Guest Forum

Multiparameter Immunophenotyping by Flow Cytometry in Multiple Myeloma: Advantages for Diagnosis and Minimal Residual Disease Monitoring

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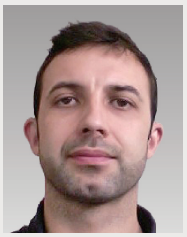

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Multiple Myeloma (MM) is a B cell neoplasia characterized by the accumulation of malignant cells in the bone marrow. Immunophenotyping has become a critical tool in the management of hematological malignancies including Multiple Myeloma (MM) with an increasingly importance with diagnosis and monitoring of Minimal Residual Disease (MRD). Here, we review the advantages of Multiparameter Flow Cytometry (MFC) to distinguish normal and malignant plasma cells, for treatment response assessment, prognostic classification and treatment management.

多発性骨髄腫(Multiple Myeloma=MM)は，悪性細胞が骨髄に蓄積するB細胞の異常増殖である。免疫型検査は，微小残留性病変(MRD)の診断とモニタリングがますます重要になってきているの伴い，MRDなどの血液病理学的の管理における重要なツールとなった。ここで我々は，治療反応評価，予後分類および治療管理のために，正常と悪性の形質細胞を区別するマルチパラメータフローサイトメトリー(MFC)の利点について検討する。

Introduction

Multiple Myeloma (MM) is a Plasma Cell (PC) neoplasm characterized by the Accumulation of Malignant PCs (MMCs) within the Bone Marrow (BM). Numerous studies have pointed out the heterogeneity of both the phenotype (CD20, CD28, CD56, CD117) and chromosomal abnormalities of MMCs in association with patient outcome.[1-3] Evaluation of MM disease is based on a variety of laboratory techniques, including BM morphology and immunophenotyping, analysis of serum and urine M-component and free light chains, hematological and biochemical parameters, cytogenetics,[4] DNA ploidy, and measurement of PC proliferative activity. These investigations are important to support the diagnosis of MM, to guide the therapy, to provide prognostic information, and to monitor treatment efficacy.[4] Many studies have shown high clinical sensitivity of flow cytometry in the analysis of MMCs when compared to conventional morphology.[5-7] This article combines a review of the literature concerning the advantages of Multiparameter Flow Cytometry (MFC) for diagnosis and Minimal Residual Disease (MRD) monitoring in MM as well as practical guidelines.

Role of Multiparameter Flow Cytometry in Multiple Myeloma

In MM, the use of MFC in clinical diagnostic laboratories becomes mandatory for the diagnosis and monitoring of the disease. MFC presents several advantages for the differential diagnosis between Monoclonal Gammopathy of Undetermined Significance (MGUS), MM and reactive conditions, the monitoring of MM patients, the assessment of prognostic markers expression, the detection of MRD after treatment and the determination of a stringent Complete Response (CR).[8], [9] MFC allows the simultaneous assessment of MM markers on rare populations of PCs as well as determination of the monoclonality. The initial studies utilized four-color MFC with a limited sensitivity.[10] More recently MFC using six-color analysis (including CD138/CD38/CD45/CD19/cytoplasmic Igκ/cytoplasmic Igλ) enables increased sensitivity, has a lower requirement of samples for detection of MMC, and ensures routine diagnosis in MM.[11], [12]

Delineation of normal and malignant plasma cells

MMCs express various markers aberrantly, compared to Normal PCs (N-PCs), and a combination of several markers is necessary to delineate optimally MMCs from N-PCs. The antigens used for detecting N-PCs and MMCs include CD19, CD56, CD20, CD117, CD28, CD33, CD27, CD81, CD31, CD39, CD40 and CD44.[5]

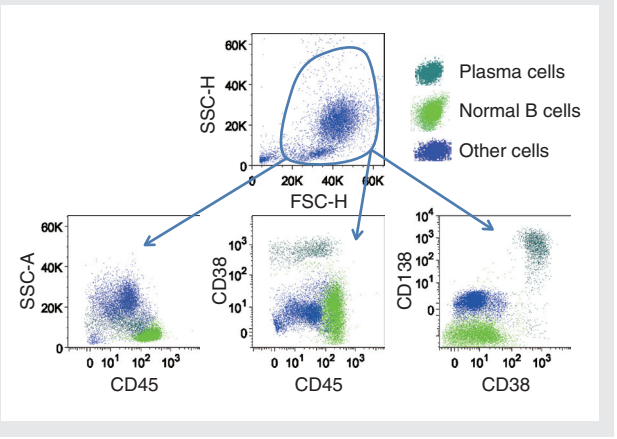

Figure 1 Primary gating strategy for plasma cells analysis
We used a combination CD38, CD138, CD45 and light scatter characteristic to identify total plasma cells ($CD38^{high}$ $CD138^{high}$ $CD45^{+}$).

The European Myeloma Network has recommended using CD38, CD138 and CD45 together with CD19 and CD56 to identify MMCs.[5] Primary gating strategy to identify PCs uses a combination of CD38, CD138, CD45 and light scatter characteristic (Figure 1). Contrary to N-PCs, MMCs from a majority of patients do not express CD19 and CD27, express at low level CD45 and express aberrantly CD56, CD28, CD81 and CD200 (Figure 2(A) and 2(B)).

Increased expression of CD20 and CD117 was also reported in a small proportion of patients (Table 1). Given these antigens are not specific to plasma cells and can yield to false plasma cell identification in case of low counts, we have developed recently a methodology to identify plasma cells based on their unique biological function: the production of immunoglobulins.[13], [14] Cells are first labelled for membrane antigens with fluorochrome-conjugated antibodies, then permeabilized and labelled with fluorochrome-conjugated kappa or lambda Ig light chain antibodies. As shown in Figure 2, plasma cells display a 10-fold higher fluorescence intensity for anti-Ig light chain antibodies than B lymphocytes (Figure. 2(A)). Using the MMC specific markers discussed above and the monoclonality of MMCs, producing either κ or λ Ig light chains, this methodology is powerful to discriminate MMCs from N-PCs, even in cases where few aberrant markers are expressed by MMCs (Figure 2(B) and 3).

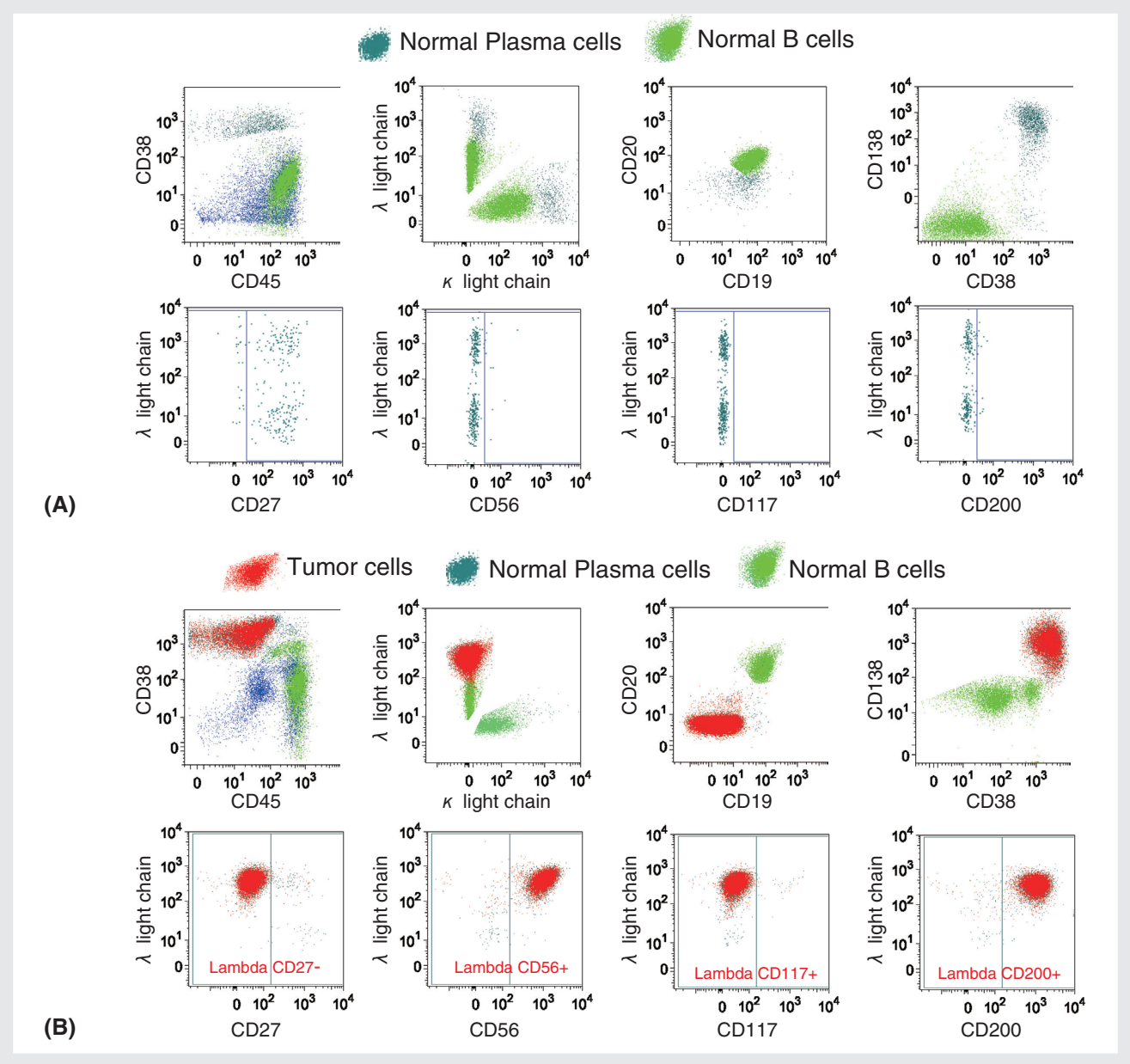

Figure 2 Immunophenotyping of normal and malignant plasma cells

(A) Cells phenotype was analyzed by gating on $CD38^{++}$, $CD45^{+}$ plasma cells (normal plasma cells in dark blue). Dotplots show FACS labeling of cytoplasmic kappa and lambda light chains, of CD19, CD20, CD138, CD27, CD56, CD117 and CD200. Normal polyclonal plasma cells are $CD38^{high}$, $CD138^{+}$, $CD45^{+}$, $CD27^{+}$, $CD19^{+}$, $CD20^{-}$, $CD56^{-}$, $CD117^{-}$ and $CD200^{-}$.

(B) Cells phenotype of malignant plasma cells was analyzed using the same gating strategy. In this patient, monoclonal (lambda light chain) malignant plasma cells are $CD38^{high}$, $CD45^{-}$, $CD19^{-}$, $CD20^{-}$, $CD138^{+}$, $CD27^{-}$, $CD56^{+}$, $CD117^{-}$ and $CD200^{+}$.

Table1 List most useful antigens for the detection of malignant plasma cells in multiple myeloma.[1], [2]* data obtained in our cohort of 70 MM patients at diagnosis.

Antigen	Expression profile on normal plasma cells	Abnormal myeloma expression profile	Percentage of MM patients with abnormal expression	Necessity for diagnosis and minimal residual disease follow-up
CD200	Negative	Strongly positive	73%*	Critical
CD56	Negative	Strongly positive	73%*	Critical
CD19	Positive	Negative	95%	Critical
CD27	Positive	Negative	50-80%	Critical
CD117	Negative	Positive	28%	Recommended
CD81	Positive	Negative	45%	Recommended
CD20	Negative	Positive	20-30%	Recommended

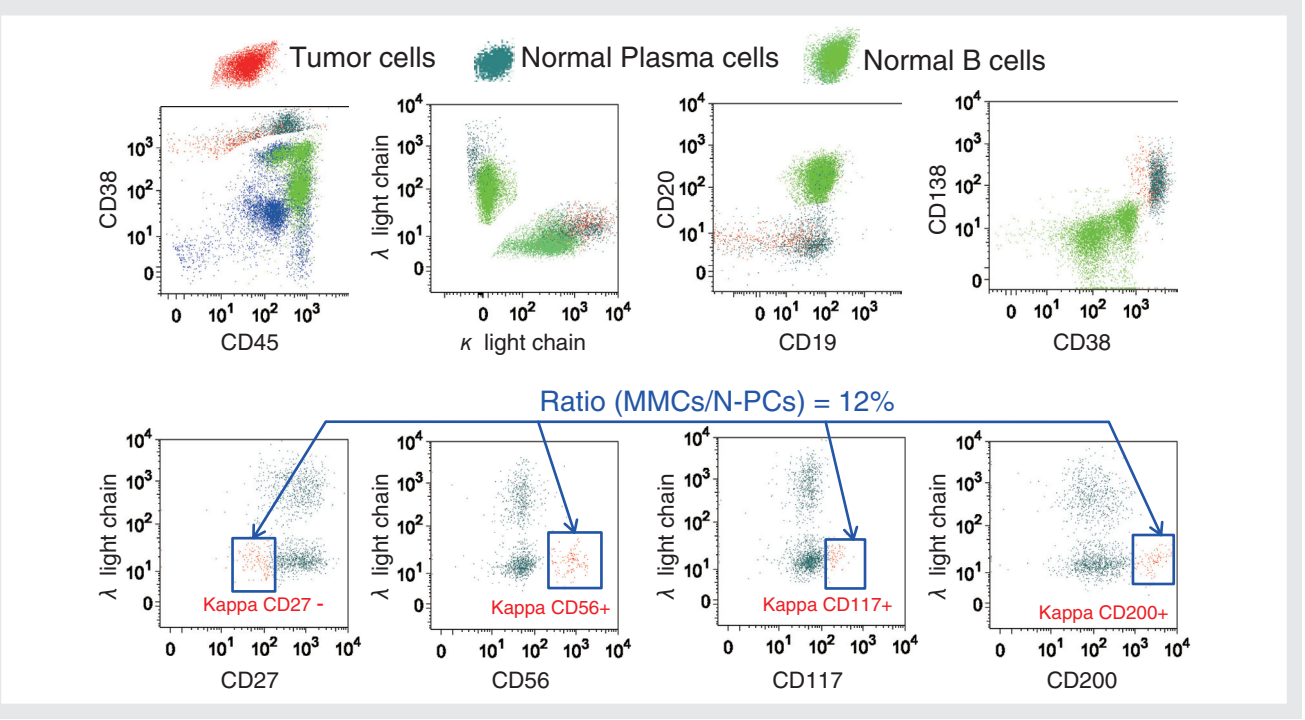

Figure 3 Detection of minimal residual disease
As previously described, malignant plasma cells were identified with the same gating strategy. In this example, we detect a small contingent of malignant plasma cells ($CD27^-$, $CD56^+$, $CD117^+$, $CD200^+$ expression with monoclonality kappa in this case) one year after allograft with a sensitivity 1/10000 (ratio: 1200 tumor cells /10000 total plasma cells in this case).

Prognostic Markers in Multiple Myeloma

CD56

CD56 (NCAM-Neural cell adhesion molecule) is a homophilic binding protein expressed on surface of neurons, glia, skeletal muscle and natural killer cells. It is aberrantly expressed on MMCs of 73% of patients, while N-PCs are negative. A poor prognosis was described to be associated with CD56 expression in previous studies including patients treated with conventional therapies. However, no prognostic value of CD56 expression was reported in patients treated with high-dose chemotherapy supported by Autologous Stem Cell Transplantation (ASCT). Modulation of CD56 expression may be involved in PCs homing since CD56 negative patients present a higher incidence of extramedullary disease. And, CD56 expression was reported to be inversely correlated with the presence of circulating MMCs.[15], [16]

CD45

CD45 is a high-molecular weight glycoprotein uniquely expressed on the surface of all leukocytes and their hematopoietic progenitors that plays a key role in lymphocyte activation.[17] In healthy individuals, CD45 is expressed on early PCs and decrease with maturation[18] while in MM patients, MMCs present a heterogeneous expression pattern with a mixture of CD45 negative and positive.[18], [19] In a study including 95 patients treated by High-Dose Chemotherapy (HDT) and ASCT, loss of CD45 expression at the surface of MMC was associated with poor survival.[20] It was reported that IL-6 is able to stimulate both $CD45^+$ and $CD45^-$ MMCs. Other growth factors including IGF-1, HGF, FGF and HBEGF stimulate the growth of $CD45^-$ MMCs.[21] More recently, Sprynski *et al.* reported an autocrine IGF-1 loop in $CD45^-$ MMCs promoting autonomous survival. At the opposite, $CD45^+$ MMCs could not survive without addition of myeloma growth factors.[22]

CD28

CD28 is a major costimulatory molecule on T cells. Its expression is aberrant in neoplastic PCs.[23] $CD28^+$ PCs are identified in 9% of patients with MGUS, 26% with newly-diagnosed MM, 59% with relapsing MM, 93% with extramedullary relapse and 100% with a secondary PCs leukemia.[24] CD28 could support the angiogenesis by the up-regulation of interleukine-8 gene transcription.[25] Whereas our group found no functional role for CD28 in MMCs [26] a recent study demonstrated CD28 is involved in interaction of MMCs and dendritic cells expressing CD80/CD86, which delivers a pro-survival signal to MMCs. This interaction induces the production of IL-6 and of the suppressive enzyme IDO by dendritic cells.[27]

CD27

CD27 is a member of tumor necrosis factor receptor superfamily found on most T-lymphocytes. CD27 expression is uniformly observed in N-PCs whereas loss of CD27 was reported in 45% of MM patients.[28], [5] And CD27 loss is associated with disease progression and shorter overall survival (OAS) in MM patients.[28], [29] Furthermore, CD27 expression is significantly lower on primary PCs leukemia compared to MMCs. And low CD27 expression correlates with plasmocytoma progression to MM.[30]

CD200

CD200 is type 1a transmembrane protein, related to the B7 family of costimulatory receptors, with immunosuppressive functions. CD200 was not expressed at the surface of N-PCs.[31], [4] We previously reported that patients with $CD200^{absent}$ MMCs have an increased event-free survival compared with patients with $CD200^{present}$ MMCs, after high-dose therapy (HDT) and stem cell transplantation.[31] These data were confirmed at protein level with a significant shorter EFS in $CD200^{+}$ patients compared to $CD200^{-}$ patients treated by HDT/ASCT (15 vs. 37 months).[32] Of note CD200 expression and International Staging System (ISS) remain independent prognostic factors for EFS in multivariate Cox proportional hazard regression model.[32]

Response Assessment and Minimal Residual Disease Follow-up

MFC has become a useful tool for MRD monitoring in MM.[33] The major reasons are that MFC could be used for MRD monitoring in 98% of the patients with a high sensitivity (detection of one MMCs among 10000 total PCs). The sensitivity is one Log less sensitive than quantitative PCR measuring MMC-specific mutated Ig genes, but it is easily applicable in almost all patients.[9]
Three months after ASCT, the detection of minimal residual MMCs is associated with shorter progression free survival (PFS).[8] The detection of MMCs by MFC in 27% patients with immunofixation negative complete response predicted a shorter PFS.[8] Furthermore, patients with at least 30% of N-PCs after treatment had a significantly longer PFS than patients with less than 30 % of N-PCs/total PCs.[34] Another retrospective study has shown that detection of more than 1.8% of MMCs prior ASCT significantly correlated with poorer PFS in MM patients.[35] More recently, Paiva *et al*. demonstrated that the MRD status 3 months after ASCT is the most powerful prognostic factor in MM patients.[36] Patients with ≤ 5% N-PCs among total bone marrow PCs had a significantly lower PFS (median 42 vs. 54 months) and OAS (median 89 months vs. not reached) than patients with > 5% N-PCs.[36] Another study on 685 newly-diagnosed patients treated by GEM 2000 protocol (HDT supported with ASCT) shows an impact of antigen expression on patient's survival. Based on CD28 and CD117 expression, MM patients with CD28 positive and CD117 negative expression had a high risk of progression and shorter survival.[2] In a study including MM newly diagnosed patients over 65 years treated according to the GEM05 PETHEMA/GEM trail, MRD monitoring by MFC is stringent to assess complete response than conventional criteria.[37] Patients with a negative immunofixation, but a positive MRD by MFC showed an early reappearance of M-component.[37] In a recent analysis combining detection of cytogenetics abnormalities by Fluorescence In Situ Hybridation (FISH) and MRD by MFC, Paiva *et al*. established a predictive score allowing delineation of three risk groups with significant different rates of disease progression in MM patients in CR after HDT/ASCT.[38] This score is also significantly predictive for OAS of patients in CR at day +100 after ASCT.[38]
All these studies have shown the advantages of MFC in the evaluation of the MRD in MM. The current challenge is the standardization of acquisition and analysis procedures[30]. The sensitivity of MFC to detect early relapse and for assessment of CR after treatment could be helpful for the follow-up of MM patients after allograft (Figure 3). MRD monitoring by MFC emerges as a promising tool for the management of treatment including donor lymphocyte infusion and/or novel agents such as Bortezomib or Immuno-Modulatory Drugs (IMiDs) in relapsed or refractory MM patients after allograft.

Assessment of Plasma Cell Proliferation

Proliferating PCs, i.e. the growth fraction of MM cells, have been evaluated by the detection of DNA synthesis using techniques including assessment of DNA content after flow cytometry or uptake of tritiated thymidine or Bromodeoxyuridine (BrdU).[40-42] The so-called PC Labelling Index (PCLI) has been shown to be a powerful and independent predictor of survival in MM[43] or flow-cytometric cell-cycle analysis using propidium iodide[44]

or assessment of Ki-67 expression.[45] Assessment of proliferation in MM is of special interest, as proliferating MMCs can be targeted by available treatments and upcoming therapeutic treatment options (e.g. aurora kinase inhibitors).[46] In our group, we combine MFC with BrdU incorporation to assess specifically the proliferation rate of MMCs at diagnosis and at the follow-up with a high sensitivity (Figure 4).

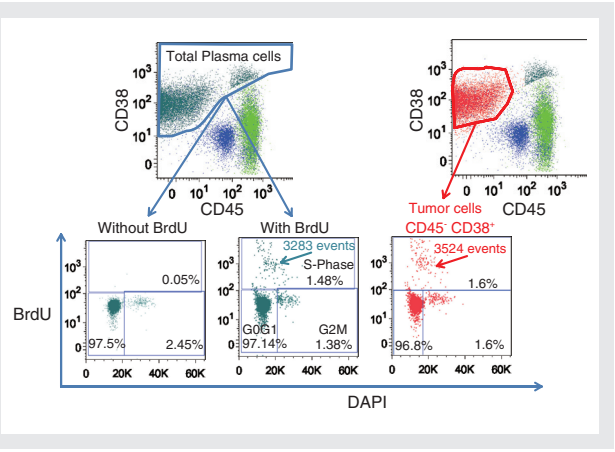

Figure 4 Assessment of plasma cell proliferation
Monoclonal plasma cell proliferation in MM patients was assessed using BrdU incorporation. Dotplots show FACS labeling of incorporated BrdU vs. DAPI (dye staining which stoichiometrically binds to DNA) in (A) total plasma cells ($CD138^+$, $CD38^{high}$, $CD45^+$ and $CD45^-$; S phase = 1.48%) and in (B) malignant plasma cells ($CD138^+$, $CD38^{++}$, $CD45^-$ in red color; S phase = 1.6%).

Individualized MRD monitoring including proliferation assessment combined with whole genome DNA copy number and gene expression profiling provide the major tools to predict the outcome of patients and to guide therapeutic management of MM patients, in particular to develop personalized medicine.

High Throughput, Highly Sensitive and Specific Automatic Flow Device for Rare Cells Detection and MRD Monitoring

Flow cytometry techniques are efficient for rare cells detection and therefore for MRD monitoring. However, MRD monitoring is a time-consuming task requiring expertized people for preparation, processing, and analysis, making this monitoring expensive.
HORIBA Medical, having a long experience in automation for hematology analyzers, can bring higher insight to deliver competitive and affordable solutions for automated specialized flow cytometry. Our company is involved in a five years collaborative project (Dat@diag) with IRB to develop prototype dedicated solutions for MRD monitoring. There are many technical challenges to be addressed, especially due to the high cell throughput required for measuring rare events.
These include leading edge technologies such as biophotonics based on supercontinuum laser source,[47] wavelength encoding as an alternative to compensation,[48] high speed acquisition systems and automatic multidimensionnal classification algorithms.[49]
This project is expected to provide fully automatic analyzers that would make MRD monitoring faster and less expensive, so that it can be widely used for Mutliple Myeloma patients.

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