Multi-probe FISH Analysis of Immunophenotyped Chronic Lymphocytic Leukemia by Imaging Flow Cytometry

Henry Y. L. Hui,[1] Jason Stanley,[1] Kathryn Clarke,[2] Wendy N. Erber,[1,3] and Kathryn A. Fuller[1,4]

[1]Translational Cancer Pathology Laboratory, School of Biomedical Sciences (M504), The University of Western Australia, Crawley, Western Australia, Australia
[2]Department of Haematology, Belfast City Hospital, Belfast, Northern Ireland, United Kingdom
[3]PathWest Laboratory Medicine, Nedlands, Western Australia, Australia
[4]Corresponding author: *kathy.fuller@uwa.edu.au*

Imaging flow cytometry is an automated method that enables cells and fluorescent signals to be visualized and quantified. Here, we describe a new imaging flow cytometry method whereby fluorescence in situ hybridization (FISH) is integrated with cell phenotyping. The method, called "immuno-flowFISH," provides an exciting new dimension for the analysis of genomic changes in cytological samples (e.g., blood, bone marrow). Cells are analyzed in suspension without any requirement for prior cell isolation or separation. Multiple antibodies and FISH probes, each with a unique fluorophore, can be added and many thousands of cells analyzed. Specific cell populations are identified by their antigenic profile and then analyzed for the presence of chromosomal defects. Immuno-flowFISH was applied to the assessment of chronic lymphocytic leukemia (CLL), a mature B-cell neoplasm where chromosomal abnormalities predict prognosis and treatment requirements. This integrated immunophenotyping and multi-probe FISH strategy could detect both structural and numerical chromosomal changes involving chromosomes 12 and 17 in CLL cells. Given that many thousands of cells were analyzed and the leukemic cells were positively identified by their immunophenotype, this multi-probe method adds precision to the cytogenomic analysis of CLL. © 2021 Wiley Periodicals LLC.

Keywords: chronic lymphocytic leukemia • CLL • fluorescence in situ hybridization • imaging flow cytometry • immunophenotype

How to cite this article:
Hui, H. Y. L., Stanley, J., Clarke, K., Erber, W. N., & Fuller, K. A. (2021). Multi-probe FISH analysis of immunophenotyped chronic lymphocytic leukemia by imaging flow cytometry. *Current Protocols*, *1,* e260. doi: 10.1002/cpz1.260

BASIC PROTOCOL

Chronic lymphocytic leukemia (CLL) is the commonest form of leukemia in the Western world. Chromosome abnormalities are detectable in up to 80% of patients, of which deletions of 11q, 13q, and 17p and trisomy 12 are the most common and of prognostic significance. Trisomy 12 occurs in 10% to 20% of patients, has an intermediate prognosis, and is associated with increased risk of progression to the aggressive Richter's transformation. Deletion of 17p13 (del(17p)) occurs in up to 10% of patients at diagnosis and 30% at relapse following treatment. It is the highest-risk prognostic category,

CURRENT PROTOCOLS
A Wiley Brand

Current Protocols e260, Volume 1
Published in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/cpz1.260

with the shortest progression-free and overall survival (Scarfò, Ferreri, & Ghia, 2016; Van Dyke et al., 2016). Further, these patients do not respond to standard chemotherapy and, to improve survival, require alternate targeted inhibitor approaches. Traditional cytogenetic analysis of CLL is performed by fluorescence in situ hybridization (FISH) on interphase cells. FISH uses fluorescently labeled single-stranded DNA probes that anneal to complementary sequences in the target cell genome (Gozzatti & Le Beau, 2000; Kearney, 2001). Fluorescent microscopy is used to examine the bound fluorescent probe in nuclei of up to 200 cells, with a positive cut-off ranging from 5% to 20% of cells. This is acknowledged to be insufficiently sensitive for clinical use and especially for disease monitoring.

"Immuno-flowFISH" is a new cytogenomic technique where multi-parametric flow cytometry immunophenotyping and FISH are performed on cells in suspension in one test (Hui et al., 2018; Hui et al., 2019). Cells are acquired using an imaging flow cytometer, with quantitative fluorescence intensity data and digital images (60× objective) collected. Cellular morphology, antigen expression, and FISH chromosomal signals ("spots") are all in focus due to the extended-depth-of-field (EDF) imaging. Digital images and quantitative data are then assessed for FISH signals within cells with the phenotype of interest. Here, we describe a protocol for the simultaneous detection of multiple antigens to positively identify the cells of interest and FISH probes, with each antibody (CD3, CD5, CD19) and FISH probe having a unique fluorescent tag. This multi-probe automated immuno-flowFISH cytogenomic technology can identify chromosomal defects of prognostic significance. We demonstrate its capability to detect both del(17p) and trisomy 12 to a limit of 0.4% positive CLL cells identified by their phenotype.

Materials

Ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood (collected in 9-ml Vacuette K3 EDTA tube, Greiner, cat. no. 455036)

1× BD Pharm Lyse [dilute 10× stock (BD Biosciences, cat. no. 555899) in MilliQ water]

1× phosphate-buffered saline [PBS; dilute 10× PBS (see recipe) in MilliQ water], room temperature and 4°C

2% FBS/PBS: 2% (v/v) fetal bovine serum (FBS; Bovogen Biologicals, cat. no. SFBSNZ) in 1× PBS

Monoclonal antibodies:
- CD3-BV605 (BD Biosciences, cat. no. 563217, clone SK7)
- CD5-AF647 (BioLegend, cat. no. 300616, clone UCHT2)
- CD19-BV480 (BD Biosciences, cat. no. 566103, clone SJ25C1)
- BV605 mouse IgG1 isotype (BD Biosciences, cat. no. 562652, clone X40)
- AF647 mouse IgG1 isotype (BioLegend, cat. no. 400130, clone MOPC-21)
- BV480 mouse IgG1 isotype (BD Biosciences, cat. no. 565652, clone X40)

BS3 cross-linking solution (see recipe; make fresh)

Quench buffer: 0.1 M Tris·HCl (diluted from 1 M Tris·HCl buffer; see recipe) and 15 mM sodium chloride (diluted from 150 mM sodium chloride; see recipe)

Fix and Perm buffer (see recipe)

0.5 M hydrochloric acid (HCl; see recipe)

Vysis LSI/WCP hybridization buffer (Abbott Molecular, cat. no. 06J67-001)

CEP17 probe [Vysis CEP 17 (D17Z1) SpectrumGreen Probe, Abbott Molecular, cat. no. 06J37-017]

SureFISH 17p12 probe (SureFISH 17p12-OrangeRed, Agilent Technologies, cat. no. G101179R-8)

CEP12 probe (CytoCell Alpha Satellite 12 Plus for CLL TexasRed custom probe, CytoCell, cat. no. LPH-069)

Stringency wash buffer 1 (see recipe)

Stringency wash buffer 2 (see recipe), pre-warmed to 55°C
Sytox AADvanced working solution (see recipe)
Sphero Rainbow Calibration Particles (8 peaks, 3.0 to 3.4 µm; BD Biosciences, cat. no. 559123)

Vortex (Vortex Genie 2, Scientific Industries, cat. no. SI-0236)
15-ml centrifuge tubes (Greiner, cat. no. 188271)
Eppendorf Centrifuge 5702 (Eppendorf, cat. no. 5702000086) with Rotor A-8-17 (Eppendorf, cat. no. 5702700002)
Automated cell counter (e.g., Countess, Thermo Fisher Scientific, cat. no. C10227)
Cell counting chamber slide kit with trypan blue (e.g., Countess, Thermo Fisher Scientific, cat. no. C10228)
5-ml Eppendorf tubes (Eppendorf, cat. no. 0030119460)
Eppendorf 5424 microcentrifuge (Eppendorf, cat. no. 05-403-93)
0.2-ml lo-bind PCR tubes (Qiagen, cat. no. 981005)
37°C and 55°C heat block (Select BioProduct, cat. no. SBD110-3)
Thermal cycler with heated lid (Applied Biosystems Veriti 96-Well Thermal Cycler, Thermo Fisher Scientific, cat. no. 4375786)
1.5-ml lo-bind microcentrifuge tubes (Sigma-Aldrich, cat. no. T4816)
ASSIST-calibrated Amnis® ImageStream®X Mk II (with INSPIRE 4.2 software; Luminex Corporation, cat. no. 100220)
Amnis® IDEAS® v6.3 image analysis software (Luminex Corporation, cat. no. CN-SW69-01)

Day 1

1. Add 1 ml EDTA-anticoagulated blood to 10 ml of 1× BD Pharm Lyse in a 15-ml centrifuge tube and gently vortex.

   Process blood within 8 hr of collection.

2. Incubate at room temperature, protected from light, for 10 min.

3. Centrifuge 5 min at 200 × *g* (Eppendorf Centrifuge 5702 with Rotor A-8-17) and remove supernatant.

4. Add 5 ml of 1× PBS, centrifuge 5 min at 200 × *g*, and remove supernatant.

5. Add 5 ml of 1× PBS, centrifuge 5 min at 200 × *g*, and remove supernatant.

6. Resuspend in 250 µl to 2 ml PBS for cell counting.

   Adjust volume to 5 ml for patients if their leukocyte count is >2 × 10^{11}/L.

7. Determine viability and cell count using an automated cell counter and cell counting chamber slide with trypan blue. Calculate volume needed to add 2–5 × 10^6 cells per test based on the calculated cell concentration (cells/ml).

   Sample may be diluted 1:10 in PBS for accurate counting.

8. Aliquot ≥5 × 10^6 viable cells (max 1.0 × 10^7 cells) per sample in 5-ml Eppendorf tubes.

   Samples should include stained/probed (test), unstained, isotype control, and single-stained or single-probed as compensation controls.

9. Add 600 µl of 2% FBS/PBS, centrifuge 3 min at 900 × *g* in a microcentrifuge, and remove supernatant.

10. Add CD3-BV605, CD5-AF647, and CD19-BV480 monoclonal antibodies (5 µl/1 × 10^6 cells) or their isotypes to cell pellet, resuspend pellet thoroughly, and incubate 30 min at 4°C.

Samples should be protected from light for all remaining protocol steps.

11. Add 800 µl of 2% FBS/PBS, centrifuge 3 min at 900 × *g*, and remove supernatant.
12. Resuspend completely in 200 µl fresh BS3 cross-linking solution and incubate 30 min at 4°C.
13. Add 1 ml Quench buffer and incubate 20 min at 4°C.

    Add buffer slowly, drop-by-drop, to the side of the tube. Do not add buffer directly to cells and do not aspirate cells at this step.

14. Add 800 µl of 2% FBS/PBS, centrifuge 3 min at 900 × *g*, and remove supernatant.

    Do not aspirate or resuspend cells when adding 2% FBS/PBS.

15. Gently vortex to loosen cell pellet and then fix samples by adding 250 µl Fix and Perm buffer, resuspend thoroughly, and incubate 10 min at room temperature.
16. Add 800 µl of 2% FBS/PBS, centrifuge 3 min at 900 × *g*, and remove supernatant.
17. Resuspend in 800 µl of 2% FBS/PBS.

    An aliquot of each test sample can be removed at this step for analysis with isotype controls to confirm immunophenotyping.

18. Centrifuge 3 min at 900 × *g* and remove supernatant.
19. Resuspend fully in 100 µl of 0.5 M HCl and incubate 20 min at room temperature.
20. Quench in 3 ml ice-cold PBS, centrifuge 10 min at 600 × *g*, and remove supernatant.
21. To prepare probe mix, add 7 µl Vysis LSI/WCP hybridization buffer and 1 µl CEP17 probe + 1 µl SureFISH 17p12 probe + 1 µl CEP12 probe to a 0.2-ml lo-bind PCR tube and pre-warm for 10 min at 37°C in a heat block.
22. After quenching (step 20), block samples in 1 ml of 2% FBS/PBS, centrifuge 3 min at 900 × *g*, and remove supernatant.

    Wash tube sides to collect all cells.

23. Add 150 µl Stringency wash buffer 1, transfer cells to a 0.2-ml lo-bind PCR tube, and centrifuge cells 3 min at 950 × *g*.
24. Remove supernatant and all excess buffer from the samples in the PCR tubes.

    It is important that no wash buffer remains, as this will change the concentration of the hybridization buffer in step 25 and affect hybridization efficiency. If needed, pulse-spin with a mini centrifuge (SPROUT Plus Mini Centrifuge, Heathrow Scientific, cat. no. 120610) and use a pipet to remove as much excess buffer as possible.

25. Resuspend cells in the pre-warmed probe mix from step 21.

    Ensure that the cells are fully resuspended.

26. Place samples in a thermal cycler with a heated lid and set reaction volume setting to 10 to 30 µl to ensure even heating. Denature at 78°C for 5 min and then hybridize at 42°C for ≥24 hr.

Day 2

27. Add 150 µl Stringency wash buffer 1, pipet gently to mix sample, and transfer to a 1.5-ml lo-bind microcentrifuge tube.
28. Centrifuge 3 min at 950 × *g* and remove supernatant.

29. Resuspend in 200 µl Stringency wash buffer 2 (pre-warmed to 55°C) and incubate for 5 min at 55°C in a heat block to degrade excess probe.

30. Add 800 µl of 2% FBS/PBS, centrifuge 3 min at 950 × *g*, and remove supernatant.

31. Resuspend in 30 µl Sytox AADvanced working solution to stain DNA and incubate 20 min at room temperature.

32. Allow an ASSIST-calibrated Amnis® ImageStream®X Mk II (with INSPIRE 4.2 software) to stabilize and then set the following acquisition parameter values:

    a. Illumination: 405-nm laser at 100 mW, 488-nm laser at 100 mW, 561-nm laser at 200 mW, 642-nm laser at 120 mW, SSC laser (785 nm) at 1.5 mW, Brightfield ON for Channels 1 and 9.
    b. Magnification and EDF: 60× objective with EDF ON.
    c. Fluidics: set to "lo" speed and "hi" sensitivity.

33. Load Sphero Rainbow Calibration particles prepared according to manufacturer's instructions. Acquire 1000 events on gate set on bead population in the Area versus Aspect Ratio plot. Display bead population in fluorescence intensity histograms to establish 6- to 8-peak histogram profiles.

    Histograms and gates may be saved as a template for longitudinal tracking of instrument performance.

34. Load a CLL sample from step 31 and acquire from 10,000 to 200,000 events from gate set for single cells. Repeat for each CLL sample prepared for analysis.

    Single cells will be located to the right of the instrument SpeedBead population in the Area versus Aspect Ratio plot.

    On average, each sample can be acquired at a rate of 1 to 300 cells per second.

35. Acquire data for unstained and isotype controls.

36. Open Compensation Wizard and acquire single-stained compensation controls.

    A reliable compensation matrix will require acquisition of at least 500 "positively stained" cells.

37. Analyze data in Amnis® IDEAS® v6.3 image analysis software (Fig. 1). Select Guided Analysis > Wizards and then select the CLL sample .rif file for analysis.

38. Create a compensation matrix with single-stained compensation controls.

    Once a compensation matrix has been created, it may be applied to subsequent samples using the "apply previously generated compensation matrix" option.

39. Set image display properties to include brightfield (Ch01), SpectrumGreen FISH probe (Ch02), OrangeRed FISH probe (Ch03), TexasRed FISH probe (Ch04), SYTOX AADvanced (Ch05), BV480 (Ch07), BV610 (Ch10), and AF647 (Ch11) (Fig. 1A).

40. Select Begin Analysis Wizard and use the Gradient Root Mean Square (RMS) feature histogram to select brightfield (Ch01) images in focus (Region 1 or R1) (Fig. 1B).

    Gate in-focus events based on a Brightfield Gradient RMS value of around 60 and above. Gating events below this threshold is discouraged, as events that are less in focus have FISH signals that are more variable or heterogeneous in quality.

41. Gate single cells with a scatterplot of the Brightfield Area versus Aspect Ratio (R2) (Fig. 1C).

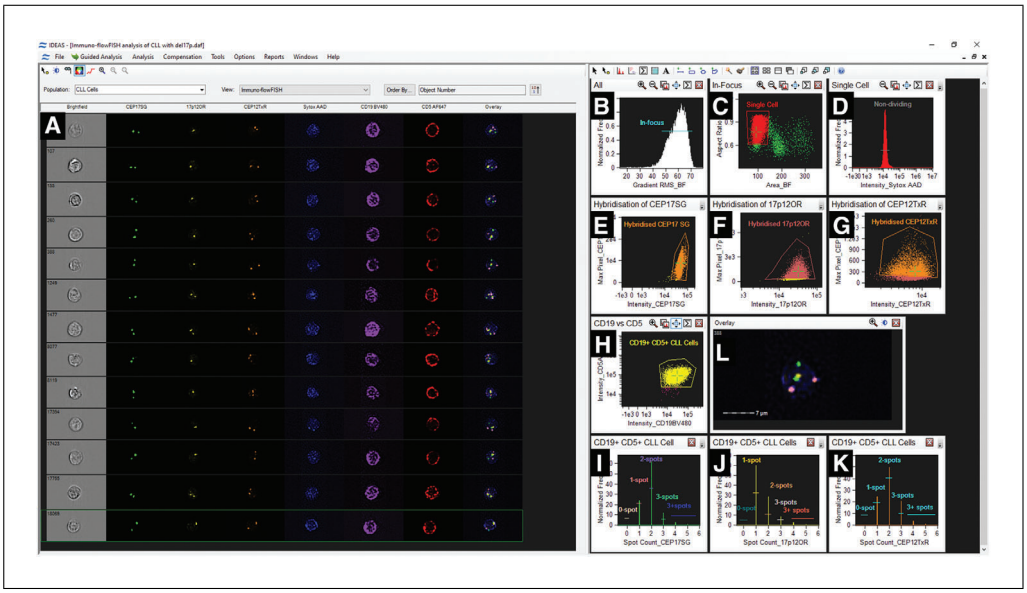

Figure 1 IDEAS® data analysis of immuno-flowFISH multi-probe assessment for deletion 17p and trisomy 12 in CLL. Brightfield (BF) and fluorescence images of cells are viewed throughout analysis in the image gallery (**A**). In-focus images are selected for analysis (**B**). Single (**C**) and nucleated non-dividing (**D**) cells with CEP17 (**E**), 17p12 (**F**), and CEP12 (**G**) hybridization signals are selected. $CD19^{+}CD5^{+}$ CLL cells are gated (**H**) and the spot counts for CEP17 (**I**), 17p12 (**J**), and CEP12 (**K**) FISH probes calculated. The overlay image review of event 388 (**L**) is a representative example of a CLL cell with two CEP17 (green) signals and one spot for 17p12 (yellow), indicating del(17p). There are two CEP12 signals (orange), indicating that trisomy 12 is not present.

42. Gate nucleated non-dividing cells using a SYTOX AADvanced fluorescence intensity histogram (R3) (Fig. 1D).

    Exclude cells with high fluorescence intensity (dividing cells, dead cells, and cell clumps).

43. Gate single nucleated cells with hybridized CEP17 probe signals with a bivariate plot of Vysis CEP17-SG probe fluorescence intensity versus Vysis CEP17-SG probe max pixel (R4) (Fig. 1E).

44. Gate single nucleated cells with hybridized 17p12 probe signals with a bivariate plot of SureFISH 17p12-OR probe fluorescence intensity versus 17p12-OR probe max pixel (R5) (Fig. 1F).

45. Gate single nucleated cells with hybridized CEP12 probe signals with a bivariate plot of CEP12-TxR probe fluorescence intensity versus CEP12-TxR probe max pixel (R6) and exit wizard (Fig. 1G).

46. Generate a scatterplot of fluorescence intensity of CD19 (B-lymphocytes including CLL) versus CD5 (CLL and normal T-lymphocytes) for the R6 population and gate $CD19^{+}CD5^{+}$ CLL population (R7) (Fig. 1H).

47. Select Guided Analysis > Wizards > Spot Wizard. For the CEP17 probe, select the focus (R1) and single-cell (R2) populations. Click Yes in response to "Do you want to analyze sub-populations" question and select CLL population (R7). Create truth populations for low (1 to 2 spots) and high (4+ spots).

48. After IDEAS® calculates Spot Count feature values and graph, click Finish to close wizard and gate 0, 1, 2, 3, and 3+ spot-count increments (R9-R13) on the *x*-axis to enumerate the percentage of cells in each category (Fig. 1I).

    The "Spot" mask for each probe can be adjusted in the mask manager. Select Function > Spot > Mask: Bright (M02 and Ch02 for SpectrumGreen, M03 and Ch03 for SpectrumOrange or OrangeRed, and M04 and Ch04 for TexasRed).

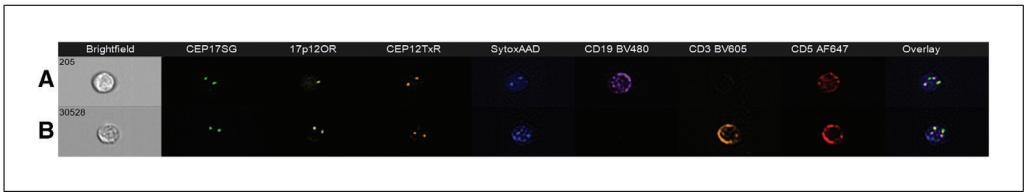

Figure 2 Representative image galleries of immuno-flowFISH multi-probe assessment for deletion 17p and trisomy 12 in CLL. (**A**) Cell 205 is a CD19-BV480$^+$/CD5-AF647$^+$ CLL cell with two CEP17 signals (CEP17SG), one spot for 17p12, and two CEP12 signals (CEP12TxR). This FISH pattern shows del(17p) in the CLL cell. (**B**) Cell 30528 is a normal CD3-BV605$^+$/CD5-AF647$^+$ T-lymphocyte with normal 2 CEP17 signals (CEP17SG) with 2 co-localized chromosome 17p12 signals (17p12OR) and 2 CEP12 signals (CEP12TxR). Merge of the probes and Sytox AADvanced nuclear marker images (overlay) shows co-location of FISH spots within the nucleus confirming specificity.

Image-based review (pixel-by-pixel) for all FISH probe signals can help assist in the setup of the automated spot-counting algorithm for Vysis CEP17-SG, SureFISH-OR, and CytoCell CEP12-TxR co-localized to Sytox AADvanced nuclear stain (Fig. 1L).

49. Repeat Spot Wizard calculations for the 17p12 probe and CEP12 probe with CLL population (R7) and gate spot-count increments (17p12: R14-R18 & CEP12: R19-R23) (Fig. 1J-K).

50. Generate a scatterplot of fluorescence intensity of CD3 (T-lymphocytes) versus CD5 (CLL and T-lymphocytes) for R6 population and gate CD3$^+$CD5$^+$ T-lymphocyte population (R8).

51. Generate histograms of spot count for the T-lymphocyte population (R8) and gate as detailed in steps 47 to 50 above for CEP17 (R24-28), 17p12 (R29-33), and CEP12 (R34-R38).

52. Calculate spot-count ratios from the spot-count statistics for each subpopulation and each probe by calculating the mean spot count of CLL cells divided by the mean spot count of normal T-lymphocytes:

$$\frac{\text{CLL}\left(\text{CD19}^+\text{CD5}^+\text{CD3}^-\right)}{\text{T}-\text{lymphocytes}\left(\text{CD19}^-\text{CD5}^+\text{CD3}^+\right)}$$

Normal T-lymphocytes will have a mean spot count of close to 2 for CEP12, CEP17, and 17p regions. A CLL/T-cell spot ratio of <1 or >1 will indicate loss or gain, respectively, of chromosomal regions under investigation. Loss of 17p will have a mean 17p12 spot count of <2 and a CLL/T ratio of <1 [e.g., del(17p)]. Cells with a ratio of >1 may indicate trisomy (e.g., trisomy 12).

53. Verify software-generated spot counts in the image gallery to further refine the gating strategy (Fig. 1A).

When data analysis is complete, representative image galleries can be downloaded for storage in a range of formats for a variety of applications, such as inclusion in reports, presentations, and publications (Fig. 2).

REAGENTS AND SOLUTIONS

BS3 cross-linking solution

40 µl 20 mM BS3 stock solution (see recipe)
760 µl 1.25× PBS (see recipe)
Prepare immediately before use

The final concentration of BS3 is 1 mM. Use within 1 hr and discard unused solution.

BS3 stock solution, 20 mM

4 mg bis(sulfosuccinimidyl)suberate (BS3; Sigma-Aldrich, cat. no. S5799)
350 µl 1.25× PBS (see recipe)
Store ≤1 year at −20°C

Fix and Perm buffer (4% formaldehyde with 0.1% Tween 20)

250 ml 16% (v/v) formaldehyde methanol-free solution (Pierce, Thermo Fisher Scientific, cat. no. 28908)
740 µl 1× PBS [dilute 10× PBS (see recipe) in MilliQ water]
10 µl 10% (v/v) Tween 20 (see recipe)
Prepare immediately before use

The buffer should be made at room temperature and the components combined for 10 min prior to use. Discard unused buffer.

Hydrochloric acid (HCl), 0.5 M

209 µl 12 M/37% (v/v) HCl SG1.18 (VWR Chemical, cat. no. 20252.420)
MilliQ water to 5 ml
Store ≤1 year at room temperature

Prepare the solution in a fume cabinet. Add HCl to water slowly.

Igepal-CA630, 10%

500 µl Igepal-CA630 (Sigma-Aldrich, cat. no. 9002-93-1)
4.5 ml 2× SSC (see recipe)
Store ≤2 weeks at room temperature

The solution is viscous, so mix well.

Phosphate-buffered saline (PBS), 1.25×

6.25 ml 10× PBS (see recipe)
43.75 ml MilliQ water
Store ≤6 months at room temperature

PBS, 10×

160 g sodium chloride (Sigma-Aldrich, cat. no. S9888)
4 g potassium chloride (Ajax Chemical, cat. no. AJA383)
28.8 g di-sodium hydrogen orthophosphate (Na_2HPO_4; Ajax Chemical, cat. no. AJA621)
4.8 g potassium dihydrogen orthophosphate (KH_2PO_4; Fisher Chemical, cat. no. P/4800/50)
1.6 L MilliQ water
Dissolve solutes in MilliQ water over low heat using magnetic stirrer
Adjust pH to 7.4 with HCl if pH>7.4 and NaOH if pH<7.4
Add MilliQ water to 2 L
Filter and autoclave
Store ≤1 year at room temperature

Sodium chloride, 150 mM

0.4383 g sodium chloride (Sigma-Aldrich, cat. no. S9888)
50 ml MilliQ water
Store ≤1 year at room temperature

SSC, 0.4×

200 µl 20× SSC stock solution (see recipe)

9 ml MilliQ water
Store ≤1 year at room temperature

SSC, 2×

1 ml 20× SSC stock solution (see recipe)
9 ml MilliQ water
Store ≤1 year at room temperature

SSC (3 M sodium chloride/0.3 M sodium citrate) stock solution, 20×

8.766 g sodium chloride (Sigma-Aldrich, cat. no. S9888)
4.9 g sodium citrate (Chem-Supply, cat. no. SA034)
50 ml MilliQ water
Adjust pH to 7.0 with HCl if pH>7.0 and NaOH if pH<7.0
Store ≤1 year at room temperature

Stringency wash buffer 1 (0.1% Igepal-CA630 in 2× SSC)

10 µl 10% Igepal-CA630 (see recipe)
9.99 ml 2× SSC (see recipe)
Store ≤2 weeks at room temperature

Stringency wash buffer 2 (0.3% Igepal-CA630 in 0.4× SSC)

30 µl 10% Igepal-CA630 (see recipe)
9.97 ml 0.4× SSC (see recipe)
Store ≤2 weeks at room temperature

Sytox AADvanced stock solution (1 µM)

1 µl Sytox AADvanced (Thermo Fisher Scientific, cat. no. S10349)
999 µl 1× PBS [dilute 10× PBS (see recipe) in MilliQ water]
Store ≤1 year at −20°C

Sytox AADvanced working solution (0.2 µM)

20 µl Sytox AADvanced stock solution (see recipe)
80 µl 1× PBS [dilute 10× PBS (see recipe) in MilliQ water]
Prepare fresh immediately before use

Use within 1 hr and discard unused solution.

Tris-hydrochloride (Tris·HCl) buffer, 1 M

6.075 g Trizma base (Sigma-Aldrich, cat. no. T6066)
35 ml MilliQ water
Adjust to pH 7.4 with HCl
Add MilliQ water to 50 ml
Store ≤1 year at room temperature

Tween 20, 10%

100 µl Tween 20 (Sigma-Aldrich, cat. no. 9005-64-5)
900 µl 1× PBS [dilute 10× PBS (see recipe) in MilliQ water]
Store ≤1 year at room temperature

The solution is viscous, so pipet slowly and mix well.

Table 1 Troubleshooting Guide for Immuno-flow FISH

Problem	Possible cause	Solution
Very few cells in sample when acquired on ImageStream$^{®X}$ Mk II	Insufficient number of cells processed	Check calculations and ensure $2-5 \times 10^6$ cells per test
	Cells lysed during addition of Quench buffer	Add Quench buffer slowly, drop by drop, to side of tube and do not aspirate. Do not add directly to cell pellet.
	Cells lost during washes	When adding buffer, wash tube sides to collect all cells
Identification of populations (immunophenotyping) difficult	Insufficient antibody	Add antibody according to manufacturer's recommendations (per 5×10^6 cells)
	Antibody fluorescence lost	Use antibody-fluorophores recommended in protocol. Traditional fluorophores (PE, APC) and their tandems are damaged during acid denaturation and hybridization.
	CD5 expression not detectable on CLL cells	CD5 expression is heterogeneous in CLL cells and in some cases may be "dim." Use antibody at manufacturer's recommended concentration and do not titrate.
High number of normal or control cells with 0 to 1 probe FISH spots, indicating low hybridization efficiency	Insufficient FISH probe	Use $2-5 \times 10^6$ cells per test, with hybridization buffer, probe, and water volumes recommended in protocol
	Hybridization buffer concentration incorrect	Remove all excess Stringency wash buffer 1 prior to adding probe master mix
	Incorrect co-denaturation or hybridization temperature	Use co-denaturation temperature of 78°C and hybridization temperature of 42°C
	Insufficient hybridization time	Hybridize for ≥24 hr when using locus-specific probes
High number of normal or control cells with ≥4 probe FISH spots	Excess FISH probe	Use hybridization buffer, probe, and water volumes listed in protocol
	Insufficient stringency wash	Pre-heat Stringency wash buffer 2 before adding to cells and incubate samples in heat block at correct temperature

COMMENTARY

Please see Table 1 for a troubleshooting guide.

The protocol is split across 2 days. On day 1, samples are immunophenotyped, fixed, and permeabilized, and cellular DNA is denatured; the time required is 3 to 4 hr. Samples are then hybridized for 24 hr. On day 2, samples undergo stringency washing, DNA is stained, and the samples are acquired on the imaging flow cytometer; the time required is 2 to 3 hr. Processing can be stopped at step 17 and samples stored ≤3 days at 4°C before completing analysis.

Acknowledgments

HYLH and JS received an LT Thean and KC Yong Memorial Medical Research Scholarship. JS received an Honours Scholarship from Cancer Council Western Australia.

Author Contributions

Henry Y.L. Hui: Data curation, Formal analysis, Investigation, Writing-review and editing. **Jason Stanley**: Formal analysis, Investigation. **Kathryn Clarke**: Formal analysis, Investigation, Writing-review and editing. **Wendy N. Erber**: Conceptualization, Funding acquisition, Resources, Supervision, Writing-review and editing. **Kathryn A. Fuller**: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing-original draft, Writing-review and editing.

Conflict of Interest

HYLH, WNE, and KAF have filed Australian (AU2018355889) and international (US20200232019, CN111448324, P2018870567) patents related to the immunoflowFISH protocol.

Data Availability Statement

Data sharing is not applicable to this article, as no new data were created or analyzed in this study.

Literature Cited

Kearney, L. (2001). Molecular cytogenetics. *Best Practice & Research Clinical Haematology*, *14*(3), 645–668. doi: 10.1053/beha.2001.0159

Gozzatti, A., & Le Beau, M. M. (2000). Fluorescence *in situ* hybridization: Uses and limitations. *Seminars in Hematology*, *37*(4), 320–333. doi: 10.1016/s0037-1963(00)90013-1

Scarfò, L., Ferreri, A. J. M., & Ghia, P. (2016). Chronic lymphocytic leukaemia. *Critical Reviews in Oncology/Hematology*, *104*, 169–182. doi: 10.1016/j.critrevonc.2016.06.003

Van Dyke, D. L., Werner, L., Rassenti, L. Z., Neuberg, D., Ghia, E., Heerema, N. A., … Kay, N. E. (2016). The Dohner fluorescence in situ hybridization prognostic classification of chronic lymphocytic leukaemia (CLL): The CLL Research Consortium experience. *British Journal of Haematology*, *173*(1), 105–113. doi: 10.1111/bjh.13933

Hui, H., Clarke, K. M., Fuller, K. A., Stanley, J., Chuah, H., Ng, T. F., … Erber, W. N. (2019). "Immuno-flowFISH" for the assessment of cytogenetic abnormalities in chronic lymphocytic leukemia. *Cytometry Part A*, *95*(5), 521–533. doi: 10.1002/cyto.a.23769

Hui, H., Fuller, K. A., Chuah, H., Liang, J., Sidiqi, H., Radeski, D., & Erber, W. N. (2018). Imaging flow cytometry to access chromosomal abnormalities in chronic lymphocytic leukaemia. *Methods*, *134-135*, 32–40. doi: 10.1016/j.ymeth.2017.11.003

Erber, W. N., Hui, H., Stanley, J., Mincherton, T., Clarke, K., Augustson, B., … Fuller, K. (2020). Detection of Del(17p) in hematological malignancies by imaging flow cytometry. *Blood*, *136*(Supplement 1), 9–10. doi: 10.1182/blood-2020-143323