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LETTER TO THE EDITOR

CLINICAL CYTOMETRY WILEY

ESCCA/ISCCA survey on the use of multicolor flow cytometry in the detection of cerebrospinal fluid involvement in hematological malignancies: How close does real-life adhere to the recommendations?

In hematologic malignancies (HM), involvement the central nervous system (CNS) remains a major limitation to achieving long-term cure and an important cause of mortality, especially when a chemotherapy-free treatment approach is used. An early detection of CNS involvement can therefore impact patients' outcome. Over the past 20 years an increasing number of publications have demonstrated that multiparameter flow cytometry (MFC) is superior to conventional cytology (CC) to detect CNS involvement in patients affected by several HM, including lymphoma, acute lymphoblastic leukemia, and acute myeloid leukemia (Subirá et al., 2001). However, the flow cytometric investigation of body fluids at non hematologic sites may present certain pitfalls, in the case of cerebrospinal fluid (CSF), these are chiefly hypocellularity and rapid loss of cell viability.

Previously, a joint working group of the European Society for Clinical Cell Analysis – (ESCCA) and the Italian Society for Clinical Cell Analysis (ISCCA) discussed the critical points of CSF handling, highlighting problems related to sample storage and processing, as well as interpretation and reporting of data, and provided recommendations (Del Principe et al., 2021). To harmonize all critical steps mentioned above and to evaluate the probable lack of standardization in CSF analysis across laboratories, Debliquis et al. (2024) recently carried out a survey to compare the real-world practices for the detection of cerebral lymphoma in CSF samples using MCF, with those recommended by ESCCA/ISSCA. The survey targeted a network of French-speaking biologists, neurologists and hematologists involved in oculocerebral lymphoma diagnostics, (CytHem/LOC French network) including the use of MCF. This study highlighted incomplete agreement between the 'real-world' practices in French-speaking countries and the ESCCA/ISCCA recommendations. This study, and the general wish to strive toward harmonizing practices across laboratories, with the view to aid and ensure a high and consistent quality of diagnostic information across laboratories for these often-complex diagnoses, led us to design another survey to reach and compare a larger number of laboratories around the world.

Our survey consisted of 16 questions, shown in full in Supplementary Material, The invitation to participate was sent to laboratories along with UKNEQAS Leucocyte Immunophenotyping programs to over 400 UKNEQAS participants during March–April 2024. Overall, one hundred and thirteen colleagues in cytometry laboratories from 23 countries answered our survey (Table 1), confirming the worldwide interest in this topic. The responses were from biologists (30%), PhD (25%), MD/PhD (15%), BS biomedical scientists/technicians (13%), and the remaining 17% from other professionals. The majority (57%) who answered our questionnaire claimed to have over 10 years' experience doing MFC, and to process >50 samples per year. A total of 93% worked in clinical laboratories, indicating that clinical labs processing CSF are more likely to be participants in UKNEQAS than non-clinical labs, and also that they are highly interested in this topic, due to its practical implications. A minority of responses were from research laboratories (7%).

Notably, while CC still remains the gold standard for diagnosis of CNS involvement in HM, 43% of our colleagues feel confident with MFC to report "occult CNS disease", namely the condition of CC negativity and MFC positivity (based on predefined criteria). As shown in Supplementary Material, about 8% of labs do not perform MFC exam in absence of CC positivity, since MFC does not play a central role in their diagnostic process. However, 24% of surveyed answers to this question were heterogeneous, indicating that in some centers CC and MFC are performed by different laboratories that do not interact very much with each other (Supplementary Material). However, the message that emerges from the responses is that the two analytical tools are mostly used in an integrated way, to confirm the diagnosis in a more robust manner.

Regarding the quality of CSF samples, nearly 40% stated that all samples delivered to the lab are adequate and processable according to their predefined requirements (i.e., sample volume, presence of ≥1000 identifiable cells, compliance to the internal quality controls). In contrast, 46% stated that sample quality is good in more than half of the cases but not in all of them. Furthermore, the amount of CSF sample sent for MFC analysis is usually less than 2 mL, and only 38% of responders receive between 2 and 10 mL of CSF. This observation is in line with the French survey. In fact, although according the "Consensus guidelines for lumbar puncture in patients with neurological diseases" (Engelborghs et al., 2017), the of collection up to 30 mL CSF is considered well tolerated and safe, most hematologists withdraw a much lower volume of CSF for fear of complications. Given the scarce cellularity of CSF samples, the ESCCA/ISCCA document recommended the acquisition of at least 1000 total events because this

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TABLE 1 Cytometry laboratories from different countries involved in survey.

Australia	4
Belarus	1
Belgium	4
Brazil	1
Canada	1
France	33
Germany	2
Greece	5
Hungary	1
Iceland	3
Italy	37
Kuwait	1
Latvia	1
Luxembourg	2
Morocco	1
Netherlands	3
Norway	1
Paraguay	1
Portugal	1
Romania	1
Spain	2
UK & Ireland	7

improves the chances of identification (Del Principe et al., 2021). However, among the clinical labs that responded to our survey, only 6%; were able to acquire at least 1000 events 90% of the times, while 55% acquire 1000 events in less than 50% of the cases, depending on the amount (and the cellularity) of the sample received. Greater agreement between ESCCA/ISCCA recommendations and the surveyed labs was observed regarding the panels used. In fact, the ESCCA/ISCCA document recommended a "have to explain all events" strategy with 8 or more color antibody panel to investigate the first diagnostic CSF extensively, especially in the absence of peripheral blood (PB) or bone marrow samples that can provide a diagnostic phenotype, or where there is a lack of comprehensive information about the patient. Our survey showed that 77% of laboratories use a monoclonal antibodies panel of 8–12 markers while 15% utilize a panel with more than 12 colors. Currently, only a minority of labs (8%) process CSF with panels of less than 8 colors. To the question of how many tubes are usually used, the answers were: (a) one tube for absolute count and one tube for immunophenotyping (23%), (b) just one tube (without cytometric absolute count) (49%), (c) two or more tubes, even without cytometric absolute count (22%), (d) about 6% of responders use one tube for absolute count and two or more tubes for immunophenotyping.

A further question in our survey regarded the analysis of cytoplasmic antigens, given that the cell loss associated with intracellular staining could limit its use. In agreement with ESCCA/ISCCA recommendations, 54% of cytometrists do not perform the analysis of cytoplasmic antigens, while 35% do it only in selected cases, such as CD3 negative T acute lymphoblastic leukemia, myeloma or diffuse large cell lymphoma with no surface light chain expression. A total of 11% of respondents always include cytoplasmic antigens.

Once collected, the viability of CSF cells is usually short. In order to obtain optimal results, delay in sample transportation and processing must be minimized. It is recommended that the CSF sample should be processed within 60 min from lumbar puncture. Comparing our recommendation with our survey, 25% of centers are able to process specimens within 60 min of collection, while 42% of centers receive specimens, 60 to 90 min after the lumbar puncture is performed. In the ESCCA/ISCCA position paper, we suggested the use of Transfix™ (Cytomark, UK) stabilizer, preferably TransFix CSF storage tubes, when the expected time between sampling and processing is longer than 90 min. However, the familiarity with TransFix CSF storage tubes is reported by only 60% of laboratories. Among the Transfix users, more than 80% do not observe changes in the expression of any cellular markers. About 17% reported the changes of the intensity of CD45, CD4, CD8, CD5, CD14, and CD7, compared to native CSF. Further, some laboratories use "other (putatively) stabilizing materials", such as RPMI, Fetal bovine serum or EDTA, which usage is however not properly validated to this aim.

As expected, one of the most critical discussion points was found to be the calculation of absolute cell count. In the ESCCA/ISCCA paper, the use of MFC and counting beads was recommended, but in the recent survey by Debliquis et al., 86% of centers obtained white blood cell (WBC) and red blood cell (RBC) concentrations by manual counting on a hemocytometer (counting chamber). Determining absolute WBC counts in CSF via MFC was an exception (4%). In our investigation, this approach was heterogeneous in the responding centers: 49% quantify the white cell content in CSF by manual counting in chamber, while only 23% utilize MFC and counting beads. Another 27% state the use of other techniques, probably automated cell counters. Certainly, MFC counting method is expensive, but as first demonstrated with leuco-reduced blood products and more recently with CSF is very accurate, linear and reliable in paucicellular samples, down to extremely low concentrations (Subirá et al., 2001).

Another crucial point in CSF analysis is to exclude the PB contamination. Most of the responders (40%) evaluate/quantify the red blood cell contamination in CSF samples during the manual counting in chamber by visual microscopy and only 6% use MFC, white and red cell markers and counting beads. As shown in Supplementary Material, 28% of responders declared to evaluate/quantify the red blood cell contamination with other techniques not further specified, and since MFC, cytospin and counting chamber were already listed, we are frankly unable to guess the alternative methods. This is a limitation of our study, worth further analysis in the future.

In our opinion, to rule out PB contamination and to increase the assay's diagnostic accuracy in HM, as leukemias or lymphomas with circulating neoplastic cells, it is best to process a PB and CSF sample from the same patient, in parallel (Del Principe et al., 2021). A total of 13% of respondents test PB in all instances, while 48% evaluate PB in

TABLE 2 Summary of major discordances.

Issue	
Scarce Cell Events in CSF	Acquisition of at least 1000 total events is carried out 90% of the time by less than 10% of the laboratories and in less than 50-of the time by 56%
White cell content in CSF	The quantification the white cell content is carried out by manual counting in chamber in 49%
Time of Processing and Transport	The CSF sample is processed within less to 60 or 60–90 min from harvesting by 68% of responders
Threshold	The threshold was more than 20 clustered events for 44.16% of responders

Abbreviation: CSF, cerebrospinal fluid.

selected cases, mainly when atypical cells are present in the PB smear, as suggested in the ESCCA/ISCCA document.

One of the points that remains a matter of debate is the choice of the threshold to define the MFC positivity. In fact, the "threshold question" showed the greatest discordance of all responses: (a) A total of 36% of participants define the sample as MFC positive in the presence of at least 10 clustered and phenotypically aberrant events, (b) A total of 44% in the presence of more 20 events and 12% set the threshold at >30 events. Eight % use other not further specified criteria. Such heterogeneous responses confirm the usefulness of our survey and warrant the need to continue to develop networks, working groups, and to adjust recommendations to better harmonize the evaluation of CSF in HMs.

The last question of our international survey asked what clinical indications are used to trigger a request for MFC, whether only for patients with current or prior HM or not. Our survey showed that the use of MFC is more widespread. 34% of responses stated MCF is used in CSF diagnostics only in the context of known current or prior HM. About half of the participants employ MFC also in hypercellular CSF samples of unclear etiology, in which bacterial or viral meningitis have been ruled out (48%), while 13% process all CSF samples with MFC, irrespective of the clinical question.

The discordance demonstrated here suggests that, for the same CSF sample, the evaluation of HM would potentially be reported differently by different laboratories. The major discrepancies are in issues concerning the acquisition of total events, the threshold for positivity, the methods for quantifying white and red blood cells, and the type of samples on which to perform the cytometric examination (see Table 2 summary). This highlights the usefulness of our study and warrants the need to continue to increase interaction between different involved subjects, such as physicians and cytometrists, and to harmonize laboratory practice. This survey highlights the need for more cooperation and better dialog among clinical laboratories in an attempt to harmonize the use of MFC in the detection of CSF involvement by HM. This may be achieved via creating inter-laboratory networks, publishing more specific guidelines, and more effective education. Agreement on the indication(s) to perform MFC, the basics of specimen processing, and on a minimal set of markers needed, would move the field closer to that goal.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest related to the submitted work.

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SUPPORTING INFORMATION

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