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How much formalin is enough to fix tissues?

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Abstract A total of sixty samples from human breast, uterus, liver, skin and abdominal fat were fixed for 8; 24 and 48 hours at a room temperature of 20 to 22°C with neutral buffered formalin (NBF) with volume to tissue ratios of 1:1; 2:1; 5:1 and 10:1 and manually processed with isopropyl alcohol and mineral oil mixtures. All the slides prepared were evaluated as suitable for diagnostic purposes by nine pathologists from three different Russian histopathology institutions. The microtomy quality differences between the samples was not statistically significant for the different fixation volume ratios tested, but the differences between fixation periods and tissues types were, with 48 hours being the optimum fixation period, with skin and fat the most difficult to infiltrate. Neither the time and volume ratio combinations affected the pH of NBF or the immunostaining for vimentin in uterus or the histochemical periodic acid reaction or reticular demonstration fibers in liver. Fixing tissues with a ratio of NBF volume to tissue volume of 2:1 for 48 hours at 20-22°C was enough to assure a proper fixation and infiltration of the tested tissues and there is no objective reason to expect that other tissues will not behave similarly. It is suggested that in order to obtain good fixation and paraffin wax infiltration in around 10 hours, the fixation with NBF at 2:1 should be at 45°C with pressure and agitation.

Keywords: NBF fixation ratios; Fixation time and volume; Fixation temperature; Histotechnology safety

1. Introduction

The volume of formalin needed to fix a specimen is not generally agreed. Histotechnology text books and journals articles dealing with this issue recommend the formalin volume to tissue volume ratios ranging from 0.5:1 to 200:1 depending on the tissue involved and the proposed study.

Out of 100 references 4 used volume ratios of less than 10:1 and 96 recommended ratios of 10:1 or more. Thus 49 used ratios from 10:1 to less than 20:1; 39 with ratios from 15:1 to 20:1 and the remaining 8 recommended ratios of more than 20:1, without any scientific justification for their preferences.

Some preferring 20:1 consider that fixatives, in general, are poor buffers, something that does not apply to 10% Neutral Buffered Formalin (NBF), in spite of which, this argument still prevails. This large ratio is also favored because some argue that the fixing molecules can be depleted which again does not apply to formalin as its concentration is not a critical factor.

Other authors favor large fixative to tissue volume ratios arguing that the fixative can be diluted while fixing, something that is mostly applicable to fixatives that do not contain water, which is not the case with NBF.

The fact remains that the ratio of NBF to tissue mostly results from personal preferences without specific scientific evidence. The qualification of “ideal amount” is applied to ratios greater than 10:1; to 20:1 or from 20:1 to 50:1; along with others like “not to exceed” 15:1; or that 5:1 “is enough”. Such dispersion of favorably considered ratios shows how little is known about this important aspect of tissue fixation.

None of these arguments consider that NBF fixation is a three steps complex process happening simultaneously although at very different rates and dependent on time and temperature rather than on concentration or availability [1,2].

In 1997, Williams et al. [3] investigated the effects of fixation on immunohistochemistry (IHC) procedures and

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concluded that there were no differences in the results obtained after fixing human tonsils with NBF at fixative to specimen volume ratios between 1:1 and 20:1 and that some IHC results were also pH and fixation time independent.

In spite of the hazards posed by NBF is still the best option for the routine fixation of tissues but it has to be used safely, employing the appropriate precautions, in well ventilated areas and using the smallest amounts possible [2].

Determining the least amount of NBF needed to provide an adequate tissue fixation was the objective of an experiment reported here.

2. Materials and methods

The tissues used were uterus, breast, liver, abdominal skin and underlying pure fat in 1cm × 1 cm × 3 mm thick slices obtained from a normal white healthy 27 years old female who died as a result of a traffic accident and was kept at 4 to 6 °C until the autopsy was performed 36 hours after death.

Except for the abdominal fat and skin samples that had to be cut manually, all the other samples were cut using a "CutMate2 Forceps" with 3mm gaps. The thickness of each slice was checked with a "CheckLite" with an accuracy of ± 0.2 mm and all the slices were between 2.8 and 3.2 mm thick. Both instruments are manufactured by Milestone s.r.l. (Italy) and were loaned for this experiment.

Parallel samples to those used in the fixation experiment were dried to constant weight at 60°C. An analog laboratory balance with ± 0.01 g accuracy was used to determine fresh and dry weights. The water contents (WC) is the percent difference between fresh and dry weights.

The NBF volume to tissue volume ratios used were 1:1; 2:1; 5:1 and 10:1. The volume of every tissue slice was determined by water displacement in a glass cylinder and the NBF was added to reach the required volume proportion for each. All samples were fixed in individual containers and the specimens in the 1:1 ratio were wrapped in gauze to assure they would not dry out.

The samples were fixed for 8; 24 and 48 hours at room temperature that ranged from 20 to 22°C during the experiment. At the end of each fixation period the 20 specimens in the group were rinsed for 15 minutes in running tap water and transferred to 70% isopropyl alcohol for at least 2 days, then manually dehydrated, cleared and embedded in paraffin, using the isopropyl alcohol and mineral oil mixtures protocol described elsewhere [4].

With 4 different NBF volume to tissue volume ratios, and 3 different fixation periods, there were 12 samples for each of the 5 tissue types, a total of 60 samples.

The pH of the NBF stock solution was determined using a pH meter with glass electrode, with a pH range of 1 to 14 ± 0.05 pH unit accuracy. At the end of each fixation period the pH from the corresponding samples was determined. For the smaller NBF to tissue ratios (1:1 and 2:1) the amount of used NBF was too small to be determined individually, so the NBF from each those ratios was aggregated to determine a common "end of fixation" pH value.

A Leica RM2245 rotary microtome was used and each block was evaluated regarding its sectioning quality as Good (=3), Fair (=2), Bad (=1) or as "0" if no sectioning was possible. This quality score represents the infiltration quality obtained for the different tissues fixed under the experimental conditions.

Sections from 7 fat and 4 skin processing combinations could not be obtained reducing the total number of slides to 49 to be blind evaluated once by 9 pathologists from 3 Russian laboratories (5 from Taganrog; 3 from Rostov-on-Don and 1 from Krasnodar). The pathologists were unaware of the NBF to tissue ratio nor the fixation time corresponding to each slide stained with hematoxylin and eosin (H&E) and had only 2 options, either the section was suitable for diagnosis, or it was not.

All 12 uterus experimental combinations, from the 1:1 ratio fixed for 8 hours, to the 10:1 ratio fixed for 48 hours, were studied for Vimentin presence to evaluate the quality and intensity of the reaction using the V9 clone from DAKO. The protocol included heat induced antigen retrieval (HIER) at high pH in PT-Link module, with Flex EnVision as detection system and diaminobenzidine (DAB) as the chromogen. All slides were tested in a single run with a DAKO autostainer. Vimentin was selected because it was used in an earlier study of the effects of fixation on immunohistochemical (IHC) tests [3].

Two liver experimental combinations, the 1:1 ratio fixed for 8 hours, and the 10:1 ratio fixed for 48 hours, were used to determine glycogen preservation with the Periodic Acid Schiff (PAS) reaction and reticulum characteristics with the Gomori's ammoniacal silver method.

The experimental data were not normally distributed and had to be transformed in order to use parametric tests. The slope of the relation between "log X_i" and "log s^2" for the microtomy quality evaluation was "2" so each value was normalized using the "**log $(X_i + 1)$**" transformation. The slope for the formalin penetration coefficients from the References was "1" and the data were normalized using the "**$(X_i + 3/8)^{1/2}$**" transformation.

All the one way ANOVAs were calculated using the on line statistical programs of Vassar University (http://facility.vassar.edu/lowry/anova1u.html) with a significance level of $P \leq .05$, an α-type error, for the null hypothesis. The Tuckey HSD Test was used to determine the source of the differences of significant ANOVA results. The regression coefficients (R^2) were calculated with the Guneric expansion of Excel 2003 Microsoft program.

3. Results

The quality of the microtomy of the 60 samples provides an evaluation of the infiltration which depends on the fixation level and the tissue type, although this last factor is frequently ignored.

The results (Table 1) show that the average quality of the sections increases from the ratio 1:1 (average of 1.7) to 10:1 (average of 2.4) for all the fixation times (8; 24 and 48 hours) combined. These values, although reflecting microtomy characteristics determined by the infiltration quality, are not statistically significant [$F(3;57) = 1.28^{ns}$; $P > .29$] but caused by the aleatoric characteristic of the tissue slice in each volume and time combination.

In spite of that statistical result, the microtomy was really more difficult for 1:1 and 2:1 ratios especially after short fixation periods but variable by tissue type. Uterus at 1:1 during only 8 hours and breast at 1:1 during 24 hours showed good results. Liver and skin, at 2:1 for 48 hours had good results also. Good fat sections were obtained only at 10:1 for 48 hours, but it is safe to assume that fixation time was the deciding factor. In general the ratio allowing a good microtomy is 5:1 for the experimental conditions of fixation and manual processing at 20-22°C. If the 8 hours fixation time is excluded, the quality differences are reduced further but still remain not statistically significant [$F(3;37) = 2.56^{ns}$; $P > .07$].

The microtomy average quality increases from 1.7 after 8 hours of fixation, to 2.7 for 48 hours fixation. These differences are statistically significant [$(F2;57) = 3.93$ **; $P < .025$] because the results after 8 hours fixation are really inferior than after 48 hours, although the fixation during 24 hours is not statistically worse than during 48 hours.

Table 1
Microtomy results

NBF to tissue Ratio	Fixation Time (hours)	Manually processed tissue					Total	Average
		uterus	breast	fat	skin	liver		
1:1	8	3	2	0	0	2	7	1.4
	24	3	3	0	0	2	8	1.6
	48	3	3	1	2	2	11	2.2
Total 1:1	8 to 48	**9**	**8**	**1**	**2**	**6**	**26**	**1.7**
2:1	8	3	2	0	1	2	8	1.6
	24	3	3	0	0	2	8	1.6
	48	3	3	1	3	3	13	2.6
Total 2:1	8 to 48	**9**	**8**	**1**	**4**	**7**	**29**	**1.9**
5:1	8	3	2	0	3	3	11	2.2
	24	3	2	0	2	3	10	2.0
	48	3	3	2	3	3	14	2.8
Total 5:1	8 to 48	**9**	**7**	**2**	**8**	**9**	**35**	**2.3**
10:1	8	3	2	0	0	3	8	1.6
	24	3	3	2	2	3	13	2.6
	48	3	3	3	3	3	15	3.0
Total 10:1	8 to 48	**9**	**8**	**5**	**5**	**9**	**36**	**2.4**
Total	for 8 hours	12	8	0	4	10	**34**	**1.7**
	for 24 hours	12	11	2	4	10	**39**	**2.0**
	for 48 hours	12	12	7	11	11	**53**	**2.7**
	8 to 48 hours	**36**	**31**	**9**	**19**	**31**	**126**	**2.1**
Average		**3.0**	**2.6**	**0.8**	**1.6**	**2.6**	**2.1**	

Microtomy quality grading: Good → 3.
Fair → 2.
Bad → 1.
No section obtained → 0.

Table 2
Water contents (WC) of tissues used

Tissue	% WC this paper	% water contents		Ref.
		Range	Average	
fat	28	1 to 50	28	[5]
liver	74	68 to 72	71	
skin	50	58 to 74	67	
breast	70	11 to 27	18	[6]
		?	45	[7]
uterus	68	?	80	[8]

The third factor to consider is the type of tissue. The tissues most difficult to be properly infiltrated are known to be fat and skin and the results are consistent with this.

The general average for all the combinations of ratios and fixation periods, ranged from "3.0" (good infiltration and microtomy) for all 12 uterus samples, to "0.8" (bad infiltration and microtomy) for the pure fat samples. The differences in infiltration and microtomy quality between the tissues [$F(4;55) = 14.0$ ***; $P < .001$] are statistically significant. The results for uterus, breast and liver are not statistically different between them but are different to fat and skin, which are not different between them also.

These results coincide with the current knowledge concerning tissue processing although it is interesting to note that breast and uterus, sometimes considered as "difficult" tissues to infiltrate, showed the same results as liver.

It is also worth noting that the water contents of the 3 tissues with similar good results, is higher than for fat and skin (Table 2) this probably influencing the results given that all slices were of the same thickness (2.8 to 3.2 mm) and were equally fixed and processed simultaneously for each volume and time combination. Additionally, NBF penetration of fat is hindered by the insolubility of fat in water.

The NBF stock solution prepared for the experiment had an initial pH of 6.9 and showed the same final value for all the NBF to tissue ratios (1:1; 2:1; 5:1 and 10:1) after all the fixation periods (8, 24 and 48 hours).

Finally, the most important result refers to the usefulness of the routine H&E sections. The 441 evaluations of the 49 sections by 9 pathologists were unanimously considered useful for diagnosis regardless of the NBF volume and fixation time combination.

Similarly the 12 uterus slides investigated for Vimentin contents showed the same reaction intensity and antigen distribution, from the least amount of NBF and fixation time (1:1 during 8 hours) to the largest (10:1 during 48 hours).

4. Discussion

The results cover two different aspects: the diagnostic value of the sections obtained and the histotechnical aspects of how fixation impacts paraffin wax infiltration and microtomy, as well as histochemical and IHC reactions.

The 49 H&E sections were unanimously considered as diagnostically equivalent and useful by the 9 pathologists from 3 different institutions who evaluated them this being the fundamental result from the pathologist's point of view and its consequences for patient care.

The usefulness extends from fixative volumes of 1:1 fixed for 8 hours to 10:1 fixed for 48 hours (Fig. 1), and this in reality should not be a surprise to anybody if we consider that one of the most difficult tissues to fix and process is colon, and once it is open and pinned down flat over a board to be fixed overnight, the amount of fixative covering it is seldom more than twice its volume.

Vimentin reaction in uterus with fixation combinations of 1:1 for 8 hours to 10:1 for 48 hours were of equal diagnostic value and characteristics (Fig. 1) meaning that Vimentin antigen was unaffected by volume or fixation time, as described elsewhere for fixation volumes of 1:1 to 20:1 during fixation periods of 5 to 24 hours for Vimentin in tonsil [3].

These results in diagnostic value and Vimentin reaction for all the volume of NBF to tissue ratios and pH independence also confirm those reported previously [3].

The microtomy results (Table 1) varied quite widely, more dependent on fixation times and types of tissues and should require specific tests in every laboratory deciding to validate the present results. The tests should concentrate in developing a good fixation protocol to allow a perfect paraffin wax infiltration even for the most difficult tissues, in particular those with high fat content.

The WC values for pure fat, liver and skin slices used (Table 2) are comparable to those calculated for a 46 years old white male [5]. On the other hand the breast WC (70%) is higher than those for premenopausal (27%) and post-menopausal (11%) subjects [6], as could be expected for the 27 years old female used in this study. The median value of 45% WC from 400 young white females ages 15 to 30 years old [7], probably includes a value similar to ours, but that is unknown because the data does not show the range for the median value.

Uterus WC for cow, guinea pig, mouse, rabbit, rat and swine abound in the literature, but the only value found for human uterus [8] is 80% which is higher than the 68% we report probably because of the characteristics of the samples used.

The first step in formalin fixation is penetration which is expressed by the penetration coefficient (k) value, and prepares the tissue for the binding and cross-linking steps. These three steps are not sequential but concomitant and time

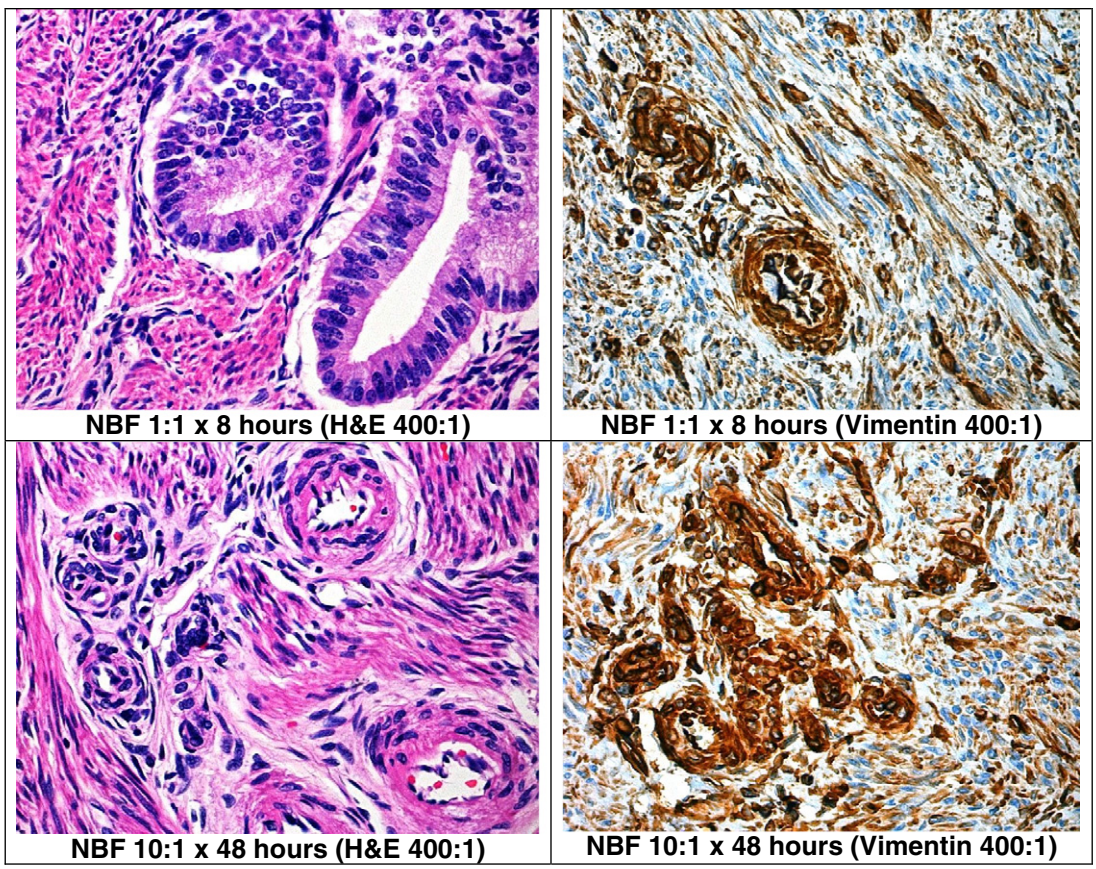

Fig. 1. Uterus fixed with NBF to tissue ratios of 1:1 for 8 hours and 10:1 for 48 hours stained with Hematoxylin & Eosin (H&E) and with Vimentin. Original magnification for all photomicrographs is 400:1.

Table 3
Penetration coefficient (k) values from different sources.

k	Subject and penetration evaluation	%F	Fixation	t°C	Ref.
0.78	Autopsy human liver, kidney, spleen and brain with visual evaluation based on color fading in not penetrated tissue.	?	?	?	[9]
0.36	7 mm Ø cylinders of guinea pig liver; after 15 min were placed in 0.02% chromic × 1 week to macerate not penetrated tissue. Processed, sectioned, and stained with Hematoxylin and acid fuchsine. Penetration measured in sections with an ocular micrometer.	4	15 min	?	[10]
6.7	Blood plasma coagulum cylinders from cockerels.	40	1hour to 5 hours	?	[11]
6.1	Phenylhydrazyne -hydrochloride added fresh to the plasma	9.5			
5.9	as penetration indicator. %F to tissue ratio = 30:1	5.9			
3.6	Gelatin/albumen or gelatin/nucleoprotein gel cylinders.	?	?	?	[12]
2.3	Chunks 1 to 2 cm thick of rabbit liver, muscle, kidney, heart and brain. After fixation were placed in 0.02% chromic during 14 hours to visually evaluate the not penetrated tissue; also frozen sections mounted in mineral oil. %F to tissue ratio = 25:1	4	10 min to several hours	21 to 25	[13]
4.1	No source for the "k" value, only a general statement that penetration is about "20 mm or more every 24 hours". Binding data from rat kidney sections.	?	?	?	[1]
0.55	10 autopsy whole human spleens. Fixed spleens were placed in 0.02% chromic during 14 days to macerate the not penetrated tissue; calculated from 3 sets of 100 vernier readings per spleen.	4	1 to 25 days	20	[14]
0.49	Penetration of 2.4 mm in 24 hours.				
0.82	Breast tissue penetrated 4 mm in 24 hours.	4	1 d	?	[15]
0.47	5 mm Ø × 5 mm high cylinders of pork liver; 0.5% w/v of Brilliant	4	15 min to 4 hours	22	[16]
1.1	Blue FCT added to the formaldehyde to mark penetration. After fixation the cylinders were paraffin embedded and sectioned at 5 μm. Penetration measured with an ocular micrometer.			42	

%F = percent formaldehyde ("pure" formaldehyde = 40%) Ø = cylinder diameter.
t°C = temperature in centigrade (°C).

dependent [1]. Consequently fixation during 8; 24 and 48 hours caused the significant differences found in the infiltration and microtomy quality of the tested tissues (Table 1).

There are different known values for the formaldehyde penetration coefficient "k" (Table 3) but their sources are not homogeneous. Four correspond to human tissues [9,14,15]; four to animal tissues [1,10,13,16] and another four were obtained while studying blood plasma coagulum cylinders from cockerels [11] or gelatin with albumen or gelatin with nucleoprotein gel cylinders [12].

Their values differ widely with an average of 0.66 ± 0.16 for "k" from human tissues; 1.81 ± 1.77 for animal tissues and 5.6 ± 1.35 for plasma or gelatin cylinders. The differences between these three averages are statistically significant [(F2;9) = 13.93*** $P < .001$] because the results from plasma and gelatin cylinders are really higher than those from animal and human tissues, although there are no differences between the latter two.

The standard deviation of "k" from animal tissues is 98% of the average indicating a large variability, but the standard deviation for "k" from human brain, breast, liver, kidney and spleen is 24% only, indicating a more stable value.

We chose the penetration coefficient of k = 0.66 as the one to apply for human tissues fixed with formaldehyde because of its lower deviation. Since all tissues slices are penetrated from at least two surfaces simultaneously, the penetration was calculated as: $d = 2k\sqrt{t}$ ($d = 1.32\sqrt{t}$) where "d" is penetration in millimeters and "t" is time in hours, as done previously [2] and also following the known positive relation between the surface to volume ratio of a tissue sample and its penetration rate [13].

The correlation between three "k" values from cockerels blood plasma coagulum cylinders and the three different formaldehyde concentrations they correspond with (from 40 to 5.9 percent) [11] is not significant ($R^2 = 0.40$; $P > .37$) pointing to the independence between formaldehyde concentration and penetration rate and with fixation by extension. The formaldehyde to tissue ratio used [11] was 30:1.

Table 4 presents penetrations of tissue slices 1 to 5 mm thick fixed during 1 to 24 hours and binding rates at 25 and 37°C both steps being temperature dependent, as any chemical reaction is. It is important to remember that binding starts while penetration is still taking place and is independent of tissue thickness because it is the result of the tissue being penetrated through [1].

The increase of "k" value from 22° to 42°C in pork liver cylinders [16] and the increased binding rate in rat kidney sections from 25°C to 37°C [1] allowed the calculation of penetration, binding and cross-linking rates for the 3 mm

Table 4
Penetration and binding rates

Time in NBF (hours)	Penetration as % of thickness					Binding level	
	Tissue thickness (mm)					% of equilibrium	
	1	2	3	4	5	25°C	37°C
1	100	66	44	33	26	5	10
2		94	62	47	37	10	21
4			88	66	53	20	40
8				93	75	39	68
12					91	57	87
18						81	100
24						100	
100% at 25°C in	0.6 h	2.3 h	5.2 h	9.2 h	14.3 h		
100% at 37°C in	0.3 h	1.3 h	3.0 h	5.3 h	8.2 h		
100% at 42°C in	0.3 h	1.0 h	2.3 h	4.0 h	6.2 h		

thick tissue slices used in the experiment at the room temperature of 20 to 22°C and higher (Table 5).

At the experimental temperature of 20-22°C the 3 mm thick tissue slices were 100% penetrated, 24% bonded and only 6% cross-linked after 8 hours. After 24 hours they were about 70% bonded and at 48 hours about 36% cross-linked causing infiltration and microtomy difficulties especially in fat and skin. Had the tissues been fixed at 37°C they would have been almost 40% cross-linked in just 24 hours or 50% cross-linked in 20 hours at 42°C and the infiltration could have been much better.

It is important to remember that tissue processing was carried out manually, without the benefit of pressure, temperature and agitation in every station although after the 5 dehydration stations with isopropyl alcohol at 20-22°C, the remaining 3 stations with the isopropyl alcohol and mineral oil mixtures and the mineral oil pre-infiltration, were completed at 50°C [4].

The histotechnique results indicate that any laboratory wanting to reduce the amount of NBF and the fixation time has to fix tissues at a minimum of 37°C to assure good fixation in short time. Fixing with NBF at room temperature and expecting for the processing protocol to deliver a good infiltration, is optimistic unless the turn around time (TAT) is extended to allow for at least 48 hour fixation.

Using mouse liver samples the fixation was equally improved using heat from microwave irradiation, from conductive and convective heating in a water bath, or with resistive heating with a low frequency (1 kHz) current passed through the fixative solution which points to the fact that the thermal effect is independent of the heat source [17].

Table 5
Fixation of 3 mm thick tissue slices.

For 3 mm thick tissues slices	Temperature			
	20°C	25°C	37°C	42°C
100% penetrated in	7.1 h	5.2 h	3.0 h	2.3 h
100% bonded in	33 h	24 h	18 h	10 h
50% cross-linked in	66 h	48 h	36 h	20 h
100% cross-linked in	132 h	96 h	72 h	40 h

Microwave energy by itself does not fix tissues. If a tissue microwave irradiated without a fixative is cooled afterwards, the results are unsatisfactory because fixation is not caused by the increase in temperature alone but by heating the tissue in the fixative. The tissue to be microwave irradiated has to be totally penetrated and immersed in the fixative [18].

Fixing under vacuum has been described as a nonsensical approach while fixing under pressure is the correct approach because chemical reactions increase their rate under pressure [1]. When pork liver cores 5 mm diameter and 5 mm high were fixed at 22°C at a pressure equivalent to 1,000 atmospheres the “k” value went from 0.47 at normal pressure to 2.2, and at 42°C “k” increased from 1.1 to 3.4 under the same pressure increment [16].

There are several ways of increasing the temperature during fixation and this is of fundamental importance if the TAT is to be minimized when using NBF as fixative because increasing the TAT to assure better fixation is unacceptable.

A recent study [19] monitoring 126 women with large core breast biopsies showed that those without an initial diagnosis had the same increased levels of salivary cortisol as those patients already diagnosed with a malignancy. The cortisol levels were significantly higher for both groups compared with the control group, also waiting for results, but knowing that their tumors were benign. Increased cortisol levels are associated with a substantial biochemical distress which may have adverse effects on immune defense and wound healing. Assuring the shortest TAT possible is of paramount importance, so optimum tissue fixation should be achieved by other means than just increasing the fixation time.

A final consideration is the time from excision to fixation or delayed fixation, known to affect mitotic cells count in some tissues, and to invalidate the discrimination between 2 sub-types of small cell carcinomas as caused by delayed fixation only and not representing two different pathological entities (Table 6).

The correlation between hours delayed and percent mitosis reduction in Table 6 is not significant ($R^2 = 0.49^{ns}$; $P > .12$) emphasizing again the important effect of the tissue type, tumor type in this case, on the results.

A delayed fixation of up to 5 hours in tonsils did not affect any of the results [3] but our 36 hours delay caused some effects. Although the diagnostic value of all sections or the vimentin contents in uterus were not affected, the reticulin stain and the PAS reaction in liver at 1:1 for 8 hours and at 10:1 for 48 hours were. The reticulum stain intensity was moderate to weak only and the PAS reaction was weak in the vessels, strong in all biliary duct cells, but failed to demonstrate glycogen, this being an expected result because glycogen is known to be lost soon after death [28].

Table 6
Percentage reduction (%) of mitosis counts in several types of tissues after delayed fixation in hours (dxh)

Type of tissue	dxh	%	Ref.
Normal colon crypts show decreased mitosis counts as fixation is delayed for 2 or 6 hours.	2 6	30 50	[20]
Xenotransplanted human osteogenic sarcoma.	1	49	[21]
Xenotransplanted soft-tissue sarcoma fixed with 5min; 3; 6; 9 and 12h delays. The % reduction was mostly caused by identification problems although the flow cytometric data did not change substantially.	3 12	10-13 39-46	[22]
5 mm cubes of breast cancer tissue with 0.5; 2; 4; 6; 8 and 24h of delayed fixation.	6	53	[23]
After 24h delay the number of observed mitotic figures was reduced in 75% in breast cancer.	24	75	[24]
Results with 20 intracranial neoplasms fixed with 0; 3 and 12h delays suggest that cell cycle ends during delayed fixation.	3 12	5 24	[25]
Lung, breast and intestinal carcinomas fixed with 0; 1; 2; 4; 6; 8;18 and 24 h delays showed deterioration of the quality of the material to determine flow cytometric % of S-phase as delay increased. Starting at 4h the % of debris increased, but the mean values of uncorrected and corrected mitotic activity showed no decreasing trend.	1 to 24	0	[26]
Small cell carcinomas were diagnosed of the "Intermediate" type if fixed immediately after removal or as "Oat cell after delayed fixation. The differences are attributed to autolysis of the "Intermediate" type which determined to propose the elimination of both subclasses.	few (?)		[27]

5. Conclusions

Fixation with NBF, and its subsequent paraffin wax infiltration, is time and temperature dependent, but is independent of the volume of NBF used. A ratio of 2:1 for the volume of NBF to that of 3 mm thick tissue samples is enough to assure a 50% cross-linking in 48 hours at an ambient temperature of 25°C which is present in most laboratories.

A fixation period of 48 hours will increase the TAT of cases beyond acceptable limits so each laboratory has to develop a fixation protocol at 45°C, ideally under pressure, to reduce the fixation time to approximately 10 hours.

Even when all the routine sections obtained in this study, from a volume ratio of 1:1 with 8 hours fixation on, were equally valuable for diagnostic purposes, there were some microtomy difficulties that would negatively impact the TAT and should be eliminated by good infiltration in a short time.

To determine the penetration level of every sample when received, each laboratory has to have in place a protocol to know when each specimen was first placed in NBF and use the fixation stations in the tissue processor at 45°C, with pressure and agitation.

Using less formalin than at the present levels of 10:1 or more, along with all the numerous personal protection measures available and with optimal ventilation, will improve the safety of the histopathology laboratory while still permitting the use of this invaluable, but potentially hazardous routine fixative.

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