DCTH - 2·2015 - 65-70

REVIEW

Novel Applications for the Flow Cytometry

Maria Chiara Lazzari, Manuele Ongari, Giulia Di Martino, Andrea Ravelli, Clara Virginia Vigano, Paola Brambilla, Pierangelo Spedini, Francesco Lanza

Section of Hematology and Flow Cytometry Unit, Hospital of Cremona, Italy

SUMMARY

Flow cytometry is a technology which measures physical properties and fluorescence of particles, for example cells, in monodisperse solution. Physical properties, like size and internal complexity, can be analyzed thanks to the interaction between particles and light which generates optical dispersion, reflection and diffraction; fluorescence can be used by binding or intercalating fluorescent molecules to cellular components or by linking them to monoclonal antibodies directed to specific surface or intracellular proteins. The labeled cells are carried by a laminar flow of isotonic fluid in front of a light source where the fluorophores can be excited by a specific wavelength: the resulting fluorescent light emissions are collected by an optical system consisting of filters and dichroic mirrors in order to isolate each wavelength and then convert it into digital signals. Flow cytometry has become an essential tool in clinical practice, particularly for diagnosis and for the classification of hematological malignancies.

ACOUSTIC FLOW CYTOMETRY AND ACOUSTOPHERESIS

One of the most important clinical applications of flow cytometry is the absolute count of CD34+ circulating cells in peripheral blood (PBSC), which renders possible auto- and allo-transplantation starting from peripheral blood (1). Due to its high sensitivity and precision, cytometry largely proved to be a powerful tool for the determination of the most suitable moment to collect PBSC in sufficient amount, as well as to indicate their value and viability during the harvest procedure (2).

The great success of cytometry is also due to the possibility of creating international standardization procedures like the well-known "ISHAGE" protocol (3, 4). Recently it has been introduced an innovative separative approach based on acoustic waves (referred to as "acoustopheresis"), which is changing the whole universe of flow cytometry thanks to the so-called "acoustic" focusing, instead of the conventional hydrodynamic system.

The traditional apheretic technology

Key words: flow cytometry, hematological malignancies, CD34+ cells.

Paper in part supported by the Charity "Beat Leukemia".

Correspondence:
Francesco Lanza
E-mail: f.lanza@ospedale.cremona.it

is based on centrifugation of a continuous flow of whole blood from which leukocytes are separated, while the residual blood components are reinfused in the donor.
This classical separative approach can cause excessive platelets depletion in the donor which may increase the risk of bleeding and, nonetheless, the excessive contamination of apheretic products may create problems for their manipulation (4).
Recently, it has been discovered that acoustic waves are able to create a field of forces which can move particles in a laminar flow, depending on their physical properties respect the surrounding medium. Based on their size, it's possible to move the selected particles from a medium to another in order to obtain highly purified products and to reduce platelet contamination and related problems in both the donor and the receiver (4, 5).
In traditional cytometers, a suspension of particles (or cells) is injected in the center of a rapid flow of a sheet fluid to create a laminar flow which carries them toward the optical system where they can be excited by laser rays. In such system, the acquisition speed is regulated by manipulating the fluid pressure, with the limitation that the more the fluid pressure increases, the more the dispersion of particles widens, causing a signal loss of stability. For this reason, in order to obtain optimal data from a conventional cytometer, the acquisition speed should not exceed 10-20 µL/min.
The acoustic focusing, instead, allows overcoming this limitation by making the alignment of particles independent from hydrodynamic forces. An acoustic cytometer includes many of the components of the conventional ones, but, unlike these, it is equipped with a piezoelectric system that generates acoustic waves which exactly align particles in the center of the capillary (5). The precise injection of the sample is possible by using a syringe which must avoid direct contact between sample and the walls of the optical cell. The main advantage offered by this new generation of instruments is increasing the acquisition speed up to 20,000,000 events, thus raising also sensitivity of analysis.
Furthermore, it becomes possible to acquire extremely diluted samples without a preliminary concentrating step. Finally, the higher ability of acoustic fields respect to the hydrodynamic force in the alignment of particles provides more precise, clear and less variable signals compared to those generated by the conventional technology (5, 6).

CELL SORTING

Flow cytometry is an extremely powerful tool allowing the measurement of a considerable amount of parameters (physical and optical) on a large number of suspended particles in a short period of time. The "flow sorting" is a process which physically separates particles of interest from a heterogeneous population using the analytical cytometer (7).
There are many cases where the isolation of a population of interest is useful or important: cell cultures, research, microbiology, and so on. The whole genome sequencing programs and, more recently, the "chromosome painting" technology, demonstrated

that also subcellular structures such as organelles and chromosomes can be successfully isolated (7, 8). Briefly, a flow sorter operates through a mechanism of electrostatic deflection of electrically charged microdroplets released into air. The formation of these microdroplets is conditioned by several factors such as the conformation of the exit hole, the fluid pressure and viscosity and by the temperature. Despite this, applying a vibration - also acoustic - of known frequency to the fluid, it's possible to stabilize the formation of drops, their size and the distance between them, in well-known and fixed working conditions. The distance between the point of drop formation and the point where it hits the laser rays is defined as "drop delay" and represents a critical value for the proper functioning of a cell sorter because it largely influences the electrical charging process of the selected droplets in the exact moment in which they are formed (9, 10). Obviously, the electrical charge can be positive, negative or neutral and it allows the deflection of each drop in different directions through the application of electric fields from 2000 up to 6000 V.

The first flow sorters were able to separate only two populations of drops (and particles) at the same time, one towards an electric pole and one towards the other, while the most recent ones can discriminate simultaneously up to four populations. Of course, no instrument has a separation efficiency of 100%, causing the loss of a variable amount of the sample, mainly due to the inevitable processes of aggregation of droplets or, anyway, to the currently unavoidable technical limitations (10-12).

FUSION PROTEINS

The presence of specific genetic aberrations is nowadays essential to the classification, stratification and treatment of many hematological malignancies. A great number of these genetic aberrations, particularly in acute leukaemias, are represented by translocations, a phenomenon involving the fusion of two different genes belonging to different chromosomes. These pathological fusion-genes can be identified through different methods, such as by karyotype evaluation, FISH, or by molecular analysis through qRT-PCR.

Over the last few years, researchers have been using flow cytometric methods to identify an abnormal fusion protein. The time required by a "flow cytometric immunoassay" can be reduced to 3-4 hours and can be performed on a traditional flow cytometer. The first assay was directed to the BCR-ABL fusion protein, which is typically found in patients affected by CML and in many cases of B-LAL (13).

The BCR-ABL fusion protein exists in different forms depending on the position of the break-points on the *BCR* gene: the most common ones are *M-bcr*, which generates the so called p210 fusion protein; *m-bcr*, which generates the p190 variant and *bcr* which is related to the p230 form. The traditional molecular biology exploits different PCR primers which are able to specifically identify the *M-bcr* and *m-bcr* variants, while the rarest ones are frequently lost.

The flow cytometric approach, in order to recognize most of the variants, uses monoclonal antibodies against BCR epitopes upstream the breaking point

and encoded by the exon 1 of the *BCR* gene and a second monoclonal antibody directed against one epitope downstream the breaking point of ABL protein (ABL-SH2 domain). The cellular lysate is first enriched with "capture beads" which are linked to BCR-specific antibodies, and in a second time with PE-conjugated-anti-ABL antibodies, in order to perform a kind of "sandwich ELISA" where the fluorescent immunocomplexes are identified just by flow cytometry. If no fusion protein is present, no PE-signal is detectable; otherwise, the PE-signal increases as the protein is present in larger amount. Many other fusion proteins can be detected by this cytometric approach, such as PML-RARα, involved in acute promyelocytic leukemia, as well as TEL-AML1, E2A-PBX1, MLL-AF4, AML1-ETO and CBFB-MYH11 (14).

Theoretically, it is also possible to mix many types of immunobeads of different size, color, or other features, to create a "multiplexing" assay (15).

As an example, *MLL* fusion gene can generate six different fusion proteins: using six different beads directed to each variant, it is possible to detect all of them at the same time in a single assay (9, 16). Based on these data, it can be stated that the flow cytometry assay is a powerful diagnostic tool for the detection of intracellular fusion proteins such as BCR-ABL in leukemic cells, and may be utilized for clinical purposes.

In conclusion, the most important advantages offered by cytometric detection of leukemic fusion proteins compared to the traditional molecular techniques are:

- independence from breaking point position;
- rapidity (3-4 hours);
- possibility of "multiplexing" assays;
- feasibility on traditional cytometers;
- simultaneous immunophenotyping.

MASS CYTOMETRY

The potential of flow cytometric assays is firstly due to the possibility of performing simultaneous multiple evaluations based on several physical or biological parameters on a large amount of particles, and moreover in a relatively short lapse of time. Multiparametric assays are essential in a wide variety of disciplines such as pharmacology, in preclinical research, or in clinical oncology, for example to evaluate the diagnostic and prognostic value of novel biomarkers (17). Immunophenotyping is an essential tool for diagnosis, classification and management of hematological malignancies, but many studies are now showing that also several solid tumors (like breast, prostate or bladder cancer) are seen to carry specific expression aberrancies of surface antigens at every stage of carcinogenesis (18). Obviously no single marker alone could ever possess the specificity and the sensitivity which are necessary for its clinical practice but, on the contrary, it is always the combination of multiple markers and the quantitative changes of their expression to determine a pattern which may have clinical significance.

A large number of parameters is also requested to identify rare populations: the higher is the number of parameters analyzed, the lower is the probability of overlapping between them. Traditional cytometers are progressively increasing the number of parameters

analyzed at the same time, but there are some technical limitations which are too difficult to overcome, mainly because of optical overlapping and interference between different wavelengths. This is the main reason at the basis of the extraordinary potential of mass cytometry: it uses monoclonal antibodies labeled with different lanthanides isotopes that offer a wide range of stable atoms with the same chemistry, which can be added at the same molecules (19). Moreover, lanthanides are not very abundant in nature and this allows minimizing the background noise in analysis.

The different isotopes are linked to monoclonal antibodies through chelating molecules which can carry up to 30 atoms, thus increasing the signal and, consequently, the sensitivity of detection. Obviously, the detection system must be completely different to the traditional cytometers, since the photomultipliers are replaced by a Time-Of-Flight (TOF) mass spectrometer that is able to separate, and then detect, a large amount of different isotopes simultaneously. The first generation of mass cytometers presents two important limitations: first, a low speed of analysis (about 1000 events/sec), and, moreover, the destruction of sample which cannot be sorted, in case. Nevertheless, it must be considered that a mass cytometer can analyze up to 30 parameters at the same time, versus the 5-10 ones of conventional instrument, and that this technology can be used for studying not only proteins, but virtually all cellular components, thus opening new frontiers in oncology, transplantation, stem cell biology, drug discovery, and related biomedical sciences (20).

REFERENCES

1. Shenkin M, Babu R, Maiese R. Accurate assessment of cell count and viability with a flow cytometer. Cytometry B Clin Cytom. 2007; 72: 427-32.
2. Lanza F, Healy L, Sutherland DR. Structural and functional features of the CD34 antigen: an update. J Biol Regul Homeost Agents. 2001; 15: 1-13.
3. Whitby A, Whitby L, Fletcher M, et al. ISHAGE protocol: are we doing it correctly? Cytometry B Clin Cytom. 2012; 82: 9-17.
4. Dreier J, Vollmer T, Kleesiek K. Novel flow cytometry-based screening for bacterial contamination of donor platelet preparations compared with other rapid screening methods. Clin Chem. 2009; 55: 1492-502.
5. Piyasena ME, Austin Suthanthiraraj PP, Applegate RW, Jr. et al. Multinode acoustic focusing for parallel flow cytometry. Anal Chem. 2012; 84: 1831-9.
6. Ferrarezi MC, Curci VC, Cardoso TC. Cellular vacuolation and mitochondrial-associated factors induced by Clostridium perfringens epsilon toxin detected using acoustic flow cytometry. Anaerobe. 2013; 24: 55-9.
7. Galbraith D. Flow cytometry and cell sorting: the next generation. Methods. 2012; 57: 249-50.
8. Kumar N, Borth N. Flow-cytometry and cell sorting: an efficient approach to investigate productivity and cell physiology in mammalian cell factories. Methods. 2012; 56: 366-74.
9. Osborne GW, Andersen SB, Battye FL. Development of a novel cell sorting method that samples population diversity in flow cytometry. Cytometry A. 2015; 87: 1047-51.
10. Want A, Hancocks H, Thomas CR, et al. Multi-parameter flow cytometry and cell sorting reveal extensive physiological heterogeneity in Bacillus cereus batch cultures. Biotechnol Lett. 2011; 33: 1395-405.
11. Muller S, Nebe-von-Caron G. Functional single-cell analyses: flow cytometry and cell sorting of microbial populations and

communities. FEMS Microbiol Rev. 2010; 34: 554-87.
12. Nelson N, Szekeres K, Cooper D, Ghansah T. Preparation of myeloid derived suppressor cells (MDSC) from naive and pancreatic tumor-bearing mice using flow cytometry and automated magnetic activated cell sorting (AutoMACS). Journal of visualized experiments: JoVE. 2012; e3875.
13. Hamilton A, Elrick L, Myssina S, et al. BCR-ABL activity and its response to drugs can be determined in CD34+ CML stem cells by CrkL phosphorylation status using flow cytometry. Leukemia. 2006; 20: 1035-9.
14. Cavazzini F, Campioni D, Luisa Ferrari, Buldini B, et al. Expression of the immunoglobulin superfamily cell membrane adhesion molecule Cd146 in acute leukemia. Cytometry Part B Clinical Cytometry 06/2015.
15. Muehlbauer PA, Spellman RA, Gunther WC, et al. Improving dose selection and identification of aneugens in the in vitro chromosome aberration test by integration of flow cytometry-based methods. Environ Mol Mutagen. 2008; 49: 318-27.
16. Li H, Mao G, Carlson J, Leng SX. A novel flow cytometry-based tool for determining the efficiency of human cytomegalovirus infection in THP-1 derived macrophages. J Virol Methods. 2015; 221: 127-30.
17. Bodenmiller B, Zunder ER, Finck R, Chen TJ, Savig ES, Bruggner RV, et al. Multiplexed mass cytometry profiling of cellular states perturbed by small-molecule regulators. Nat Biotechnol. 2012; 30: 858-67.
18. Hemmer J, Kraft K, Van Heerden WF. Correlation between DNA ploidy by flow cytometry and chromosome 3 aberration in oral squamous cell carcinoma. Oncol Rep. 2006; 15: 243-6.
19. Silverman DH, Delpassand ES, Torabi F, et al. Radiolabeled antibody therapy in non-Hodgkins lymphoma: radiation protection, isotope comparisons and quality of life issues. Cancer Treat Rev. 2004; 30: 165-72.
20. Hansmann L, Blum L, Ju CH, et al. Mass cytometry analysis shows that a novel memory phenotype B cell is expanded in multiple myeloma. Cancer Immunol Res. 2015; 3: 650-60.