A rapid manual processing technique for resource-limited small laboratories

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ABSTRACT

Background: Surgical pathology is an integral part of diagnosis and management planning in patient care. In the absence of widespread automation, many small laboratories are unable to provide this service due to lack of time. Currently, the shortest processing schedule for formalin-fixed paraffin-embedded tissues is 16 working hours; most small laboratories cannot complete the rapid schedules within the average 8–9 working hours. Thus, the availability of an 8–9 h processing schedule that provide satisfactory results can help many small laboratories in routinely providing surgical pathology services. Objectives: To evaluate the effectiveness of a new rapid processing schedule and compare it with two existing rapid processing schedules. Materials and Methods: This animal study tested a new rapid processing schedule suggested by authors with overnight preprocessing in 60% isopropyl alcohol followed by an 8 h processing schedule. This was tested and compared with the rapid processing schedules described by Godkar’s (11 h) and Bancroft’s (2 working days). A routinely used automatic tissue processor long cycle (17 h) was the control. Each schedule was used on 20 tongue specimens. The prepared slides were evaluated for surface area and linear tissue shrinkage, ease of sectioning, quality of hematoxylin and eosin staining, histological appearance and artifacts. Results: No statistical differences were found between schedules. Overall total average performance ranking placed Bancroft’s schedule as the best with only 27.9% of the sections processed and stained showing any shortcomings, followed closely by the test schedule suggested by authors (28.7%), Godkar’s (31.8%) and the automatic processor schedule/control (33.3%). Conclusion: The test results indicated that the schedule devised by authors is an effective rapid processing cycle that produces diagnostic quality histological results when compared with other conventional processing schedules for small tissue blocks (average 6 mm × 8 mm).

Key words: Artifacts, medical laboratory technique, rapid tissue processing, tissue processing shrinkage

INTRODUCTION

Rapid diagnosis that enables timely intervention and management planning is largely dependent on an efficient and easily accessible surgical pathology service. Surgical pathology is based on microscopic examination of formalin-fixed paraffin-embedded (FFPE) tissue sections. It is a technology and time intensive service which is currently not provided by many small laboratories. The method depends on preservation of tissues as close to the living state as possible by formalin fixation, followed by a processing cycle aimed...
at replacing the tissue water (dehydration) with a paraffin miscible agent (clearing) to facilitate paraffin impregnation. As a component of surgical pathology services, there are obvious time constraints on the process.

The rapid processing schedules currently available require a minimum of 16–48 working hours for completion. Processing times that extend beyond the working hours of a laboratory require automation for uninterrupted processing to continue. The high cost of automation and unavailability of uninterrupted power supply in the developing countries makes automation unsuitable for small laboratories. This poses a challenge to these laboratories which have to complete tissue processing during working hours limited to 8–9 h a day. An extensive search of the currently available rapid processing techniques shows that there are no processing schedules that can be completed within 8–9 working hours.

This study was therefore designed to assess the quality of the processed tissues and stained sections by a new rapid manual processing technique devised by the authors for 8 working hours with two of the shortest processing schedules previously described.

**MATERIALS AND METHODS**

**Study design and sample source**

Comparison of processing schedules required fresh samples of equal size and type to avoid variations and technical confounders. This necessitated the design as an animal study. Ethical clearance was obtained from the concerned committee of the institute.

Tissue specimens were sourced from the animals housed in the medical college animal house. Twenty-two Wistar rats nearing the end of their natural lifespan (2–3 years) were selected. The animals weighed 400–600 g and were 2–2½ years in age. They were euthanized humanely by the administration of chloroform inhalant. Tongue was chosen as a soft tissue organ that can provide uniformly firm tissue. The whole mandible with the tongue was dissected with the tongue excised at the base.

**Grossing, trimming and fixation**

Each tongue (cylindrical in shape) was cut into an average of 4 bits [8 mm × 8 mm × 6 mm] as shown in Figure 1 with sections randomly assigned to the depicted schedules. The natural variation in the size of the animal’s tongue meant that some specimens were too small to use. Thus, 22 tongues were included to harvest a total of 80 tissue samples of the desired size. The specimens were fixed in freshly prepared 10% neutral buffered formalin for 24 h.

**Figure 1: The schematic representation of grossing of rat tongue**

**Preprocessing preparation and measurements**

After fixation, all specimens were washed with water and pierced through with a thin Begg’s wire. Two different sets of measurements were performed for each sample. First, linear length in millimeters was measured as the length of the tissue specimen from the point of entry to the point of exit of the Begg’s wire. Second, a two dimension measurement of surface area in square millimeters was done. This was accomplished by morphometric analysis of the specimen images captured with a 3 chip CCD camera on Trinocular stereo microscope (Olympus SZX7, Japan) calibrated for millimeter under × 10 magnification [Figures 2 and 3].

**Tissue processing**

Tissue samples were processed using Godkar’s (schedule I) and Bancroft’s (schedule II) rapid processing techniques, the test schedule (schedule III) and a routine automatic processor schedule (schedule IV) used as a control [Table 1]. The chemicals were chosen based on a combination of performance, availability, safety and cost. Thus, isopropyl alcohol, which is easily available to purchase and store, was used for dehydration. Xylene, the main clearing agent has been highly recommended for its penetrative power and ease of removal during impregnation. It is easy to procure, has a good shelf life (not as volatile as chloroform) and possesses relatively low toxicity with short-term exposure limit of 150 ppm. Acetone is a rapid acting agent that is miscible with both xylene and alcohol. With xylene, acetone achieves complete clearing in a short time without an increase in tissue hardness. It was used as an additional clearing agent in the processing schedule III.

Paraffin tissue blocks were prepared and sectioned using soft tissue microtome. Quality of tissue blocks by means of adequate ribbon formation (without tears) as well as folds obtained was observed and recorded during sectioning and mounting [Figures 4-6]. The mounted sections were subsequently stained using hematoxylin and eosin stains.
All sections were examined for diagnostic adequacy. Poststaining (PS) surface area of the sections was measured using stereomicroscope [Figure 7]. The dimensional changes relating to shrinkage were calculated for changes in length and surface area measured between preprocessing (PrP) and postprocessing (PoP) and in surface area between PrP and PS. Linear shrinkage was not measured after staining because the two points through which the thin wire passed before processing could not be recognized on the stained slide.

Preservation of tissue architecture by identifying fragmentation, epithelial stripping and irregular voids were examined [Figures 8-10] and recorded after staining by a single observer since there was no expectation of inter-observer variability in this parameter. Remaining parameters were examined by two observers.

**Statistical analysis**

Quantitative data are expressed as mean ± standard deviation and number/percentages for discrete data. Shrinkage was calculated as the mean difference between PrP and PoP or PS.

![Figure 2: Measurement for linear shrinkage](image1)

![Figure 3: Measurement for linear shrinkage after processing](image2)

**Table 1: The processing schedules used and their details**

| Schedules                          | Steps         | Reagents          | Number of changes | Duration       |
|-----------------------------------|---------------|-------------------|-------------------|----------------|
| Schedule I - suggested by Godkar, [4] working time 11 h | Dehydration | 80% alcohol | 2 station | 1 h each |
|                                   | Clearing     | 90% alcohol | 1 station | 1 h |
|                                   | Impregnation | 100% alcohol | 3 station | 1 h each |
|                                   | Xylene       | 2 station | 1 h each |
|                                   | Paraffin     | 2 station | 1 h each |
| Schedule II - suggested by Bancroft, [2] 16 h working in 2 days with overnight time | Dehydration | 70% alcohol | 1 station | 1 h |
|                                   | Clearing     | 95% alcohol | 2 station | 1 h, 2 h |
|                                   | Impregnation | 100% alcohol | 3 station | 1½ h each |
|                                   | Xylene & Xylene | 1 station | Overnight 1 h, 1½ h |
|                                   | Paraffin     | 2 station | 1 h, 1½ h, 2 h |
| Schedule III - test schedule, 8 h following preprocessing overnight in 60% alcohol | Dehydration | 60% alcohol | 1 station | Overnight |
|                                   | Clearing     | 70% alcohol | 1 station | 1 h |
|                                   | Impregnation | 90% alcohol | 1 station | 1 h |
|                                   | 100% alcohol | 1 station | 1 h |
|                                   | Acetone + xylene | 1 station | 1 h |
|                                   | Xylene       | 2 station | 1 h each |
|                                   | Paraffin     | 2 station | 1 h each |
| Schedule IV - 17 h working schedule in automatic processor | Dehydration | 60% alcohol | 1 station | 1½ h |
|                                   | Clearing     | 70% alcohol | 1 station | 1½ h |
|                                   | Impregnation | 80% alcohol | 1 station | 1½ h |
|                                   | 90% alcohol | 1 station | 1½ h |
|                                   | 100% alcohol | 2 station | 1½ h each |
|                                   | Xylene       | 4 station | 1 h each |
|                                   | Paraffin     | 2 station | 2 h each |

*Overnight in this study has been from 18:00 to next morning 09:00 (total of 15 h). Alcohol: Isopropyl alcohol, Paraffin: Paraffin with ceresin (MP 57°C)
One-way ANOVA was used for multiple group comparisons followed by Tukey’s test for group-wise comparisons. Chi-square test was used for analyzing categorical data. Interobserver reliability was tested by Kappa measure of agreement. *P* ≤ 0.05 was considered statistically significant.

**RESULTS**

**Effect of processing schedules on shrinkage**

Tissues processed in all the schedules showed some amount of linear shrinkage [Table 2]. The mean percentage of the shrinkage ranged from 10.51% in the schedule II to 14.08% in schedule IV. The difference between the rapid processing techniques was insignificant.

PoP surface area shrinkage [Table 3] ranged from 21.94% to 29.36% and from 10.91% to 13.33% PS [Table 4], with schedule II showing the least and schedule IV the most shrinkage. Summary of the mean percentages [Table 5] calculated from overall shrinkage values demonstrated a range of 14.4–19% shrinkage of specimens by the Schedules and showed schedule II to have the least overall shrinkage followed by schedule III, schedule I and schedule IV.

**Effect of processing schedules on ease of sectioning and quality of sections**

None of the schedules produced inadequate ribbons or an inadequate consistency for cutting. The schedules showed slight variation in their performance in terms of section tears, folds and difficulty in section handling [Table 5]. The overall performance of the schedules also found them in a narrow range of performance with the best schedule being schedule III with 48.3% total score in terms of shortcomings, followed by schedule II at 50%, schedule I at 51.7% and schedule IV at 56.7%.

**Effect of processing on staining**

Sections from all schedules were of acceptable diagnostic quality. However, some degree of cellular shrinkage,
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Differences in staining, loss of detail and artifacts was seen in sections processed by all schedules [Table 6]. The overall range [Table 7] of performance was narrow and no significant differences were found between the resulting stained sections.

Schedule II performed the best with only 19.4% of the stained sections having any shortcomings, followed by schedule III with 20%, schedule I with 25.6% and schedule IV with 24.4%.

The overall performance based on total average performance in each category showed Bancroft’s schedule (II) as the best with only 27.9% of the sections processed and stained showing any shortcomings, followed closely by the test schedule suggested by authors’ (28.7%), Godkar’s schedule (31.8%) and the automatic processor schedule/control (33.3%).

DISCUSSION

The practice of anatomical pathology and, therefore, surgical pathology is widely dependent on the study of FFPE tissue sections. Manual processing of tissues in small resource-limited laboratories can be challenging. It requires schedules that can be completed within the working hours of the laboratory without compromising on the quality of the stained sections. A number of rapid processing protocols for FFPE have been described in the literature. While widely accepted by pathologists around the world, even the shortest of these methods require 11 working hours. This study compared a new schedule (III) that required 8–9 working hours with two standard rapid processing schedules (I and II) and a routinely used automatic processor long cycle protocol (IV) as control. The tissues processed were screened for the adequacy of sections for diagnosis and examined for degree of shrinkage in length and surface area, ease of sectioning, preservation of architectural and cytological structure, staining properties and artifacts.

Godkar’s rapid processing schedule (schedule I) begins dehydration at the highest initial concentration of 80% alcohol among the tested schedules and maintains the tissue at 100% alcohol for 3 h. The tissues then go through a 2 h clearing and 3 h impregnation cycle. Bancroft’s schedule (II) begins with immersion in 70% alcohol. It goes on to have the longest dehydration cycle of 7 stations in alcohol (approximately 19½ h) and the longest stay in 100% alcohol the tissues then go through 3½ h clearing and 4½ h impregnation cycles. The third schedule introduced by the authors (schedule III) begins with overnight PrP dehydration in 60% alcohol followed by 3 h of progressive dehydration, ending with an hour in 100% alcohol the tissues then go through 3½ h clearing and 4½ h impregnation cycles. The schedule IV was the routinely used automatic processor schedule. It starts with 60% alcohol, and then progresses through a total 6 stations in alcohol for a total dehydration time of 9 h that includes 3 h in 100%. It is then followed by the longest clearing and impregnation cycles of 4 h each.

Shrinkage is one of the most significant tissue changes in FFPE tissues. All the schedules tested in this study caused some shrinkage in the tissues. The amount of linear
shrinkage in the test schedule III (12.95%) was intermediate between schedule I (13.68%) and schedule II (10.51%) with no significant differences between these schedules. A significant difference was found between schedule II and IV (14.08%); this difference did not relate to the performance of the test schedule. All schedules caused PoP and PS shrinkage in surface area. PoP shrinkage (21.17–29.36%) was larger than PS shrinkage (10.91–13.33%). The differences in the surface area shrinkage between the schedules were not statistically significant. The overall score based on linear and surface area shrinkage showed least shrinkage by schedule II (14.4%), followed by schedule III (17.8%), schedule I (17.8%) and schedule IV (19%). Paraffin solidification can cause postfixation shrinkage of up to 15% in FFPE tissues\cite{9,10} and could have been contributory to this level of shrinkage. The overall shrinkage volume PS was lesser than PoP shrinkage. Thus, some quantum of PoP shrinkage was reversed during staining; this may be due to removal of paraffin and rehydration of sections during the staining process.
The schedules did not show any significant differences in the ease of sectioning and quality of sections produced. None of the schedules showed inadequate ribbon formation and cutting consistency. Schedule III (5%) showed the least amount of tears and schedule II (20%) the highest amount while schedules I and IV (15% each) showed an intermediate amount of tears. The higher number of tears in schedule II tissues might have been caused by longer dehydration. Optimal dehydration is achieved by removal of interstitial water. Excessive dehydration causes a progressive elimination of bound water molecules from the tissue. The removal of these molecules, which are parts of structural configuration of the amino acids, makes the over dehydrated tissues more dry and brittle. These tissues are difficult to manage easily and are damaged during microtomy.\textsuperscript{[11]}

Both small (35%) and large folds (25%) were least in schedule I and most in schedule IV (55% and 35%), respectively. Other two schedules were intermediate between these. Folds may be caused by remaining traces of processing fluid in the specimen making the specimen hydrophilic, with rapid water absorption with the hydrophobic wax around causing tissue folds in water-bath.\textsuperscript{[12,13]} Thus, the short processing time in schedule I as against the long time in schedule IV can explain the distribution of section folds in different schedules.

Schedules III and II (15% each) showed the least number of sections that were difficult to handle while schedules IV and I (35%) did not perform, as well. This ranking is not in agreement with previously reported increasing tears with increasing dehydration.\textsuperscript{[11]}

Overall score in ease of sectioning and adequacy of sections showed the schedules to be marginally different. Schedule III was the best with only 48.3% of sections showing any defect. Schedule II was the second with 50%; schedule I was the third with 51.7% and schedule IV performed the worst at 56.7%. While the differences are not significant, they do demonstrate that the three rapid processing schedules provide similar quality of sections, and the excessively long cycle chosen for the automatic processor leads to poor quality.

The schedules showed nonsignificant differences in the microscopic quality of the stained sections. While the schedules performed variably under each criterion, the best overall performance was by schedule II with only a total mean of 19.4% of the stained sections showing any shortcoming, followed by schedules III (20%), IV (24.4%) and I (25.6%).

Cell shrinkage followed a pattern similar to overall shrinkage with the least quantity of shrinkage in schedule II (10%) and the most in schedule IV (25%). Schedules I (15%) and III

Table 6: H and E staining (with Chi-square or $\chi^2$ values and kappa statistics values for inter observer variability wherever applicable)

| Criteria                                      | Schedule I (%) | Schedule II (%) | Schedule III (%) | Schedule IV (%) | $P$   |
|-----------------------------------------------|----------------|-----------------|------------------|----------------|-------|
| Defects in the integrity of tissue            | 9 (45)         | 11 (55)         | 8 (40)           | 11 (55)        | $\chi^2=1.35$ $P=0.72$ |
| Shrunken size of cell                         | 3 (15)         | 2 (10)          | 4 (20)           | 5 (25)         | $\chi^2=1.73$ $P=0.63$ |
| Clumped cytoplasmic details                  | 3 (15)         | 0 (0)           | 0 (0)            | 2 (10)         | $\chi^2=5.76$ $P=0.124$ |
| Dark/unclear nucleus                          | 5 (25)         | 5 (25)          | 7 (35)           | 7 (35)         | $\chi^2=3.8$ $P=0.698$ |
| Dark/unclear nucleolus                        | 6 (30)         | 4 (20)          | 6 (30)           | 7 (35)         | $\chi^2=5.97$ $P=0.098$ |
| Distribution of chromatin details not clearly seen | 7 (35)     | 3 (15)          | 4 (20)           | 5 (25)         | $\chi^2=2.42$ $P=0.491$ |
| Over staining/under staining                  | 4 (20)         | 5 (25)          | 3 (15)           | 1 (5)          | $\chi^2=6.43$ $P=0.377$ |
| Other possible artifacts - smooth voids and micro-vibrations | 4 (20)      | 1 (5)           | 0 (0)            | 1 (5)          | $\chi^2=8.49$ $P=0.21$ |

Table 7: Overall performance of schedules

| Criteria            | Schedule I (%) | Schedule II (%) | Schedule III (%) | Schedule IV (%) |
|---------------------|----------------|-----------------|------------------|-----------------|
| Shrinkage           | 18.1           | 14.4            | 17.8             | 19.0            |
| Section             | 51.7           | 50.0            | 48.3             | 56.7            |
| Staining            | 25.6           | 19.4            | 20.0             | 24.4            |
| Overall             | 31.8           | 27.9            | 28.7             | 33.3            |

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(20%) occupied the second and third place, respectively. Cellular shrinkage has been linked to overall length of stay in Xylene.\(^\text{[8]}\) Schedules I, II and III had varying xylene exposure times of <4 h which did not correlate with the cell shrinkage values. Thus, it may be assumed that other unknown factors may be responsible for shrinkage.

Cytoplasmic details were the least affected by schedule II and III (0% each) followed by schedule IV (10%) and schedule I (15%). Over stained or dark nuclei were found to be equal and least common in schedule I and II (25% each), followed by an equal amount in schedule III and IV (35%). Dark or unclear nucleoli were least in schedule II (20%), followed by an equal number in schedule I and III (30%) and a maximum number in schedule IV (35%). Overall suboptimal staining (over/under staining) was least in schedule IV (5%), followed by schedules III (15%), I (20%) and II (25%). Over-stained or darkly stained sections are linked to conformational tissue changes that result from excessive dehydration. They lead to the formation of stable proteins, into which dye aggregates get locked.\(^\text{[14]}\) A literature search did not find any currently accepted processing linked causes for under-staining.

Lack of clarity of chromatin distribution was the least in schedule II (15%), followed by schedule III (20%), IV (25%) and I (35%). Increased xylene exposure has been linked to reduced nuclear chromatin details and unclear nucleoli.\(^\text{[8]}\) This study showed a poorer performance by schedule I with the shortest Xylene exposure than schedule IV with the longest exposure, thereby contradicting the previous findings.

Sections showed no artifacts in schedule III while schedules II and IV (5% each) and schedule I (20%) showed some artifacts.

The overall performance of the schedules based on all the parameters showed them to be closely comparable. All the sections by all the schedules were of sufficient diagnostic quality. Bancroft’s rapid processing schedule was the best with only 27.9% of the sections processed and stained showing any loss of quality. Bancroft’s rapid processing schedule was the best with only 27.9% of the sections processed and stained showing any loss of quality.

CONCLUSION

The rapid processing schedule suggested by this study is an effective method for FFPE tissues. It produces pre- and post-staining sections that are comparable with those produced by Bancroft and Godkar’s rapid processing schedules. The suggested rapid processing schedule can be effectively used by small laboratories in providing surgical pathology service.

The new technique can be beneficially used in the short cycle of the automatic processor to reduce both material cost and time.

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Conflicts of interest

There are no conflicts of interest.

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