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.3–8 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 > 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.

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
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)\(^2\) 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.\(^2\) For this purpose, development and implementation of new software tools for fast and easy handling of large data files,\(^2\)^\(^2\),\(^2\)\(^3\) combining multiple tubes and mapping of leukemia samples against templates of normal and pathological reference samples for fast multidimensional pattern recognition,\(^2\)\(^3\) 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).\(^2\)\(^4\)–\(^2\)\(^6\)

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 Marveld, H Wind, B van Bodegom | Erasmus MC, Rotterdam, NL | JJM van Dongen | VHJ van der Velden, AW Langerak, J te Marveld, 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 Português de Oncologia, Lisboa, PT | P Lucio | M Gomes da Silva, J Caetano, T Faia |
| Cytognos, Salamanca, ES | M. Martin-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 Thümer |
| 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 Sluij-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).
WHY DID IT TAKE SEVERAL YEARS TO DEVELOP THE EUROFLOW ANTIBODY PROTOCOLS?

With a few exceptions focused on specific diseases, 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. 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.

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 staining that constitute the EuroFlow 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.

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). 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 redesign 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.

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. 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.

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.
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). 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 ≥8 fluorescence emissions, as described by Kalina et al. However, by the end of 2009, new multi-color instruments became commercially available. 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, 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,
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. 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 CD14hi cells, and thereby providing a powerful tool for the discrimination 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. A third example is CD305 (LAIR1), 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. 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 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. 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. 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).

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. 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; in turn, if only CD20 would be used, CLL cells would frequently not be detected because of the low CD20 expression.

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.

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. 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 CD38hi 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). 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                                                                 | Achievements/deliverables                                                                 |
|----------------------------------------------------------------------------|-----------------------------------------------------------------------------------------|
| 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 | 1. Development of new antibodies directed to oncogene proteins and tumor-associated markers |
| 2. Novel immunobead technology for fast and easy classification of acute leukemias via detection of oncogenic fusion proteins in cell lysates | 2. Novel immunobead assays for detection of fusion proteins                               |
| 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 | 3. Novel software for integration of list mode data files and multivariate data analysis |
| 4. Evaluation and selection of fluorochromes suited for 8-color flow cytometric immunostainings | 4. Standardized procedures for instrument set-up and 8-color flow cytometry immunostaining optimized for immunophenotyping of leukemias and lymphomas |
| 5. Development of standardized procedures for instrument settings and immunostaining procedures to guarantee reliable interlaboratory comparability of flow cytometric immunophenotyping | 5. New evaluated EuroFlow antibody panels for diagnosis and classification of leukemias and lymphomas |
| 6. Design, standardization and clinical evaluation of novel 8-color immunostaining protocols for diagnosis and classification of hematopoietic malignancies | 6. Educational program (EuroFlow workshops5, web page6 and scientific publications) |

*Please see Table 4. 5www.euroflow.org.

Software tools for the classification of diseases using EuroFlow reference databases and PCAs 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 criteria1 (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 > 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 detection of chromosomal translocations.25 The 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 detection of fusion proteins, that is, 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: ‘Analise 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 Linfoproiferativos Crônicos 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 Onc-Hematologia por Citometria 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 | 1º 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 Cytnos SL particularly worked in the innovations of the Infinicyt software tools, which are now commercially available. The SME Dynamics (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 Dynamics to BD Biosciences (San Jose, CA, USA) for commercialization. The BCR-ABL immunobead assay24 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 hemat–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 COMPARISON 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 hemat–oncology is ahead of the other fields in medicine.

The EuroFlow antibody panels and technical protocols have been developed as application for 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

JNMvd 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 Cytnos 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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REFERENCES

1. Swerdlov SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H et al. WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues, 4th edn. International Agency for Research on Cancer: Lyon, 2008, 439 pp.
2. Szczepanski T, Orfao A, van der Velden VH, San Miguel JJ, van Dongen DJ. Minimal residual disease in leukaemia patients. Lancet Oncol 2001; 2: 409–417.
3. Orfao A, Schmitz G, Brando B, Ruiz-Aruguelles A, Basso G, Braylan R et al. Clonally useful information provided by the flow cytometric immunophenotyping of hematological malignancies: current status and future directions. Clin Chem 1999; 45: 1708–1717.
4. Szczepanski T, van der Velden VH, van Dongen JJ. Flow cytometric immunophenotyping of normal and malignant lymphocytes. Clin Chem Lab Med 2006; 44: 775–796.
5. Davis BH, Holden JT, Bene MC, Borowitz MJ, Braylan RC, Cornfeld D et al. 2006 Bethesda International Consensus recommendations on the flow cytometric immunophenotypic analysis of hematolymphoid neoplasia: medical indications. Cytometry B Clin Cytom 2007; 72(Suppl 1): 55–51.
6. Craig FE, Foon KA. Flow cytometric immunophenotyping for hematologic neoplasms. Blood 2008; 111: 3941–3967.
7. Kraai J, Gratama JW, Haion J, Orfao A, Blauwet A, Porwit A et al. Flow cytometric immunophenotyping of cerebrospinal fluid. Curr Protoc Cytom 2008; Chapter 6 Unit 6.25.
8. Paiva B, Almeida A, Perez-Andres M, Mateo G, Lopez A, Rasillo A et al. Utility of flow cytometry immunophenotyping in multiple myeloma and other clonal plasma cell-related disorders. Cytometry B Clin Cytom 2010; 78: 239–252.
9. Bene MC, Nebe T, Bettelheim P, Baldini B, Bunseh H, Kern W et al. Immunophenotyping of acute leukemia and lymphoproliferative disorders: a consensus
proposal of the European LeukemiaNet Work Package 10. Leukemia 2011; 25: 567–574.

10 Borowitz MJ, Guenther KL, Shults KE, Stelzer GT. Immunophenotyping of acute leukemia by flow cytometric analysis. Use of CD45 and right-angle light scatter to gate on leukemic blasts in three-color analysis. Am J Clin Pathol 1993; 100: 534–540.

11 Knapp W, Strob H, Majdic O. Flow cytometric analysis of cell-surface and intracellular antigens in leukemia diagnosis. Cytometry 1994; 18: 187–198.

12 Groeneveld K, te Velde MJG, van den Beemd MW, Hooijkaas H, van Dongen JJM. Flow cytometric detection of intracellular antigens for immunophenotyping of normal and malignant leukocytes. Leukemia 1996; 10: 1383–1389.

13 Porwit-MacDonald A, Bjorklund E, Lucio P, van Lochem EG, Mazur J, et al. BIOMED-I concerted action report: flow cytometric characterization of CD7+ cell subsets in normal bone marrow as a basis for the diagnosis and follow-up of T cell acute lymphoblastic leukemia (T-ALL). Leukemia 2000; 14: 816–825.

14 Lanza F. Towards standardization in immunophenotyping hematological malignancies. How can we improve the reproducibility and comparability of flow cytometric results? Working Group on Leukemia Immunophenotyping. Eur J Haematol 1996; 40(Suppl 1): 7–14.

15 Rothe G, Schmitz G. Consensus protocol for the flow cytometric immunophenotyping of hematopoietic malignancies. Working Group on Flow Cytometry and Image Analysis. Leukemia 1996; 10: 877–895.

16 Wood BL, Arroz M, Barnett D, DiGiuseppe J, Greg B, Kussick SJ et al. 2006 Bethesda International Consensus recommendations on the immunophenotypic analysis of hematologic neoplasia by flow cytometry: optimal reagents and reporting for the flow-cytometric diagnosis of hematopoietic neoplasia. Cytometry B Clin Cytom 2007; 72(Suppl 1): S14–S22.

17 Greg B, Oldaker T, Warzymski M, Wood B. 2006 Bethesda International Consensus recommendations on the immunophenotypic analysis of hematopoietic neoplasia by flow cytometry: recommendations for training and education to perform clinical flow cytometry. Cytometry B Clin Cytom 2007; 72(Suppl 1): S23–S33.

18 van Dongen JJM, Orfao A, Staal FJ, Martin-Ayuso M, Parreira A, Kneba M et al. EuroFlow: Flow cytometry for fast and sensitive diagnosis and follow-up of hematological malignancies. In: European Commission. Genomics and Biotechnology for Health – Diagnostics. Office for Official Publications of the European Communities: Luxembourg, 2008, pp 54–56.

19 van Dongen JM, Orfao A, Staal FJ, Martin-Ayuso M, Parreira A, Kneba M et al. EuroFlow: Flow cytometry for fast and sensitive diagnosis and follow-up of hematological malignancies. In: European Commission. Genomics and Biotechnology for Health – Diagnostics. Office for Official Publications of the European Communities: Luxembourg, 2008, pp 54–56.

20 van Dongen JM, Lhermitte L, Böttcher S, Almeida J, van der Velden VJH, Flores-Montero J et al. EuroFlow antibody panels for standardized n-dimensional flow cytometric immunophenotyping of normal, reactive and malignant leukocytes. Leukemia 2012; 26: 1908–1975.

21 Kalina T, Flores-Montero J, van der Velden VJH, Martin-Ayuso M, Böttcher S, Ritgen M et al. EuroFlow standardization of flow cytometry instrument settings and immunophenotyping protocols. Leukemia 2012; 26: 1986–2010.

22 Pedreira CE, Costa ES, Barrena S, Lecrevisse Q, Almeida J, van Dongen JJ et al. Generation of flow cytometry data files with a potentially infinite number of dimensions. Cytometry A 2008; 73: 834–846.

23 Costa ES, Pedroza CE, Barrena S, Lecrevisse Q, Flores J, Quijano S et al. Automated pattern-guided principal component analysis vs expert-adopted immunophenotypic classification of B-cell chronic lymphoproliferative disorders: a step forward in the standardization of clinical immunophenotyping. Leukemia 2010; 24: 1937–1933.

24 Weerkamp F, Dekking E, Ng YY, van der Velden VH, Wai H, Böttcher S et al. Flow cytometric immunobead assay for the detection of BCR-ABL fusion proteins in leukemia patients. Leukemia 2009; 23: 1106–1117.

25 Dekking E, van der Velden VH, Böttcher S, Bruggemann M, Sonneveld E, Koning-Goedheer A et al. Detection of fusion genes at the protein level in leukemia patients via the flow cytometric immunobead assay. Best Pract Res Clin Haematol 2010; 23: 333–345.

26 Dekking EHA, van der Velden VH, Varro R, Wai H, Böttcher S, Kneba M et al. Flow cytometric immunobead assay for fast and easy detection of PML-RARA fusion proteins for the diagnosis of acute promyelocytic leukemia. Leukemia 2012; 26: 1976–1985.

27 Rawstron AC, Villamor N, Ritgen M, Bottcher S, Ghia P, Zehnder JL et al. International standardized approach for flow cytometric residual disease monitoring in chronic lymphocytic leukemia. Leukemia 2007; 21: 956–964.

28 Rawstron AC, Orfao A, Beksa M, Bedzickova L, Brooijmans RA, Bumbea H et al. Report of the European Myeloma Network on multiparametric flow cytometry in multiple myeloma and related disorders. Haematologica 2008; 93: 431–438.

29 Bene MC, Castoldi G, Knapp W, Ludwig WD, Matutes E, Orfao A et al. Proposals for the immunological classification of acute leukemias. European Group for the Immunological Characterization of Leukemias (EGIL). Leukemia 1995; 9: 1783–1786.

30 Stewart CC, Behm FG, Carey JL, Cornbleet J, Duque RE, Hudnall SD et al. U.S. Canadian Consensus recommendations on the immunophenotypic analysis of hematologic neoplasia by flow cytometry: selection of antibody combinations. Cytometry 1997; 30: 231–235.

31 Ruiz-Arreguies A, Duque RE, Orfao A. Report on the first Latin American Consensus Conference for Flow Cytometric Immunophenotyping of Leukemia. Cytometry 1998; 34: 39–42.

32 Basso G, Buldini B, De Zen L, Orfao A. New methodologic approaches for immunophenotyping acute leukemias. Haematologica 2001; 86: 675–692.

33 Ruiz-Arreguies A, Rivadeneyra-Espinoza L, Duque RE, Orfao A. Report on the second Latin American consensus conference for flow cytometric immunophenotyping of hematological malignancies. Cytometry B Clin Cytom 2006; 70: 39–44.

34 Stetler-Stevenson M, Ahmad E, Barnett D, Braylan R, Diguiseppi J, Marti G et al. Clinical flow cytometric analysis of neoplastic hematolymphoid cells: Approved guideline. 2nd edn. CLSI document H43-A2 ed. Clinical and Laboratory Standards Institute: Wayne, PA, 2007.

35 Beckman Coulter Inc. Beckman Coulter Flow Cytometers. Clinical Flow Cytometry Instruments. Available from http://www.beckmancoulter.com/wsportal/wsi/diagnostics/clinical-products/flow cytometry/flow cytometers/index.htm (cited 2 September 2011).

36 Wilson WH. International consensus recommendations on the flow cytometric immunophenotypic analysis of hematological neoplasia. Cytometry B Clin Cytom 2007; 72(Suppl 1): S29–S32.

37 van de Loosdrecht AA, Alhan C, Bene MC, Della Porta MG, Drager AM, Feuilard J et al. Standardization of flow cytometry in myelodysplastic syndromes: report from the first European LeukemiaNet working conference on flow cytometry in myelodysplastic syndromes. Haematologica 2009; 94: 1124–1134.

38 Serke S, Schwaner I, Yordanova M, Szczepak A, Huhn D. Monoclonal antibody FMIC7 detects a conformational epitope on the CD20 molecule: evidence from phenotyping after rituxan therapy and transfectant cell analyses. Cytometry 2001; 46: 98–104.

39 Aguilar H, Alvarez-Errico D, Garcia-Montero AC, Orfao A, Sayos J, Lopez-Botet M. Molecular characterization of a novel immune receptor restricted to the monocytic lineage. J Immunol 2004; 173: 6703–6711.

40 Palumbo GA, Panninello N, Fargione G, Cardillo K, Chirenda A, Berretta S et al. CD200 expression may help in differential diagnosis between mantle cell lymphoma and B-cell chronic lymphocytic leukemia. Leuk Res 2009; 33: 1212–1216.

41 van der Vuurst de Vries AR, Clevers H, Lagtenberg T, Meylaard L. Leukocyto-associated immunoglobulin-like receptor-1 (LAIR-1) is differentially expressed during human B cell differentiation and inhibits B cell receptor-mediated signaling. Eur J Immunol 1999; 29: 3160–3167.

42 Almassi NM, Duque RE, Rijnweides J, Everett E, Braylan RC. Reduced expression of CD20 antigen as a characteristic marker for chronic lymphocytic leukemia. Am J Hematol 1992; 40: 259–263.

43 Matutes E, Owusu-Ankomah K, Morilla R, Garcia Marco J, Houihane A, Que TH et al. The immunological profile of B-cell disorders and proposal of a scoring system for the diagnosis of CLL. Leukemia 1994; 8: 1640–1645.

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