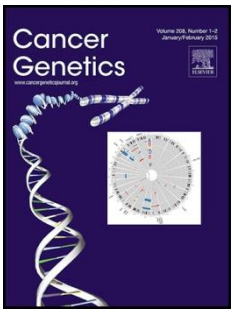
Flinders UNIVERSITY

Accepted Manuscript



Title: Trisomy 12 assessment by conventional fluorescence *in-situ* hybridization (FISH), FISH in suspension (FISH-is) and laser scanning cytometry (LSC) in chronic lymphocytic leukemia.

Author: Cuc H. Do, Karen M. Lower, Cindy Macardle, Bryone J. Kuss

PII: S2210-7762(16)30287-3
DOI: http://dx.doi.org/10.1016/j.cancergen.2017.07.009
Reference: CGEN 541

To appear in: *Cancer Genetics*

Received date: 15-11-2016
Revised date: 3-6-2017
Accepted date: 26-7-2017

Please cite this article as: Cuc H. Do, Karen M. Lower, Cindy Macardle, Bryone J. Kuss, Trisomy 12 assessment by conventional fluorescence *in-situ* hybridization (FISH), FISH in suspension (FISH-is) and laser scanning cytometry (LSC) in chronic lymphocytic leukemia., *Cancer Genetics* (2017), http://dx.doi.org/doi: 10.1016/j.cancergen.2017.07.009.

Trisomy 12 assessment by conventional fluorescence *in-situ* hybridization (FISH), FISH in suspension (FISH-IS) and laser scanning cytometry (LSC) in chronic lymphocytic leukemia.

Cuc H. Do[3], Karen M. Lower[3], Cindy Macardle[2], Bryone J. Kuss[1,3].

[1] Hematology, Molecular Medicine and Pathology;

[2] Department of Immunology, Allergy and Arthritis, Flinders Medical Centre.

[3] School of Medicine, Flinders University, Adelaide, South Australia, Australia.

Correspondence to**:** Associate Professor Bryone Kuss,

Hematology, Molecular Medicine and Pathology,

Flinders University and Medical Centre, Bedford Park, SA 5042, Australia.

Email: bryone.kuss@flinders.edu.au

Funding: This research is funded by Faculty of Medicine Seeding Grant, Nursing and Health Sciences, Flinders University, Australia.

Research highlights:

- First repetitive data illustrated the combining conventional FISH with flow cytometry (FISH-IS) to aneuploidy detection in chronic lymphocytic leukemia patients' samples for a range of chromosome centromere probes (Y, X,9,12).

- This study proved that FISH-IS method is able to accurately differentiate between monosomy, disomy and trisomy at the sensitivity threshold of 1% in CLL.

- A comparison of three current cytogenetic methods (FISH, laser scanning cytometry, FISH-IS) with different abilities in detecting low frequency trisomy 12 clones in chronic lymphocytic leukemia samples by investigating the

Abstract

Chronic lymphocytic leukemia (CLL) has an extremely heterogeneous clinical course, and prognostication is based on common genetic abnormalities which are detected by standard cytogenetic methods. However, current methods are restricted by the low number of cells able to be analyzed, resulting in the potential to miss clinically relevant sub-clonal populations of cells. A novel high throughput methodology called fluorescence *in situ* hybridization in suspension (FISH-IS) incorporates a flow cytometry-based imaging approach with automated analysis of thousands of cells. Here we have demonstrated that the FISH-IS technique is applicable to aneuploidy detection in CLL samples for a range of chromosomes using appropriate centromere probes. This method is able to accurately differentiate between monosomy, disomy and trisomy with a sensitivity of 1% in CLL. An analysis comparing conventional FISH, FISH-IS and laser scanning cytometry (LSC) is presented.

Key terms:

FISH in suspension, laser scanning cytometry, centromere, chronic lymphocytic leukemia.

Introduction

Chronic lymphocytic leukemia (CLL) results from a clonal overgrowth of B lymphocytes in the blood and bone marrow, but also involves other compartments such as lymph nodes and spleen. It is the most common adult leukemia in western countries, usually affecting individuals in their 7th decade of life and beyond (1) . CLL is an extremely heterogeneous disease, and is characterized by a highly variable disease course where survival can range from months to decades. The current clinical challenge in CLL is differentiating between these different clinical courses at diagnosis and also at time of first therapy. To date, the best prognostic and predictive indicators in CLL are the presence or absence of chromosomal abnormalities. Patients are likely to have a favorable disease course with monosomy del(13q), whereas patients with a normal karyotype or trisomy 12 have intermediate outcomes and patients with chromosomal deletions of (11q) or (17p) are likely to suffer the most aggressive disease course (2, 3). The current treatment for fit CLL patients is a combined chemotherapy (Fludarabine and cyclophosphamide) and monoclonal antibody (Rituximab, an anti-CD20 monoclonal antibody) approach: FCR (4). The majority of individuals initially respond well to this treatment. However, a proportion of patients will eventually relapse, some relatively quickly following treatment. Early relapses are considered refractory to treatment, are associated with clonal evolution and a form of aggressive CLL that most often results in the death of the patient.

Clearly, the use of chemotherapy in these patients as first line therapy may not be in the best interest of the patients, particularly in the era of

targeted therapies. Being able to identify these patients prior to using DNA damaging agents would be preferable. One area of interest in CLL research is the role of sub-clonal evolution in the course of this disease. Due largely to the recent advances in sequencing technologies, it is now appreciated that an individual cancer, such as CLL, consists of populations of genetically heterogeneous cells (5-8). The cancer itself evolves and develops as a result of not only the interactions between these genetically different sub-clones, but also on the response of each sub-clone to any given treatment. Chemotherapy intervention may eradicate treatment-sensitive sub-clones, whilst enabling an expansion of the treatment-resistant cell populations (9-11), leading to disease relapse and/or chemo-refractoriness in some patients. Therefore, there is a pressing clinical need to identify and to better understand the biology of small sub-clonal populations which may contain common chromosomal alterations at diagnosis, as well as monitoring the dynamic changes in these populations during the disease course. This would inform us about the clonal evolution of the disease, and may also guide prognostication.

Currently, cytogenetic tests including both conventional FISH and karyotype analysis play a vital role in identifying these chromosomal abnormalities. However, they are not suited to detecting small sub-clones or repeated monitoring of the disease course. Conventional FISH is analyzed by two independent scorers via microscope visualization, and is the current gold standard technique for determining cytogenetic abnormalities in CLL. This method assays approximately 200 cells per patient sample, meaning that sub-clones of cells with a potentially clinically relevant chromosomal abnormality

may be missed. This is also operator dependent. Therefore, although conventional FISH is able to identify high-frequency chromosomal abnormalities, its sensitivity precludes analysis of sub-clonal populations of cells, which may evolve to eventually represent the bulk of the patient's disease at relapse (12, 13).

An alternative technique to conventional FISH is FISH in suspension (FISH-IS), which uses flow cytometry combined with imaging to generate high-resolution digital images of individual cells. FISH-IS is able to analyze thousands of cells per second, enabling it to generate information on 100-fold more cells from an individual patient sample than conventional FISH. The images which are generated are computer captured, and the associated IDEAS software enables automated analysis of this data, quantifying characteristics such as cell shape, cell size, fluorescence intensity of the hybridized signal, and co-localization of signals (14).

Another high-throughput FISH method is laser-scanning cytometry (LSC), a slide FISH-based technique, which allows high-throughput automated quantitative measurements of fluorescence signals captured from single cells at a high resolution. The computer-controlled analysis also enables investigation of thousands of cells per sample.

This study is the first to report the FISH-IS methodology for CLL samples and to provide a working comparison of the three methodologies outlined above. The relatively common CLL chromosomal aneuploidy, trisomy 12, has been used to compare the three current FISH technologies for relative sensitivity, accuracy, time, cost, and clinical applicability.

Materials and Methods

Cells

Blood was taken from CLL patients with the written consent forms (FCREC 216.56). Peripheral blood mononuclear cells (PBMC) were isolated by density gradient with Ficoll (Lymph prep), centrifuged at 500 x g for 20 minutes. The buffy coat cells were collected and centrifuged at 1000 x g for 5 minutes. Pellets were resuspended in 500 µL RPMI 1640 media supplemented with 5% fetal calf serum (FCS) prior to Carnoy's fixation.

Conventional FISH

Conventional FISH was carried out on PBMCs extracted from CLL patients as described previously. Samples were hybridised with the following Chromosome Enumerate Probes (CEP): CEP Y (DYZ1) Spectrum Orange probe, CEP 9 Spectrum Orange probe, CEP X (DXZ1) Spectrum Green probe, and CEP 12 (D12Z3) Spectrum Green probe. All probes and CEP hybridization buffer were obtained from Abbott Molecular (USA). Samples were processed according to manufacturer's instructions. Hybridized slides were manually scored by two independent scorers through visualization with the appropriate fluorescence channel on an Olympus BX50 fluorescence microscope. Conventional FISH analysis of 200 nuclei was conducted by two independent scorers. The average of the two scores was taken as the final result.

Laser scanning cytometry (LSC)

The slides were prepared for conventional FISH were analyzed on a CompuCyte iCys™ laser scanning cytometer as follows. Initial low-resolution scan with 405 nm wavelength was performed to determine a suitable region. Within this region, an area was selected at random for high-resolution scanning with the appropriate wavelength for the hybridized probe e.g. 488 nm for Spectrum Green. The threshold, laser voltage, offset, and any required

additional filtering was adjusted on a slide-by-slide basis. The selected area of the slide was automatically scanned in 0.2 µm sections overnight. The data was analyzed by the iCys software providing a detailed statistical analysis of the data collected from the scans, and displaying these data using scatterplots and histograms (similar to flow cytometry analysis). Single cells were first gated on the nuclear marker (DAPI) based on their size and perimeter. In the single cells gate, using statistical analysis software, each DAPI stained cell and the number of "green" spots were contoured and enumerated**.** This software provides in-slide position of each cell, therefore, allowing the user to cross check the morphology of individual nuclei and spots. This permitted the exclusion of those cells in which spots were outside the contour of DAPI. Based on the data of more than 2000 nuclei, the percentage of two spots and three spots were calculated, therefore identifying the trisomy 12 proportion.

FISH-IS

The published FISH-IS methods (14, 15) were applied with several modifications (Supplementary Table 1). To collect data, the settings were applied on the Image StreamX MkII (Amnis, Seattle, USA): channel 1 and 9, bright-field was applied to visualize morphology of cells. Channel 2 (480-560 nm) with laser 488 nm (50 mW) detected Spectrum Green probes (Excitation/Emission 496/520 nm). Channel 3 (560-595 nm) with laser 561 nm (200 mW) detected Spectrum Orange probes (Excitation/Emission 552/576 nm). Channel 7 (420-505 nm) with laser 405 nm detected DAPI (Excitation/Emission 358/460 nm). Twenty thousand cell events were detected at 60x magnification using the lowest flow speed of events. All

imaging data was collected with extended depth of field, which enables analysis of in-focus spots. To analyse data, the hierarchical gating strategies were established using spot wizard in IDEAS Software version 6.1 by: cells were gated for the best focus, followed by single cells, and then fluorescence spots were assigned with both low and high range intensity. Software automatically calculated the number of spot counts by scoring the fluorescence FISH signals inside the intact cells.

Results

FISH-IS is applicable to CLL patient samples

To apply this technique to CLL cells, a model of monosomy was used. FISH-IS was carried out using the centromere Y probe on male CLL samples (n=6). Modified steps to those already published were required to prevent cell damage and reduce background and false hybridization (Supplementary Figure 1). Following this protocol, cells discriminated into one population based on fluorescence, representing 100% of cells with a single spot when analyzed with Spot Wizard, confirming that CLL cells are able to be analyzed using this technique to accurately detect monosomy in CLL samples (Supplementary Figure 2).

FISH-IS is able to accurately determine ploidy by fluorescence intensity on autosomes and sex chromosomes

Centromeric probe for chromosome 9 (unaffected by the common genetic aberrations in CLL), chromosome 12 (common trisomy in CLL) and the X chromosome were evaluated using the FISH-IS protocol established above. FISH-IS was able to accurately detect disomy and trisomy in CLL samples using these centromere probes (Supplementary Figure 3 and 4).

The sensitivity of this detection method was then determined using a known ratio mixture of male and female CLL cells hybridized with an X chromosome probe, allowing discrimination between monosomy (male CLL cells) and disomy (female CLL cells) within the same sample. To ensure the observed difference in fluorescence intensity was specifically due to hybridization signal and not intrinsic differences affecting hybridization within different samples, 50:50 and 80:20 mixtures of two male CLL samples were analyzed with the X chromosome probe. This demonstrated 100% monosomic cells as expected (Supplementary Figure 5).

Analysis of these mixed samples with FISH-IS following hybridization with the X chromosome centromere probe was performed. An example of FISH-IS carried out on a 10% male: 90% female mixed CLL sample is shown in Figure 1. It is clear that cells discriminate into two populations based on their chromosomal content when analyzed by fluorescence intensity (Figure 1A-C). This analysis demonstrated that FISH-IS is indeed able to discriminate between monosomy and disomy by fluorescence intensity. Furthermore, FISH-IS was able to accurately differentiate between monosomic and disomic cells down to 1 in 100 cells (1%) (Figure 2 and Supplementary Table 2).

The ability to detect trisomy 12 in CLL was also confirmed using a CLL patient sample previously diagnosed with 95% trisomy 12 by conventional FISH (n=200) was analyzed by FISH-IS. The results for this patient sample were comparable to standard FISH results, identifying 95.1% trisomy 12 and 4.9% disomy 12 (n=20,000) (Supplementary Figure 4).

Spot count needs manual correction to detect ploidy

The spot count (Spot Wizard or algorithm spot count) was compared with fluorescence intensity as a means of discriminating between monosomic, disomic and trisomic CLL sub-clones. However, in all cases, except for the detection of 100% monosomy with the Y chromosome centromere probe on male samples, the calculated spot count did not accurately estimate the expected signal (Figure 3).

Cell-by-cell images visually inspected revealed two limitations in enumerating spots by the spot count software. Firstly, the software was unable to discriminate two juxtaposed spots or partially or completely superimposed spots (Figure 4D). This issue can be resolved by observing the overall fluorescence intensity being greater than for a true single spot. The second issue was that spots located outside of the cell were occasionally incorrectly counted by spot count. This can be improved by manually inspecting the “3-spots” gate (Figure 4E).

In the example of 10% : 90% male/female mixing experiments, spot count demonstrated that 21.8% of cells were classified as having 1-spot (Figure 1D and 4F). Analysis of the overall fluorescence intensity of cells within the 1-spot gate revealed that there were in fact two populations of cells contained within this group: (Figure 4A) those with a fluorescence intensity consistent with 2 spots and those consistent with 1 spot. (Figure 4A-C). Therefore, it was found that 54.2% of these cells had either partially or entirely overlapping signals (R1 accounting for 54.2%), resulting from mistaken identification as a single spot by the Spot Wizard software. Additionally, the fluorescence intensity was discernible for both of these populations of cells when analyzed by a flow cytometry analysis software (FlowJo_V10) (Figure 4C). In addition,

manual curation of the 7.3% of cells classified as having 3-spots found that 93% of these cells actually had 2 spots within the nucleus (marked by DAPI staining) in addition to one or more spots outside of this nuclear region (Figure 4E). After carrying out the manual corrections as described above, the percentage of cells contained within the 1-spot population based on fluorescence intensity was 10.0%, as expected (Figure 4F). However, it is clear that there were a remarkable difference between Wizard analysis and manual correction, therefore, our separations of monosomic, disomic and trisomic CLL sub-clones identified based on fluorescence intensity of probes.

This manual re-analysis was applied to the centromere 9 probe and demonstrated that 96.4% of cells analyzed using the centromere 9 probe showed the expected diploid signal in 100% diploid CLL samples. Similarly, the manual correction of centromere 12 recalled 4.89% of the 2-spots and 94.91 % of 3-spots populations, becoming comparable to the fluorescence intensity based analysis (Supplementary Figure 6).

Comparison of detection of trisomy 12 in CLL samples using three different methods

Trisomy 12 is well established as a common aneuploidy occurring in CLL patients and has prognostic relevance. Six Trisomy 12 patient samples were analyzed by conventional FISH, LSC and FISH-IS. The CLL samples had varying frequencies of trisomy 12 by conventional FISH as the current laboratory standard: sample 1 (95% trisomy 12), sample 2 (80% trisomy 12), sample 3 (75% trisomy 12), sample 4 (50% trisomy 12), sample 5 (5% trisomy 12) and control sample 6 (100% diploidy 12). Although LSC and FISH-IS analyzed 10-times to 68-times more cells per sample, all methods were found

to be comparable in the percentage of trisomy cells. (Supplementary Table 3). With high frequency sub-clones, the percentages of trisomy 12 were comparable using the three methods, while greater disparity in the estimated frequency of small sub-clones was evident. FISH-IS showed lower estimates while LSC showed higher estimates compared with conventional FISH (Figure 5A, B).

Discussion

This study has analyzed the applicability of the high throughput method FISH-IS in accurately identifying monosomy (chromosome Y centromere) and disomy (chromosome 9 centromere) and trisomy (chromosome 12 centromere) in CLL patient samples.

In analyzing the FISH-IS data, it was clear that the overall fluorescence of the cell gave an accurate representation of the hybridization signal, and therefore this method could accurately discriminate between monosomy/disomy and disomy/trisomy. Unfortunately, whilst the current software is able to discriminate between cells with a different number of signals by fluorescence intensity, it is less able to correctly determine the exact number of signals within these cells, using the 'spot count' feature.

There are several aspects of this system which may be causing this. The software records a 2-dimensional image representation of a 3-dimensional object (the cell). Therefore, if spots are at different depths along the same axis as the camera, the conversion to a 2-dimensional image may cause the two spots to be partially or entirely merged. When manually curating the "1-spot" cells, some were easily discernible by eye as being two spots (Figure 4D). However, the software was unable to discriminate them as two distinct spots.

These issues no doubt contribute to the inaccuracy of the current spot count wizard. Previous studies have found a similar level of miscounting spots with the current software (14, 16, 17). There is a pressing need for further development of this software so that spot counting is an accurate and reproducible analysis step, without requiring manual curation and confirmation which is a significant limitation of this methodology.

Conventional FISH is the current standard method for determining clinically relevant chromosomal abnormalities in CLL, including trisomy 12. Here we have undertaken a limited comparison between conventional FISH and two high throughput methods, FISH-IS and LSC, using trisomy 12 as our model. Firstly, it is important to recognize that all of these methods rely on the successful and accurate hybridization of a labelled probe to the genetic material within a cell. Therefore, it follows that these methods, whether high-throughput or not, are all affected by factors such as probe size, success of probe labelling, hybridization technique, and the quality of the cells being analyzed.

The main advantage of conventional FISH is that it is technically simple, only requiring access to basic tissue culture facilities and a fluorescence microscope. However, due to the lack of automation and significant reliance on manual scoring, results are highly operator dependent and the process of data acquisition is relatively laborious and time consuming. Data misinterpretation and scoring inconsistencies may be the result of operator fatigue and inexperience. In addition, the sensitivity of this method is limited by the low number of cells able to be evaluated (approximately 200 nuclei per slide), representing only a small sample of the potentially complex mixture of

cytogenetically abnormal cells often found in CLL samples. Therefore, conventional FISH is limited in its capacity to detect small sub-clones, which may become clinically relevant during the course of the disease.

In an attempt to overcome these limitations, the microscope-based laser scanning cytometer (LSC) emerged in the mid-1990s (18-20), and has since been updated with the new iGeneration of LSC Research Imaging Cytometer (e.g. iCys by Compucyte) and complemented by analysis software (21-23). These advances have allowed large-scale automated quantification of conventional FISH data. The two main advantages of the LSC method, compared to the conventional FISH method, is that LSC is able to analyze a significantly larger number of cells, and the process of automated spot counting solves the problem of scorer fatigue and human error.

The LSC method is not however without its limitations. Firstly, the LSC machine itself represents expensive and highly specialized equipment; thereby access will be the limiting factor for most researchers or diagnostic centers in applying this method. In terms of the actual method of signal detection, LSC detects cells by the primary contour (visualized as DAPI-stained nuclei) and enumerates any hybridized probes by the secondary contour. However, both the hybridized probes and the nuclei can vary greatly in relative fluorescence intensities and size, requiring a large training range of acceptable contours to be established to ensure correct detection of all cells and hybridized probes. The fluorescence signals can also occur at variable depths along the optical axis, which can also lead to incorrect spot counting per cell. Therefore, the accuracy of the spot counting analysis needs to be checked by manually scoring a random sample of the cells, in order to ensure

that these factors are not resulting in a flawed automated analysis. In addition, there are several parameters, which need to be established prior to scanning the slides, for example, the focal length of the camera needs to be adjusted according to the thickness of the covering glass and the sample itself. Considering these factors, the application of LSC in FISH analysis is considered a semi-automated procedure (20, 24).

By contrast, FISH-IS is able to provide accurate data generated from thousands of cells by analyzing the fluorescence intensity of the samples. However, this method relies heavily on maintaining the original morphology of the cells, as broken or disrupted cells are automatically excluded from the analysis. For our CLL samples, analysis was compromised when using previously frozen and/or long term fixed samples. This may be a feature of CLL cells specifically, or a more general issue with this method. Regardless, the applicability of the FISH-IS method may be limited by sample availability.

With respect to the financial cost of these three methods, the obvious difference is the specialized equipment required for the two high-throughput methods. That factor aside, all methods cost approximately the same for consumables, however the LSC and FISH-IS methods are less labor-intensive than conventional FISH. Experienced scorers spend approximately 2 hours to score 200 nuclei on conventional FISH, whilst the LSC and FISH-IS need 30 minutes to 1 hour for analysis up to 3000 cells in LSC and up to 10,000 cells in FISH-IS. Therefore, the labor time of the later methods reduces significantly and the results do not depend heavily on experienced scores.

While the role of trisomy 12 in CLL itself is not yet fully understood, Dohner *et. al*. (2) reported that trisomy 12 is the third most frequent chromosomal

aberrations in CLL, found in 16% of all cases, and in isolation, confers an intermediate outcome. Other studies have since found that trisomy 12 may actually be associated with either a good or a poor prognosis (25, 26). These conflicting findings may be due to differences in the proportion of CLL cells carrying trisomy 12 and additional mutations present in the sub-clones. Gonzalez-Gascon *et al.* found that trisomy 12 had to be present in >60% of CLL cells to confer a poor outcome (27). Moreover, trisomy 12 often emerges in early stage CLL therefore may act as a driver mutation for secondary genetic alterations (e.g. NOTCH1 and TP53 mutations) (8, 10, 28, 29). Therefore, the detection of sub-clones carrying trisomy 12 appears important in the understanding of the biology of the disease eventually resulting in diagnostic and prognostic information which ultimately effects treatment decisions.

It is noteworthy that the signal intensity generated using a centromere probe is vastly different from single allele probes making analysis of important chromosomal aberrations such as del17p and del11q difficult to demonstrate by FISH-IS. The next focus for this analysis is to further modify the high-throughput methodologies to be applicable for smaller intra-chromosomal aberrations commonly found in CLL, such as del11q, del13p, and del17p. This is the subject of our ongoing research.

FISH-IS is a dynamic methodology which is able to accurately analyze whole chromosome genetic aberrations, and hence provides an important research tool. While the diagnostic clinical laboratory would benefit from automation of cytogenetic analysis the best means of accomplishing this remains to be determined. Additionally, this methodology may well have other applications in

medicine such as the sensitive detection of fetal cell aneuploidy in maternal blood samples. Irrefutably however, the ability to detect low frequency clones in CLL is a vital part of prognostic determination and treatment decision making therefore we must continue to investigate the best means of providing that information to the treating clinicians.

Acknowledgements

The authors acknowledge Dr Randall Grose, the Flow and Laser-Scanning Cytometry Facility, SAMHRI, Australia. Dr Orla Maguire and Dr Hans Minderman, Roswell Park Cancer Institute, Buffalo, New York, USA. Dr Kathy Heel, University of Western Australia, Crawley, Australia. Ms. Christine Probst and Dr Brian Hall, Amnis Corporation. Dr Sinoula Apostolou, Flinders Centre for Innovation in Cancer. Jasmine Wannop, School of Biological Sciences, Flinders University, Australia.

Disclosures and authors contribution

The authors declare no conflict of interest and research was conducted without any commercial financial supports. All experiments conducted by C.H.D. Analysis performed by C.H.D, K.M.L, B.J.K. Supervision provided by K.M.L, C.M and B.J.K. All authors contributed to manuscript writing and editing.

Reference

1. National Cancer Institute. ; Available from: http://seer.cancer.gov/csr/1975_2013/ (accessed 17 October 2016) SEER cancer statistics review (1975–2015). .
2. Dohner, H., et al., *Genomic aberrations and survival in chronic lymphocytic leukemia.* N Engl J Med, 2000. **343**(26): p. 1910-6.
3. Davids, M.S., et al., *Controversial fluorescence in situ hybridization cytogenetic abnormalities in chronic lymphocytic leukaemia: new insights from a large cohort.* Br J Haematol, 2015. **170**(5): p. 694-703.

4. Oscier, D., et al., *Guidelines on the diagnosis, investigation and management of chronic lymphocytic leukaemia.* Br J Haematol, 2012. **159**(5): p. 541-64.
5. Gerlinger, M., et al., *Intratumor heterogeneity and branched evolution revealed by multiregion sequencing.* N Engl J Med, 2012. **366**(10): p. 883-92.
6. Mullighan, C.G., et al., *Genomic analysis of the clonal origins of relapsed acute lymphoblastic leukemia.* Science, 2008. **322**(5906): p. 1377-80.
7. Navin, N., et al., *Tumour evolution inferred by single-cell sequencing.* Nature, 2011. **472**(7341): p. 90-4.
8. Landau, D.A., et al., *Evolution and impact of subclonal mutations in chronic lymphocytic leukemia.* Cell, 2013. **152**(4): p. 714-26.
9. Greaves, M. and C.C. Maley, *CLONAL EVOLUTION IN CANCER.* Nature, 2012. **481**(7381): p. 306-313.
10. Landau, D.A., et al., *Mutations driving CLL and their evolution in progression and relapse.* Nature, 2015. **526**(7574): p. 525-30.
11. Rossi, D., et al., *Clinical impact of small TP53 mutated subclones in chronic lymphocytic leukemia.* Blood, 2014. **123**(14): p. 2139-47.
12. Best, G., P. Thompson, and C.S. Tam, *Diagnostic techniques and therapeutic challenges in patients with TP53 dysfunctional chronic lymphocytic leukemia.* Leuk Lymphoma, 2012. **53**(11): p. 2105-15.
13. Gozzetti, A. and M.M. Le Beau, *Fluorescence in situ hybridization: uses and limitations.* Semin Hematol, 2000. **37**(4): p. 320-33.
14. Minderman, H., et al., *Image cytometry-based detection of aneuploidy by fluorescence in situ hybridization in suspension.* Cytometry A, 2012. **81**(9): p. 776-84.
15. Maguire, O., P.K. Wallace, and H. Minderman, *Fluorescent In Situ Hybridization in Suspension by Imaging Flow Cytometry.* Methods Mol Biol, 2016. **1389**: p. 111-26.
16. Fuller, K.A., et al., *Development of a robust immuno-S-FISH protocol using imaging flow cytometry.* Cytometry A, 2016. **89**(8): p. 720-30.
17. Grimwade, L.F., K.A. Fuller, and W.N. Erber, *Applications of imaging flow cytometry in the diagnostic assessment of acute leukaemia.* Methods, 2016.
18. Kamentsky, L.A. and L.D. Kamentsky, *Microscope-based multiparameter laser scanning cytometer yielding data comparable to flow cytometry data.* Cytometry, 1991. **12**(5): p. 381-7.
19. Kamentsky, L.A., et al., *Slide-based laser scanning cytometry.* Acta Cytol, 1997. **41**(1): p. 123-43.
20. Kamentsky, L.A., *Laser scanning cytometry.* Methods Cell Biol, 2001. **63**: p. 51-87.
21. Luther, E., et al., *Next-generation laser scanning cytometry.* Methods Cell Biol, 2004. **75**: p. 185-218.
22. Holden, E., E. Luther, and M. Henriksen, *New developments in quantitative imaging cytometry.* Nat Meth, 2005. **2**(7).
23. Henriksen, M., et al., *Laser scanning cytometry and its applications: a pioneering technology in the field of quantitative imaging cytometry.* Methods Cell Biol, 2011. **102**: p. 161-205.

24. Pozarowski, P., E. Holden, and Z. Darzynkiewicz, *Laser scanning cytometry: principles and applications-an update.* Methods Mol Biol, 2013. **931**: p. 187-212.
25. Rossi, D., et al., *The prognostic value of TP53 mutations in chronic lymphocytic leukemia is independent of Del17p13: implications for overall survival and chemorefractoriness.* Clin Cancer Res, 2009. **15**(3): p. 995-1004.
26. Gunnarsson, R., et al., *Array-based genomic screening at diagnosis and during follow-up in chronic lymphocytic leukemia.* Haematologica, 2011. **96**(8): p. 1161-9.
27. Gonzalez-Gascon, Y.M.I., et al., *A high proportion of cells carrying trisomy 12 is associated with a worse outcome in patients with chronic lymphocytic leukemia.* Hematol Oncol, 2015.
28. Balatti, V., et al., *NOTCH1 mutations in CLL associated with trisomy 12.* Blood, 2012. **119**(2): p. 329-31.
29. Schnaiter, A., et al., *NOTCH1, SF3B1, and TP53 mutations in fludarabine-refractory CLL patients treated with alemtuzumab: results from the CLL2H trial of the GCLLSG.* Blood, 2013. **122**(7): p. 1266-70.

Figure Legends

Figure 1. FISH-IS is able to accurately differentiate monosomy from disomy by the fluorescence intensity of an X centromere probe in CLL samples. (A) Fluorescence intensity and normalized frequency indicate two populations of cells. (B) Single CLL cells discriminate into two populations based on raw max pixel and fluorescence intensity of the Spectrum Green X-chromosome centromere probe. Each spot represents one cell. (C) Examples of CLL cells with monosomy X (top panel) and disomy X (bottom panel). Nuclear staining (DAPI) is shown in purple, centromere X probes are shown in green (Spectrum Green). (D) FISH-IS algorithm spot count (Spot Wizard) with centromere X probes from IDEAS software. The percentages of 1-spot gate were 21.8%, 2-spots 70.9% and 3-spots 7.3%. Representative results shown are from mixed 10% male CLL and 90% female CLL PBMCs. Data is representative of three separate experiments per ratio, for all ratios analyzed in Supplementary Table 2.

Figure 2. Correlation of observed versus expected monosomy X percentages in mixing experiments (male: female). Samples analyzed by fluorescence intensity using FISH-IS.

Figure 3. Correlation of the observed proportions of spot-count (Spot Wizard) analysis and the expected percentage spots of centromere Y, 9, X and 12 probes using FISH-IS.

Figure 4. FISH-IS is able to enumerate centromere X spots in CLL cells merely by manual curation of the Spot Wizard. (A) Histogram of fluorescence

intensity of hybridized probe gated for cells automatically identified as having 1-spot by Spot Wizard. (B) Histogram of fluorescence intensity of hybridized probe gated for cells automatically identified as having 2-spots by Spot Wizard. (C) Overlapping fluorescence intensity of (A) and (B), indicating that the majority of cells automatically identified as having 1-spot actually have 2-spots based on fluorescence intensity. (D) Examples of cell images in R1 gate, two spots closed (left panel), two spots overlapping partially (center panel) or completely (right panel). (E) Examples of cells that were inaccurately recorded as having three hybridization signals by Spot Wizard software as the spot(s) located outside the nuclei. (F) Manual correction of gating based on Spot Wizard shows 10.0% of cells have one spot and 89.9% of cells have two spots. Nuclear staining (DAPI) is shown in purple, centromere X probes are shown in green (Spectrum Green). Data is representative of three different experiments.

Figure 5. Correlation of three methods with centromere 12 probes results in detecting CLL samples with different percentages of trisomy 12. (A) Comparing iCys and FISH-IS with standard slide FISH. (B) Linear regression and R^2 values > 0.99 of LSC and FISH-IS while comparing with conventional FISH.