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Imaging flow cytometry to assess chromosomal abnormalities in chronic lymphocytic leukaemia

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ABSTRACT

Chronic Lymphocytic Leukaemia (CLL), the most common leukaemia in the Western world, has a characteristic phenotype and prognosis largely defined by the presence of cytogenetic aberrations. The gold standard for detecting these cytogenetic abnormalities is interphase fluorescence *in situ* hybridisation (FISH) performed on cell smears or tissue sections on glass slides. Fluorescently labelled DNA probes bind to specific chromosomal regions and the signal detected by fluorescent microscopy. Generally only 200 cells are assessed and the limit of sensitivity is 3% positive cells.

Here we report the development and use of imaging flow cytometry to assess chromosomes by FISH in phenotyped CLL cells in suspension. Thousands of CLL cells, identified by their phenotype, are assessed for specific FISH probe signals using an automated, high throughput imaging flow cytometer. The "extended depth of field" capability of the imaging flow cytometer enables FISH probe signals ("spots") to be resolved and localised within the (stained) nucleus of the immunophenotyped cells. We report the development of the automated "immuno-flowFISH" on normal blood using the Amnis ImageStreamX mark II platform and illustrate the clinical application of the method for the assessment of chromosome 12 in CLL. It is a powerful new method which has potential to be applied at diagnosis for disease stratification, and following treatment to assess residual disease. These applications will assist clinicians in optimising therapeutic decision making and thereby improve patient outcome.

1. Introduction

Chronic Lymphocytic Leukaemia (CLL), the most common leukaemia in the Western world, is a chronic lymphoproliferative disorder characterised by the accumulation of dysfunctional clonal CD5-positive mature B-lymphocytes [1–4]. It is clinically heterogeneous varying from indolent requiring no treatment to aggressive with short survival [1–5]. Precise and sensitive stratification is critical to deliver appropriate risk-adapted therapy [1,2,5–9]. CLL is defined by a combination of clinical features, morphology, immunophenotype, and genetics as determined by the World Health Organisation (WHO) [8,10–14]. Immunophenotyping tends to form the basis of the diagnosis with cytogenetics used to stratify disease based on specific chromosomal defects [4,8,13,15]. Multicolour flow cytometry immunophenotyping is most commonly used at diagnosis since CLL cells have a characteristic phenotype. They are mature B lymphoid cells (CD19, CD20- positive) that characteristically co-express CD5 and CD23 antigens and show light chain restriction. This phenotype enables clonal expansions to be quantitatively detected by flow cytometry with high routine sensitivity (0.01–1%) [2–4,12,14,16–19]. CLL is also characterised by heterogeneous genetic instability. Cytogenetic aberrations are present in more than 80% of cases, the most common being deletions of 11q, 13q or 17p and trisomy 12 (15% of cases) and these can be used to stratify patients into high, intermediate, low and very low prognostic risk categories. Detection of these genomic aberrations

Abbreviations: AF, AlexaFluor; BB, Brilliant Blue; BV, Brilliant Violet; CEP, chromosome enumeration probe; Ch, channel; EDF, extended depth of field; FICTION, fluorescence immunophenotype and interphase cytogenetics as a tool for investigation of neoplasms; FISH, fluorescence *in situ* hybridisation; ISXmkII, ImageStreamX mark II; MFI, mean fluorescence intensity; PBMC, peripheral blood mononuclear cells; SG, SpectrumGreen; SO, SpectrumOrange.

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also assists in clinical decision-making and therapeutic choice [1–5,7,9,20]. Small "sub-clones" (0.5–7.5% of cells) may also be detected within the CLL population [20–25]. These include translocations (e.g. t(14;18)(q32;q21) or t(2;18)(p11.2;q21)) and deletions of *TP53*/del(17p) [21–24]. These sub-clones are associated with disease progression or relapse and can therefore be independent risk factors [20–26]. The emergence of del(17p) is of particular note as it is a strong predictor of refractoriness to treatment and poor survival [22,24,25].

The gold standard for detecting these cytogenetic abnormalities is interphase fluorescence *in situ* hybridisation (FISH). This utilises fluorescently labelled DNA probes to detect chromosomal specific aberrations on cell smears or tissue sections on glass slides [4,5,9,15,27–33]. Analysis involves fluorescent microscopy to examine the location and number of fluorescent "spots" (bound probe) per counterstained nucleus; generally 200–400 cells are analysed per case. The sensitivity of FISH can be further improved by incorporating immunophenotyping in the FICTION method (i.e. fluorescence immunophenotype and interphase cytogenetics as a tool for investigation of neoplasms) [13,30,31,33]. This adds sensitivity through the simultaneous assessment of cell immunophenotype so that chromosomal FISH probe signals are only assessed in the relevant phenotypically-identified cells [13,27,30,31,33]. FICTION is also a manual slide-based method and generally only a few hundred cells are analysed. The limit of sensitivity for both FISH and FICTION are reported as 1–3 positive cells per 100 analysed. These methods are therefore only applied at diagnosis; they are not sufficiently sensitive for ongoing follow-up disease monitoring where small but clinically significant cytogenetically abnormal populations may persist [27,31,32].

Here we report the development and use of imaging flow cytometry to assess chromosomes in phenotyped CLL cells. The CLL cells, identified by their phenotype, are assessed for specific FISH probe signals using an imaging flow cytometer. In contrast to traditional FISH, where cells are analysed on a glass slide, the cells are in suspension. In addition, thousands of cells (not hundreds) are analysed at a flow rate of 1000–2000 cells per second. The "extended depth of field" capability of the imaging flow cytometer enables FISH probe signals ("spots") to be localised within the (stained) nucleus of the immunophenotyped cells. In addition to automated digital analysis, imagery allows for manual inspection of each cell. This new method enables FISH probe signals to be analysed in a large number of cells in suspension at high throughput, providing accurate analysis of chromosomal abnormalities, and has the potential to improve the sensitivity for the detection of small CLL sub-clones, including the highly prognostically significant del(17p). We report this automated "immunoflowFISH" (integrated immunophenotyping and FISH by imaging flow cytometry) as established on normal blood using the Amnis ImageStreamX mark II platform and illustrate the clinical application of the method for the assessment of chromosome 12 in CLL.

2. Materials and methods

2.1. Peripheral blood mononuclear cell (PBMC) sample preparation

Blood from healthy volunteers (n = 42) was collected by antecubital venepuncture into VACUETTE EDTA vacuum tubes (Greiner Bio-One Preanalytics, Frickenhausen, Germany). Development of this protocol was approved by the Sir Charles Gairdner Hospital Human Research Ethics Committee (#2014-023, #2016-145) and the University of Western Australia Human Research Ethics Committee (#RA/4/1/6708), in accordance with the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMC) were prepared by incubating whole blood with BD PharmLyse (BD Bioscience, Sydney, Australia). Cells were washed with phosphate buffered saline (PBS) and resuspended at a concentration of 5×10^6 cells/mL prior to further assay.

2.2. Immunophenotyping, fixation and permeabilisation

PBMC were stained with an immunophenotyping antibody cocktail that contained: AF647 conjugated mouse anti-human CD3 (clone SK7, Australian Biosearch), BD Horizon BB515 conjugated mouse anti-human CD5 (clone UCHTC2, BD Biosciences), and BD Horizon BV480 conjugated mouse anti-human CD19 (clone SJ25C1, BD Biosciences); or BD Horizon V500c conjugated mouse anti-human CD3 (clone SK7, BD Biosciences), AF647 conjugated mouse anti-human CD5 (clone UCHTC2, Australian Biosearch) and BD Horizon BV480 conjugated mouse anti-human CD19 (clone SJ25C1, BD Biosciences) (Table 1); or appropriate isotypic control as previously described [25]. To improve the stability of fluorophore-conjugated antibody binding to cell surface antigens during acid denaturation, cells were incubated for 30 min in 1 mM bis(sulfosuccinimidyl)suberate (BS3) (Thermo Scientific, Sydney, Australia) at 4 °C, as per manufacturer's recommendations, before quenching in 100 mM Tris-HCl pH7.4/150 mM NaCl. Fresh 4% formaldehyde with 0.1% Tween20 was added to the cell solution and mixed by gentle aspiration before incubation for 10 min at RT to fix cell proteins and permeabilise the cell membrane.

2.3. DNA denaturation and FISH probe hybridisation conditions

DNA was denatured with 0.5 M hydrochloric acid (37% AnalaR, SG1.18, Normapur, VWR Sydney, Australia) for 20 min at RT followed by quenching in ice-cold PBS. Cells were washed and resuspended in PBS/1%BSA to block non-specific probe binding. Cells were washed in 0.1% Igepal CA-630 (Sigma-Aldrich, Sydney, Australia) in 2× standard sodium citrate (SSC) buffer and resuspended in Vysis CEP hybridisation buffer and Vysis CEP12-SpectrumOrange or Vysis CEP12-SpectrumGreen FISH probe (Abbott Molecular, Sydney, Australia) as per manufacturers recommendations. Cells were heated to 73 °C for 5 min to denature DNA and ensure specific probe annealing before hybridisation at 37 °C in an automated thermocycler for 16–24 h. Following hybridisation, cells received two stringency washes in 0.1% Igepal in 2×SSC then in 0.3% Igepal 0.4×SSC for 5 min at 42 °C. Cells were stained with SYTOX® AADvanced DNA stain (Thermo Fisher Scientific) for 30 min at RT and analysed on an Amnis ImageStreamX mark II (ISXmkII).

2.4. Imaging flow cytometry

Imaging flow cytometry was performed on an Amnis ISXmkII, with INSPIRE v4.1 acquisition software (Amnis Merck, Seattle, USA). Excitation lasers used for analysis include 100 mW 405 nm, 50 mW 488 nm, 150 mW 561 nm and 120 mW 642 nm as detailed in Table 1. A 1.5 mW 785 nm laser provided a scatter signal and measurement of SpeedBeads for internal calibration. All images were captured with the 60× objective using extended depth of field (EDF) imaging which uses specialised optics and image processing to extend the in-focus range from 4−16 μm (30). Cells were identified in a scatter plot of the Aspect Ratio versus brightfield Area (Ch01) and 10,000–20,000 cells were recorded per sample. Single stained Simply Cellular anti-mouse compensation standard controls (Bangs Laboratories Inc., Indiana, USA) and SYTOX AADvanced stained cells were analysed using identical laser settings in the absence of brightfield and 785 nm laser illumination to calculate a compensation matrix using INSPIRE v4.1 software (Amnis). A minimum of 1000 Simply Cellular compensation particles or SYTOX AADvanced stained cells were recorded per control sample.