Single-Tube Flow Cytometry Assay for the Detection of Mature Lymphoid Neoplasms in Paucicellular Samples

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Key Words

Multicolor flow cytometry · Paucicellular samples · Non-Hodgkin lymphomas

Abstract

Objectives: Flow cytometry (FC) has become a useful support for cytomorphologic evaluation (CM) of fine-needle aspirates (FNA) and serous cavity effusions (SCE) in cases of suspected non-Hodgkin lymphoma (NHL). FC results may be hampered by the scarce viability and low cellularity of the specimens. **Study Design:** We developed a single-tube FC assay (STA) that included 10 antibodies cocktailed in 8-color labeling, a cell viability dye, and a logical gating strategy to detect NHL in hypocellular samples. The results were correlated with CM and confirmed by histologic or molecular data when available. **Results:** Using the STA, we detected B-type NHL in 31 out of 103 hypocellular samples (81 FNA and 22 SCE). Of these, 8 were not confirmed by CM and 2 were considered to be only suspicious. The FC-negative samples had a final diagnosis of benign/reactive process (42/72), carcinoma (27/72), or Hodgkin lymphoma (3/72). **Conclusions:** The STA approach allowed obtainment of maximum immuno-

phenotyping data in specimens containing a low number of cells and a large amount of debris. The information obtained by STA can help cytomorphologists not only to recognize but also to exclude malignant lymphomas.

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Introduction

Several studies have demonstrated the usefulness of flow cytometry (FC) in the diagnosis of non-Hodgkin lymphoma (NHL) in samples such as fine-needle aspirates (FNA), cerebrospinal fluid, vitreous biopsies, and serous cavity effusions (SCE), in which cytomorphology evaluation (CM) may be insufficient to detect neoplastic involvement [1–10]. Multiparameter FC analysis, which makes it possible to obtain reliable information about cellular composition, immunophenotype, and lymphocyte cell size, could help reduce the overall number of inconclusive results that may be obtained by evaluation of specimens based on morphology alone. FC investigations may, however, be hindered by the low cell number, limiting the number of tests that can be performed. Tailored

panels and appropriate sample processing are mandatory in such cases [11]. This study reports our experience with paucicellular samples using an FC approach that employs a nuclear dye to recognize viable cells, antibodies cocktail in 8-color labeling to detect lymphomas, and a Boolean gating strategy to analyze data.

Patients and Methods

Between December 2011 and December 2015, FNA ($n = 674$) and SCE ($n = 264$) samples were sent for FC evaluation.

Among 938 specimens, 103 (10.9%) belonging to 96 patients (mean age 59 years, range 17–92) were considered hypocellular (cellularity $<0.5 \times 10^6$ total cells) and were selected for evaluation with a single-tube FC assay (STA). This study was approved by the local institutional review board. Informed consent was obtained from all participants. The STA was applied to 40 FNA (29 lymph nodes and 11 extranodal lesions), 41 endoscopic ultrasound (EUS)-FNA (13 deep lymph nodes, 27 extranodal lesions, and 1 pancreatic cyst fluid), and 22 SCE (15 pleural fluids, 5 ascites, and 2 pericardial fluids). FNA were placed in Vacutainer lithium heparin tubes (BD Biosciences, San Jose, Calif., USA) containing RPMI 1640 with 10% fetal calf serum (holding medium); fluids were collected directly into Vacutainer lithium heparin tubes. All detailed procedures for FNA collection and cell processing have been previously published [4, 11].

The STA used the following monoclonal antibody (MoAb) combinations:

- for samples evaluated between 2011 and 2014 (74 specimens): (κ +CD8) fluorescein isothiocyanate (FITC)/(λ +CD4) phycoerythrin (PE)/7-aminoactinomycin D (7-AAD)/CD10 phycoerythrin-cyanin7 (PE-cy7)/CD5 allophycocyanin (APC)/(CD19+CD20) APC-H7/CD3 Horizon V450 (HV450)/CD45 Horizon V500 (HV500); all MoAbs were from BD Biosciences; 7-AAD was from Beckman Coulter, Marseille, France.
- for samples evaluated in 2015 (29 specimens): (κ +CD8) FITC/(λ +CD4) PE/7-AAD/(CD19+CD20) PE-cy7/CD10 APC/CD3 allophycocyanin-Alexa Fluor 750 (APC-A750)/CD5 Pacific Blue (PB)/CD45 Krome Orange (KrO); κ and λ were from BD Biosciences; the remaining MoAbs were all from Beckman Coulter.

All antibodies were previously titrated for optimal staining. After washing and resuspension, the samples were immediately acquired on FACSCanto II cytometers (BD Biosciences; configured with 3 lasers to detect up to 8 colors and analyzed with Diva software) for specimens evaluated between 2011 and 2014, or on Navios cytometers (Beckman Coulter; configured with 3 lasers to detect up to 10 colors and analyzed with Kaluza software) for specimens evaluated in 2015. The instrument fluidic was accurately washed prior to sample acquisition to minimize the risk of carryover and/or false-positive events; ungated flow-cytometric runs were continued to achieve maximum cellular acquisition, leaving no residual fluid in the collection tube. Cytometer performance was tested by applying daily quality control protocols as per the manufacturer's instructions. Verification of the compensation matrix was performed by analyzing normal peripheral blood cells labeled with CD8 in different fluorochrome conjugations depending on the instrument used for acquisition.

Viability assessment was made by FC using a 7-AAD dye that intercalates into double-stranded nucleic acids, employed to distinguish between viable cells (7-AAD negative) and apoptotic cells (7-AAD dimly positive) or late apoptotic-dead cells (7-AAD brightly positive) [12]. Viable cells were analyzed in a forward scatter (FS) versus a 7-AAD dot plot and, to accurately and reproducibly set the gate, the fluorescence intensity value of the control samples was considered. Cell suspensions from reactive lymph nodes with a cell viability $\geq 95\%$, determined by Trypan Blue exclusion, served as control samples. To obtain objective and reproducible information about the size of B lymphocytes, the FS signal distribution of B cells was expressed as a ratio (FSr), with the FS distribution obtained for T cells kept as an internal reference. The FSr was previously determined in the laboratory in reactive lymphadenopathy and NHL to be ≤ 1 for small cells, between 1.1 and 1.29 for medium cells, and ≥ 1.3 for large cells (all NHL used for the calculation of FSr had a histologic diagnosis). Samples were considered positive for lymphoma when a discrete population of cells, with phenotypic characteristics of a disease entity, was identified [13]. FC data were independently interpreted by the authors (A.S., A.D., and S.A.) blinded to the cytomorphology findings. All detailed procedures for the cytomorphologic analysis of FNA have been previously described [4]. In the case of effusions, fresh specimens were centrifuged at 2,000 rpm for 10 min. Two to 4 drops of the sediment were used for the preparation of 1 Papanicolaou-stained slide and 1 H&E-stained slide. The resulting cell pellet was fixed in a test tube containing 95% alcohol for the subsequent preparation of cell blocks.

Statistical Methods

Median values were calculated for the quantitative variables. The Mann-Whitney U test was used to analyze the values of viable cells in EUS-FNA versus FNA and versus BF, as well as the values of lymphocytes [total; T, B, and (natural killer) NK cells, and CD4/CD8 ratio] in NHL versus benign/reactive processes (B/R) and versus carcinoma (Ca) samples. $p \leq 0.05$ was considered statistically significant.

Fig. 1. Example of the STA and gating strategy applied to FNA in a follicular lymphoma (case 11) acquired on a FACSCanto II flow cytometer (BD Biosciences). 7-AAD-negative cells (orange) were gated in order to exclude dead cells. FSC-W^{low}/CD45⁺ events were gated to define the leukocyte area and to exclude glued cells (doublets; green). Gated cells were examined in the SSC/CD45 diagram for the identification of lymphocyte clusters (gray). A region drawn on (CD19+CD20)⁺/CD3⁻ events identified the B cells (blue) and allowed the analysis of surface light chains and CD5 and CD10 expression on B lymphocytes. A region drawn on CD3⁺/(CD19+CD20)⁻ events identified T cells (purple) and allowed the quantification of CD4 and CD8 subpopulations and CD5 expression. CD3⁻/(CD19+CD20)⁻ events (non-T/non-B) were considered to be NK cells. The ratio of FSC-A signal distribution between B and T lymphocytes was used to evaluate B-cell size. Population hierarchy (bottom right). Gating on B cells displayed polyclonal and surface light chain-negative elements corresponding to CD10⁺ events (red; 25% of the total lymphocytes). FSC-A = Forward-scatter area; FSC-W = forward-scatter width; SSC-A = side-scatter area.

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Table 1. Summary of the parameters analyzed in the 103 samples

	Samples, n	Viable cells, %	Lymphocytes, %	B cells, %	T cells, %	CD4/CD8 ratio	NK cells, %	FS-r, n		
								≤1	1.1–1.29	≥1.3
<i>Samples</i>										
EUS-FNA	41	71.00 (18–99)								
FNA	40	77.00 (4–97)								
SCE	22	70.00 (6–99)								
<i>Diagnosis</i>										
NHL	31	71.50 (5–95)	94.00 (6–99)	47.50 (3–97)	45.50 (3–94)	1.90 (0.5–13.7)	3.00 (0–16)	17	6	8
B/R	42	70.00 (4–99)	74.50 (1–98)	10.50 (0–52)	73.00 (22–98)	2.55 (0.1–31.7)	9.00 (0–59)	39	3	–
Ca	27	74.00 (18–99)	38.00 (2–95)	11.00 (1–41)	75.00 (53–96)	2.30 (0.8–4.4)	10.00 (2–28)	27	–	–
HL	3	73.00 (69–97)	85.00 (62–97)	12.00 (7–27)	86.00 (71–91)	5.80 (3.9–7.3)	2.00 (2)	2	1	–
Total	103	72.50 (4–99)	74.00 (1–99)	16.00 (0–97)	70.00 (3–98)	2.30 (0.1–31.7)	6.00 (0–59)	85	10	8

Values are presented as medians (ranges) unless otherwise stated.

Results

The overall rationale of the analysis and of the gating strategy applied has been previously published [11]. Examples of the STA protocol with the different cytometers utilized in this study are showed in figures 1 and 2.

A total of 31 out of 103 (30%) samples positive for lymphoma were detected by FC; 72 out of 103 (70%) samples were considered negative by FC and were classified as B/R (n = 42), Ca (n = 21 of 27), or Hodgkin lymphoma (HL; n = 1 of 3) by CM; 6 Ca and 2 HL had subsequent histologic confirmation. Of note, FC signaled the presence of CD45-negative cells, not belonging to a hematopoietic lineage, in 5 patients who had a final diagnosis of Ca. A summary of the parameters analyzed for each sample type is reported in table 1. A comparison of the different variables among groups is reported in table 2; no comparison was attempted with HL due to low numbers. The percentage of viable cells ranged from 4 to 99% of the total cells recovered, with no statistically significant difference among the groups. The total lymphocyte number was significantly higher in NHL compared to B/R and Ca. Given that all NHL detected were of the B-cell type, this group

had the highest percentage of B cells and the lowest percentage of T cells. No lymphomas of the T-cell type were detected in this study. Between B/R and Ca, a significant difference was observed regarding the total lymphocyte number, but no significant differences were measured among lymphocyte subsets. No significant differences in CD4/CD8 ratio were observed in any comparison.

A summary of the findings in FC-positive specimens is reported in table 3. Viable cells represented less than 50% of the total cellularity in 7 specimens. Neoplastic B cells comprised less than 30% of the total lymphocytes recovered in 15 samples. B-cell size, evaluated based on the normalized FSr, was <1.3 in all samples belonging to low-grade lymphoma cases. The normalized FSr was ≥1.3 in 7 high-grade lymphoma cases and in Mantle cell lymphoma (MCL); only sample 30, i.e. a pericardial fluid belonging to a patient with a diagnosis of diffuse large B-cell lymphoma (DLBCL) in the pharynx, displayed an FSr <1.3.

CM was unable to detect lymphoma cells in 8 out of 31 (25.8%) samples considered positive by FC, and 2 out of 31 were considered only suspicious for NHL. Samples 1 and 14 displayed a high level of necrosis, with only isolated small lymphocytes. Sample 2b belonged to a patient with an MCL evaluated via analysis of 4 specimens taken from different sites. Results were concordant in FC and CM investigations on EUS-FNA biopsy of gastric mucosa of the body/antrum and fundus (both negative for lymphoma) and on ileum mucosa (positive for lymphoma). The discordant result concerned the EUS-FNA sample of duodenum mucosa, in which FC provided a clearly positive result while CM was able to detect only a small lym-

Fig. 2. Example of the STA and gating strategy applied to an FNA (rectum lesion) in a marginal-zone lymphoma (case 27) acquired on a Navios flow cytometer (Beckman Coulter). The STA and gating strategy described in figure 1 allowed the detection of a small cluster of κ^{weak} -positive B cells, consisting of 2.5% of the total lymphocytes. FS INT = Forward-scatter integral; FS TOF = forward-scatter time of flight; SS INT = side-scatter integral.

Table 2. Comparison of data obtained among the groups

	Samples, n	Group	Viable cells	Lymphocytes	B cells	T cells	NK cells	CD4/CD8 ratio
<i>Sample</i>								
EUS-FNA	41	A	A vs. B: 0.93	–	–	–	–	–
FNA	40	B	A vs. C: 0.95	–	–	–	–	–
SCE	22	C	B vs. C: 0.83	–	–	–	–	–
<i>Diagnosis</i>								
NHL	31	D	–	D vs. E: 0.0002	D vs. E: 0.0001	D vs. E: 0.0001	D vs. E: 0.0002	D vs. E: 0.98
B/R	42	E	–	D vs. F: 0.0001	D vs. F: 0.0001	D vs. F: 0.0002	D vs. F: 0.0001	D vs. F: 0.91
Ca	27	F	–	E vs. F: 0.002	E vs. F: 0.67	E vs. F: 0.74	E vs. F: 0.48	E vs. F: 0.92

Table 3. Summary of the findings in the FC-positive samples

Case No.	Stage	Sample	Viable cells, %	Lymphocytes, %	B cells/clonal cells, %	FSr	FC	CM	Histological or molecular data	Final diagnosis
1	R	EUS-FNA pelvic lymph node	75	96	97/97	<1	(CD19+CD20)+/CD5+/λ+	CM–	ND	SLL
2	N	EUS-FNA ileum	62	96	66/66	1	(CD19+CD20)+/CD5+/λ+	CM+	EB	MCL
2b	N	EUS-FNA duodenum	31	95	47/47	<1	(CD19+CD20)+/CD5+/λ+	CM–	EB	MCL
3	R	EUS-FNA lung	88	30	29/29	1.2	(CD19+CD20)+/Igs–	CM+	ND	MALT
4	R	EUS-FNA pancreatic lymph node	84	81	62/62	<1	(CD19+CD20)+/CD5+/λ+	CM+	ND	SLL
5	N	EUS-FNA adrenal gland	98	96	77/77	1.1	(CD19+CD20)+/κ+	CM+	EB	MALT
6	N	FNA laterocervical lymph node	96	97	17/6	1.7	(CD19+CD20)+/κ+	CM+	EB	TCRBCL
7	R	FNA hepatic	87	99	57/57	1	(CD19+CD20)+/CD5+/λ+	CM+	ND	SLL
8	N	FNA laterocervical lymph node	18	75	21/21	1.2	(CD19+CD20)+/CD10+/κ+	CM+	EB	FL
9	N	Ascites	96	93	3/1	<1	(CD19+CD20)+/CD10+/λ+	CM–	EB	FL
10	R	FNA scapular lesion	95	87	86/86	<1	(CD19+CD20)+/CD10+/κ+	CM+	ND	FL
11	N	FNA laterocervical lymph node	94	99	58/25	1	(CD19+CD20)+/CD10+/Igs–	CMi	EB	FL
12	N	EUS-FNA hepatic lesion	99	59	14/14	1	(CD19+CD20)+/CD5+/κ+	CM+	BMB	SLL
13	N	EUS-FNA hepatic hilum lymph node	85	80	34/20	1	(CD19+CD20)+/CD5+/κ+	CMs	BMB	SLL
14	N	FNA laterocervical lymph node	6	74	72/72	1	(CD19+CD20)+/CD10+/λ+	CM–	EB	PTLD
15	R	FNA laterocervical lymph node	60	37	38/38	1.3	(CD19+CD20)+/κ+	CM+	ND	DLBCL
16	N	FNA breast	58	98	23/1	1.9	(CD19+CD20)+/Igs–	CM+	EB	DLBCL
17	R	EUS-FNA perigastric lymph node	94	96	63/63	1.7	(CD19+CD20)+/CD5+/λ+	CM+	FISH	MCL
18	R	FNA axillary lymph node	45	82	62/62	1.2	(CD19+CD20)+/CD5+/κ+	CM+	ND	SLL
19	R	FNA laterocervical lymph node	49	94	22/22	1	(CD19+CD20)+/CD10+/κ+	CM+	ND	FL
20	R	FNA rectum lymph node	88	99	28/11	1	(CD19+CD20)+/CD10+/λ+	CM+	ND	FL
21	R	FNA liver	59	94	91/91	1.5	(CD19+CD20)+/CD10+/κ+	CM+	ND	DLBCL
22	R	Pleural fluid	61	98	26/26	<1	(CD19+CD20)+/CD5+/κ+	CM+	ND	LPL
23	N	FNA submandibular	35	90	70/70	1.5	(CD19+CD20)+/CD5+/κ+	CMs	EB	DLBCL
24	R	Ascites	21	67	47/47	1.4	(CD19+CD20)+/κ+	CM+	ND	DLBCL
25	N	FNA thyroid	63	98	97/97	1.8	(CD19+CD20)+/CD10+/Igs–	CM+	EB	BL
26	N	Pleural fluid	68	83	26/26	<1	(CD19+CD20)+/κ+	CM–	BMB	MZL
27	N	FNA rectum lesion	83	97	5/2.5	1	(CD19+CD20)+/κ+	CM–	EB	MZL
28	R	FNA paravertebral lesion	88	99	81/81	<1	(CD19+CD20)+/λ+	CM+	ND	FL
29	R	FNA lung	87	19	15/12	1.1	(CD19+CD20)+/Igs–	CM+	ND	FL
30	R	Pericardial fluid	79	78	6.5/6.5	1.1	(CD19+CD20)+/λ+	CM–	ND	DLBCL

BL = Burkitt lymphoma; BMB = bone marrow biopsy; CM+ = cytomorphology positive for lymphoma; CM– = cytomorphology negative for lymphoma; CMs = suspicious cytomorphology; CMi = inconclusive cytomorphology; EB = excisional biopsy; FL = follicular lymphoma; LPL = lymphoplasmacytic lymphoma; MALT = extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue; MZL = marginal zone lymphoma; N = newly diagnosed; ND = not done; PTLD = posttransplant lymphoproliferative disorder; R = relapse; SLL = small lymphocytic lymphoma; TCRBCL = T-cell-rich B-cell lymphoma.

phoid aggregate in chronically inflamed mucosa, not suggestive of lymphoma involvement. Sample 11 was hematologic and was considered insufficient for a diagnostic conclusion. Sample 27 displayed a heterogeneous pattern of small to medium lymphocytes without significant morphologic alterations. SCE specimens No. 9, 26, and 30 were all considered negative by CM, being composed of a heterogeneous pattern of lymphocytes, inflammatory cells, and reactive mesothelial cells.

The absence of an altered immunophenotype was observed in 72 out of 103 (70%) samples, including 42 B/R cases and 21 specimens in which a Ca was detected by CM, confirmed by a subsequent surgical biopsy in 13 out of 42 and 15 out of 27 cases, respectively. The FSr value of B cells was, in these cases, always ≤1.1. As expected, no immunophenotypic abnormalities were detected by FC in 3 cases of HL.

Fig. 3. Example of the STA and gating strategy applied to an FNA sample (case 14) with a high number of dead cells. The STA and gating strategy described in figure 1 allowed the detection of a small number of viable CD10⁺ λ B cells. FSC-A = Forward-scatter area; FSC-W = forward-scatter width; SSC-A = side-scatter area.

Discussion

One of the factors that hamper immunophenotyping investigations in FNA and body fluids is the low number of cells that can be processed. Therefore, the set-up of a single tube, based on patient information and clinical suspicion, is frequent in FC laboratory practice. In this study, we report our experience with an FC assay that was developed in order to gather the greatest amount of information from hypocellular samples sent for lymphoma suspicion or monitoring [11]. The method used different MoAbs cocktailed in one tube to detect and identify lymphocytes, as well as a logical gating strategy to gate out dead cells and get rid of debris and clumped cells (doublets). We have already used a similar approach to detect occult lymphoma cells in cerebrospinal fluid with good results [14].

Multicolor FC evaluation of a single tube for paucicellular samples has been previously proposed by others in the literature [15–18]. Langerak et al. [15], using 8-color labelling (small sample tube) for screening of lymphomas, detected 13 positive cases in a cohort of 164 small cell samples consisting of cerebrospinal fluid and vitreous biopsies. Preijers et al. [16] proposed a 10-color panel to detect leukemia and lymphoma cells in small cell samples, but they did not report data on specimen types.

Rajab and Porwit [17] and Rajab et al. [18] developed a 10-color 14-antibody tube for screening of abnormal lymphoid populations and myelodysplasia in bone marrow samples, which was also reported to be useful in the evaluation of paucicellular cytologic specimens [17, 18]. None of these studies included a vital dye in the protocol.

Our study was mainly done on specimens like FNA and EUS-FNA, which may contain high amounts of debris, dead cells, or aggregates, particularly if coming from extranodal sites (39% of the samples in this study). Debris may originate from the damage and disintegration of cells following apoptosis or fragmentation associated with FNA and body fluid aspiration procedures, even if they are minimally invasive. Dead cells may be the result of cell fragility and necrosis that may occur in neoplastic cells. Aggregates may derive from the presence of nonhemopoietic cells. All of these aspects could affect the overall quality of the specimens and the result of the FC analysis, and then adequate gating strategies must be applied in order to remove unwanted events [11]. A viability dye and a doublet discrimination gate were introduced for these reasons into the assay. In this way, we were able to obtain meaningful results also in samples with a viability <50%, usually discarded for FC investigations; an exam-

ple is showed in figure 3. Using the STA, we detected NHL of the mature B-cell type in 31 samples; 7 had a viability <50%. In these cases, CD19 and CD20 allowed the identification of B cells; κ and λ allowed the detection of clonally restricted B cells (fig. 1); CD5 and CD10 allowed either subtyping of lymphomas or the detection of neoplastic cells in cases where clonal restriction was not evident, as neoplastic cells were mingled with normal B cells (fig. 2). The calculation of FSr gave a reliable estimation of B-cell size in all but one case, i.e. pericardial fluid in a patient with high-grade lymphoma in the pharynx. The neoplastic population described in the histologic report consisted of a mixture of medium to large cells. We cannot exclude that delays in delivery or improper conservation may have affected the cell size in this sample. On the other hand, we could hypothesize that medium-sized neoplastic cells selectively colonized the pericardial serous cavity in this patient.

Using the STA, we detected lymphomatous cells in 8 samples in which CM gave a negative or inconclusive result and in 2 cases considered only suspicious. Difficulties in morphologic detection of lymphomas in FNA and EUS-FNA may be related to sampling errors, tissue necrosis, a scarcity of tumor cells, a loss of tissue architecture, and difficulties in recognition of malignancy due to a morphologic overlap between neoplastic and nonneoplastic lymphocytes [19]. Analogously, in SCE morphologic distinction between reactive and malignant cells can be difficult in cases of hypocellular and bloody specimens, or if malignant cells are sparse, so that even experienced cytomorphologists are unable to render a conclusive diagnosis [20, 21].

In this study, FC excluded a neoplastic lymphoproliferative process in 69 cases in which no evidence of monotypic B cells or atypical T cells was found. FC has been proven to be of great value when there is a diagnostic dilemma between a hematologic and a reactive or inflammatory condition in which the lymphocyte infiltrate mimics lymphoma [22, 23]. Small round blue neoplasms such as neuroendocrine tumors or poorly differentiated Ca and melanoma may be another diagnostic dilemma in which FC can contribute to a differential diagnosis towards lymphomas [24, 25]. The presence of CD45-negative elements of nonhematopoietic origin was signaled by FC in 5 patients who received a final diagnosis of Ca. The emerging role of FC for screening of Ca metastasis has been recently evidenced [26], but that is beyond the scope of this study.

We did not attempt to detect Reed-Sternberg cells by FC. It must be noted, however, that cases with a final di-

agnosis of HL had a prevalence of the CD4⁺ T-cell subset; this findings has been previously reported to be highly suggestive of this type of lymphoma [27].

Optimization of FNA collection techniques, submission of larger volumes of body fluids, and a fast specimen delivery may contribute to reducing the number of paucicellular samples. The results here presented are evidence of the efficacy of the STA approach for the detection of neoplastic cells in paucicellular samples, also when these are of poor quality. The STA was able to detect or exclude NHL in hypocellular samples by using different antibody cocktails and different instrumentations and software for the analysis. Using the described approach, we have demonstrated that, even in the case of specimens usually considered inadequate for FC analysis, appropriate sample processing, gating strategies, and multicolor protocols

can help to reduce false-negative or nondiagnostic cases in the workup of lymphomas. The increase in the number of fluorescence channels available for immunophenotyping and the use of targeted antibodies will further improve the detection of hematologic malignancies, as well as their follow-up and monitoring.

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Disclosure Statement

The authors have no conflicts of interests to declare.

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