Journal of Immunological Methods 475 (2019) 112704

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Editorial

EuroFlow and its activities: Introduction to the special EuroFlow issue of The Journal of Immunological Methods

1. The EuroFlow Consortium and its activities

The EuroFlow Consortium officially started its activities in April 2006 as a Specific Targeted Research Project (STREP) in the FP6-2004-LIFESCIHEALTH-5 program of the European Commission (grant LSHB-CT-2006-018708). The project originally focussed on "*Flow cytometry for fast and sensitive diagnosis and follow-up of hematological malignancies*" (van Dongen et al., 2012a). The major goals were to innovate flow cytometry applications via the development of new tools and strategies for the diagnosis and classification of hematological malignancies and to generate reliable and reproducible results across individual laboratories and countries, via assay standardization and automation at multiple levels (van Dongen et al., 2012b). Despite the great challenge of such broad and ambitious objectives, the results of this very first EuroFlow project were actually completed six years later (Kalina et al., 2012; van Dongen et al., 2012b). This was achieved via extensive collaboration between (initially) eight public European universities and two small-medium enterprises (SMEs), enrolled in the project. During the course of the formal project period (April 2006 – Oct 2009) additional university partners joined the project to support the novel developments and to contribute to the validation of the technologies. The work accomplished translated into the development of standardized instrument set-up, standardized sample preparation and standardized data acquisition procedure, with a full set of diagnostic and disease-oriented panels of antibody reagents (van Dongen et al., 2012b) all of which became publicly available in 2012 (van Dongen et al., 2012b; Kalina et al., 2012), including the corresponding Standard Operating Procedures (SOPs), which are continuously being updated and can be downloaded from the EuroFlow website (www.EuroFlow.org). In addition, innovative software tools and strategies were developed for flow cytometry data analysis (da Costa et al., 2010; Pedreira et al., 2013). These innovations translated into the development of several innovative products, including proprietary Infinicyt™ software (Cytognos SL, Salamanca, Spain) and novel EuroFlow data bases (Costa et al., 2010; Pedreira et al., 2008a; Pedreira et al., 2008b). The EuroFlow Consortium also developed novel flow cytometric immunobead assays for fast and reliable detection of the most common and clinically relevant fusion gene proteins for oncogenetic classification of acute leukemia patients, including the BCR-ABL1 (Weerkamp et al., 2009), ETV6-RUNX1, TCF3-PBX1, KTM2A-AF4 (Dekking et al., 2010) and PML-RARA fusion proteins (Dekking et al., 2012). This innovative work of the EuroFlow Consortium was recognized in 2012 by dr. Nicole Muller Berat-Killmann, Editor-in-Chief of *Leukemia* who published the EuroFlow special issue with the statement "*EuroFlow revolutionises flow cytometric immunophenotyping*".

Since then, EuroFlow continued its activities in the field of diagnosis and classification of leukemia and lymphoma. Examples of the ongoing EuroFlow activities are the construction of large data bases with both normal and leukemia & lymphoma patient samples analysed with the EuroFlow antibody panels and SOPs, which serve as templates for prospective expert-guided automated data analysis, interpretation and reporting (Flores-Montero et al., 2019a, 2019b; Lhermitte et al., 2018). EuroFlow also developed 8 to 12-color antibody panels for minimal (or measurable) residual disease (MRD) monitoring in several types of hematological malignancies such as multiple myeloma (Flores-Montero et al., 2017) and B cell precursor acute lymphoblastic leukemia (BCP-ALL)(Theunissen et al., 2017). The design of the MRD antibody panels takes advantage of detailed studies on the corresponding normal counterparts of the malignant cells, preferably according to normal differentiation and maturation pathways with inclusion of markers that detect the deviations from normal. Development of MRD antibody panels for other hematological malignancies, are in progress.

Since March 2012, EuroFlow has expanded the development of tools and strategies into other fields of flow cytometric immunophenotyping, including diagnosis, classification and monitoring of primary and secondary immunodeficiencies (Blanco et al., 2019, 2018; Criado et al., 2018; van der Burg et al., 2019; van Dongen et al., 2019), analysis of the monocyte and macrophage compartment for evaluation of disruption of normal tissue homeostasis (Damasceno et al., 2019; van den Bossche et al., 2018), and immune monitoring of adaptive and innate (myeloid and NK) cell populations, in distinct physiologic states, disease conditions and immune therapies, (in collaboration with the PERISCOPE (PERtussIS COrrelates of Protection Europe at http://periscope-project.eu/) and INCAR Consortia (Botafogo et al., 2019; Diavatopoulos et al., 2019).

2. EuroFlow as independent scientific foundation

Upon completion of its formal STREP project duration (per October 2009), the EuroFlow consortium was incorporated into the European Scientific Foundation for Laboratory Hemato-Oncology (ESLHO at http://www.ESLHO.org/), together with the sister consortia EuroClonality (http://www.EuroClonality.org/) and EuroMRD (http://www.EuroMRD.org/). All three scientific consortia aim at innovation and standardization of laboratory diagnostics as well as quality assessment and education & training. The long-term sustainability of these scientific consortia is merely based on intellectual property (IP) and related patents, which are licensed to companies, such as Invivoscribe (San Diego, CA), Cytognos (Salamanca, ES), and BD Biosciences (San José, CA), which pay royalties to the EuroClonality

https://doi.org/10.1016/j.jim.2019.112704
Received 19 November 2019; Accepted 20 November 2019
Available online 20 November 2019

Table 1
Participant institutions and members of the EuroFlow Consortium (status November 2019).

EuroFlow Institution	Participants	Status in EuroFlow Consortium
Leiden University Medical Center, Leiden, NL; Dept of Immunohematology and Blood Transfusion	Prof. J. J. M. van Dongen, MD, PhD	Chairman
	W. M. Bitter	Management Team
	B. R. Lubbers, PhD	Management Team
	A. S. M. van der Meij	Management Team
	C. I. Teodosio, PhD	Member
	M. Zlei, PhD	Member
	A. J. van der Sluijs-Gelling, MSc	Member
	F. de Bie, MSc	Member
	S. de Bruin-Versteeg, BSc	Member
Leiden University Medical Center, Leiden, NL; Dept of Pediatrics	M. van der Burg, PhD	Member
Erasmus MC, Rotterdam, NL; Dept of Immunology	Prof. A. W. Langerak, PhD	Member
	V.H.J van der Velden, PhD	Board member
	J. te Marvelde, BSc	Member
	J. Schilperoord-Vermeulen, BSc	Member
	R. Jugooa, BSc	Member
	K.C. Heezen, BSc	Member
	A. Bijkerk, BSc	Member
University of Salamanca, ES; Dept of Medicine, Cancer Research Center	Prof. A. Orfao, MD, PhD	Co-chairman
	Prof. J. Almeida, MD, PhD	Member
	M. B. Vidriales, MD, PhD	Member
	J. Flores-Montero, MD, PhD	Member
	M. Pérez-Andrés, PhD	Member
	S. Matarraz, PhD	Member
	E. Blanco, PhD	Member
	L. Martín, PhD	Member
	Q. Lecrevisse	Member
	J.J. Pérez-Morán BSc	Member
	N. Puig, MD, PhD	Member
Instituto Portugués de Oncologia, Lisbon, PT; Hemato Oncology Laboratory	Prof. A. Medina Almeida, MD, PhD	Member
	Prof. M. Gomes da Silva, MD, PhD	Member
	T. Faria, PhD	Member
University of Schleswig-Holstein - Campus Kiel, Kiel, DE; 2nd Dept of Medicine	Prof. M. Brüggeman, MD, PhD	Board member
	M. Ritgen, MD, PhD	Member
	M. Szczepanowski, PhD	Member
	S. Kohlscheen, PhD	Member
	A. Laqua, PhD	Member
	E. Harbst	Member
	J. Finke	Member
Hôpital Necker-Enfants Malades, Paris, FR; Laboratoire d'Hematology	Prof. V. Asnafi, PhD	Member
	L. Lhermitte, PhD	Member
	E. Duroyon	Member
Charles University, Prague, CZ; Dept of Hematology/Oncology	J. Trka, PhD	Member
	O. Hrusak, PhD	Member
	T. Kalina, PhD	Board member
	E. Mejstrikova, PhD	Member
	M. Novakova, PhD	Member
	D. Thurner	Member
	V. Kanderova, PhD	Member
Medical University of Silesia, Zabrze, PL; Dept of Pediatric Hematology and Oncology	Prof. T. Szczepanski, MD, PhD	Member
	L. Sedek, PhD	Member
	J. Bulsa, PhD	Member
	L. Slota	Member
	J. Kulis	Member
Federal University of Rio de Janeiro	Prof. C.E. Pedreira, PhD	Member
	Prof. E. Sobral da Costa, MD, PhD	Member
Centro Hospitalar do Porto/University of Porto, Porto, PT; Department of Hematology, Cytometry Lab	Prof. M. Lima, MD, PhD	Member
	A.H. Santos,	Member
Universitätmedizin Rostock - Rostock, DE; Abteilung für Hämatologie, Onkologie und Palliativmedizin,	Prof. S. Böttcher, MD, PhD	Member
	S. Lange, PhD	Member
	R. Engelmann, PhD	Member
Medizinische Klinik III, Zentrum für Innere Medizin	D. Paape	Member
	C. Machka	Member
Dutch Childhood Oncology Group, Utrecht, NL	S. Nierkens, PhD	Member
	A. de Jong	Member
	A. de Koning	Member
Centro Ricerca Tettamanti, Clinica Pediatrica Universitá di Milano, Monza, IT	G. Gaipa, PhD	Member
	C. Burracchi, PhD	Member
	C. Bugarin, PhD	Member
University Hospital La Paz-IdiPAZ, Madrid, ES; Clinical Immunology Department	E. Lopez-Granados, MD, PhD	Member
	L. del Pino Molina, MSc	Member
St Anne's Faculty Hospital, Brno, CZ; Dept. of Immunology and Allergology/ MU Brno Faculty of Medicine	M. Vlkova, PhD	Member
	J. Nechvatalova, PhD	Member
CHU de Saint-Etienne, Saint-Etienne, Fr	Prof. L. Campos-Guyotat, MD, PhD	Member
Hematology Laboratory	C. Aanei, PhD	Member

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Table 1 (*continued*)

EuroFlow Institution	Participants	Status in EuroFlow Consortium
Universidad de Navarra, Pamplona, ES	Prof. J. F. San Miguel, MD, PhD	Member
Clinica Universidad de Navarra	B. Paiva, PhD	Member
Centro de Investigaciones Medica Aplicadas	L. Burgos, PhD	Member
Hospital Clínic de Barcelona, Barcelona, ES; Unitat d'Hematopathologia	N. Villamor-Casas, MD, PhD	Member
	L. Magnano, PhD	Member
University Hospital Ghent, Ghent, BE; Laboratory of Clinical Biology	Prof. J. Philippé, MD, PhD	Board member
	Prof. C. Bonroy, MD, PhD	Member
	B. Denys	Member
	A. Willems	Member
	P. Breughe	Member
	J. de Wolf	Member
University of Lisbon, Lisbon, PT; Institute of Molecular Medicine	Prof. A.E. Sousa, MD, PhD	Board member
	Prof. S. L. Silva, MD, PhD	Member
Kantonsspital Aarau, Aarau, CH; FACS/Stem Cell Laboratory	P. Fernandez, PhD	Member
	D. Morf	Member

and EuroFlow Consortia, respectively. These royalties are exclusively used for continuation of the collaboration and sustainability of the consortia. In order to guarantee full scientific independence, the EuroClonality, EuroMRD and EuroFlow consortia have been transformed in 2017 into independent scientific foundations (see: www.EuroFlow.org), still supported by ESLHO as umbrella organization for meetings and management. As independent scientific organization, the aims of EuroFlow remain the same: research and innovation in flow cytometric diagnostic patient care, standardization of laboratory diagnostics, quality assessment and continuous education in the field of flow cytometric immunophenotyping.

Because of their leading position in the field, the European Hematology Association (EHA) (https://ehaweb.org/) has recognized the EuroFlow, EuroClonality and EuroMRD consortia as part of the ESLHO Scientific Working Group within EHA.

During the last 10 years, EuroFlow has stepwise expanded from the initial 10 member institutions and SMEs, to the current 22 member institutions (Table 1). Of the 10 early members three left the consortium by the end of the EU-funded project, i.e. University of Leeds and the two SMEs Cytognos SL and Dynomics BV. Fifteen new members were selected according to their potential to contribute to the different EuroFlow activities, resulting in a total current membership of 22 EuroFlow member institutions with ~85 individual participants (Fig. 1). All current EuroFlow members belong to European institutions, except for one member institution, located in Brazil (Federal University of Rio de Janeiro, Rio de Janeiro, Brazil).

3. Innovative EuroFlow tools and strategies

Since the start of the consortium, EuroFlow has developed multiple innovative tools and assays for advanced flow cytometric immunophenotyping. These include validated antibody panels and standard operating procedures (SOPs) in the fields of leukemia & lymphoma (L&L) diagnosis and classification (Kalina et al., 2012; van Dongen et al., 2012b) and MRD monitoring (Flores-Montero et al., 2017), as well as for the diagnosis and classification of primary immune deficiencies (PID) (van Dongen et al., 2019) and immune monitoring (IMM) (Fig. 2). These antibody panels were designed for unequivocal identification and full dissection of lymphocyte subsets and their differentiation and maturation-associated pathways, in parallel to other leukocyte subpopulations. Specific combinations of fluorochrome-conjugated reagents were selected based on the need for brightness, stability, limited fluorescence spill-over and compensation requirements. These antibody combinations were evaluated in parallel in multiple centers (at least 3 centers per testing round) and they were optimized via multiple consecutive cycles of design-testing-evaluation-redesign. In each testing cycle, the Infinicyt software was used to identify antibodies for optimal recognition and clear-cut separation of the target cell

Fig. 1. EuroFlow members at the 33rd EuroFlow meeting in Kiel, DE.

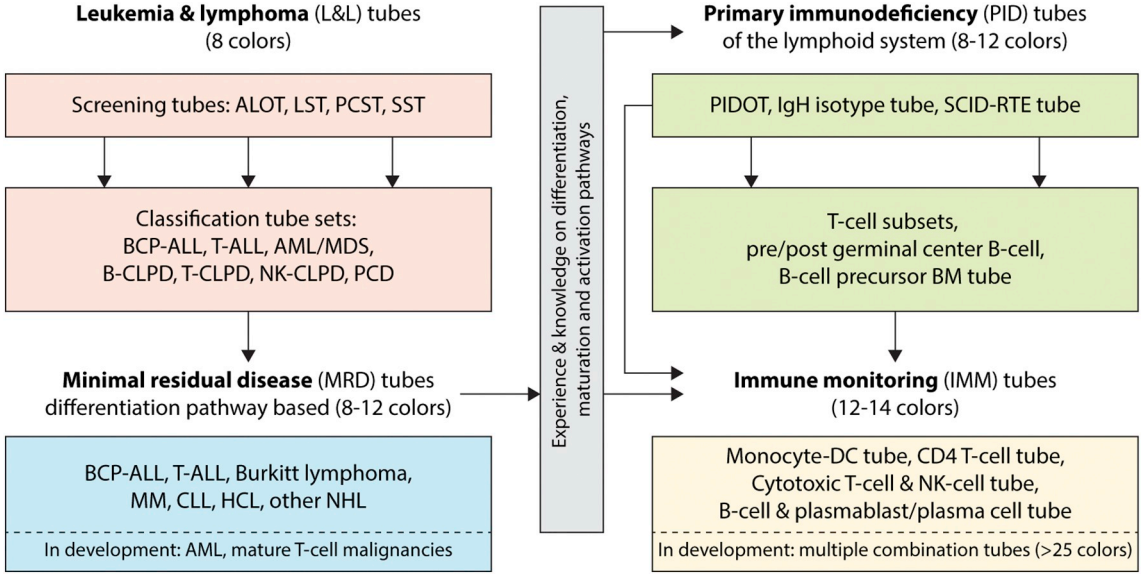

Fig. 2. EuroFlow tube sets and their interrelationship. Since the start of the EuroFlow consortium in March 2006, stepwise several sets of antibody tubes have been designed for specific applications, starting with the Leukemia & lymphoma (L&L) tubes, consisting of Screening tubes and Classification tube sets. As an extension of the L&L tubes, antibody tubes were designed for monitoring of treatment effectiveness. The design of these minimal residual disease (MRD) tubes was based on detailed knowledge of normal differentiation and maturation pathways. The experience and knowledge on differentiation, maturation and activation pathways was also used for the design of the primary immunodeficiency (PID) tubes as well as for the subsequent design of immune monitoring (IMM) tubes.

subsets, while other antibodies were discarded because of insufficient separation of target cell subsets. Optimal recognition and clear-cut separation of target cell populations avoids arbitrary marker settings between cell populations with vague cutoff values, which easily vary between different laboratories, particularly when different antibody clones and fluorochrome conjugates are used.

Of note, the SOPs developed by EuroFlow for instrument set-up, sample preparation, data acquisition and data analysis, have been validated across a wide range of ≥8 flow cytometry instruments (Nováková et al., 2017), including the more recent > 20-color flow cytometers, from distinct manufacturers (de Bie et al., 2019). Such validation has proven that identical results can be obtained across different platforms, including FACS-CANTO II (BD Biosciences, San José CA), LSR II (BD Biosciences), Cyan (Beckman Coulter, Hialeth, FL), Navios (Beckman Coulter), MACSQuant (Myltenyi, Bergisch Gladbach, Germany) instruments (Glier et al., 2019; Nováková et al., 2017) and more recent cytometers including the 10–12-color FACSLyric (BB Biosciences) (Glier et al., 2019), for which standardized instrument specific set-up and calibration protocols are available from the EuroFlow webpage (www.EuroFlow.org). Evaluation of other multi-color flow cytometry instruments for the development of standardized protocols, such as the 11-color Omnicyte (Cytognos, SL), 14-color Attune (Thermo Fisher Scientific, Waltham, MA), and the ≥25-color 3-laser Aurora (Cytek, Fremont, CA) are being developed (de Bie et al., 2019). SOPs for each of these instruments along with SOPs for bulk lysis, surface and/or cytoplasmic staining, and antibody panels have been published and are currently available on the EuroFlow website (Flores-Montero et al., 2017; Kalina et al., 2012).

Standardized EuroFlow antibody panels consist of discrete sets of 8 to 12-color antibody reagents (Fig. 2). Such panels include screening tubes, such as the lymphocyte screening tube (LST), acute leukemia orientation tube (ALOT), plasma cell disease screening tube (PCST) and a small sample tube (SST), together with more extensive disease-oriented 8-color antibody panels for the characterization of acute myeloid leukemia/myelodysplastic syndromes (AML/MDS panel), B-cell precursor acute lymphoblastic leukemia (BCP-ALL panel), T-cell acute lymphoblastic leukemia (T-ALL), plasma cell neoplasias (PCST tube), B-cell chronic lymphoproliferative disorders (B-CLPD panel), and both T- (T-CLPD panel) and NK-cell chronic lymphoproliferative disorders (NK-CLPD panel) (van Dongen et al., 2012b). Similarly, standardized panels for the diagnosis and classification of primary immune deficiencies (PID), include three screening tubes: primary immune deficiency orientation tube (PIDOT), immunoglobulin (Ig) heavy chain (H) isotype and subclass tube (IgH isotype) and the severe combined immune deficiency/recent thymic emigrant T-cell tube (SCID-RTE tube)- together with another four tubes - the T-cell maturation tube, the pre-germinal center (pre-GC), post-GC and B-cell precursor (BCP) tubes- for more in depth characterization of the altered T- and B-cells in blood and bone marrow of PID patients (Blanco et al., 2019; 2018; van der Burg et al., 2019). For both the leukemia & lymphoma and PID tubes, EuroFlow algorithms have been proposed with precise definition of the entries, recommended for the application of each tube (van Dongen et al., 2019; van Dongen et al., 2012a).

More recently, the EuroFlow leukemia & lymphoma diagnostic panels have been extended with MRD antibody panels specific for e.g. BCP-ALL MRD (Theunissen et al., 2017) and MM MRD panels (Flores-Montero et al., 2017). In addition, four 12- to 14-color EuroFlow tubes have been built for detailed dissection of the innate cell (monocyte/dendritic cell), T-CD4 (TCD4), Cytotoxic T & NK-cell (TCD8), B-cell and plasma cell (B-cell) compartments, for immune monitoring purposes, i.e. the EuroFlow immune monitoring (IMM) tubes (Fig. 2).

The design of every EuroFlow antibody tube required multiple cycles of design-testing-evaluation-redesign for optimal performance once combined with the EuroFlow SOPs and the EuroFlow software tools. This implies that for each tube, its specific expected performance is known. No single EuroFlow laboratory could have afforded such extensive efforts on its own. Solely thanks to intensive collaboration and frequent exchange of results and information during the multiple EuroFlow meetings (35th EuroFlow meeting in November 2019), the here described results could be achieved. As an example, validation of ALOT showed optimal performance with selection of the appropriate disease-oriented panels in 99.7% of > 700 acute leukemia patient samples tested (Lhermitte et al., 2018). Furthermore, the EuroFlow MM MRD and BCP-ALL MRD tubes reach an unprecedentedly high-sensitivity $< 2 \times 10^{-6}$ (Flores-Montero et al., 2017; Theunissen et al., 2017). Similarly, the IgH subclass tube allowed identification of predominantly antibody deficiency (PAD) in 100% of patients with IgA-deficiency, IgG-isotype deficiency and common variable immune

deficiency (CVID) (Blanco et al., 2019). Finally, the four EuroFlow IMM tubes have demonstrated robust identification and enumeration of > 250 immune cell populations, including 23 innate cell populations, 89 T-CD4 cell subsets, 48 subpopulations of cytotoxic T/NK-cells and 135 B-cell and plasma cell subsets in (normal) blood (Botafogo et al., 2019; Morán-Plata et al., 2019; van der Pan et al., 2019).

During the early phases of development of EuroFlow antibody panels, a need for innovative software tools emerged. This was mainly due to the greater complexity of the data generated by the increased number of markers evaluated simultaneously, the analysis of the exponentially larger number (> 10 million) of cells per sample, and the increasingly higher number and complexity of the cell populations identified with a single antibody combination. These complexities increased the risk for more subjective interpretation and reporting of flow cytometry data in diagnostic patient care. Based on these needs, innovative software tools linked to properly annotated data bases were developed for control samples and for patient samples on a per disease category and per type of sample basis (Flores-Montero et al., 2017; Lhermitte et al., 2018). In parallel, for the PID antibody tubes and the IMM antibody tubes normal reference ranges for blood subsets (e.g. per age group) were generated (Blanco et al., 2018; van der Burg et al., 2019).

Implementation of these innovative *Big Data* tools, has permitted an unprecedentedly fast and objective evaluation of the performance of single-tube and multi-tube antibody combinations, e.g. relative contribution of one antibody vs. another in a panel (van Dongen et al., 2012a). Standardization and these carefully composed databases allowed to implement automated gating for identification and labelling of normal vs. pathological tumor cells coexisting in a sample. The "pathological tumor cells" were followed by expert-guided interpretation of their clinical meaning and automated reporting of the results (Flores-Montero et al., 2017; Lhermitte et al., 2018). At the same time, the new data bases and software tools implemented in Infinicyt™ provide unique and innovative external quality assurance tools on a per sample basis, via direct comparison of each interrogated data file against a reference data base composed of large numbers of data files with corresponding sample types (e.g. bone marrow or blood) each stained with the same standard EuroFlow panels, prepared and measured in distinct instruments in multiple laboratories, all using the EuroFlow SOPs (Kalina et al., 2018, 2015).

4. Diagnosis and classification of hematological malignancies

The introduction of the standardized EuroFlow panels for the diagnosis and classification of hematological malignancies in 2012 (van Dongen et al., 2012a), represented the only antibody panels that had undergone a full technical and clinical multi-center validation. Because of this thorough standardization, the proposed antibody panels and corresponding SOPs stimulated laboratories worldwide to evaluate the EuroFlow methods and tools against their local panels and immunophenotyping strategies. Since then, many diagnostic laboratories have fully or partially adopted the EuroFlow panels and SOPs. This resulted in more than 40,000 downloads of EuroFlow SOPs from the EuroFlow webpage by > 4,000 distinct institutions worldwide (status per November 2019; Fig. 3).

In turn, other laboratories have evaluated the EuroFlow panels and SOPs and *adapted* them to their settings. As an example, the same combination of CD-markers included in the LST, but with distinct clones and fluorochrome conjugated reagents, was first compared to the EuroFlow LST in 2014, confirming the optimal performance of LST (Preijers et al., 2014). Subsequently, an extended variant of the EuroFlow LST was proposed in a 10-color format (i.e. an LST tube containing CD10, CD14, CD33 and CD34 in the absence of TCRγδ and CD38) (Rajab and Porwit, 2015). Such EuroFlow-inspired variant tube is a stand-alone tube and does not fit in the EuroFlow diagnostic algorithm for leukemia & lymphoma diagnosis and classification. Interestingly, a similar variant of LST including CD10 instead of CD38 had already been tested by EuroFlow, but proved not to be cost-effective (Flores-Montero et al., 2012). Moreover, the inclusion of CD10 did not provide further accurate and efficient diagnosis of CD10+ vs CD10-negative B-CLPDs, while the deletion of CD38 reduced the utility in plasma cell dyscrasias, including lymphoplasmacytic lymphoma. In addition, lack of anti-TCRγδ in the modified LST versions leads to a need to repeat the assay for appropriate classification of T-cell malignancies in around 3% of T-CLPD. Of utmost importance, such LST variants cannot be used in combination with the LST data bases for fast, and reproducible automated gating, diagnostic orientation and reporting with the Infinicyt™ software (Flores-Montero et al., 2019a). This is of utmost relevance as the standardized EuroFlow approaches take advantage of combined use of optimal antibody combinations and antibody panels together with standardized instrument set-up and sample preparation SOPs, for fast and objective automated gating, classification and labelling of the normal and tumor cell populations that coexist in a sample, through direct comparison against well-established data bases of normal and patient samples (Pedreira et al., 2019). More recent studies have also evaluated the dried LST kits manufactured by Cytognos SL and by BD Biosciences (LST OneFlow reagent kit) showing optimal performance in distinct settings (van der Velden et al., 2017). Altogether, this reinforces the relevance of the EuroFlow panels for the diagnosis and classification of hematological malignancies in the field.

5. MRD monitoring

EuroFlow has finalised the design and validation of the MM MRD (Flores-Montero et al., 2017) and BCP-ALL MRD (Theunissen et al., 2017) panels. Final validation of T-ALL and CLL antibody panels is ongoing (Böttcher et al., 2019), while MRD panels for other B and T cell chronic lymphoproliferative disorders (CLPD) and acute myeloid leukemia (AML) are under development.

Since the first reports of the MM MRD panels and BCP-ALL MRD panels with SOPs, many groups have incorporated them into their routine practice. Recently, the EuroFlow MM MRD approach has been adopted by the International Myeloma Working Group (IMWG) as the reference method to define complete response (CR) and sustained MRD-negativity by flow cytometry (Flow-MRD negativity) (Kumar et al., 2016). More recently, the EuroFlow MM MRD antibody panel and approach proved to be of great sensitivity for detection of circulating tumor plasma cells (CTPC) in patients with monoclonal gammopathies, particularly in patients with MM and amyloidosis, both at diagnosis and after therapy, with significant potential for blood-based diagnosis, monitoring, prognostic and therapeutic ramifications (Puig et al., 2019; Sanoja-Flores et al., 2019; Sanoja-Flores et al., 2018). In an effort to reduce reagent costs, a few groups also modified the 2-tube 8-color panels proposed by EuroFlow into a single 8- (e.g. tube 2 only) and 10-color tube panel (Blum et al., 2019; Carulli et al., 2019; Roshal et al., 2017; Takamatsu et al., 2019). However, detailed analysis of the results of these comparisons show a systematically reduced sensitivity for the modified assays (up to a 70% decreased sensitivity) (Blum et al., 2019). Use of these modified 8-color (Carulli et al., 2019; Takamatsu et al., 2019) and 10-color (Blum et al., 2019) variants of the EuroFlow MM MRD antibody combinations, including lack of or replacement of CD117 by CD200, hampers adequate evaluation of sample quality (e.g. hemodilution) and early detection of false MRD-negative results (Flores-Montero et al., 2017, 2016; Pojero et al., 2015).

Similar to the EuroFlow MM MRD panel, the EuroFlow BCP-ALL MRD panel has been incorporated in many laboratories worldwide, including the new Spanish MRD-directed protocol PETHEMA (Protocolo Español para el Tratamiento de Hemopatias Malignas) 2019 protocol for adult non-*BCR-ABL1* ALL (available at: www.Clinicaltrials.gov).

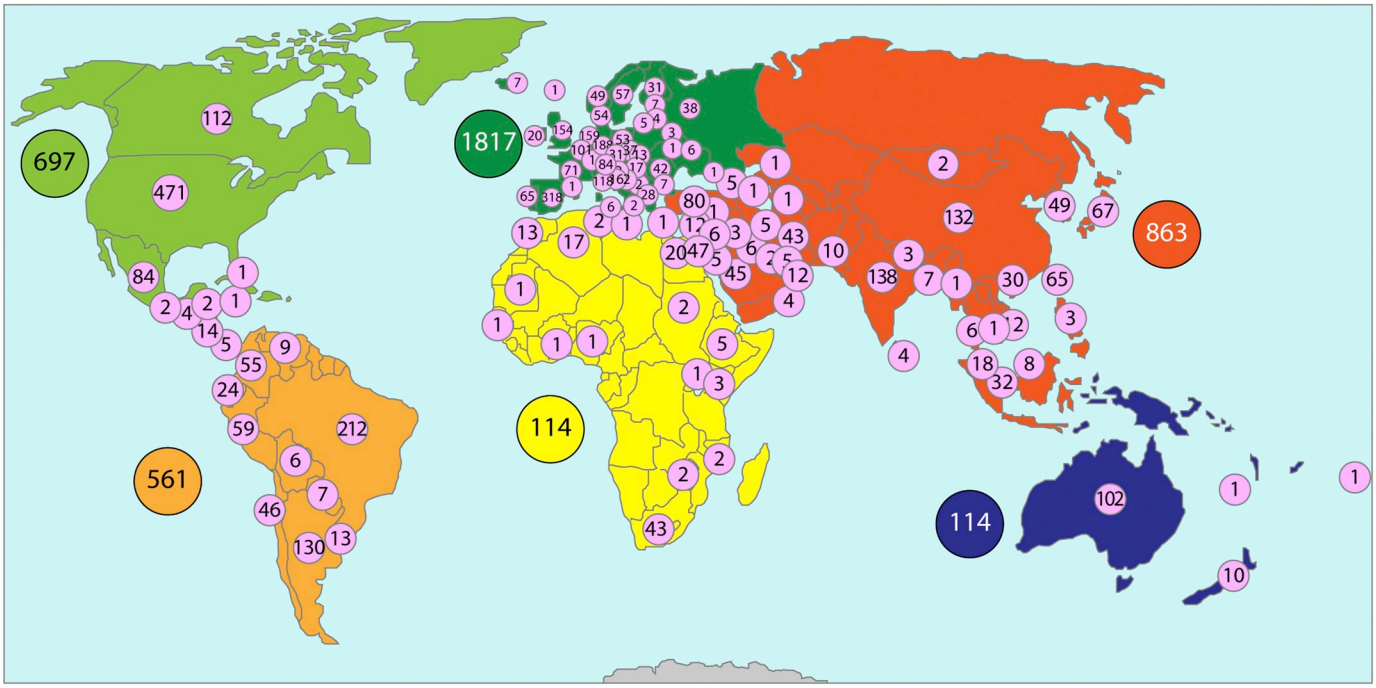

Fig. 3. Overview of registered recipients of EuroFlow documents and SOPs. > 40,000 downloads of the EuroFlow SOPs by > 4000 EuroFlow users and/or institutions worldwide were registered. (Spring 2013 — Autumn 2019).

6. Diagnosis and classification of primary immunodeficiencies (PID)

In 2019, EuroFlow introduced a flow cytometry-based system for the diagnosis and classification of PID of the lymphoid system. The system includes a primary immunodeficiency orientation tube (PIDOT), an IgH isotype tube as well as analysis algorithms based on the automated identification of relevant immune cell subpopulations (Blanco et al., 2019; van der Burg et al., 2019; van Dongen et al., 2019). The EuroFlow PID algorithm includes age-related reference ranges for both the EuroFlow PIDOT (van der Burg et al., 2019) and IgH-isotype tubes (Blanco et al., 2018). The EuroFlow PID algorithm provides a new and more solid approach to the diagnosis and classification of PID of the lymphoid system, including novel criteria for sub-classification of antibody deficiencies (PAD), such as IgA deficiency, IgG-subclass deficiencies and common variable immunodeficiency (CVID)(Chapel and Patel, 2019). Other EuroFlow PID tubes contribute to the characterization of other PID such as severe combined immunodeficiencies (SCID) and agammaglobulinemia (van der Burg et al., 2019).

7. Immune monitoring

Monitoring of CD4+ T-cell counts in blood of individuals infected with the human immunodeficiency virus (HIV) represents one of the first and most well-established clinical applications of flow cytometry (Kestens and Mandy, 2017). In addition, detailed monitoring of both the immune status and the effects of innovative immune modulatory treatments on immune cells has become critical in many distinct immune dysregulated conditions and diseases such as auto immune diseases, auto inflammatory diseases, and immunocompromised patients (Behnam Sani and Sawitzki, 2017; Boyd et al., 2017; Cogdill et al., 2017; Fridman et al., 2017). This is particularly true in case of intervention via immune modulatory treatments, including: i) classical immune suppressive drugs (corticosteroids, cyclosporine, methotrexate); ii) immune cell-based treatments, such as gene therapy, stem cell transplantation, CAR T-cell therapy; iii) the many different antibody treatments (with many different antibody effector functions); and iv) vaccination, whether against microorganisms, insect venoms, allergens or tumor antigens (Boyd et al., 2017; Cebon, 2018; Fridman et al., 2017; Furman and Davis, 2015).

Because the cells of the immune system have the unique capability of reaching any compartment of the body via migration through blood and homing, blood can be regarded as a crossroad where many relevant leukocytes and immune cell subsets can be identified. The immune response is inordinately complex, involving networks of distinct subsets of myeloid and lymphoid cells, with distinct maturation stages, activation stages and unique functional roles, all dependent of the timing of the assessment.

In order to provide standardized approaches and antibody panels for detailed dissection of innate and adaptive immune responses in blood, the EuroFlow group has developed 12- to > 30-color antibody panels (van der Pan et al., 2019) for immune monitoring purposes. These novel EuroFlow IMM antibody panels can be used for blood leukocyte subsetting with classical multi-parameter flow cytometric technologies, using ≥12 to > 30 different fluorochromes. Currently, several multicentric studies are ongoing to explore the clinical utility of the new IMM panels in vaccination, distinct disease conditions and in patients undergoing immunotherapies such as antibody- and CAR-T cell targeted therapies, the actual contribution of the novel EuroFlow IMM panels still remaining to be fully established.

8. EuroFlow educational activities

Starting in 2009, the EuroFlow consortium has contributed to the field with multiple seminars, lectures and workshops around the world in order to support laboratories and personnel interested in adopting EuroFlow antibody panels, SOPs and software tools (Fig. 4). More recently (2015), an external quality assurance program was initiated which now includes over 50 different institutions distributed worldwide (Kalina et al., 2018). In addition, all EuroFlow publications are Open Access and each EuroFlow SOP is available at the EuroFlow webpage (www.EuroFlow.org).

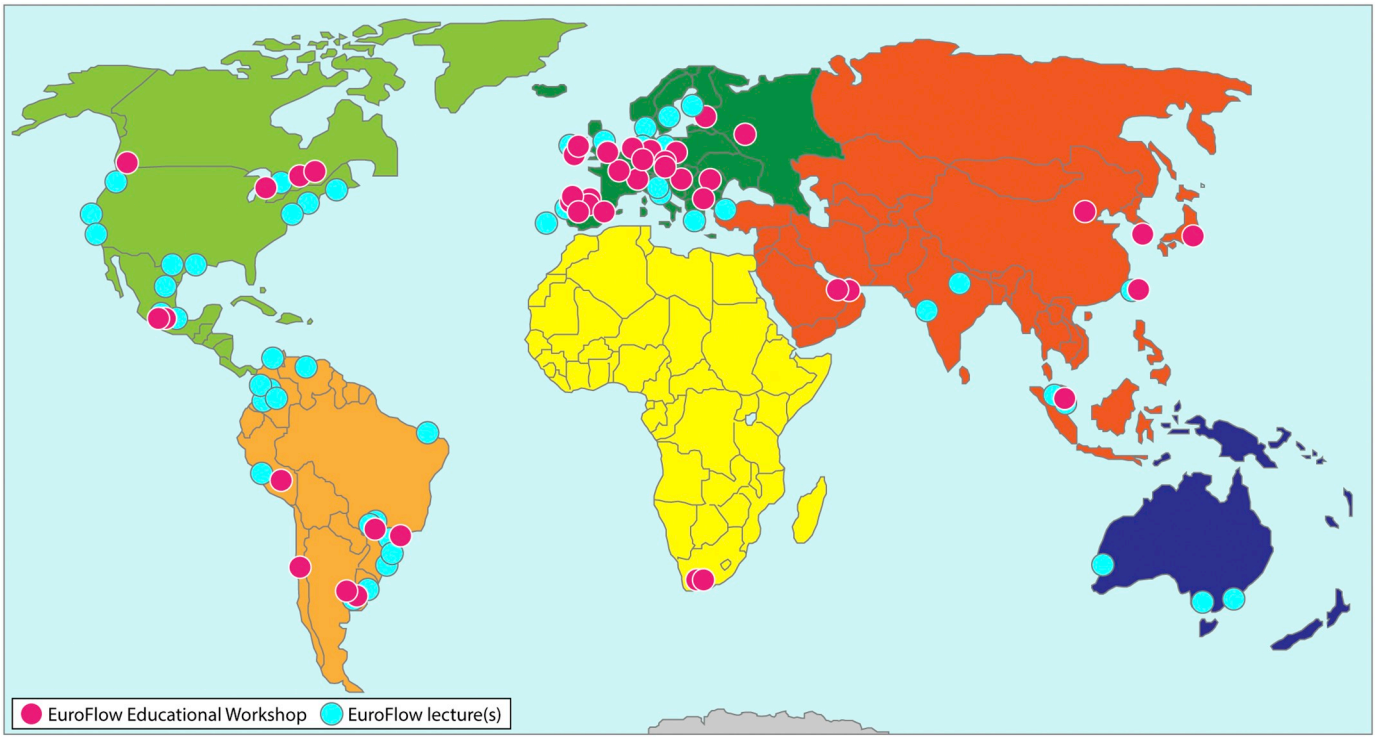

Fig. 4. EuroFlow Educational Workshops ($n = 55$) and EuroFlow invited lectures ($n = 105$) on multidimensional flow cytometry, organized from September 2009 till October 2019.

9. Journal of immunological methods special issue on EuroFlow

This special issue of the Journal of Immunological Methods is dedicated to EuroFlow. In total it contains 14 manuscripts describing several aspects of the work and achievements of EuroFlow and some of the critical underlying concepts (including this brief overview). The manuscripts are divided into three sections. Section 1 consists of five manuscripts describing technical aspects related to the implementation of the EuroFlow methods in distinct 8- to 10-color instrument platforms (Glier et al., 2019; Nováková et al., 2017), the strategies used for fluorochrome selection (Flores-Montero et al., 2019b) and the evaluation of the anti-IgH isotype and subclass antibody reagents (Blanco et al., 2017), together with the EuroFlow approach for determining lot-to-lot stability of fluorochrome-conjugated antibody reagents (Böttcher et al., 2017). Section 2 includes another four manuscripts devoted to the work performed by EuroFlow in developing the flow cytometry data bases (Flores-Montero et al., 2019a), and the strategies upon which the novel EuroFlow software tools were developed (Orfao et al., 2019; Pedreira et al., 2019; Sędek et al., 2018). Finally, Section 3 consists of four manuscripts which focus on different aspects of standardization and quality assessment in diagnostic flow cytometry. The contents of these last four manuscripts include lessons learned from the EuroFlow quality assurance program (Kalina et al., 2018), the evaluation of the impact of blood storage and sample handling on the quality of flow cytometry data (Diks et al., 2019), implementation of EuroFlow strategies and tools in diagnostic practice using the optimization and testing of the EuroFlow LST and PIDOT dried antibody tubes as an illustrating example (van der Velden et al., 2017) and standardization of 8-color flow cytometry across different laboratories (Glier et al., 2017).

We hope that the combination of comprehensive and complementary manuscripts provided here, along with the previously published EuroFlow manuscripts and detailed protocols available at the EuroFlow webpage (www.EuroFlow.org), will contribute to a better understanding of the EuroFlow concepts and provide complementary educational tools necessary for any worldwide laboratory that has started to implement the EuroFlow concepts and tools in the fields of hemato-oncology, immunodeficiencies and immune monitoring.

Acknowledgements

EuroFlow activities have been supported by Specific Targeted Research Project (STREP) in the FP6-2004-LIFESCIHEALTH-5 program of the European Commission (grant LSHB-CT-2006-018708)

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