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Research Article

Evaluation of a Novel Technique of Frozen Sections of Cell Blocks and Immunocytochemistry Protocol for Fine Needle Aspirates and Fluid Cytology Specimens

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Abstract

Aim and objectives: Turnaround time is the main issue for formalin fixed-paraffin embedded-cell block diagnosis. The main objectives of this study were to develop fast and simple method of cell blocks preparation using frozen section technique, to develop a protocol of immunocytochemistry (ICC) on them and to evaluate its utility in clinical practice.

Material and method: In this case series, we made frozen cell blocks (F-CB) from fine needle aspirates (FNA) and fluid samples using cryostat and performed ICC on them. Results were compared with histopathology diagnosis using Cohen's Kappa agreement and Fisher Exact test.

Results: Out of 25 cases, F-CB from FNA and fluid samples were made in 15 and 10 cases, respectively. ICC was performed on 20 cases. Only 40% of the cases had confirmed diagnosis on cytology smears. Addition of F-CB + ICC test could give a definitive diagnosis in 24/25 (96%) of cases.

Conclusions: Newly developed method of frozen sectioning of cellblocks and ICC on them can give rapid and accurate cell block diagnosis.

Keywords: frozen section; cellblocks; turnaround time; immunocytochemistry

Introduction

Cell blocks are important as adjuvant to cytology because of preservation of architecture and availability of cellular material for immunohistochemistry and molecular analysis [1]. They are like mini-biopsies and can avoid invasive biopsy procedure [2, 3]. Formalin-fixed Paraffin- embedded Cell blocks (FFPE-CB) are now integral part of cytology, as they are now routinely indicated on most of the cytology specimens [4, 5, 6]. Cell blocks are usually processed as routine histopathology specimens. Depending on tissue processing time, it takes 2-3 days' time for generation of final examination reports [4, 7]. To reduce the overall turnaround time, some researchers have used different processing methods using microwaves [8], ultrasound energy [9] and vaccum [10]. Frozen sections or cryosections of the tissue are being used for rapid preliminary diagnosis and they have been gold standard for immunohistochemical analysis from the inception of these methods [11]. Method of frozen sectioning of cell blocks and immunocytochemistry (ICC) on them in clinical practice is not very well developed. Only Lee et al [12]. Have used agar based frozen cell blocks (F-CB) to reduce the turnaround time of FFPE cell blocks. However, this is a preliminary study involving few cases only. Method is technically complex and involves multiple steps. To reduce overall turnaround time for final cellblock diagnosis and handle the excessive work load in a busy laboratory, we have developed a novel fast, simple technique and device for preparation of frozen cell blocks made from fine needle aspirates and fluid sediments. We also describe immunocytochemistry protocol for cryosections of F-CB and evaluated this technique in routine clinical practice.

Material and methods:

After ethical committee approval, we prospectively observed series of cytology cases and its diagnostic ability when F-CB with ICC was performed. In this case control study, we included the cases that came for the diagnosis by fine needle aspiration and fluid cytology on OPD bases, and which had confirmed histopathological diagnosis. We excluded the cases which were lost to follow up or had no histopathological confirmation. In those cases that came for fine needle aspiration cytology (FNAC) diagnosis; we took the second sample for frozen cell block preparation (F-CB) after patient consent to avoid split sampling error. For fluid samples, we prepared two to three optimal cytology smears and then processed residual samples as F-CB. Frozen remaining tissue was

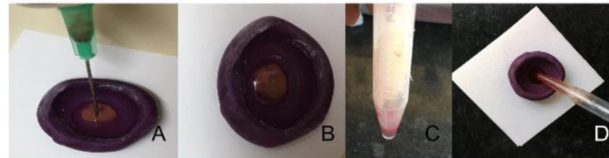
processed as routine histopathological specimens (FFPE-CB). Biopsies with or without IHC were carried out for diagnostic confirmation in indicated cases.

Frozen cell block (F-CB) preparation from fine needle aspirates. (Time duration: 2-5 minutes)

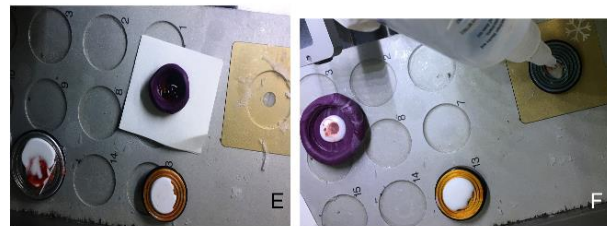
After taking consent, we aspirated the material from swelling using 21-gauge needle and 10-ml syringe with 10-12 passes with continuous suction. We dispensed the material into a cup containing a drop of OCT (Optimal cutting temperature) compound. Figure 1 A and B demonstrate this step for fine needle aspirates (FNA) samples. After that, we layered the OCT compound on it and put it into the cryostat at -20^0 C temperature till it was frozen. (Figure 1 E). After that we removed the frozen tissue and embedded upside down on the cryo chuck layered with OCT and put OCT on it again (Figure 1 F, G) and kept for freezing. Then chuck was mounted on the cryostat microtome and trimmed at 20 micron (Figure 1 G). Final frozen sections were cut with 4 micron thickness at -20^{0}C. (Figure 1 G, H) We fixed the cut sections into NBF (neutral buffered formalin), methanol and cold acetone, respectively. Formalin and methanol fixed smears were stained by haematoxylin and eosin stain (H&E) and acetone fixed smears were processed for ICC

Method of Preparation of cryoblocks and cryosectioning

Step-1: Sample preperation: Preparation of cryoblocks in cryogel



Step 2: Freezing & Mounting: Cryoblocks are frozen in cryostat chamber at -20 degree C. immediately. Than mounted upside down on cryochucks



Step-3: Sectioning: After minor trimming of cryochucks at 25 micron, sections are cut at 4 micron and taken on slides for staining.

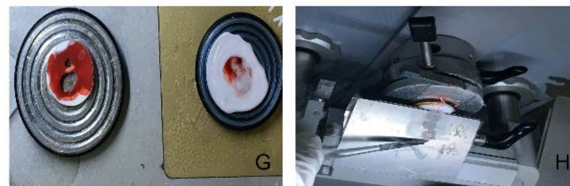


Figure 1: *[A] FNA F-CB sampling [B] FNA material with OCT compound [C] Fluid sediments [D] Fluid F-CB sampling [E] F-CBs and cryo-chucks in cryostat [F] F-CB, mounted upside on cryo chucks [G] Trimmed F-CB [H] frozen section of F-CB at 4 micron thickness Abbreviations; FNA: fine needle aspirate F-CB : frozen cell block*

Frozen cell block (F-CB) preparation from fluid sediments. (Time duration: 12-15 minutes)

All the fluids (processed within one hour) were centrifuged 2000 rpm for 10 minutes and supernatant was discarded. The supernatant fluid was removed completely. The sediments were aspirated from the bottom and dispensed gently on the cup with OCT compound as showed in figure 1 C and D. After layering OCT compound again, cup was put into cryostat chamber at -20^0 C (figure 1-E). After freezing, we removed the frozen block and mounted upside down (figure 2 F) on cryo chuck already with OCT. After layering OCT compound, the block was frozen again and sections were cut. (Figure G, H). We fixed the cut sections into formalin, methanol and cold acetone, respectively. Formalin and methanol fixed smears were stained by H&E and acetone fixed smears were processed for immunocytochemistry.

Protocol for H &E stain and Immunocytochemistry [13]:

Haematoxylin and Eosin stain: After washing slides in tap water, we immersed the slides in haematoxylin in 1-2 minutes. After tap water wash, slides were stained by eosin for 1-2 minutes. Slides were immersed in 80 %, 90% and 100% alcohol (1 dip each) for differentiation, xylene and then mounted in DPX. (Average time: 10-12 minutes)

Immunohistochemistry stain (Time duration: 1.5-2 hours)**:** After pouring of the fixative, slides were washed in deionised water for five minutes and then in distilled water for 1 minutes. Slides were rinsed in 300 ml of Triple buffered saline (8.7 gm Sodium chloride + 1.21 gm trisbase in 1 litre of distilled water) at a7.4 pH for 2 changes, 5 min each. After that the slides were incubated in 0.3% H_2O_2 solution at room temperature for 10 min to block endogenous peroxidase activity. Then immunohistochemistry was performed routinely according to manufacturer's protocol. We used CK 5/6 (clone EP24, EP67) Ki67 (clone MIB-1), TTF-1(clone EP229), CK 7(clone EP 16), Ck 20 (Clone EP 23), Pax 8 (clone EP 331) , WT-1(clone EP 122) as primary antibodies and polyExcel HRP/DAB detection system (PathnSitu, Pleasanton, CA,USA) as detection kit. Histological sections from lung, oral cavity and ovarian cancers were used as positive control. We omitted primary antibody as negative control.

Examination and reporting: (Time duration: 10 -15 minutes)

We gave opinion on cytology smears according to published guidelines and simultaneously prepared F-CB in all cases. Immunocytochemistry was advised when diagnosis was equivocal or suspicious.

Immunocytochemistry was also performed on cases with already known diagnosis as control. IHC markers were reported as strong (+3), moderate (+2), weak (+1) and negative (-).Cytology diagnosis with or without IHC were compared with final histopathological diagnosis with or without immunohistochemistry. Details of studied cases are given in table 1. Expression of some of IHC markers (detailed in table 1) are presented in figure 2, 3.

	Age/ Sex	Clinical presentation*	Site of FNAC/Fluid *	Microscopic Diagnosis*	F-CB diagnosis*	IHC markers on F-CB (Expression +1,+2,+3,-)	Diagnosis after IHC*	Histopathological confirmed cases*
1	64/M	MUO	Matted mass Rt II,III	Met PDC	Met PDSCC	CD 56 - CK5/6 +3	Met PDNKSCC	Met SCC UNP
2	58/F	Rt breast lump	Right breast lump	IDC	IDC	None	-	Ca breast IDC
3	42/M	Rt BM growth	Rt cheek	MDSCC	Mets scc	-	-	Ca Rt BM SCC
4	44/F	Ca ovary post NACT	Ascitic fluid	MetPDC	ADC	CK 7 +3	ADC	HGSADC
5	38/F	Lt breast lump	Left breast lump	Fibroadenoma? LGIDC	benign	Ki 67 -	benign	fibro adenoma
6	59/M	MUO with ulcer	Left level II LN	PDC	PDC	CK 5/6 +3	PDNKSCC	Met SCC UNP
7	61 /F	Ca ovary post NACT	Ascitic fluid	Met ADC	Met ADC	Pax 8 +3 CEA +1	Met ADC	Ca ovary HGSADC
8	37/F	Ca Stomach	Ascitic fluid	Met PDADC	Met adenoca	CDX2 +2 CEA +3	Met PDADC	Ca stomach ADC
9	42/M	Ca stomach	Pleural fluid	Met PDADC	Met PDADC	CK 7+3 CDx2+2	Met PDADC	Ca stomach ADC
10	40/F	MUO	supraclavicular region	SmCC	Small cell ca	Synaptophysin +3 TTF-1 +3 Ki67 +3 LCA-	Small cell ca	Oesophagus small cell ca
11	50 /M	MUO	Left level II LN	Met SCC	Met SCC	CK 5/6 +3	Met SCC	Metastatic SCC UNP
12	40/M	MUO fungating	Rt level Ib LN	Met SCC	Met scc	None	Mets scc	Met SCC UNP
13	42/M	Lt BM growth	Submandibular swelling	Met SCC	Met SCC	-	Met SCC	Ca Lt BM SCC
14	28/F	Lung lesion	Pleural fluid	Reactive	Reactive	Calretinin +3	Reactive	Reactive
15	37/M	Rt arm swelling	fluid	Met SCC	Met SCC	CK5/6 +3	Met SCC	Ca right tongue SCC
16	43/M	Ca colon	Ascitic fluid	Suspicious	suspicious	CEA - LCA +3	Negative for MC	Ca colon ADC
17	50/M	MUO	Left level II	PDC	PDSCC	inadequate	-	Ca Lt PFS SCC
18	68/F	Ovarian Mass	Ascitic fluid	Met ADC	Met ADC	CK 7 +3	Met ADC	Ca Lt ovary HGSADC CA 125 1540.83
19	42/M	MUO	Matted mass Rt L II,III,IV	PDC	PDCC	CK 5/6 +3	PDNKSCC	Metastatic SCC UNP
20	49/M	MUO	Cervical swelling II,III	PDC	PDCC	CK 5/6 +3	PDNKSCC	Ca Rt BM SCC
21	55/M	MUO	Left level II LN	MetPDC	MetPDSCC	P63 +3	MetPDSCC	Metastatic SCC UNP
22	35/M	MUO	Right level II LN	Met PDC	Met PDSCC	P63 +3	Met PDSCC	Ca right BM SCC
23	67/M	Tongue growth	Bilateral level II LN	Met SCC	Met SCC	Ki 67 +3	Met SCC	Case tongue SCC
24	45/M	Lung mass	Pleural fluid	Met ADC	Met ADC	Ck 7+3 Synaptophysin -	Met ADC	Ca lung ADC
25	52/F	Ca breast	Pleural fluid	Met ADC	Met ADC	ER -PR -Her2neu +	Met ADC	Ca breast IDC

*Abbreviations: Rt: right, Lt: left, BM: bucal mucosa, SCC: squamous cell carcinoma PDC: poorly differentiated carcinoma, PDSCC poorly differentiated squamous cell carcinoma PDNKSCC: poorly differentiated nonkeratinising squamous cell carcinoma, ADC : adenocarcinoma, IDC: invasive ductal carcinoma, Met: metastatic, Ca: carcinoma, LN: lymphnode, SmCC: small cell carcinoma, UNP: unknown primary, + : positive, -: negative, LG: low grade
a : Figure 1 A,B,C,D b: figure 1 E,F,G,H c: figure 1: I,J,K,L d: figure 2 A-F, e: figure 2 G,H,I f: figure

Table 1: *Summary of the cases diagnosed by immunocytochemistry on frozen cell block sections.*

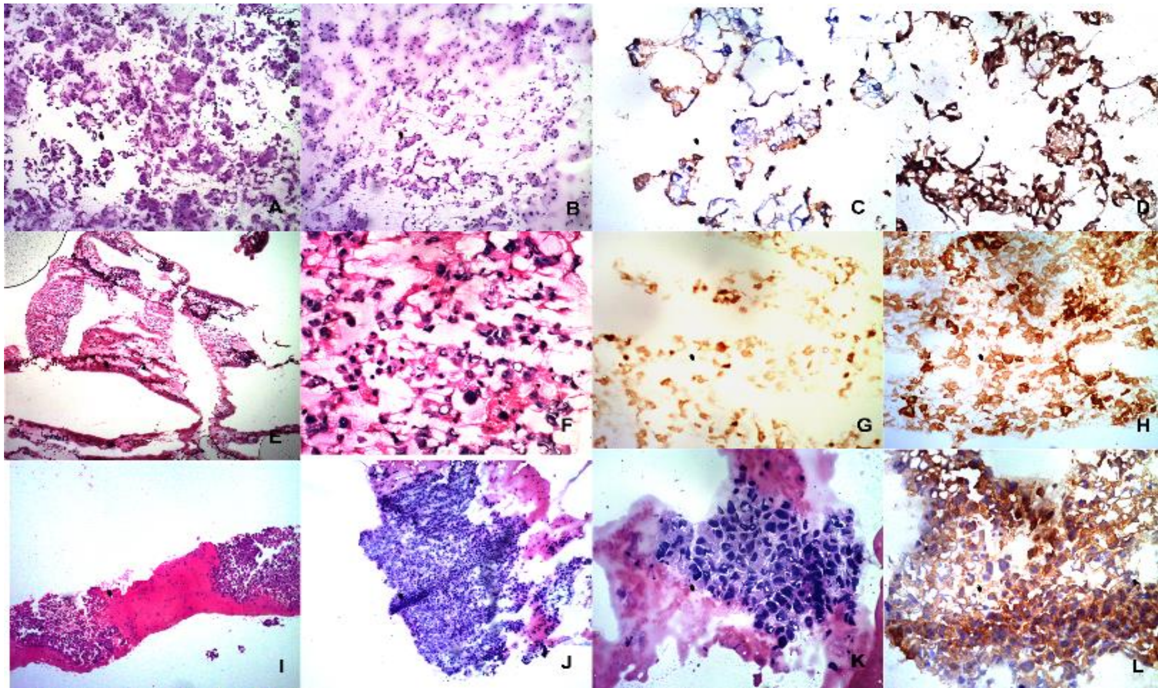
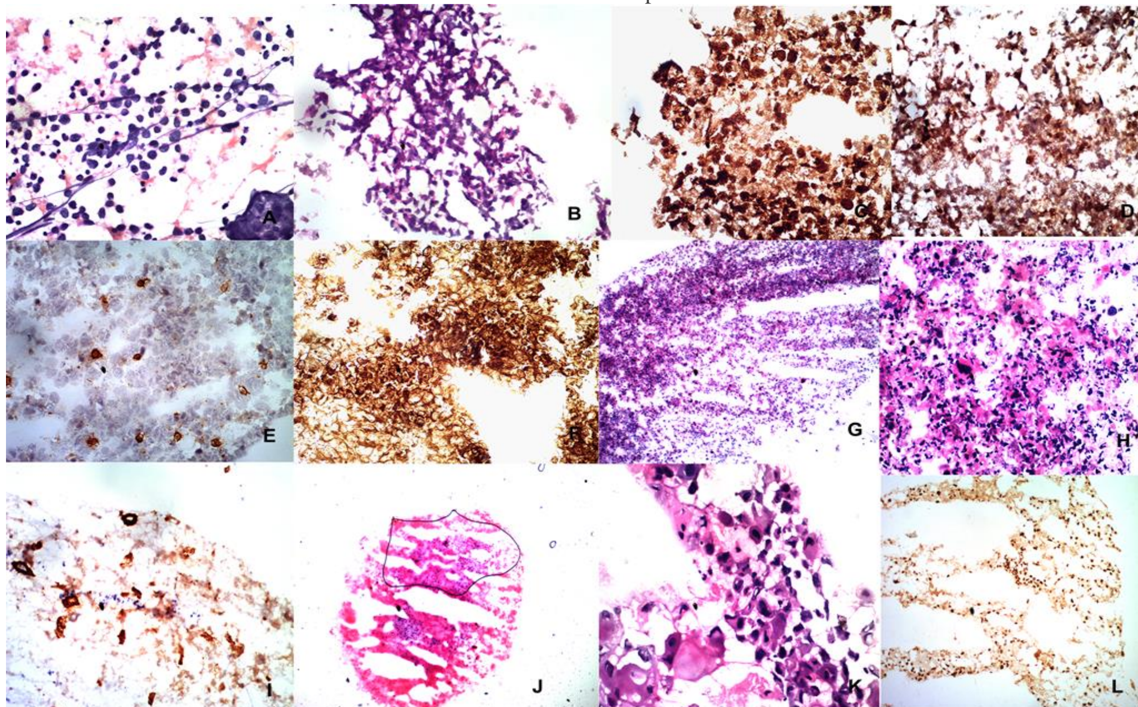


Figure 2: *[A] Ascitic fluid, ADC, FFPE CB,10x,H& E [B] Ascitic fluid ADC, F-CB,4x,H&E [C] Ascitic fluid,ADC, F- CB, 40x,CEA [D] Ascitic fluid, ADC,F- CB, 40x,Pax 8 [E] Ascitic fluid,PDADC,F-CB,4x,H&E [F] Ascitic fluid, PDADC,F- CB, 40x, H &E [G] Ascitic fluid,PDADC,F-CB,40x,CDX2 [H] Ascitic fluid ,PDADC,F-CB,40x,CK7 [I]FNA, PDSCC, FFPE CB,10 x,H & E [J] FNA,PDSCC,F-CB,40x,H &E [K]FNA, PDSCC,F-CB,40x,CK 5/6*

Abbreviations: ADC: adenocarcinoma PDADC: poorly differentiated adenocarcinoma PDSCC poorly differentiated squamous cell carcinoma. F-CB: Frozen cell block FFPE CB: formalin fixed paraffin embedded cell block



Abbreviations: Ca: carcinoma F-CB: Frozen cell block FFPE CB: formalin fixed paraffin embedded cell block. KSCC: Keratinising squamous cell carcinoma

Figure 3: *[A] FNA, Small cell Ca,10x,Pap smear [B] FNA, small cell ca,F-CB,10 x, H& E [C] FNA, Small cell ca, F-CB,40x,Ki 67 [D] FNA, small cell Ca,F-CB,40x,TTF-1 [E] FNA, small cell ca, F-CB, 40x,LCA [F] FNA, small cell ca,F-CB,40x,Synaptophysin [G] FNA, KScc,F-CB,10x,H&E [H] FNA, KScc,F- CB ,40x,H& E [I] FNA,KScc,F-CB,40x,C K 5/6.[J] FNA, KScc, F- CB,4x,H&E [K] FNA, KScc, F-CB ,40x,H& E [L] FNA,KScc,F- CB,40x,p63*

Statistical analysis: The statistical analysis was performed using IBM SPSS Statistics 21. In our study, we used chi squared and Fisher's exact test to compare the variables and Cohen's kappa agreement to identify the similarity between diagnostic tests and histopathological reference standard. We could not do diagnostic test evaluation as none of the test was interpreted independently.

Results

We studied 30 cases. Maximum time duration for preparation of F-CB and ICC for FNA samples was 2 hour and 20 minutes and that for fluid samples was 2 hours and 30 minutes. Five cases were excluded due to lack of histopathological confirmation. Out of 25 cases, FNAC and fluid cytology were performed in 15 (60%) and 10 (40 %), respectively. Diagnosis was known in 10 (40%) cases and unknown in 15 (60%) cases. On FNA smears, we found 5 squamous cell carcinoma (10%), poorly differentiated carcinoma 7 (46.7%), Invasive ductal carcinoma 1 (6.7%). We gave suspicious in 1 (6.7%) case. On fluid sediment cytology smears, we gave one squamous cell carcinoma (10%), 6 adenocarcinoma 6 (60%), one poorly differentiated carcinoma (10%), one reactive (10%), two suspicious (8%). IHC was performed on F-CB of 20 (80%) cases (10 fluid and 10 FNA F-CB). Details of IHC markers performed are given in table 1. After IHC markers, definitive diagnosis was given in 24/25 (96%) of cases. Cohen's kappa agreement value for Cytology and histopathological standard was 0.329. ($p \approx 0.000$). The kappa value for Cytology + F-CB+ IHC and histopathological standard was 0.694. ($p \approx 0.000$)

Discussion

Even though there are some drawbacks, like lack of standardisation of preparatory methods and lack of availability for rapid diagnosis, cell blocks still widely being used as non-invasive technique for immunocytochemistry and molecular testing [14]. Immunocytochemistry on cellblocks is also challenging and depends on the preparation method of cell blocks [6, 15, 16, 17] Average time for such a diagnosis is usually 2-3 days. To reduce this time, researchers have used different methods of tissue processing. These methods require special equipments and still can reduce the processing time to 3-4 hours [8, 9, 10] Frozen sections can give rapid preliminary tissue diagnosis, but frozen sections of cell blocks in clinical practice is hardly used [12]. We have observed that the present method of frozen sectioning and immunocytochemistry of cell blocks is fast, cheap, easy, reproducible and can improve diagnostic ability of cytology smears (Fisher exact $p \approx 0.000$). Kappa agreement for Cryoblock+IHC was 0.694, suggesting good agreement with histopathological diagnosis. One case we could not confirm was fibrocystic disease of the breast. Confirmed diagnosis was possible only on biopsy.

Major advantage of the method is that, final cell block diagnosis with IHC interpretation is ready in two to three hours. It is possible to handle multiple samples simultaneously (Figure 1 E), thus suitable for high throughput situations. Though we did not perform cost analysis in detail, we observed that method does not require investment of special equipments and processing fluids, overall cost is reduced. In our study, we could give rapid confirmed cell block diagnosis with IHC on OPD basis and treatment was started on the same day, thus reducing overall stay and expenditure in the hospital. Technically, our method of frozen section of cell blocks differs from that described by Lee et al [12], who used agarose gel for cell block embedding. In our cases, we used OCT compound as embedding medium that kept the cellular material together. In our method, because of density gradient, malignant cells were sedimented at the bottom in OCT gel. When the F-CB remounted upside down on cryo chucks, it faced the representative tissue and did not require deep trimming. We immediately got the representative sections on minimum 4-5 slides. More, Lee et al [12]. Observed that, many times it is difficult to remove agarose embedded CB from the conical tube before mounting on chucks. The silicone cup or mould that we used immediately separated the F-CB from the surface and remounting on chuck was easy.

There are many limitations to the study. One of them is a small sample size. In initial few cases we got high background staining and we had to repeat the staining. This was actually because of acetone fixation. Adequate perodixase blocking and optimal wash at each step could reduce the background staining. We did not compare formalin and acetone fixation and its effect on immunocytochemistry nor signal intensity of immunolabling on frozen sections of cell blocks. Formalin fixed cryosections require antigen retrieval and reported giving better signal enhancing [11]. We also observed that the architecture is better preserved in formalin fixation (Figure-2 B), however did not perform any statistical analysis. Choice of fixative and immunocytochemistry protocol needs to be validated in the laboratory before going for clinical use. Freezing artefacts (Figure 2 E, Figure 3B, G, J) were another issue. When the water content of the cellular material was high, ice crystals were formed. When the slides were stained at room temperature, these crystals were melted breaking the tissue block apart. This was more common in fluid samples; however, micro architecture was preserved in most of the cases and did not affect immunocytochemistry. Another issue was limited cryo-sections available, especially in FNA F-CB. When a large panel of IHC markers for diagnosis is required, it is better to go for biopsy. Multiple FNA material and preparation of CBs also can give good number of sections.

Conclusions

Thus, method and device for frozen section of cell blocks made from FNA and fluid samples and immunocytochemistry, presented in this method, can give rapid and accurate cell block diagnosis with good Cohen's kappa agreement than cytology alone. ($p \approx 0.000$)

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