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EDITORIAL

EuroFlow: Resetting leukemia and lymphoma immunophenotyping. Basis for companion diagnostics and personalized medicine

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Laboratory diagnostics in patients with a hematological malignancy has three major applications: establishing the diagnosis, prognostic classification and evaluation of treatment effectiveness.[1,2] Immunophenotyping is currently recognized to provide essential information for all three applications.[2–9] Expression of individual immunophenotypic markers was initially assessed by microscopic techniques, but since the 90's multiparameter flow cytometric immunophenotyping has become the technique of choice, as it is the sole technique that fulfills the requirements for high speed, broad applicability at diagnosis and during follow-up, and accurate focusing on the malignant cell population using membrane-bound and intracellular proteins as targets.[9–14] Despite the objectivity of flow cytometric measurements, flow cytometry is perceived as a technique that is highly dependent on expertise and is regarded to have limited reproducibility in multicenter studies.[15–18] This probably relates to the increasing number of antibodies and fluorochromes that are used and the corresponding progressively larger complexity of the multivariate data analyses of both major and minor cell populations, together with limited standardization of the laboratory procedures and instrument settings. In this regard, the weakest points of multiparameter flow cytometry relate to: (i) the design of the panels of markers to be applied, (ii) the evaluation of new versus 'classical' markers, (iii) the analysis of the data obtained from the flow cytometric measurements and (iv) interpretation of the results. In addition, it is a technological field that is continuously evolving, but where many traditional procedures are still in use, for example, for data analysis. Whereas industry invested significantly in developing and implementing further innovation of the flow cytometry instruments, the innovation in immunophenotyping reagents and in software for analysis of the progressively larger and complex data sets was much more limited or virtually absent, particularly in the area of leukemia and lymphoma typing.

Consequently, we concluded in 2005 that major innovations are required to adequately advance the field of flow cytometric immunophenotyping. Therefore, we initiated the European Union (EU)-supported EuroFlow Consortium. The original objectives of the EuroFlow Consortium were: the development and evaluation of novel antibodies, the introduction of novel immunobead technology, the development of novel flow cytometry software tools and data analysis approaches for recognition of complex immunophenotypic patterns, and the design of novel multicolor immunostaining protocols and carefully balanced antibody panels. In this editorial, we critically comment on the most relevant aspects of the EuroFlow activities through a series of frequently asked questions from the field.

WHAT IS THE EUROFLOW CONSORTIUM?

EuroFlow is an independent scientific consortium that aims at innovation and standardization of flow cytometric immunophenotyping to further improve and progress diagnostic patient care. The EuroFlow Consortium was formed in 2005 to initiate the EU-FP6 funded EuroFlow project (LSHB-CT-2006-018708), which started in April 2006. The group was initially composed of >40 researchers from eight different public university hospital-based institutions in eight distinct European countries, and two small/medium enterprises (SMEs), with complementary experience and knowledge in the field of flow cytometry immunophenotyping of hematological malignancies (Table 1, Figure 1). More recently, the EuroFlow Consortium has become a Scientific Working Group of the European Hematology Association and it has expanded to a total of 11 institutions in Europe and America (Table 1, Figure 2). Whereas the EU-FP6 funded EuroFlow project required the active and crucial contribution of the two SMEs, they left the consortium per 2012 to retain the full scientific independence of the EuroFlow Consortium. In parallel, the scientific activities of the group have extended to other clinical diagnostic areas in the format of well-defined workpackages and projects. This includes the development of $\geqslant$8-color antibody panels for lymphocyte subset studies in blood and bone marrow of patients suspected to have a primary immunodeficiency (PID Workpackage). Consequently, several additional affiliated participants are currently being included for this new workpackage.

WHAT WERE THE INITIAL AIMS OF THE EUROFLOW PROJECT?

The general aims of the EuroFlow project were the development and standardization of fast, accurate and highly sensitive flow cytometry approaches for diagnosis and (sub)classification of hematological malignancies, as well as for the evaluation of treatment effectiveness during follow-up. In the first 4 years (2006–2010) activities were exclusively focused on the diagnosis and (sub)classification of hematological malignancies. Five specific aims were addressed in this period: (1) to evaluate the utility of new antibodies developed either by the EuroFlow members or other institutions and companies, (2) to introduce novel flow cytometry immunobead assays for the detection of fusion proteins to be used for characterization of acute leukemias, (3) to define multicolor flow cytometry protocols and comprehensive antibody panels for the diagnosis and classification of hematological malignancies, (4) to create novel software tools for recognition of complex immunophenotypic patterns and multivariate analysis of flow cytometric data and (5) to promote standardization of flow cytometric immunophenotyping.[19]

WHAT ARE THE CURRENT ACHIEVEMENTS OF THE EUROFLOW CONSORTIUM?

After a period of 5 years, the EuroFlow Consortium has reached most of its initial goals, a large part of the results obtained being presented in this issue of the *Leukemia* journal. In this period, 8-color antibody protocols for the diagnosis and classification of hematological malignancies have been developed.[20] Such protocols consist of a sequential combination of (i) screening

Table 1. List of initial and current EuroFlow members

Initial EuroFlow members (April, 2006)			*Current EuroFlow members (January, 2012)*		
Institute	*Senior scientist*	*Other participants*	*Institute*	*Senior scientist*	*Other participants*
Erasmus MC, Rotterdam, NL	JJM van Dongen	VHJ van der Velden, J te Marvelde, H Wind, B van Bodegom	Erasmus MC, Rotterdam, NL	JJM van Dongen	VHJ van der Velden, AW Langerak, J te Marvelde, H Wind, B van Bodegom, WM Comans-Bitter
University of Salamanca, ES	A Orfao	JF San Miguel, J Almeida, J Flores-Montero, MB Vidriales, JJ Pérez-Morán, Q Lecrevisse	University of Salamanca, ES	A Orfao	JF San Miguel, J Almeida, J Flores-Montero, MB Vidriales, JJ Pérez-Morán, Q Lecrevisse
Dynomics, Rotterdam, NL	F Weerkamp	K Brouwer-de Cock	Instituto Portugues de Oncologia, Lisbon, PT	P Lucio	M Gomes da Silva, J Caetano, T Faria
Cytognos, Salamanca, ES	M Martín-Ayuso	J Hernández, M Muñoz, J Bensadón	University of Schleswig-Holstein – Campus Kiel, DE	M Kneba	S Böttcher, M Ritgen, M Brüggemann, E Harbst, L Falck
Instituto Medicina Molecular, Lisbon, PT	A Parreira	P Lucio, M Gomes da Silva, J Parreira, A Mendonça	Hôpital Necker-Enfants Malades, Paris FR	E Macintyre	L Lhermitte, V Asnafi, A Trinquand
University of Schleswig-Holstein – Campus Kiel, DE	M Kneba	S Böttcher, M Ritgen, M Brüggemann, V Krull	Charles University, Prague, CZ	J Trka	J Hrusak, T Kalina, E Mejstrikova, V Kanderova, D Thürner
Hôpital Necker-Enfants Malades, Paris, FR	E Macintyre	L Lhermitte, V Asnafi	Medical University of Silesia, Zabrze, PL	T Szczepanski	L Sędek, J Bulsa, A Sonsala
St James University Hospital, Leeds, UK	S Richards	AC Rawstron, PA Evans, R de Tute, M Cullen	Federal University of Rio de Janeiro, BR	CE Pedreira	ES da Costa
Charles University, Prague, CZ	J Trka	J Hrusak, T Kalina, E Mejstrikova, M Vaskova	Dutch Childhood Oncology Group, The Hague, NL	E Sonneveld	AJ van der Sluijs-Gelling, A Koning-Goedheer
Medical University of Silesia, Zabrze, PL	T Szczepanski	L Sędek	University Hospital Gasthuisberg, Leuven, BE	N Boeckx	
			University of Porto, PT	M Lima	AH Santos

Abbreviations: BE, Belgium; BR, Brazil; CZ, Czech Republic; DE, Germany; ES, Spain; FR, France; NL, The Netherlands; PL, Poland; PT, Portugal; UK, United Kingdom.

Figure 1. EuroFlow members attending the first EuroFlow meeting held in Salamanca (April, 2006).

tubes adapted to address distinct clinical questions and specific medical indications of immunophenotyping and (ii) multi-tube panels for the diagnosis and classification per disease category. The development of the new 8-color protocols was paralleled by a set of standard operating procedures (SOP)[21] to assure full technical standardization of multicolor flow cytometry based on 3-laser flow cytometry instruments, selection of appropriate fluorochromes, standardization of instrument settings and laboratory protocols, and detailed testing and comparison of antibody clones and fluorochrome-conjugated antibodies from multiple companies.[20] For this purpose, development and implementation of new software tools for fast and easy handling of large data files,[22,23] combining multiple tubes and mapping of leukemia samples against templates of normal and pathological reference samples for fast multidimensional pattern recognition,[23] appeared to be crucial. Finally, new antibody clones were developed against carefully selected epitopes of proteins involved in chromosomal translocations, to be used in immuno-bead assays for detection of the most frequent fusion proteins in acute leukemias and chronic myeloid leukemia (CML).[24–26]

Figure 2. EuroFlow members attending the 19th EuroFlow meeting in Prague (October, 2011).

WHY DID IT TAKE SEVERAL YEARS TO DEVELOP THE EUROFLOW ANTIBODY PROTOCOLS?

With a few exceptions focused on specific diseases,[27,28] most antibody panels that have been proposed so far by consensus groups consist of lists of markers with limited or no information about reference clones or about the most adequate fluorochrome conjugates.[9,17,29–34] Also no guidelines are provided on how such markers should be combined in single-tube or multi-tube multicolor antibody panels. The composition of such lists of markers most frequently relies on 'expert opinions', based on experience and knowledge shared during meetings that run for a few days, where 'consensus' is reached by majority voting among the experts. Consequently, agreement about the informative and relevant markers is reached in a relatively fast way and the lists of consensus markers can be rapidly transferred to the public domain, for example, through one or more publications.[9,17,29–34] As consensus recommendations are based on longstanding experience of a major fraction of the group, markers with the lowest CD numbers (for example, CD1 to CD50) are more likely to be included as being informative, than the later defined antibody reagents (for example, CD100–CD400).[17]

During the first two meetings of the EuroFlow group in 2006 (Table 2), a preliminary list of consensus markers was composed for evaluation of informativity. The selected markers had to be combined in panels and arranged in multicolor combinations that, once applied to a given set of patient samples, would be capable of answering specific clinical questions with an acceptable degree of efficiency, greater than reached with the routinely applied panels in the EuroFlow centers. In other words, they had to be tested in parallel with the local panels, and their utility objectively evaluated to prove their informativity and superiority over existing panels. In practice, such evaluation of the preliminary consensus panels showed a need for improvement for every antibody panel. Consequently, this lead to multiple cycles (2–7) of (re)design and (re)evaluation of the 8-color antibody panels, in which new antibody clones and fluorochrome conjugates were evaluated on multiple cell samples per testing cycle.[20] On top of this, we carefully evaluated new (potentially informative) markers as well as classical markers for their added value or redundancy in the diagnosis and classification of hematological malignancies. The multiple cycles of antibody panel testing appeared very demanding and required a lot of effort in terms of reagents, personnel and logistics. This explains why the design of the EuroFlow antibody panels took more than 3 years.

HOW WERE THE EUROFLOW ANTIBODY PANELS DESIGNED AND TESTED?

The strategy used to design and test the different markers and 8-color combinations arranged in single tubes or multi-tube panels that constitute the EuroFlow antibody panels are described in detail in this issue of *Leukemia*.[20] The design process followed general rules and criteria. Overall two groups of markers were selected to be combined in each multicolor staining: (i) markers devoted to the identification of distinct cell populations in a sample (so-called *backbone markers*) and (ii) markers aimed at the characterization of particular cell populations (*characterization markers*). Backbone markers should efficiently identify both normal and malignant cells of interest with a high sensitivity and specificity. In multi-tube panels, backbone markers should be placed at the same fluorochrome position in every multicolor antibody combination, to provide identical multidimensional localization of the target cell population(s). If application of a screening tube was envisaged in the diagnostic algorithm before a multi-tube panel, the backbone markers of the screening tube were arranged at the same fluorochrome positions as in the related multi-tube panel, whenever possible. Through such strategy, automated gate setting for the definition of the target cell population(s) becomes possible. At the same time, the calculation procedures based on the nearest neighbor principle allow generation of data files, where each cellular event contains information about all parameters measured in the total set of multicolor antibody combinations.[22] In contrast to the backbone markers, each characterization marker is present in only one tube of a panel. Selection of characterization markers was based on experience and knowledge from the literature about the physiological role of the protein in normal cells, its expression pattern and its clinical utility in immunophenotyping of leukemia and lymphoma cells. For these markers, positioning in a specific combination was evaluated with respect to the diagnostic utility

Table 2. Summary of EuroFlow meetings and their main topics addressed

	City, Country	*Dates*	*Main topics of meeting*
1	Salamanca, ES	6–9 April 2006	Harmonization of informed consent forms and procedures List of consensus markers for evaluation of informativity Priority list of novel antibodies to be developed Introduction to multicolor flow cytometry Brainstorming on antibody protocols
2	Rotterdam, NL	20–23 September 2006	Standardization of instrument setup Choices of fluorochromes for the antibody protocols Introduction to Infinicyt software Development of immunobead assays for fusion gene proteins Preliminary proposals for backbone antibody testing per disease category
3	Prague, CZ	25–27 January 2007	First test results of BCR-ABL cytometric immunobead assay Final proposal for fluorochrome choices First proposals for choices of backbone markers First design for ALOT and LST
4	Kiel, DE	28–30 June 2007	First testing results of ALOT and LST and proposal for adjustments First design for PCD panel Confirmation of backbone markers and first proposal for B-, T- and NK-CLPD panels Final proposal for backbone markers for the AML/MDS and T-ALL panels
5	Lisbon, PT	22–24 November 2007	Fine tuning of ALOT, LST and PCD panels New design of B-, T- and NK-CLPD panels First testing results of BCP-ALL, T-ALL and AML/MDS panels and proposal for adjustment
6	Paris, FR	24–26 April 2008	Final proposals for the ALOT and PCD panels Fine tuning of BCP-ALL, T-ALL, AML/MDS, T- and NK-CLPD panels First results of standardization of immunostaining protocols Final SOP for instrument settings and compensation
7	Roosendaal, NL	25–26 June 2008	First proposal for standardized immunostaining protocols Ongoing testing of all panels First results of the testing of the BCR-ABL RUO immunobead assay
8	Kraków, PL	2–4 October 2008	First results of standardization of FCS and SSC scatter patterns Ongoing testing of all panels BCP-ALL and T-ALL panels ready to be used in prospective routine diagnostic testing *versus* conventional onsite panels
9	Schiphol, NL	14–15 December 2008	Final proposal for B-CLPD panel
10	York, UK	11–13 February 2009	Final proposal for standard sample preparation protocol Standard proposal for titration of antibodies First design of SST panel All other panels ready for collecting large series of samples for the EuroFlow database Introduction of multivariate analysis of testing results using the Infinicyt software PML-RARA immunobead assay ready for testing
11	Salamanca, ES	14–16 May 2009	Collection of samples for reference data files for the EuroFlow database for all panels Final proposal for SST panel Use of EuroFlow panels in routine diagnostics
12	Schiphol, NL	22–24 September 2009	Collection of samples for reference data files for the EuroFlow database for all panels
13	Lisbon, PT	14–16 January 2010	Collection of samples for reference data files for the EuroFlow database for all panels
14	Salamanca, ES	14–17 April 2010	Collection of samples for reference data files for the EuroFlow database for all panels First design and testing of MRD panels for various disease categories
15	Hoofddorp, NL	13–15 October 2010	Collection of samples for reference data files for the EuroFlow database for all panels First design and testing of MRD panels for various disease categories
16	Paris, FR	13–14 January 2011	Brainstorm meeting on ALL MRD panels
17	Lisbon, PT	20–21 January 2011	Brainstorm meeting on B-CLPD MRD panels
18	Leuven, BE	16–18 March 2011	Testing of adjusted MRD antibody panels for various disease categories Collection of samples completed for most EuroFlow panels
19	Prague, CZ	5–7 October 2011	Discussion on final draft of EuroFlow Antibody Panel manuscript Discussion on results of adjusted MRD antibody panels
20	Katowice, PL	21–23 March 2012	Discussion on results of adjusted MRD antibody panels

Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; ALOT, acute leukemia orientation tube; BCP, B-cell precursor; BE, Belgium; CLPD, chronic lymphoproliferative disorder; CZ, Czech Republic; DE, Germany; ES, Spain; FR, France; FSC, forward scatter; LST, lymphoid screening tube; MDS, myelodysplastic syndrome; MRD, minimal residual disease; NL, The Netherlands; PCD, plasma cell disorders; PL, Poland; PT, Portugal; RUO, research use only; SOP, standard operating protocol; SSC, sideward scatter; UK, United Kingdom.

of the combined markers. Each combination of backbone markers and backbone plus characterization markers was objectively evaluated using multivariate analysis strategies through the Infinicyt software (Cytognos SL, Salamanca, Spain).[23] Based on the results of the above described (re)design and (re)evaluation strategy, characterization markers were included or excluded.

CAN THE EUROFLOW ANTIBODY PANELS BE USED IN ANY FLOW CYTOMETER INSTRUMENT?

The EuroFlow panels were designed in such a way they would work in all 3-laser flow cytometry instruments, available at the moment the project started in 2006 and capable of simultaneously reading $\geqslant$8 fluorescence emissions, as described by Kalina *et al.*[21] However, by the end of 2009, new multi-color instruments became commercially available.[35] At that time, the design of most antibody panels was completed or in an advanced phase of testing. Consequently, it was not affordable for the EuroFlow Consortium to restart the testing of the antibody panels on the new instruments. However, the EuroFlow group is willing to advise or guide such testing. This requires close collaboration with the users and active involvement of the manufacturers of the new instruments.

DO THE EUROFLOW PANELS CONTAIN ALL 'CLASSICAL' OR WHO-RECOMMENDED ANTIBODIES?

The EuroFlow panels do contain virtually all 'classical' and WHO-recommended markers,[1,8,9,17,29,32,33,36,37] but some markers were left out from, for example, the acute leukemia panels (for example, CyCD22, CD11c and CyLysozyme) and the B-cell chronic lymphoproliferative disorder (B-CLPD) panels (for example,

FMC7). The deletion of these markers was based on their redundancy or inferior information, when compared with the set of other markers in the panel. As an example, markers like CyLysozyme and CD11c were found to be inferior compared to the combination of CD64, CD36, CD14 and CD300e (IREM-2) together with the expression pattern of CD117 and HLADR, for definition of early commitment to the monocytic lineage. Similarly, FMC7 is known to recognize a specific epitope on the CD20 molecule[38] and was found to be redundant and of no added value to the selected combinations of markers in the B-CLPD multi-tube antibody panel.

WHAT IS THE RELEVANCE OF THE NEW MARKERS IN THE EUROFLOW ANTIBODY PANELS?

Selection of a given marker to be included in the EuroFlow antibody panels was based on the type and quality of diagnostic information provided in combination with the other markers of the same panel. The contribution of the new markers is discussed in detail in the sections of the EuroFlow antibody panel manuscript.[20] Nevertheless, we here provide some typical examples. A first example, is CD300e (IREM-2), which is currently known to be specific for the monocytic lineage, being expressed only at the later stages of maturation among $CD14^{hi}$ cells,[39] and thereby providing a powerful tool for the discrimination between $CD14^{+}$ monoblasts/promonocytes and more mature monocytic cells. This may particularly be useful for the distinction between acute monocytic leukemias (AML) and myeloproliferative/myelodysplastic syndromes (MDS) like chronic myelomonocytic leukemia. A second example is CD200, which was included in the B-CLPD multi-tube antibody panel because of its added value in the differential diagnosis between mantle cell lymphoma (typically CD200 negative) and chronic lymphocytic leukemia (CLL) and other $CD200^{+}$ B-CLPD.[40] A third example is CD305 (LAIR1),[41] which proved not only to be a reliable marker for hairy cell leukemia but also to be particularly useful in other relevant differential diagnoses of B-CLPD, such as CD10-negative follicular lymphomas. More detailed information is provided by Böttcher *et al.*[20] in Section 8 of the EuroFlow Antibody Panel Report in this issue of *Leukemia*.

CAN THE SAME RESULTS BE OBTAINED WITH FEWER MARKERS?

EuroFlow antibody panels[20] seem to consist of an extremely large list of reagents. However, it should be noted that such panels aim at addressing most clinical questions where multiparameter flow cytometry immunophenotyping has proven to be of clinical utility in the diagnosis and classification of all different types of hematological malignancies, including the (very) rare disease entities.[20] Some diagnostic questions might not apply in individual laboratories and several antibody combinations are not required to answer the most frequent diagnostic questions. Consequently, appropriate algorithms can be built for sequential usage of distinct antibody tubes in a multi-tube panel. For example, the first tube of the B-CLPD multi-tube panel together with the lymphoid screening tube (LST) is sufficient for differential diagnosis of CLL from other B-CLPD.[20] Similarly, the first four tubes of the AML/MDS panel will provide all required information for full immunophenotypic characterization of the vast majority of AML and MDS cases. Application of the other three tubes of the AML/MDS panel is only needed in a minority of rare leukemias and myeloid disorders (see Van der Velden *et al.*, in Section 7 of the EuroFlow Antibody Panel Report).[20]

Furthermore, not every marker might seem essential for the diagnosis or classification in each individual case, but it should be noted that the combination of markers is essential in a group of patients. A clear example is the need for both CD19 and CD20 as backbone markers for the B-CLPD panel.[20] If only CD19 would be used, neoplastic B cells from a subgroup of B-CLPD (for example, follicular lymphomas) would not be identified by the common backbone;[42] in turn, if only CD20 would be used, CLL cells would frequently not be detected because of the low CD20 expression.[42,43]

Finally, T-CLPD and NK-CLPD are relatively rare diseases that can be detected by the LST, but which are frequently further characterized in specialized centers with a strong focus on these disease categories.

Therefore, each section of the EuroFlow Antibody Panel Report clearly explains the contribution of each marker and the contribution of each tube to the diagnostic process, so that individual laboratories can decide which tubes and panels are relevant for their own diagnostic practice.[20]

CAN OTHER FLUOROCHROMES, ANTIBODY CLONES OR ANTIBODY CONJUGATES BE USED?

The EuroFlow antibody panels have been designed with some flexibility. However, because of the need of full standardization and reproducibility of the results, reference reagents were defined for each marker in each multicolor combination (www.euroflow.org). The selection of a given reagent was based on its unique staining pattern of well-defined normal and aberrant cells. Therefore, the quality of a reagent was not solely evaluated on the basis of its brightness, but also on its discrimination potential between different cell populations present in a sample.[23] For example, if the brightest fluorochrome would have been selected for the CD38 reagents in the plasma cell disorders antibody panel, it would be virtually impossible to represent on scale simultaneously the $CD38^{hi}$ plasma cells and the CD38 negative cell populations coexisting in the same sample (see Flores-Montero *et al.*, in Section 1 of the EuroFlow Technical Report).[21,28]

Despite all the above, the presented reference reagents should not be viewed as the sole exclusive reagents that can be used in the EuroFlow antibody panels. In fact, reagents from different manufacturers that are conjugated to highly comparable fluorochromes or that use other antibody clones might also be used instead of the corresponding reference reagent, as long as identical or very similar staining patterns are obtained in a series of patient samples. If individual laboratories prefer to use alternative antibody clones and/or fluorochrome conjugates in the EuroFlow protocols, the performance of these potentially equivalent reagents and the complete new set of markers should be tested against the reference reagents before their acceptance. Consequently, usage of other fluorochromes or antibody clones and conjugates is possible after careful evaluation of their performance against the available reference reagents, following stringent criteria for the definition of comparable performances.

CAN ADAPTATIONS OR EXTENSIONS OF THE EUROFLOW ANTIBODY PANELS BE EXPECTED?

The EuroFlow antibody panels clearly contribute to answer most current clinical diagnostic questions regarding characterization of hematological malignancies. However, we also identified some diagnostic questions, which the currently used antibody panels cannot fully answer. Therefore, we anticipate that further improvements can be expected in the future. In turn, we also anticipate that EuroFlow antibody panels can answer additional (new) clinical diagnostic questions based on the introduction of the new markers and new concepts.

EuroFlow antibody panels can be reproduced in any laboratory with appropriate flow cytometers and a large database of fully characterized cases, such as those that have already been acquired at the EuroFlow centers using these panels. It is therefore easily possible to evaluate the added diagnostic utility of novel markers by measuring such markers in addition to the original

Table 3. Main objectives and achievements of the EuroFlow project

Objectives
1. Development of novel antibodies, particularly against intracellular proteins, such as oncoproteins and newly defined classification markers, as identified by gene expression profiling and molecular cytogenetic findings
2. Novel immunobead technology for fast and easy classification of acute leukemias via detection of oncogenic fusion proteins in cell lysates
3. Novel flow cytometry software for easy and fast handling and integration of list mode data files of multi-tube 8-color immunostainings and for automated pattern recognition of novel, reactive/regenerating and malignant cell populations
4. Evaluation and selection of fluorochromes suited for 8-color flow cytometric immunostainings
5. Development of standardized procedures for instrument settings and immunostaining procedures to guarantee reliable interlaboratory comparability of flow cytometric immunophenotyping
6. Design, standardization and clinical evaluation of novel 8-color immunostaining protocols for diagnosis and classification of hematopoietic malignancies
7. Design, standardization and clinical evaluation of 8-color immunostaining protocols for assessment of treatment effectiveness via monitoring the malignant cells during and after treatment

Achievements/deliverables
1. Development of new antibodies directed to oncogene proteins and tumor-associated markers
2. Novel immunobead assays for detection of fusion proteins
3. Novel software for integration of list mode data files and multivariate data analysis
4. Standardized procedures for instrument set-up and 8-color flow cytometry immunostaining optimized for immunophenotyping of leukemias and lymphomas
5. New evaluated EuroFlow antibody panels for diagnosis and classification of leukemias and lymphomas
6. Educational program (EuroFlow workshops[a], web page[b] and scientific publications)

[a]Please see Table 4. [b]www.euroflow.org.

panel and subsequently apply multivariate analyses—for example, principal component analysis (PCA)—to the standard panel plus the proposed marker. If such marker is of added value, it would rank highly for the proposed differential diagnosis.

DO THE EUROFLOW ANTIBODY PANELS REQUIRE USAGE OF THE EUROFLOW SOP FOR INSTRUMENT SET-UP AND THE NEW SOFTWARE TOOLS?

The EuroFlow antibody panels and tools are flexible in terms of how they can be applied, but optimal performance is achieved when used in combination with the EuroFlow SOP for instrument set-up and software tools.

In fact, the deliverables of the EuroFlow project can be seen as a comprehensive set of novel tools that can be combined according to the needs of different laboratories. Consequently, one may choose to use the EuroFlow antibody panels[20] together with the EuroFlow instrument set-up, sample preparation and data analysis procedures,[21] including EuroFlow reference data files and templates for fast multivariate recognition of complex protein expression patterns of suspected cells in a sample.[23] Alternatively, one might decide just to use some of the newly proposed markers or some multicolor antibody combinations or even only those disease-oriented panels that have immediate utility in the routine diagnostic work of a laboratory. Similarly, the new software tools may be implemented without using the panels or the instrument set-up procedures may be selected to be used alone.

The EuroFlow group has developed a broad set of new tools that contribute to improve routine diagnostic work in the field of flow cytometric immunophenotyping in general and of hematological malignancies in particular. Obviously, the complete combination of the EuroFlow tools provides more added value than one tool on its own. For example, if the EuroFlow antibody panels are used without the new multidimensional data analysis tools of the Infinicyt software (and consequently without the sets of reference data files), it will be difficult to rapidly evaluate the complex immunophenotypic profile of the gated malignant cells. On the other hand, the same software tools may be used to objectively evaluate the diagnostic value of the local in-house antibody panel in comparison with the EuroFlow antibody panel for each disease category.

Software tools for the classification of diseases using EuroFlow reference databases and PCA can be used only if the investigator strictly adheres to the EuroFlow SOP for staining and instrument set-up and if the original EuroFlow panels (or a comprehensively evaluated replacements) are used.

WHAT WOULD BE THE ADVANTAGES OF USING THE EUROFLOW 8-COLOR PANELS AND PROTOCOLS?

Usage of the EuroFlow panels and protocols has multiple advantages and only few limitations. The main advantage is that many deliverables of the EuroFlow project did not exist before and provide new opportunities for improved, more objective and standardized flow cytometric diagnosis and classification of hematological malignancies in individual laboratories around the world. These EuroFlow deliverables include: (i) new highly informative diagnostic markers, (ii) new marker combinations for better characterization of specific populations of normal and neoplastic cells in blood, bone marrow and other types of samples, (iii) new antibody panels and algorithms objectively evaluated for multiple diagnostic questions with well-defined performance and clinical utility, (iv) standardized instrument set-up and sample preparation procedures proved to allow full intra- and inter-laboratory comparisons, (v) new software tools for reliable and reproducible multivariate analysis of complex immunophenotypic patterns of both normal and aberrant cell populations, (vi) new templates of multiparameter flow cytometry data from normal reference samples as well as leukemia/lymphoma reference samples classified according to the WHO 2008 criteria[1] (Table 3). These normal and malignant reference samples can now be used for rapid comparative assessment of the nature of suspected phenotypic profiles in individual patient samples.[23]

If the EuroFlow antibody panels and protocols are fully adopted by a significant number of laboratories and linked to (inter)national clinical treatment protocols, they will significantly progress the field in terms of standardization, reproducibility and clinical impact.

WHAT DOES EUROFLOW STANDARDIZATION MEAN?

The EuroFlow Consortium believes that harmonization is not sufficient to progress the field and to obtain truly comparable

results among different laboratories. Therefore, full standardization is required. The EuroFlow standardization includes: (i) usage of comparable 3-laser $\geqslant$8-color flow cytometers; (ii) selection of appropriate and compatible fluorochromes; (iii) full standardization of instrument settings (for example, based on bead standards); (iv) standardization of laboratory protocols and immunostaining procedures (SOPs); (v) careful selection of optimal reference antibody clones per marker/CD code; (vi) design of combinations of multiple 8-color tubes; (vii) new multivariate software tools for the comparison of reference data files for specific diagnostic questions; (viii) recognition of normal subsets, including definition of complete normal and regenerating differentiation pathways, using the same immunostaining protocols; and (ix) mapping of new patient samples against large databases of earlier collected patient samples and normal/regenerating differentiation pathways, analyzed with the same immunostaining protocol in different diagnostic centers.

Noteworthy, these are diagnostic techniques that may influence the treatment decision process and accordingly they must be conducted or at least supervised by specialized and well-trained personnel who has not only technical skills but also the ability and knowledge to perform interpretation of the results according to the state of the art in the diagnostic and clinical field of hematological malignancies.

HOW SHOULD THE IMMUNOBEAD ASSAYS FIT INTO THE DIAGNOSTIC ALGORITHMS?

EuroFlow has developed several immunobead assays for fast flow cytometric detection of fusion proteins in lysates from leukemic cells carrying specific chromosomal translocations.[25] The immunobead assay has been designed to be applied for rapid and easy identification of the most frequent subtypes of acute leukemias associated with specific recurrent cytogenetic translocations that result in a fusion gene and consequently in a fusion protein.[24–26] Therefore, the immunobead assay fits with the aim of (sub)classification of acute leukemias at diagnosis.[25] Once an acute leukemia is diagnosed and the lineage of the suspected blast cells has been assessed by use of the acute leukemia orientation tube (for example, non-lymphoid *versus* T- or B lymphoid, see Lhermitte and colleagues,[20] in Section 1 of the EuroFlow Antibody Panel Report), identification of the relevant fusion proteins may start, that is, *PML-RARA* versus *AML1-ETO* and *CBFB-MYH11* versus *TEL-AML1*, *BCR-ABL*, *E2A-PBX1* and *MLL-AF4* for cases suspected of acute promyelocytic leukemia, AML and B-cell precursor acute lymphoblastic leukemia, respectively. In addition, detection of the BCR-ABL fusion protein may also be used for fast diagnosis and confirmation of CML.[24] This type of immunobead assay is particularly suited for centers where rapid molecular detection of chromosomal translocations is not implemented or routinely performed, but where a standard flow cytometer is readily available. The immunobead assay is fast (results are obtained in a few hours) and easy to perform, and allows reliable detection of the most relevant and common fusion proteins, independently of the breakpoints involved. The assay may be used in a multiplex format where each bead population is labeled differently. Finally, the assay may be run in parallel to standard immunophenotyping with the EuroFlow acute leukemia antibody panels. This will save technician time.

WHY ARE SOME OF THE EUROFLOW DELIVERABLES LINKED TO SPECIFIC COMPANIES?

In line with the EU-FP6 guidelines, the 'Specific Targeted Research Project' (STREP) of the EuroFlow Consortium included two SMEs as formal members of the Consortium. The two SMEs were involved from the start of the project onwards in the development of products that were not available in the market and that could be used for the aims of the EuroFlow project. Logically, such novel products were linked to the companies that actively participated in their development. These products contained innovative solutions from the individual members of the EuroFlow group.

Table 4. Summary of EuroFlow educational symposia and workshops

Number	*City, Country*	*Date*	*Workshop title*
1	Berlin, DE	4 June 2009	Innovation in flow cytometry symposium: 'Presentation of the latest results of the EuroFlow EHA Scientific Working Group', 14th EHA Congress
2	Paris, FR	28 October 2009	First EuroFlow Educational Workshop: 'Atelier d'information EuroFlow'
3	Coimbra, PT	22 January 2010	Second EuroFlow Educational Workshop: 'Análise de dados da citometria.'
4	Rotterdam, NL	13 March 2010	Third EuroFlow Educational Workshop: 'EuroFlow Antibody panels and Infinicyt Software'
5	Salamanca, ES	16–17 April 2010	Fourth EuroFlow Educational Workshop
6	Barcelona, ES	10 June 2010	Innovation in flow cytometry Symposium: 'Presentation of the latest results of the EuroFlow EHA Scientific Working Group', 15th EHA Congress
7	Dublin, UK	26 November 2010	EuroFlow Educational Workshop in association with the Academy of Medical Laboratory Sciences: 'EuroFlow Antibody panels and Infinicyt Software'
8	Paris, FR	9 March 2011	Fifth EuroFlow Educational Symposium: 'EuroFlow meets FranceFlow'
9	Coimbra, PT	31 March–1 April 2011	Sixth EuroFlow workshop: 'Desenho e aplicação dos paineis Euroflow de Síndromes Linfoproliferativos Crónicos de Células B e de Gamapatias Monoclonais'
10	Cape Town, SA	13–14 April 2011	EuroFlow Infinicyt Workshop
11	St. Petersburg, RU	18 May 2011	EuroFlow session: 'Modern approaches to the diagnosis of lymphoproliferative diseases'
12	Buenos Aires, AR	30 May–1 June 2011	'Curso Avanzado de Actualización en Onco-Hematología por Citometría de Flujo'
13	London, UK	9 June 2011	Innovation in flow cytometry symposium: 'Presentation of the latest results of the EuroFlow EHA Scientific Working Group', 16th EHA Congress
14	Rio de Janeiro, BR	25–27 August 2011	1° Workshop do Consórcio EuroFlow no Rio de Janeiro
15	Prague, CZ	8 October 2011	Seventh EuroFlow Educational Symposium and Workshop
16	Katowice, PL	24 March 2012	Eighth EuroFlow Educational Symposium and Workshop

Abbreviations: AR, Argentina; BR, Brazil; CZ, Czech Republic; DE, Germany; EHA, European hematology association; ES, Spain; FR, France; NL, The Netherlands; PL, Poland; PT, Portugal; RU, Russia; SA, South Africa; UK, United Kingdom.

The SME Cytognos SL particularly worked in the innovations of the Infinicyt software tools, which are now commercially available. The SME Dynomics (Rotterdam, The Netherlands) focused on the development of new antibodies particularly for the immunobead assay for detection of fusion proteins. The immunobead technology has been transferred by Dynomics to BD Biosciences (San Jose, CA, USA) for commercialization. The BCR-ABL immunobead assay[24] was launched in Autumn 2008 and the other immunobead assays will follow soon, such as the PML-RARA immunobead assay.[26]

EuroFlow is a consortium of scientific institutes and does not have production facilities or a distribution network for products related to its activities. However, to achieve production and distribution of the novel products and make the public investment money return into public (research) activities, the *EuroFlow Consortium Agreement* (signed by all parties) indicated that intellectual property derived from the deliverables of the EuroFlow project should be patented, and licensed to commercial companies that might be interested in large-scale (quality-controlled) production and distribution of the EuroFlow deliverables for rapid availability to the field. In parallel, all institutions and individual EuroFlow members declined their rights on revenues (such as royalty rights) in favor of the EuroFlow Consortium, to provide sustainability for future activities and projects of the group, including Educational Workshops and Educational Symposia (Table 4).

WHICH EUROFLOW ACTIVITIES ARE STILL ONGOING?

The current activities of the EuroFlow Consortium concern:

(1) Building the reference databases and templates for the whole set of EuroFlow antibody panels to be linked to the software tools (Infinicyt software) that are already available.
(2) Design of innovative strategies for the detection of minimal residual disease (MRD) during and after therapy in patients that have reached complete remission according to conventional criteria. The new strategies search for disease-oriented single-tube combinations instead of patient-specific multicolor antibody panels. This new MRD strategy takes advantage of all new data analysis software tools and reference databases collected previously.
(3) Because of the successful innovation and standardization in the hemato–oncology field, the EuroFlow Consortium has now decided to extend its activities to flow cytometric diagnosis for other diseases such as primary immunodeficiencies. In this context, more detailed studies on normal lymphocyte subsets are being performed. These studies show that more than eight colors might be needed to fully unravel all relevant B- and T-cell subsets and their memory and effector pathways.

CONCLUSION: EUROFLOW TOOLS FOR COMPANION DIAGNOSTICS IN PERSONALIZED MEDICINE

In the current era of personalized medicine, many different new treatment options are being evaluated to further improve treatment outcome while increasing quality of life, such as treatment with antibodies and small (blocking) molecules. The implementation and evaluation of such new treatment modalities requires accurate diagnosis and classification of the disease and careful monitoring of treatment effectiveness. Consequently, the applied diagnostics should be optimally suited for the management of the involved patients. Such companion diagnostics is currently particularly needed for patients with a hematological malignancy, because the field of hemato–oncology is ahead of the other fields in medicine.

The EuroFlow antibody panels and technical protocols have been developed for application as companion diagnostics for (inter)national clinical treatment protocols, where standardization and reproducibility are of utmost importance. In this way, the EuroFlow achievements can contribute to advanced comparability of innovative clinical treatment protocols and thereby to further improvement of diagnostic and therapeutic patient care.

CONFLICT OF INTEREST

JJMvD and AO are the coordinators of the EuroFlow Consortium and are inventors of the EuroFlow patent '*Methods, reagents and kits for flow cytometric immunophenotyping*' (PCT/NL2010/050332), together with 13 other EuroFlow members. The patent describes the composition of the EuroFlow antibody combinations for diagnosis and classification of hematological malignancies. The patent has been licensed to the companies BD Biosciences and Cytognos for making the antibody combinations commercially available as full-tube combinations in order to speed up the immunostaining process. The patent is collectively owned by the EuroFlow Consortium and the revenues of the patent are exclusively used for EuroFlow Consortium activities, such as for covering (in part) the costs of the Consortium meetings, the EuroFlow Educational Workshops and the purchase of custom-made reagents for collective experiments.

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