_\_\_

**Scale representation:** 1=Poor, 2=Fair, 3=Good, 4=Excellent, 5=Outstanding

The following table shows the scales of the parameter used to evaluate the slides of the tissue fixed with microwave and conventional method.

Dear pathologist: you are kindly asked to put your evaluation as **X mark** under the scale of each parameter

		Scale of the parameter				
		1	2	3	4	5
Evaluation Parameters	Cellular outline,					
	Cytoplasmic detail,					
	Nuclear detail,					
	Erythrocyte integrity,					
	Lymphocyte appearance,.					
	Overall morphology					
	Overall staining					
*Grade of the slide						

\*Grade of the slide will be the sum total of the scale of each parameter rating 7 to 13 =Poor, 14 to 20=Fair, 21 to 27=Good, 28 to 34=Excellent, and above 34=Outstanding.

**Result/Dx** \_\_\_\_\_

## 10.2. Annex II: Tissue processing and staining procedures

### 1. Procedures of conventional automated tissue processing method

No.	Reagent	Time duration	Actions to be done
1	Formalin I	60 min	Further fixation of the tissue
2	Formalin II	60 min	
3	70% ethanol	60 min	Dehydration
4	85% ethanol	90 min	
5	96% ethanol	90 min	
6	Absolute ethanol I	90 min	
7	Absolute ethanol II	90 min	
8	Absolute ethanol III	90 min	
9	Xylen I	120 min	Clearing
10	Xylen II	90 min	
11	Molten paraffin wax I	120 min	Impregnation/infiltration
12	Molten paraffin wax II	120 min	

### 2. Procedures of automated hematoxylin and eosin tissue staining method

No	Reagent	Time Duration in minutes	Activities to be done
1	Xylen I	10	Clearing
2	Xylen II	10	
3	Absolute alcohol	5	Hydration of the tissue
4	96 % alcohol	5	
5	70% alcohol	5	
6	Tap water	5	
7	Mayers haematoxylin	10	Primary stain (basic stain)
8	Tap water	10	Bluing
9	Eosin Y	2	Counter stain (acidic stain)
10	Tap water	1	Washing
11	70% alcohol	2	Dehydration of the tissue
12	96 % alcohol	2	
13	Absolute alcohol	2	
14	Xylen I	2	Clearing of the tissue
15	Xylen II	2	