Past and present concepts in flow cytometry: A European perspective

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ABSTRACT: The development of flow cytometric instrumentation, methods and research concepts in Europe has been a continuous driving force for the general scientific advancement in this area over the years. This review addresses early European concepts of continuing interest with regard to instrumentation, data analysis, clinical and eperimental DNA analysis, cell function and microbiology at their worldwide first appearence while flow cytometric immunology and immunophenotyping will be covered separately. Flow cytometry represents an efficient approach to the enormous complexity of molecular cell architecture and cell function by the analysis of apparent molecular cell phenotypes in heterogeneous cell samples. The present merger of flow and image cytometry into the method independent cytomics discipline increases the potential of cell analysis very significantly. It opens the way for predictive medicine as well as for predictive cytopathology and predictive cytology in everyday clinical and medical practice. Current progress is driven by joint advances in molecular fluorescence technologies and instrument development. This complements the analysis of genome sequence information in an efficient way. (J Biol Regul Homeost Agents 2003; 17: 213-22)

KEY WORDS: *Flow cytometry, Instrumentation, DNA, Cell function, Data analysis, Predictive medicine, Cytomics, Cytology*

Background

The cytometric discipline is increasingly impacting on the development of modern bioscience. A major reason for this is that the very detailed knowledge on the myriades of molecules coded by the genome does by itself presently not explain how molecules associate to constitute the architecture and function of living cells. The high number of theoretical possibilities for molecular interactions to form structures or highly ordered and compartimentalized complex metabolic pathways is prohibitive for an understanding of living cells by top-down hypothesis and experimentation.

Alternatively, the cytometric bottom-up strategy of observing molecular presence and molecular action in intact cells as the elementary building units of tissues, organs and organisms, constitutes a very promising conceptual and experimental approach. It comprises the potential to uncover essential parts of the molecular puzzle in cells and cellular systems by a hypothesis driven inductive reverse engineering like process.

Cytophotometry

The origin of cytometric measurements came through the early efforts of cytophotometric instrument development for the determination of molecular properties of single cells in health or disease (1). Cells for cytophotometric studies were typically immobilized on slides, fixed and screened for photometric absorption in predetermined areas by a stepwise moving small pinhole within the focal plane of a microscope objective. Light absorption at the nucleotide absorption maximum around 260nm was summed up over an entire cell nucleus and expressed as integrated optical density to obtain a measure of cellular DNA content. Stoichiometric Feulgen staining (2) and light absorption measurement between 550-570nm (3) as an alternative did not require UV-optics.The protein content of the cytoplasm was determined from the light absorption of aromatic amino acids (tyrosine, tryptophane, phenylalanin) around 280nm. The Zeiss UMSP-1 cytophotometer (Oberkochem, Germany) was frequently used in the early days for such determinations although the low measuring speed of 5-10 min per cell nucleus or cytoplasmic compartment was prohibitive for larger studies.

Pulse cytophotometry

A very significant improvement for the cytophotometry concept of molecular cell research was introduced (4) by keeping the microscope's pinhole fixed while moving fluorescence stained cells in a closed cuvette within a narrowly focused epi-illuminated laminar fluid stream at ca 1 m/sec speed below the pinhole through the focal plane of the microscope objective. The resulting fluorescence light pulses were amplified by photomultipliers and classified according to amplitude or area in nuclear physics multichannel analyzers. Several thousand cells/sec were typically measured in

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the worldwide first commercially available flow cytometer, the ICP-11 Impulscytophotometer (Phywe 1969, Göttingen, Germany). Two photomultiplier tubes collected the fluorescence light within different emission wavelength ranges. Doublet discrimination (5, 6) was achieved via pulse height over pulse area ratios. This pulse analysis mode is today routinely implemented in most current flow cytometers or cell sorters. The ICP-11 pulse cytophotometer was the first fluorescence oriented (7) flow cytometer despite the still ongoing discussion at this time whether fluorescence (4, 7) or absorption (8, 9) was better suited for flow cytometric measurements. The Phywe ICP instrument development was purchased in 1976 by Ortho-Diagnostics (Raritan, NJ, USA) to disappear from the market. PARTEC company, Münster (Germany) continued, however, the Impulscytophotometer (7) development with a piezo crystal driven closed cuvette cell sorter to sort cells (10-12) but also larger particles like pancreatic islets (13). The sorter became available in 1986 together with other flow cytometric instrumentation.

Nomenclature

The early nomenclature for the new technology was very heterogeneous. It varied between Impulsfluorimetrie (7, 14), Impulsfluorometrie (15), Impulscytophotometrie (16), Impulszytophotometrie (17), Impulsmicrophotometrie (18), impulsecytophotometry (19), pulse cytophotometry (20), micro-flow fluorometry (21), microflow fluorometry (22) in Europe as opposed to flow cytometry (23) or flow microfluorimetry (24) being mostly used in the USA. The term flow cytofluorometry (25) was used in a shared way, a further term was flow DNA analysis (26).

The term flow cytometry was finally adopted at the 5th American Engineering Foundation Conference on Automated Cytology in Pensacola, FL, USA in 1976. Due to nomenclature change and the very distributed publishing habits of the early adepts of the new technology, a significant part of the groundbreaking work remains usually unconsidered. A literature screen for the time period 1969-1978 turns up 60 journal articles for the European and shared nomenclature as opposed to 44 journal articles for flow cytometry, flow microfluorimetry and flow cytofluorometry in majority from US authors. This indicates the considerable early activities of European research in this newly emerging scientific domain despite the fact that the search does not account for a significant number of relevant book articles or articles in journals which are not listed by the Institute for Scientic Information (ISI).

European pulse cytophotometry

A first meeting of the growing community of scientists and clinicians interested in pulse cytophotometry was organized by Michael Andreeff in Heidelberg 1972. Consecutive meetings in Nijmegen 1974, Münster 1975, Vienna 1977, Voss 1979 and Rome 1980 were organized by Clemens Haanen, Wolfgang Göhde, Dieter Lutz, Ole Laerum and Francesco Mauro. Proceedings of these meeting are available as Pulse Cytophotometry I edited by CAM Haanen, HFP Hillen, JMC Wessels, Pulse Cytophotometry II edited by W Göhde, J Schumann, Th Büchner, Pulse Cytophotometry III edited by D Lutz. Pulse Cytophotometry I-III were printed by European Press, Ghent, Belgium 1975, 1976, 1978. Flow Cytometry IV was edited by OD Laerum, T Lindmo, E Thorud and printed by Universitetsforlaget, Bergen 1980. A page identical duplicate of this book is available in the Acta Pathol Microbio Immunol A, 1981, Supplement 274: 1-535. The abstracts of the Rome meeting 1980 were edited by F Mauro and G Mazzini. They are printed in Basic and Applied Histochemistry 1980; 24: 229-398 but no proceedings of this meeting are available. The importance of the early European meetings is apparent from the fact that 20 of the cited references in this review article are localized in the proceedings of the meetings between 1973 and 1980. Seven of the cited articles are printed in the Journal of Histochemistry and Cytochemistry. They derive from European work presented at the Automated Cytology meetings organized during approximately the same time period intermittently in the US and Europe by the American Engineering Foundation (http://www.isac-net.org/ history/isachistory.htm).

Finally the Society for Analytical Cytology (SAC) was founded at the 6th American Engineering Foundation Conference on Automated Cytology at Schloß Elmau, Germany in 1978. This generated the initially not undisputed tendency amongst European cytometric scientists to discontinue the European meetings after 1980 and to become scientifically active within SAC. As a consequence, SAC meetings were organized in the USA and Europe according to an agreed schedule and the Cytometry journal was founded. This structure contributed very essentially to the fast development of scientific impact of the cytometric discipline altogether during the last decades.

National cytometric societies were founded in Italy, France, Portugal/Spain, Germany, Denmark, UK (Royal Microscopic Society), Belgium, Switzerland, Sweden, Poland and Austria between 1985 and 2000. The foundation of the European Society for Analytical Cellular Pathology (ESACP) in 1986 together with its Analytical Cellular Pathology (ACP) journal in 1989 and of the European Working Group on Clinical Cell Analysis (EWGCCA) in 1996 together with its affiliation to the *Journal of Biological Regulators and Homeostatic Agents (JBRHA)* since 2002 indicates the necessity for national and regional associations in Europe. These structures permit to cope with the fast progress in scientific and clinical cytometry. The issues of quality assessment, quality control and training in the field of clinical cytometry in Europe are addressed by EWGCCA, by EuroStandards (Sheffield, UK) as the central facility for the production of refer-

ence standards and by UK-NEQAS for Leukocyte Immunophenotyping (Sheffield, UK).

Instrumentation

During the fast international spread of ICP-11 Impulscytophotometers, a number of European research groups developed their own instrumentation because the ICP-11 and its successor instrument ICP-22 covered only parts of their interests. The mercury arc lamp was useful for fluorescence excitation in the UV and blue light range but no light scatter or electrical cell volume determination was initially possible and weak immunofluorescences were difficult to detect due to the low FITC excitation energy of the HBO-100 mercury arc lamp between 480-500nm. The Cytofluorograf (Ortho-Diagnostics 1970) contained an argon laser for fluorescence excitation at 488nm, including light scatter determination but no possibility for UV excitation or cell volume measurement was provided.

The requirements for more fluorescence parameters led to the development of the first dual laser instrument at the Deutsches Krebsforschungszentrum (DKFZ) in Heidelberg (27). The need for fast and precise length measurements of cells and cell aggregates generated a laser based instrument (28, 29) at the Gesellschaft für Strahlenforschung (GSF), Hannover which was later commercialized by Kratel-Instrumente (Leonberg-Stuttgart, Germany).

The determination of analyte concentrations or average surface densities of molecules for cell biochemical purposes prompted the development of the Metricell (30) and Fluvo-Metricell instrumentation (31). A hydrodynamically focused Coulter orifice for absolute volume measurements was initially combined for this purpose with the optical part of an ICP-11 instrument at the Max-Planck-Institut für Biochemie in Munich-Martinsried. A multichannel analyzer with attached computer for on-line list mode data storage and analysis (32) were characteristic for this instrument. The Fluvo-Metricell was manufactured by HEKA Elektronik (Forst/Weinstraße, Germany) between 1985-1990.

The artificial right skew of cell volume distribution curves recorded with Coulter counter electrical sizing orifices was during a certain time prohibitive for exact cell volume distribution measurements. The considerable efforts to prove that the skew was indeed an artifact generated initially the droplet cell sorter (33). The exact nature of the artifact became, however, only clear by high speed flow cytometric imaging using pulse laser flashes (34) or microsecond argon arc illumination (35). The skew is caused by M-shaped electrical pulses leading to an overestimation of cell size during pulse height analysis. M-shaped pulse are caused by cells traveling close to the entrance edge of the sizing orifice through zones of increased electrical field strength (34, 36, 37). Hydrodynamic focusing (36, 38, 39), electronic rejection of M-shaped pulses (40) or conical shaping of the orifice efflux zone (41) reliably avoids or reduces the artifact. AEG Telefunken company (Ulm, Germany) exploited the hydrodynamically focused electrical sizing (36) with its Telefunken Partikelanalysegerät (1972). The AEG development was subsequently bought by Coulter company (Miami, FL, USA) to disappear from the market. The observation that transcellular ion fluxes at increased transmembrane voltage of cells passing through sizing orifices were caused by a dielectric breakdown of the cell membrane (42-45) provided the basis for the transmembrane transport of extracellular analytes into cells by electroporation. Electroporation has subsequently developed into a widely used transfection technology in molecular biology.

High speed flow cytometric imaging gave rise to the concept of fast imaging in flow (46), an idea that has recently been taken up for high throughput screening (47) in the ImageStream100 (Amnis, Seattle, USA) instrument.

The necessity for fast signal processing and histogram display, including software driven fast ratio calculations prompted the early use of microprocessors (48-50) for flow cytometric purposes instead of hardware circuits or main frame computers. The modular instrumentation was commercialized by Dr. O. Ahrens Meßtechnik (Bargteheide, Germany) a company being focused today on the production of DNA image analysis systems.

The requirements for carefully time and temperature controlled flow cytometric experiments at low level fluorescence led to the development of a sophisticated flow cytometer and cell sorter (51). It was equipped with several lasers, fast computer data evaluation and flow chambers with highly efficient light collection (52, 53).

The need for the analysis of microorganism such as bacteria, yeast cells (54) or other microorganisms was at the origin of a particularly sensitive epi-illumination mercury arc lamp system (55). The instrument was consecutively produced under the names MPV flow cytometer by Leitz (Wetzlar, Germany), Argus100 by Skatron (Tranby, Norway) and is presently distributed as Bryte HS by Biorad-Laboratories (Hercules, USA).

The Bruker-Odam company, Wissembourg, France, produced the ATC3000 flow cytometer and cell sorter between 1990-1993. It was equipped with a hydrodynamically focused Coulter sizing orifice in combination with multilaser fluorescence excitation and developed under the auspices of the French Commissariat à l'Enérgie Atomique (CEA). Fast graphics and the possibility for a high number of simultaneous polygonal or elliptical multiparameter gates constituted a particular feature of this instrument.

Experimental and clinical DNA cytometry

Ethidiumbromide (EB, 2,7-diamino-10-ethyl-9-phenylphenantridiniumbromide, 7, 14), DAPI (4',6-diamidino- 2-phenyindole, 56), EB+mithramycin (57),

Hoechst 33342 and 33258 (2,6-bisbenzimidazol-derivatives, 58) for DNA or DANS (1-dimethyl-aminonapthalin-5-sulfochlorid (14) and FITC (fluoresceinisothiocyanate, 59) for protein were used for one or two parameter flow cytometric measurements. A DNA against protein double staining represented the first flow cytometric two parameter fluorescence measurement (14). The resulting histograms were subject to mathematical analysis (60, 61).

Biological material was typically digested with 0.5% acid pepsin pH 1.8 (19, 62) or pronase (63) for the preparation of cell nuclei. Alternatively its was treated with 0.1-1% RNase (7, 19, 20, 64) for RNA elimination in entire cells. Furthermore, high and low salt procedures at pH 10 and 5.8 in the presence of RNase and detergent (65) or trypsin together with detergent (66) were useful for the determination of DNA distribution curves of cells or cell nuclei. The successful enzymatic cell nuclei preparation from paraffin block material (63) gained its widespread popularity only significantly later (67).

The low coefficients of variation (CV = 100 x standard deviation/mean) of DNA distribution curves recorded by the mercury arc operated Phywe and Partec instruments in conjunction with EB+ mithramycin stain enhancement (57, 68) opened the way into the analytical and preparative separation of human and animal x and y spermatids (69, 70) as well as into the non laser based flow cytometric chromosome DNA analysis (71, 72).

The clinical interest in DNA content measurements concerned the detection of DNA aneuploidy as a sign of tumor neoplasia. In addition, DNA aneuploidy was frequently used for the characterization of tumor prognosis in form of the DNA index. The S-phase fraction of the cell cycle (19, 20, 62, 73-75) characterized the proliferative activity of malignant cells. The detection of precancerous lesions (76), stomach cancers (17, 77), leukemias and lymphoma (73, 78-80) or abnormal granulo- and erythropoiesis (81) as well as the measurements on synovial (82), skin (22) or bladder tumor cells (83, 84) indicate an immediately widespread clinical interest in the new technology.

The use of DNA aneuploidy or S-phase determinations for everyday clinical decisions remained, however, limited (85-89) despite substantial efforts of many scientists resulting in worldwide more than 1.000 clinically oriented journal articles between 1969 and 2003.

Cell cycle analysis, hematopoietic stem cells

An essential part of the initial work was conceptually oriented towards experimental models for DNA cell cycle analysis in unperturbed or pharmacologically perturbed situations by cytostatic drugs like VELBE (14), daunomycin (16), bleomycin (90), combinations of adriamycin and bleomycin (59) as well as by ionizing radiation (59, 91-93). Duration of cell cycle phases (94), cell cycle synchronization by x-irradiation and daunomycin (95), contact inhibition (96, 97) and ConA cell agglutination (98) were other topics of early interest. The flow cytometric bromodeoxyuridine (BUDR) Hoechst 33258 quenching technique (99) provided a fast and excellent non radioactive alternative for the study of cell cycle phase duration while the KI-67 antibody has been extensively used for cell proliferation studies (100). Hematopoietic stem cells were characterized by light scatter properties (101) and FITC-antibody binding (102) following enrichment by centrifugal elutriation while flow cytometric micronuclei determination indicated the mutagenic potential of ionizing irradiation (103) or cytotoxic drugs.

Predictive cytology and cytopathology by DNA image cytometry

A very informative part of the cytometric DNA analysis work consists in the DNA image analysis of potentially malignant cells within dysplastic tissue areas. DNA aneuploid cells in such lesions indicate that a significant number of initially dysplastic lung (104), larynx (105) and uterine cervix (106) epithelial lesions will turn later into cancers after variable time intervals. DNA image analysis was capable of predicting malignant tumor recurrence (107) and is today increasingly in use as reference method for the detection of cytological malignancy (108, 109). It has become apparent that DNA image cytometry is more accurate than the traditional definition of cytological malignancy by morphological features. The DNA image analysis concept was recently extended to predictions with >95% accuracy for cancer development in oral leukoplakias (110-113). Quantitative DNA image cytometry opens the way for predictive cytology and predictive cytopathology. This emphasizes the importance of early rather than late chromosome instabilities for the prediction of neoplasia development in the human organism.

Fluorescence anisotropy, fluorescence resonance energy transfer (FRET), light scatter polarization and Raman scatter

Fluorescence anisotropy (114) and fluorescence resonance energy transfer (FRET) (27, 68, 115-118) permitted the study of membrane fluidity and spatial closeness of interacting biomolecules by flow cytometry. Light scatter polarization (119) opened a way for the rapid discrimination of unstained lympho-, mono- and granulocytes as well as baso- and eosinophils (120) in diluted blood. Confocal Raman microspectroscopy accesses the functionality of a significant number of molecules in unstained living cells (121).

Cell functions

The interest in cell functions as informative and rapidly reactive parameters for cell biochemical, cell physiological and clinical studies prompted the devel-

opment of fluorescent indicator dyes for specific molecular cell functions. Such dyes frequently permeate the cell membrane in form of uncharged precursor molecules by diffusion. Following intracellular enzymatic cleavage, oxidation or reduction, they are converted into fluorescent function indicator molecules. The indicator molecules are frequently electrically charged. This favors their intracellular autoaccumulation, especially at positive charge due to the negative transmembrane potentials from outside over the cell membrane towards the cell interior and into cell organelles like mitochondria.

The flow cytometric determination of esterase (fluorescein-diacetate (FDA), 122) and phosphatase activities and kinetics (umbelliferone-phosphate, 123, 124), represented initial challenges for enzyme activity measurements in viable cells while simultaneous DNA staining with Hoechst 33662 and propidium iodide (PI) discriminated viable from dead cells (125). Phagocytosis of fixed FITC labeled bacteria (126), cell function changes upon phagocytosis of monosized fluorescent microparticles (127) and live bacteria (128) provided insight into phagocytosis associated alterations of cell functions.

The interest in the metabolic situation of living cells prompted the use of dyes with pH-dependant excitation spectra at constant emission wavelength. Sequential fluorescence excitation at two different wavelengths provides a ratio change of the emitted fluorescence intensities at an intracellular pH change. Cells were initially excited in a single laser flow cytometer during two runs at different excitation wavelengths (fluorescein, 129). The ratio of the mean fluorescences of both measurements provided the mean pH value for all cells but not the intracellular pH of individual cells. Single cell pH measurements were achieved by the instrumentally more demanding dual laser excitation (4-methylumbelliferone, 130). The simpler approach was to use a dye with a pH dependent fluorescence emission wavelength shift (1,4-dicyano-hydroquinone (DCH), 131). This permitted the determination of a fluorescence emission ratio for individual cells from the simultaneously collected light in two fluorescence channels of a standard flow cytometer, equipped with a mercury arc lamp or a single laser. The use of fluorescence emission ratios from various light channels (132) has become current practice in many cytometric applications.

Further challenges were the flow cytometric measurement of the degree of cellular excitation by Ca^{2+} ion fluxes (INDO1, 133), stopped-flow calcium kinetics (134, 135), oxidative burst activities (dihydrorhodamine123 (DHR123), 136, hydroethidine (HE), 137), intracellular glutathione levels (ortho-phthaldialdehyde (OPT), 138 or: monobromobimane with N-ethylmaleimide protein thiol group blocking, 139) as indicators of the reductive cell potential as well as the use of fluorescinated (FITC) polycations like polylysine or polyornithine as indicators of the electrophoretic mobility of cells (140).

The determination of protease activities in vital cells was of interest for granulocyte function studies in intensive care patients being potentially in danger of imminent sepsis or shock development (141). The efforts for an increase of sensitivity and specificity of the originally used endopeptidase protease substrate (Z-Arg 2 -4-trifluoromethyl-coumarinyl-7-amide (AFC),142) initiated the development of rhodamine110 proteinase substrates for cysteine and serine proteinases (143-145) as well as for aminopeptidases (146).

Cell function assays were furthermore useful for pretherapeutic cytostatic drug testing in individual cancer patients (147) but also for the simultaneous determination of lympho-, mono-, granulo-, erythro- and thrombocytes counts in conjunction with reticulocytes and reticulated platelet (148) counts in peripheral blood. The longtime lack of volumetric counting capabilities of standard flow cytometers prompted the development of single platform blood cell counting in the presence of known concentrations of monosized fluorescent microbeads (149). This methodology is currently in wide use for the determination of absolute cell counts in clinical flow cytometric immunophenotyping assays. Recently available multiparameter flow cytometers for accurate absolute cell counting (150) may continue to utilize admixed monosized particles as internal fluorescence standards to monitor instrument performance.

Apoptosis

In situ nick translation with DNA polymerase I in the presence of either fluorescein-12-UTP or digoxigenin-labeled 11-dUTP opened a way for the flow cytometric detection of the degree of DNA fragmentation during programmed cell death (151).

Microbiology and biotechnology

The interest in microorganisms led to the determination of DNA, RNA and protein in yeast cells (152, 153) and bacteria (54). These studies represent early flow cytometric efforts directed towards biotechnology, food quality and sensitivity or resistance phenomena in the presence of antibiotics.

Data analysis

Data analysis was of primordial importance from the very beginning of flow cytometry on. Initial interests concerned cell cycle phase determinations (154, 155) in one parameter DNA histograms but also the mathematical analysis of two parameter DNA/protein measurements (156). The interest concerned furthermore the fitting of one (157, 158), two (140) or three parametric (159) linear or logarithmic Gaussian distributions to flow cytometric single or multiparametric measurements for result simplification and facilitation of subsequent scientific hypothesis development.

Predictive medicine by cytomics

The development of software for automated, self gating flow cytometric list mode analysis and result input into databases as well as the unattended self-learning multiparameter data classification (DIAGNOS1, 160, CLASSIF1,161) was essential for disease diagnoses but also for individualized disease course predictions in clinical patients. The latter development followed the observation that cell function parameters provided >80% correct three day in advance extrapolations for the occurrence of posttraumatic shock, transitional state or normal recovery in intensive care patients (141). Multiparameter flow cytometric or other multiparametric data from DNA or proteomics chip arrays or from multiplex bead arrays in conjunction with algorithmic data sieving has been essential for the development of the predictive medicine by cytomics concept (162-166). It provides a generalized approach to individualized disease course prediction at a >95% accuracy level. The resulting therapeutic leadtime may permit preventive therapy in many instances and avoid or reduce irreversible tissue damage. In other cases the earlier reduction of therapeutic intensity may lower unwanted therapeutic side effects.

Outlook

Rapid technical progress is presently leading to a merger of flow and image cytometry into the cytomics discipline. Technical examples of this are the development of laser scanning microscopes (167) or of fast imaging in flow instrumentation (47). At the same time chip or bead array and nanotechnologies are of increasing interest for cellular studies. It seems important to consider past and present concepts of flow cytometric developments in Europe as footholds for the advent of an important new era of experimental, medical and clinical cytomics.

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