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Review Article

Flow cytometry potential applications in characterizing solid tumors main phenotype, heterogeneity and circulating cells

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Abstract

Flow cytometry (FCM) is a unique technique that allows rapid quantitative measurement of multiple parameters on a large number of cells at the individual level. FCM is based on immunolabelling with fluorochrome-conjugated antibodies, leading to high sensitivity and precision while time effective sample preparation. FCM can be performed on tissue following enzymatic or mechanical dissociation. The expression of epithelial antigens and cytokeratin isoforms help in distinguishing tumor cells from adjacent epithelial cells and from tumor infiltrating leukocytes. Tumor phenotypes can be characterized on expression intensity, aberrancies and presence of tumor-associated antigens as well as their cell proliferation rate and eventual heteroploidy. FCM can measure quantitative expression of hormone or growth factor receptors, immunoregulatory proteins to guide adjuvant therapy. Expression of adhesion molecules tells on tumor's capacity for tissue invasion and metastasis seeding. Tumor heterogeneity can be explored quantitatively and rare, potentially emerging, clones with poor prognosis can be detected. FCM is easily applicable on fine needle aspiration and in any tumor related biological fluids. FCM can also be used to detect circulating tumor cells (CTC) to assess metastatic potential at diagnosis or during treatment. Detecting CTC could allow early detection of tumors before they are clinically expressed although some difficulties still need to be solved. It thus appears that FCM should be in the pathologist tool box to improve cancer diagnosis, classification and prognosis evaluation as well as in orientating personalized adjuvant therapy and immunotherapy. More developments are still required to better known tumor phenotypes and their potential invasiveness.

More Information

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Abbreviations: CTC: Circulating Tumor Cells; ESA: Epithelial Specific Antigen; EMA: Epithelial Membrane Antigen; EMT: Epithelial to Mesenchymal Transition; FCM: Flow Cytometry; MRD: Minimal Residual Disease; MDSC: Myeloid-Derived Suppressor Cell; TAA: Tumor-Associated Antigens; PCR: Polymerase Chain Reaction; TIL: Tumor-Infiltrating Lymphocytes; TAM: Tumor-Infiltrating Macrophages

Introduction

Flow cytometry (FCM) is a unique technique in medical diagnosis that allows rapid quantitative measurement of multiple parameters on a large number of cells and at the individual level [1]. The expression of each parameter is reported for each single cell, in a quantitative way. The use of multiple parameters simultaneously produces extremely precise definitions and extensive classification of cell. Hundreds thousands to millions of cells can be easily analyzed bringing good resolution in determination of very small subsets. FCM is quantitative in different ways. Measuring the level of expression of each marker on each cell makes possible to detect fine changes as compared to normal cells. The relative number of cells is provided for each different subsets. Comparatively, Polymerase Chain Reaction (PCR) is an extremely sensitive technique to detect and quantify genes and transcripts from a cell population. However PCR cannot deduce information at single-cell level. FCM is not as sensitive as PCR but is extremely rapid, precise and specific.

FCM detection is based on fluorescence. Detectors are photomultiplicators that amplify exponentially the labelling, enabling the detection of very low fluorescence intensity. Nowadays, most common instruments have 5 to 12 detectors that can measure different colors according to the filters used for selecting the light. Thus, numerous labelings can be measured simultaneously and multiple combinations of labelling can define a large range of phenotypes.

Cells produce very low fluorescence (auto-fluorescence) but can be stained with fluorochromes. Lot of fluorescent

chemicals is available to stain cell components with strong affinity like nucleic acids or lipids. However, antibodies are more specific to detect cell components and many are already commercially available but homemade polyclonal anti-serum or monoclonal antibody can also be used. The primary labelling is revealed using secondary antibodies like goat or rabbit anti-mouse antibody. A biotin-streptavidin system can be used. Secondary tools conjugated with fluorochromes are available. Steps are separated by washing procedures and blocking steps may be necessary. This is indirect immuno-labelling. However, the primary antibody can be directly conjugated with a fluorochrome. The direct immuno-labeling spares technical steps and multiple antibodies conjugated with fluorochromes of different colors can be used simultaneously. Membrane, cytoplasmic or nuclei components can be labelled. A very large portfolio of conjugated antibodies, with different colors is now commercially available. Monoclonal antibodies are preferentially used because of their specificity and quality assurance. Recently, bioengineered antibodies have been proposed with better purity, consistency and reproducibility.

Sample preparation is very simplified. Immunostaining is better on fresh cells and is preferentially performed within few hours of cell collection. Cells in suspension are incubated with a panel of antibodies for 10 to 20 minutes. The excess of antibody is washed away and the sample is ready for analysis. Tube holders can keep up to 10 until 40 samples in waiting for automatic loading. Data acquisition of each sample takes 2 to 5 minutes depending on cell concentrations and number of cells to be analyzed. Data analysis and interpretation may take some time, depending on the complexity of the analysis but in most of the time can be ready for report the same day. This may encourage clinicians to get impatiently a prompt feedback.

Cell membrane components are easy to label but cytoplasmic and even nuclei components can be analyzed after cell permeabilisation. The cell phenotype can first be identified using membrane labeling. Then, cells are fixed to keep their global shape and detergent is used to dissolve membrane lipids. Cytoplasmic and nuclear components are then accessible for immuno-labeling. Intercalating fluorochromes are used to measure nucleic acid content. DNA amount duplicates during the G2-M phases of the cell cycle or is partially increased in case of heteroploidy.

The most frequent applications of FCM analyses are applied in cell biology, hematology and immunology. Consequently, most available antibodies, conjugated with fluorochromes of different colors, validated and ready to use, focus on leukocytes components, red blood cells and platelets. The lineage and maturation stage of each leukocyte can be precisely determined. The expression of adhesion molecules informs on the destination of cells, going to lymph node or different tissues (homing). Functional tests explore the activation pathways, cytokine production, proliferation, regulatory or cytotoxic activity, phagocytosis, oxidative burst etc. In case of clonal proliferation or partial deficiency, FCM allows precise identification of the lineage and the stage where the maturation process has failed. This brings precious information for diseases classification, stratification and prognosis supporting treatment adaptation to the person. The capacity of monitoring pathological cell populations, even greatly diluted, makes FCM a precious tool for the clinician in making treatment decisions. Quantitation of residual pathological populations not yet completely destroyed by the treatment (Minimal Residual Diseases, MRD) is precious to make the decision of stopping the treatment.

Pathological analysis of solid tumors is essentially based on tissue architecture and cell morphology. Immunostaining, through indirect immunolabelling of tissue section, can be used to detect markers of interest. As an example, some tumors are dependent of growth factors and the expression of growth factor or hormone receptors helps predicting response to adjuvant treatment. In another example, the expression of immunoregulatory proteins by the tumor predicts the efficacy of biotherapy. However, immuno-histology is time consuming and not quantitative.

FCM analysis can be applied on solid tumors. As a basic rule, FCM only analyzes small particles in suspension. This explains why FCM applications have been extensively developed for cell analyses on cell culture or in blood and biological fluids including bone marrow. So at first glance, FCM is not convenient for the analysis of cells in tissues. This explains why fluorochrome-conjugated antibodies targeting epithelial cells are rare. However, FCM have been used other cells than leukocytes like tumor cell lines in biological research but also on clinical cancer: 1) Some tumor cells are available in suspension, from fine needle aspiration or tumor associated biological fluids like pleural effusions or ascites or even in bone marrow for micro metastasis [2-4]. 2) But tumor cells can be available in suspension after extraction from tissue using enzymatic or mechanical dissociation. Of course, tissue structure and part of the cells are lost but resuspended cells can be immuno-labelled and quantitatively analyzed. As a matter of fact, cells extracted from spleen or lymph nodes are frequently analyzed for immunological studies or to complete classification of lymphoproliferative disorders. Furthermore, this method is the best way to precisely characterize cells staying (resident) or actively recruited in tissue like tumor infiltrating lymphocytes (TIL) or tumor infiltrating macrophages (TAM) [5].

FCM could complete histological analysis in different ways. Tumor contains normal epithelial cells, tumor cells, interstitial and vascular cells and leukocytes for the blood stream or actively recruited like TIL, TAM.

Immunostaining can distinguish epithelial cells from

leukocytes on FCM [2]. Leukocytes express high levels of CD45 while epithelial cells express some epithelial specific antigens. There are few antibodies available that recognize epithelial antigens like Ep-CAM (now classified as CD326 ; clones: Ber-EP4; MOC31 ; VU-1D9); Epithelial Specific Antigen (ESA; Clone: HEA125); Epithelial Membrane Antigen (EMA/MUC1/CD227) [2,6-8]. Epithelial cells also express most exclusively cytokeratins under different isoforms regarding the tissue they originate from [9,10]. Several monoclonal antibodies recognize either several cytokeratins (pancytokeratin, e.g. clone AE-1/AE-3) or are specific for a single isoform (e.g. E3; MNF116; DC10 and much more). Few of these antibodies are already conjugated with fluorochromes ready for flow cytometry applications. On the opposite, antigens specific for other types of tissues like mesenchyme, vessels, neurons or other types are rarely identified.

One characteristic of cancer is a defect in differentiation. Thus the level of expression of epithelial antigens is reduced. FCM quantitative analysis can show a difference in epithelial antigen expression between normal and tumor epithelial cells. It is then very helpful to compare cells extracted from the tumor and from normal tissue around the tumor. Epithelial-specific antigens are gradually decreasing from full expression observed on normal tissue to almost non-detectable in extremely poor differentiation as seen in Epithelial to Mesenchymal Transition (EMT) [11]. Usually, the poorer the differentiation is, the more aggressive is the cancer process with increasing tissue remodeling, tumor progression and detachment and migration of tumor cells. As a matter of fact, epithelial antigens and cytokeratins play a role in building up and maintaining cell shape and tissue structure [12,13].

Cell changes may be progressive and usually start in one or a few clones that, at the end, can become predominant in tumor progression and/or in metastasis. As FCM analyze cells individually, it is possible to approach the tumor heterogeneity and identify emerging clones with poor prognosis.

Measuring the level of expression of other adhesion molecules or metalloproteases indicates the cell ability to detach from the tissue, participating in the tissue disorganization and giving the chance for the cell to invade peripheral tissue or to migrate and eventually seed metastases [12].

Measuring the level of expression of hormone or growth factor receptor indicates the cell dependence to endocrine factors opening opportunities for adjuvant therapy. On the other hand, the loss of such expression used as one of the strategies used by emerging clones to escape the treatment can also be detected.

Epithelial phenotype: Another characteristic of cancer is to express aberrantly antigens usually expressed on other lineages or at different maturation stages. A large effort has been done to clearly established phenotypes significance of leukemic cells but this is still to be explored for solid tumors.

As part of aberrancies in antigen expression, tumor cells can express new molecules (neoantigens) [14]. Several Tumor Associated Antigens (TAA) have been identified according to the site of the tumor like Embryonic Carcinogen Antigen, Prostate Specific Antigen, Cancer antigen CA125, CA15.3. Until now, most of known TAA are secreted while only TAA anchored in cell structure would be useful for FCM cell analysis. This is because research programs for TAA identifications have focused on candidates that could lead to new diagnostic tests in serum. Discovering new aberrant anchored TAA would not only be very useful for identifying tumor cells but would also become potential targets for *in vivo* imaging and immunotherapy [15]. In a way to escape anti-tumor immunity or TAA specific biotherapy, emerging tumor clones can stop expressing TAA and such clone can be detected by FCM analysis.

One more characteristic of cancer is a default in apoptosis that secure cell homeostasis. The expression of proteins involved in regulating cell apoptosis like BCl2, p53 can be altered. Apoptosis has been extensively studied in cell biology using, in a great part FCM. These protocols are applied in tumor characterization.

Tumoral cell connections with its environment are frequently altered. Growth receptor signaling can be aberrantly overacting. Signaling aberrancies can also be detected by FCM [16,17]. This eventually indicates the potential efficacy of new specific treatments like kinase inhibitors [18,19].

One more characteristic of cancer is the increased cell proliferation rate. Dividing cells express proteins involved in cell cycle like ki-67, PCNA, Cyclins and more [20,21]. The nucleic acid content is doubling during mitosis [22,23]. Measuring the amount of nucleic acid and the expression of these proteins reflects the proliferation rate of the cells. A lot of works have been done on evaluating the prognosis value of proliferation index by measuring simply the nucleic acid content on cell nucleus extracted from frozen or paraffin embedded tissues [24]. The first results were disappointing but the question should be reconsidered taking advantage of the capacity of FCM to analyze the proliferation rate on individual tumor cells taking into account the tumor heterogeneity using multiple labeling.

Another strategy for the tumor cells to escape anti-tumor immunity is to express immunoregulatory proteins (PD-L1, CTLA-4) [25-27]. Tumor expression of these these check point inhibitors have poor prognosis but these molecules brings an opportunity for specific biotherapy.

FCM also helps in characterizing anti-tumor immunity,

measuring TIL and TAM infiltrate [28-31] and detecting eventual immunosuppressive cells like myeloid-derived suppressor cells (MDSC) [32].

Finally a good knowledge of tumor phenotypes could help in early diagnosis of small tumors using biopsy or fine needle aspiration and in deciding whether the tumor present signs of malignancy.

FCM technology is in constant progress and new tools are already available in research or pre-clinical fields. Fluorescence molecular gene probes can be used to label cells and detect chromosome duplication or translocation or mutations ... Some instruments give the possibility to rapidly sort selected cell subsets or even single cells for further analyses like PCR, advanced gene profiling, single cell RNA-sequencing (scRNA-seq) and eventually Whole Genome Exon Sequencing [33]. On one disruptive instrument, mass spectrometry has replaced fluorescence detection using metal probes. Mass cytometry allows simultaneous analyses of more parameters with less interference between probes. Furthermore, mass cytometry has been coupled with laser microdissection making possible to analyze tissue sections cell by cell, with a very large panel of immuno-labelings. The tissue architecture is then reconstituted virtually by the software [34].

Furthermore, many attempts are trying to detect Circulating Tumor Cells (CTC) in peripheral blood for multiple applications. 1) Cell detachment and migration from one known tumor reflects the risk for metastasis [12]. In particular this can be explored during tumor manipulations, for example during surgical removal. 2) There are also attempts to detect tumors in a simple blood sample improperly named liquid biopsy. Detection of CTC has been attempted with different approaches including molecular biology or FCM [35]. In a first step, this raised the challenge of detecting very rare events. As FCM is very precise and sensitive and can analyze high amounts of cells in a short period of time, several applications have been successful in detecting in blood very rare cells such as antigen specific T cells or Minimal Residual Disease (MRD). MRD method can reach a sensitivity of 10 or even one cell per milliliter of blood which is equivalent to one cell per million of leukocytes. This expertise can be applied for the detection of very rare tumor cells in biological fluids. Secondly, the detection of CTC in blood also raises the challenge of locating the primary tumor by looking for tissue specific phenotypes [2, 3]. This is not so difficult for endocrine tumors like prostate or thyroid but it is more difficult for most adenocarcinoma that could have developed in colon, liver, lung... [36-38]. However, one must keep in mind that tumor cells with highest capacity for migration are probably the ones that are less differentiated and more difficult to identify. Therefore, looking for CTC with high potential to seed metastasis may more likely become a chase for cancer stem cells [39]. Despite the important difficulties in detecting very rare CTC in the blood stream, several diagnosis tools and not only FCM are now under clinical evaluation. They usually include preliminary steps to enrich epithelial cells, frequently using immunocapture with anti ESA monoclonal antibody bound to a solid phase like polystyrene or magnetic beads [40]. Bispecific immunoglobulin complexes aggregating epithelial cells to red blood cells give encouraging results too. There are also ancillary methods looking for circulating tumor derived nucleic acid or microvesicles in serum, which might be more sensitive than FCM but definitely less precise [41-43].

There are still challenges in implementing FCM for solid tumors characterization. First, the cells must be extracted from pieces of the tumor and normal tissue surrounding the tumor. The selection of these pieces must be performed on fresh tissue, under pathological examination, after the evaluation of tissue organization and the limits of the tumor. Frequently, the extracted material must be shared with molecular biology analyses. This is difficult to do with very small tumors, as it is frequent on breast cancer. Ideally, different sites in the tumor must be individually characterized to better understand the tumor heterogeneity. Optimal panels of immunolabelling still need to be designed. They would probably all include CD45 and a few antibodies to epithelial antigens in order to discriminate leukocytes and epithelial cells. More antibodies can be selected to measure the tissue-specific differentiation and eventual default in cell maturation process. Useful information with prognosis value can be obtained by measuring growth receptor and adhesion molecules expression, proliferation rate, immunoregulatory proteins and aberrant signaling. Instruments are generally available in hematology and immunology laboratories where they are usually daily used. As instruments are more and more user-friendly and cost effective, pathologists should now have their own for rapid access to spare time and gain precision in their pathological reports.

In conclusion, a very large number of FCM applications have been developed in the last few decades, improving considerably the precision and quality of understanding and diagnostic in hematology and immunology. FCM contributed significantly in improving the classification of hematologic malignancies, leading to better adaptation of the treatments. FCM has also considerably contributed to the better understanding of fundamental cell biology, tumorigenesis, metastatic process and drug activity. Sample preparation and data acquisition up to the final interpretation can be achieved within few hours. In pathology, thanks to its unique capacity to characterize cells individually, FCM opens the way to improve diagnosis precision, characterizing growth rate, differentiation, invasiveness and heterogeneity of tumors, to the search for potentially emergent clones with poor prognosis. This will help clinicians in setting personalized treatment. FCM also brings precious information on anti-tumor immunity that could help in the prescription of

adjuvant biotherapies. Technology is more and more user friendly. Biotechnology develops high quality antibodies, opening a large opportunity to focus on new targets. So FCM will probably become an essential tool for pathologists to optimize tumor characterization.

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