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Cryoembedder, automatic processor/stainer, liquid nitrogen freezing, and manual staining for frozen section examination: A comparative study

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ABSTRACT

Frozen section examination (FSE) reshaped surgical pathology and is characterized by a high accuracy. Nonetheless pathologists can experience artefacts that can compromise or defer the diagnosis. We compared a commercial system, composed by a cryoembedder and a processor/stainer, to our FSE protocol. Feasibility of diagnosis as well as overall architecture, cytology, and staining, were scored under the following conditions: Traditional (liquid nitrogen freezing and manual staining), Only-Presto (liquid nitrogen freezing and commercial processor/stainer), Only-PrestoCHILL (cryoembedder and manual staining), and PrestoSystem (cryoembedder and processor/stainer). Scores were compared across the different experimental conditions. PrestoSystem had significantly higher scores than Traditional, Only-Presto or Only-PrestoCHILL in all categories (Wilcoxon test; all P-value < .001); similarly, also Only-Presto and Only-PrestoChill had significantly higher scores than Traditional in all categories. Only-PrestoCHILL had significantly higher scores than Only-Presto in Cytology and Architecture. In conclusion the control of pre-analytical variables provided reproducible results, of a higher quality.

1. Introduction

Intraoperative Frozen Section Examination (FSE) allows rapid histological evaluation to make therapeutic decisions (Ackerman and Ramirez, 1959). At the beginning of the twentieth century, patients began to arrive well before malignant disease could be “easily recognized clinically” (Bloodgood, 1927a): since cancer became a microscopic disease there was a need for a microscopic diagnosis (Bloodgood, 1927b). Except the pioneering attempts in the late 1800 at Johns Hopkins, laboratory protocols for FSE are substantially derived from the original 1905 paper of Louis Wilson (Wilson, 1905). Basically, this procedure consists of three phases: 1) tissue embedding and freezing, 2) cutting and slice retrieving, 3) fixation, and staining. Classical series report FSE accuracy higher than 95% (Ackerman and Ramirez, 1959; Nakazawa et al., 1968; Ferreiro et al., 1995; Kaufman et al., 1986).

Each phase can produce artefacts that can prevent correct diagnosis; the freezing artefact, caused by ice crystals formation during freezing, is the most characterized and is related to water content and freezing time (Peters, 2010). Reduction of freezing time and limited sample re-heating reduce ice crystal formation. Reduction of freezing time is often

achieved by snap freezing; sample re-heating can be reduced by face-down embedding in wells bar, reducing the need of block trimming (Peters, 2010, 2003). Also staining variability can limit the results of the procedure; the first described procedure employed methylene blue staining (Wilson, 1905), nowadays the most common staining procedures for FSE are toluidine blue stain and the hematoxylin and eosin (H&E) stain, the former takes few seconds, but is unfamiliar to many pathologist, the latter, take few minutes and produces slides very similar to the H&E derived from formalin fixed paraffin embedded (FFPE) samples (Peters, 2010); automatic processor and stainers are the current standard in FFPE; a similar approach could hence be implemented to limit staining variability.

The primary aim of the study is to compare the technique currently employed in our laboratory (liquid nitrogen freezing and manual staining) with a controlled freezing system and a controlled processor/staining system.

2. Materials and methods

Study design is summed up in Supplementary Fig. 1. During routine FSE quality control for 30 biobank cases, two samples (A, B) were

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gathered from the same specimen to obtain four experimental FSE conditions: Traditional (face-up embedding, liquid nitrogen freezing and manual staining), only-Presto (face-up embedding, liquid nitrogen freezing and commercial processor/stainer), only-PrestoCHILL (cryoembedder and, manual staining), and PrestoSystem (cryoembedder and processor/stainer).

Sample A underwent standard embedding and freezing procedure currently in use in our institution: a face-up embedding was employed: tissue was placed on top of a chuck preloaded of embedding medium (Tissue-Tek O.C.T. Compound, Sakura Finetek, Mestre (VE), Italy), more embedding medium was applied liberally over the chuck surface, the chuck was then placed on a support and dipped into liquid nitrogen until homogeneous freezing of the surface occurred. Sample B underwent face down cryoembedding in well bars (Peters, 2010, 2003) hold in a -40 °C, temperature controlled, timer equipped cryoembedder (PrestoCHILL, Milestone, Sorisole (BG) Italy); tissue was embedded with a dedicated medium (MCC Milestone Cryoembedding Compound, Milestone).

Samples underwent cryostat cutting (Microtome Cryostat Microm HM 525, Thermo Fisher Scientific, Walldorf, Germany) in order to produce two slides from each (A1, A2, B1, and B2). Slides A1 and B1 underwent the standard staining procedure currently in use in our institution: a counting based, manual staining; slides A2 and B2 underwent automatic processing-staining (Presto, Milestone). Staining procedures are depicted in Supplementary Table 1.

Two pathologists, blindly evaluated the slides and scored with a three-levels scoring system (Inadequate: scoring 1; Satisfactory: scoring 2; and Excellent: scoring 3) the Diagnosis Feasibility (Diagnosis), as well as the overall Architecture, Cytology, and Staining Quality (Staining). A detailed description of the scoring system is provided in Supplementary Table 2.

Inter-pathologist agreement was calculated comparing Inadequate scores vs all others (Satisfactory and Excellent) using unweighted Cohen's kappa test. Strength of agreement was evaluated as described by Landis and Koch. Comparison between Diagnosis, overall Architecture, Cytology, and Staining quality was done between the following experimental conditions: 1) PrestoSystem Vs Traditional; 2) Traditional Vs a) Only-Presto, b) Only-PrestoCHILL; 3) Only-PrestoCHILL Vs Only-Presto; 4) PrestoSystem Vs a) Only-Presto, b) only-PrestoCHILL. Statistics were performed with R3.2.3 and R Commander. Differences between scores were calculated with Wilcoxon Signed Rank Test. A P-value < .05 was considered statistically significant.

3. Results

Twenty-seven cases out of 30 had all the 4 slides available for the evaluation (Table 1). Pathologists had substantial agreement in Diagnosis Feasibility (Cohen's Kappa: 0.654, P-value < .001), Cytology (Cohen's Kappa: 0.651, P-value < .001), Architecture (Cohen's Kappa: 0.649, P-value < .001) and Staining Quality (Cohen's Kappa: 0.737, P-value < .001). Diagnosis scored Satisfactory or Excellent in 211/220 cases (96%); Architecture, Cytology, and Staining scored Inadequate 12 (5%), 23 (10%), and 24 (10%) cases, respectively. All specimens that scored Inadequate in Diagnosis (N = 9) were frozen in liquid nitrogen, 6 (6/9) were in the Traditional group.

3.1. PrestoSystem vs traditional

PrestoSystem scores were almost always Satisfactory or Excellent: only one case was Inadequate in staining; in contrast Traditional scored Inadequate in Diagnosis (6 cases), Cytology (18 cases), Staining (13 cases) and Architecture (9 cases) (Fig. 1). PrestoSystem had significantly higher scores than Traditional in Diagnosis, Cytology, Staining, and Architecture (Wilcoxon test; all P-value < .001). Moreover, PrestoSystem scores were always equal or better than Traditional,

Table 1
Scores of the Different Experimental Conditions.

Scores	No. of Cases (Percentage)							
	Excellent		Satisfactory		Inadequate		NA	
Traditional								
Architecture	20	33%	25	42%	9	15%	6	10%
Cytology	7	12%	29	48%	18	30%	6	10%
Staining	6	10%	35	58%	13	22%	6	10%
Diagnosis	21	35%	27	45%	6	10%	6	10%
Only-PrestoCHILL								
Architecture	30	50%	18	30%	6	10%	6	10%
Cytology	12	20%	39	65%	3	5%	6	10%
Staining	19	32%	30	50%	5	8%	6	10%
Diagnosis	29	48%	22	37%	3	5%	6	10%
Only-Presto								
Architecture	37	62%	19	32%	0	0%	4	7%
Cytology	29	48%	25	42%	2	3%	4	7%
Staining	17	28%	34	57%	5	8%	4	7%
Diagnosis	34	57%	22	37%	0	0%	4	7%
PrestoSystem								
Architecture	43	72%	13	22%	0	0%	4	7%
Cytology	44	73%	12	20%	0	0%	4	7%
Staining	38	63%	17	28%	1	2%	4	7%
Diagnosis	47	78%	9	15%	0	0%	4	7%

Abbreviation: NA, not available.

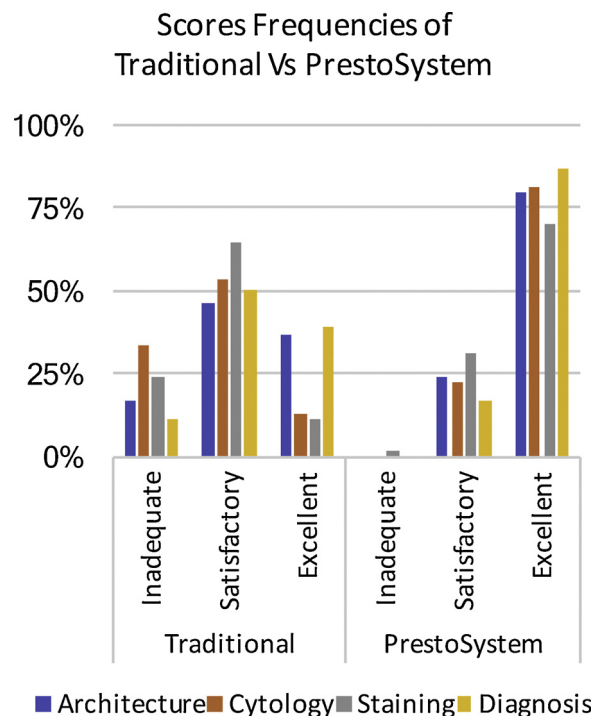


Fig. 1. Score Frequencies of Traditional Vs Presto System.

improving the Diagnosis scores in 27 cases (45%); similarly, Cytology, Staining and Architecture had better scores in 41 (76%), 41 (76%), and 27 cases (45%) respectively.

3.2. Only-Presto OR Only-PrestoCHILL vs Traditional

Only-Presto had significantly higher scores than Traditional in Diagnosis (Wilcoxon test; P-value = .005), in Cytology (Wilcoxon test; P-value < .001), in Staining (Wilcoxon test; P-value < .001), and in Architecture (Wilcoxon test; P-value = .003). Only-PrestoCHILL had significantly higher scores than Traditional in Diagnosis (Wilcoxon test; P-value < .001), in Cytology (Wilcoxon test; P-value < .001), in Staining (Wilcoxon test; P-value < .001), and in Architecture

(Wilcoxon test; P-value < .001). Only-Presto and Only-PrestoCHILL had better scores in Diagnosis than Traditional in 11 (20%) and 16 (30%) cases, respectively. Compared to Traditional, Only-Presto had improved scores in Architecture and Cytology in 24 (44%), 14 (26%) and 21(39%) cases respectively. Similarly, Only-PrestoChill, compared to Traditional, had improved Architecture, Cytology and Staining scores in 22 (41%), 32 (59%), and in 21 (39%) cases respectively.

3.3. Only-PrestoCHILL vs only-Presto

Only-PrestoCHILL had not substantial difference in Diagnosis and Staining scores compared to Only-Presto Only-Presto (Wilcoxon test; P-value = .14); however significantly higher scores in Cytology (Wilcoxon test; P-value < .001), and in Architecture (Wilcoxon test; P-value = .013) were found. Compared to Only-Presto, Only-PrestoChill improved Diagnosis score in 9 cases (17%) conversely Only-PrestoCHILL improved the Diagnosis scores in 4 cases (7%) compared to Only-Presto.

3.4. PrestoSystem vs Only-Presto or Only-PrestoCHILL

PrestoSystem had significantly higher scores than Only-Presto or Only-PrestoCHILL in Diagnosis, Cytology, Staining, and Architecture (Wilcoxon test; all P-value < .001). PrestoSystem always scored equal to or better than Only-Presto or Only-PrestoCHILL in Diagnosis. PrestoSystem also had equal or better scores than only-Presto in Staining, Architecture and Cytology in 51 (94%), 53 (98%) and 53(98%) cases respectively. Similarly, PrestoSystem had equal or better scores than only-Presto in Staining, Architecture and Cytology in 51 (94%), 53 (98%) and 53 (98%) cases respectively.

4. Discussion

We compared our current FSE procedure with a controlled freezing system coupled with a controlled processor-staining system (PrestoSystem) (Figs. 2 and 3). PrestoSystem never produced an inadequate Diagnosis and scores were always equal or better than all other settings. These results highlight how pre-analytical variables are critical in determining the quality of results.

Artefacts can be considered as limitations of pre-analytical phase. Well recognized artefacts of FSE are drying artefacts, derived from delaying fixation of the slide, and freezing artefacts (Peters, 2010). The latter is caused by formation of ice crystals that alter the morphology. Ice crystals formation depends by the water content and the rapidity of freezing. Controlled freezing as well as reduced time between freezing and cutting, due to reduced trimming time, probably account for the fact that all the specimens Inadequate for diagnosis underwent liquid nitrogen freezing, this is also supported by the fact that specimens that underwent cryoembedder and manual staining (only-PrestoCHILL) did better than the ones that did liquid nitrogen freezing and automatic processor/stainer (only-Presto). These results could be of interest especially in these settings where a better cytological detail can assist the pathologist in the differential diagnosis, as in assessing primary Vs metastasis in solitary lung nodule or in grading of endometrial cancer (Sienko et al., 2005; Baker and Oliva, 2008).

Surprisingly even if architecture, cytology and staining were inadequate up to 10% of cases, rarely diagnosis was not feasible, in fact cases can show preserved tissue quality focally and this could be enough to establish a diagnosis; moreover, diagnosis is often done relying on more than one parameter and even in absence of a satisfactory quality, other features, as clinical can compensate, in fact accuracy in FSE is improved by a clinically oriented pathologist (Ackerman and Ramirez, 1959).

Technical advancements are often incorporated in production workflow without testing and pathology is not an exception, this is probably related to the amount of human judgement still involved in this process; to create evidences to change our current practice, we compared the technique currently employed in our laboratory with a controlled freezing system and a controlled processor/staining system.

Compared to a paper recently published using the same system (Orchard et al., 2017), our study design allowed us to make a comparison between our current technique and a controlled freezing system combined to an automatic processor/stainer; moreover, the cross-over design also allowed to highlights the contribution of the different systems to the final results.

FSE transformed surgical pathology, moving the pathologist inside the operating room (Rosai, 1997); we demonstrated that controlling pre-analytical procedural variables of embedding, freezing, fixation and

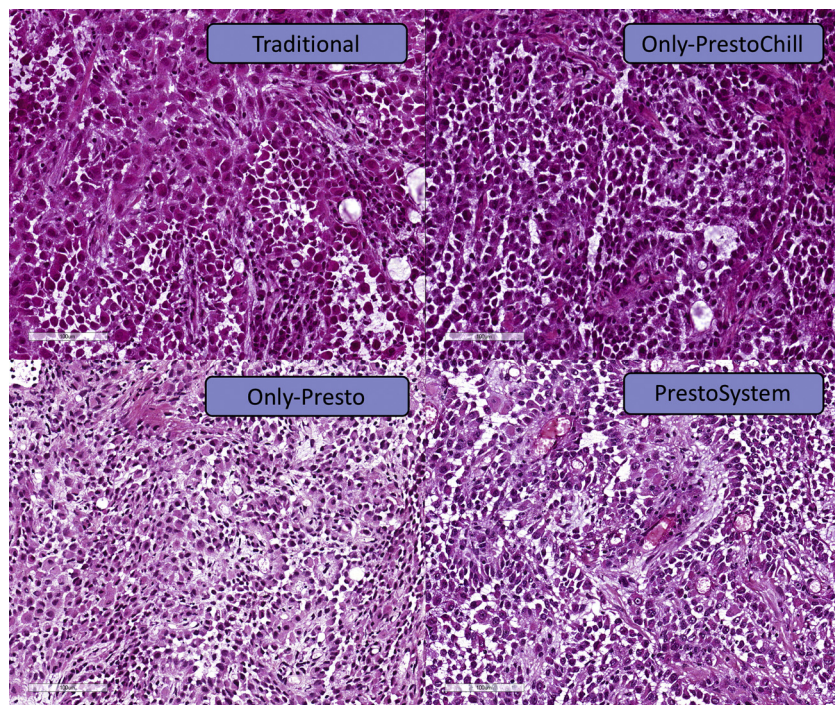


Fig. 2. Microphotograph of a malignant mesothelioma of the peritoneal cavity (case 19). PrestoSystem scored excellent in all parameters by both pathologists. Diagnosis was considered Satisfactory or Excellent for all conditions. Only-Presto and PrestoSystem had a higher score in Cytology and Staining compared to Traditional and Only-PrestoChill. Scale-bars indicate 100 μ m.

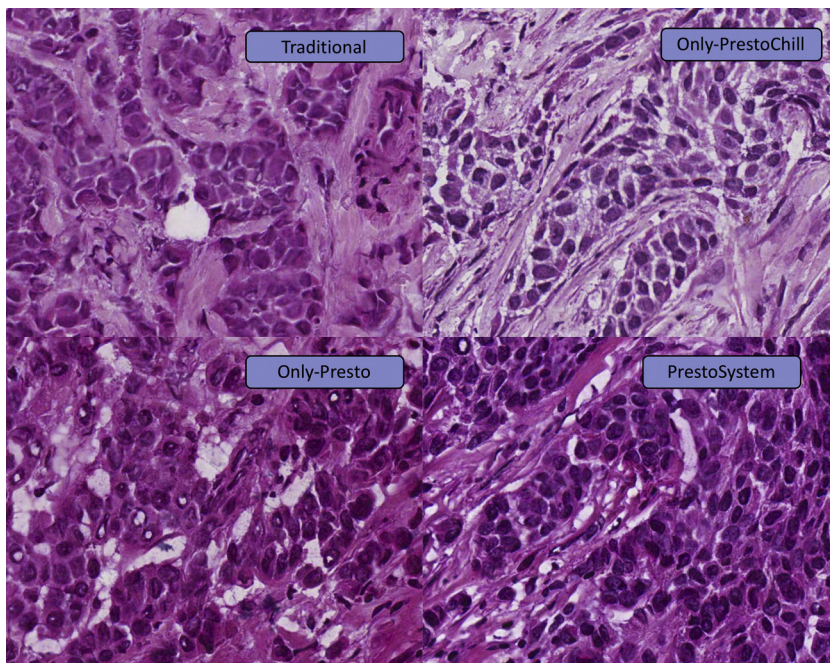


Fig. 3. Microphotograph of a carcinoma of the breast (case 10). Even if Diagnosis was considered Satisfactory or Excellent in all experimental conditions a great variability in parameters between conditions was recorded. Several artefacts can be appreciated: delayed fixation artefacts (smudged chromatin, poorly defined nuclear and cytoplasmic membrane) are seen in Traditional and Only-PrestoChill conditions, probably reflecting the distance between the cryostat and the formalin under-hood. On the other hand, only-Presto shows freezing artefacts (scalloping and compression neoplastic cells resulting in clefts between cells), probably prevented by snap-freezing in Only-PrestoChill and PrestoSystem and masked by delayed fixation artefact in Traditional. Original magnification 400 \times .

staining improved slide quality, giving more consistent results.

Disclosure of potential conflicts of interest

Presto and PrestoCHILL were kindly lent by Milestone SRL for the duration of the study. No funding, honoraria, or financial support was obtained for the study. The authors declare that they have no conflict of interest. This research did not receive any specific grant from funding agencies in the public, commercial, or non-for-profit sectors.

Research involving human participants and/or animals

The system was used in the standard procedure of morphologic control of bio-banked tissue.

Informed consent

Written informed consent for bio-banking was obtained.

CRediT authorship contribution statement

Salvatore Lorenzo Renne: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - original draft. **Silvia Redaelli:** Data curation, Investigation. **Biagio Paolini:** Conceptualization, Data curation, Investigation, Writing - original draft.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.acthis.2019.05.002>.

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