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Applications of imaging flow cytometry in the diagnostic assessment of acute leukaemia

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Automated imaging flow cytometry integrates flow cytometry with digital microscopy to produce high-resolution digital imaging with quantitative analysis. This enables cell identification based on morphology (cell size, shape), antigen expression, quantification of fluorescence signal intensity and localisation of detected signals (i.e. surface, cytoplasm, nuclear). We describe applications of imaging flow cytometry for the diagnostic assessment of acute leukaemia. These bone marrow malignancies are traditionally diagnosed and classified by cell morphology, phenotype and cytogenetic abnormalities. Traditionally morphology is assessed by light microscopy, phenotyping by conventional flow cytometry and genetics by karyotype and fluorescence *in situ* hybridisation (FISH) on interphase nuclei/metaphase spreads of cells on slides. Imaging flow cytometry adds a new dimension to the diagnostic assessment of these neoplasms. We describe three specific applications:

1) Assessment of PML bodies in acute promyelocytic leukaemia,
2) The nuclear and cytoplasmic localisation of NPM antigen in acute myeloid leukaemia, and,
3) The ability to detect cytogenetic abnormalities (i.e. aneuploidy) by automated FISH on intact whole cells in suspension.

From this we conclude that imaging flow cytometry offers benefits over conventional diagnostic methods. Specifically the ability to visualise the cells of interest, the pattern and localisation of expressed antigens and assess cytogenetic abnormalities in one integrated automated high-throughput test. Imaging flow cytometry presents a new paradigm for the diagnostic assessment of leukaemia.
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1. Introduction

Leukaemia is a malignancy of the bone marrow and the 11th most common type of cancer worldwide [1]. There are many subtypes which can be broadly classified into "acute" and "chronic" forms. This determination is based on the stage of cell differentiation at which the neoplasm arose. Acute leukaemias are derived from early progenitor cells in the bone marrow and the neoplastic cells have an immature ("blast") appearance. Chronic leukaemias are also derived from a clonally defective early cell but retain the capacity to differentiate into cells that resemble normal end-stage mature cells as seen in the blood. The distinction between acute and chronic forms can generally be made on morphological assessment of stained blood and bone marrow specimens. Further sub-classification is then made on antigenic profile which can define the cell lineage (e.g. B-lymphoid, T-lymphoid or myeloid) and stage of differentiation or maturity. This is performed by flow cytometry on fresh blood or bone marrow cells or by performing immunohistochemistry on fixed tissue sections. The distinction between the various subtypes of leukaemia is important because of different treatment regimens available and prognosis.

Acute leukaemia is the proliferation of abnormal immature white blood cells. The World Health Organisation (WHO) categorises acute leukaemia into two broad categories: acute myeloid leukaemia (AML) affecting cells of the myeloid lineage (including monocytes and neutrophils) and precursor lymphoid neoplasms, also known as acute lymphoblastic leukaemia (ALL) affecting cells of the B- or T-lymphoid lineage [2]. The diagnosis of acute leukaemia requires an integrated workup including morphology, immunophenotyping, cytogenetics (karyotype and fluorescence *in situ* hybridisation) and molecular genetics. This holistic assessment gives a complete diagnostic picture and WHO classification, indicates likely prognosis, identifies potential markers to be used for disease monitoring and can guide therapy. Immunophenotyping using flow cytometry is a vital tool in this diagnostic workup. It can be performed rapidly on freshly aspirated bone marrow or a blood sample, is multi-parametric enabling multiple antigens to be assessed simultaneously and can detect both surface and intracellular antigens. However, the cells are not directly visualised in standard flow cytometry and so it can only be used to determine the number of cells expressing an antigen and the intensity of expression (i.e. mean fluorescence intensity). The pattern (i.e. punctate) and localisation (i.e. nuclear versus cytoplasmic) of expression cannot be determined.

Imaging flow cytometry has the capacity to address these limitations of standard flow cytometry (Table 1). Although this technology has been accepted in research settings to assess antigen localisation, it remains a relatively novel technology in the clinical field. This is in spite of its potential benefit in the routine diagnostic assessment of some types of acute leukaemia where knowing the pattern of antigen expression can lead to a specific WHO diagnosis and/or generate information of prognostic and therapeutic significance. In this manuscript we describe scenarios where imaging flow cytometry offers superior information to standard flow cytometry in the diagnostic assessment of acute leukaemia. Two specific WHO-defined subtypes of acute myeloid leukaemia (AML) are described to illustrate the phenotypic applications, i.e., acute promyelocytic leukaemia and acute myeloid leukaemia, *NPM1* mutated. Since imaging flow cytometry has the capacity to resolve small signals, or "spots", it also offers the opportunity to assess chromosomal signals. This is a new field of endeavour and has potential applications for leukaemia diagnostics. The principles of this approach as well as other potential clinical applications of imaging flow cytometry are discussed.

Table 1
Comparison of imaging flow cytometry to standard flow cytometry.

	Standard flow cytometry	Imaging flow cytometry
Visualise cell morphology	No	Yes
Analyse differences in cell size and shape	No	Yes
Immunophenotype cells based on antigen expression detected with fluorescent antibodies	Yes	Yes
Assess multiple fluorescent parameters simultaneously	Yes	Yes
Determine cellular location of antigen expression, i.e. cytoplasmic versus nuclear	No	Yes
Determine the pattern of antigen expression, i.e. diffuse versus punctate	No	Yes
Ability to perform all tests required in leukaemia diagnosis in one analysis, as defined by the WHO Classification, i.e. morphology, phenotype and chromosomal analysis	No	Yes
Multiparametric analysis of small volume (<100 μL) or low cell number	Limited	Yes
Return any residual sample after you have finished acquiring	Yes	Yes
Automated data analysis software available	Yes	Yes
Ability to use software "masks" to calculate similarity or co-localisation of expression	No	Yes
High throughput analysis, i.e. >100 cells per second	Yes	Yes
In Vitro Diagnostic (IVD) compliant	Yes	Pending

2. Acute promyelocytic leukaemia

Acute promyelocytic leukaemia (APML) is a subtype of acute myeloid leukaemia characterised by a proliferation of atypical promyelocytes and the presence of a genetic translocation involving the *RARa* gene on chromosome 17 predominantly with the *PML* gene on chromosome 15 resulting in t(15;17)(q22;12) *PML-RARa* translocation. This results in production of a fusion protein which causes a block in the normal granulocyte differentiation pathway at the promyelocyte stage by disrupting the normal function of the *RARa* gene as a transcription factor. APML is associated with coagulopathy which leads to an increased risk of haemorrhage including intracranial. The basis of this coagulopathy is complex and thought to involve the production of proteases, cytokines and other activators of coagulation and/or fibrinolysis by the malignant cell population. This is compounded by reduced platelet production due to marrow infiltration by the malignant cells. Since the coagulopathy can lead to potentially life-threatening haemorrhage prompt diagnosis of APML is crucial in ensuring that these patients survive to receive therapy. An integrated approach to diagnosis is applied and in cases of suspected APML a preliminary diagnosis can often be made on morphology and immunophenotype (CD9+, CD33+, CD34−, CD117+, HLA-DR−). This allows treatment to commence whilst genetic confirmation of the t(15;17) translocation is performed.

An additional antibody-based fluorescent microscopy test can be used to confirm the diagnosis of APML. This uses a fluorescently-labelled antibody to PML protein, an oncoprotein produced by the *PML* gene, to assess the distribution and staining pattern of PML protein bodies within the nucleus of the leukaemic cells. In normal and non-APML cells, PML protein is contained within a small number (usually less than 20) of large distinct "bodies". In contrast, in APML, when the t(15;17) translocation is present, there are numerous small PML bodies which give a diffuse pattern of staining (Fig. 1A and B). This PML protein stain has high specificity for APML [3]. Despite this, as a fluorescent microscopy

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