Imaging Flow Cytometry and Leukaemia

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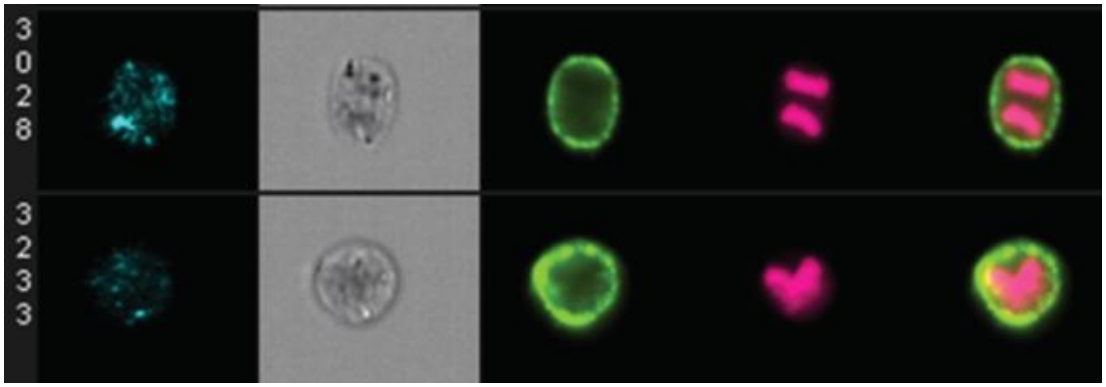
Outline

1. Imaging flow cytometry: introduction
2. Applications for leukaemia
3. “Immuno-flowFISH”: introduction
4. Technical aspects
5. Chronic lymphocytic leukaemia
6. Plasma cell myeloma
7. Summary

1. Imaging Flow Cytometry

- Standard high-throughput flow cytometry with imagery
- Brightfield, darkfield (SSC) & 10 fluorescent markers
- Magnification: x20, x40, x60 objectives; EDF
- Imagery: photographs all events (cells)
- Direct visualisation of cell features & analysis software
- AMNIS ImageStream Mark II



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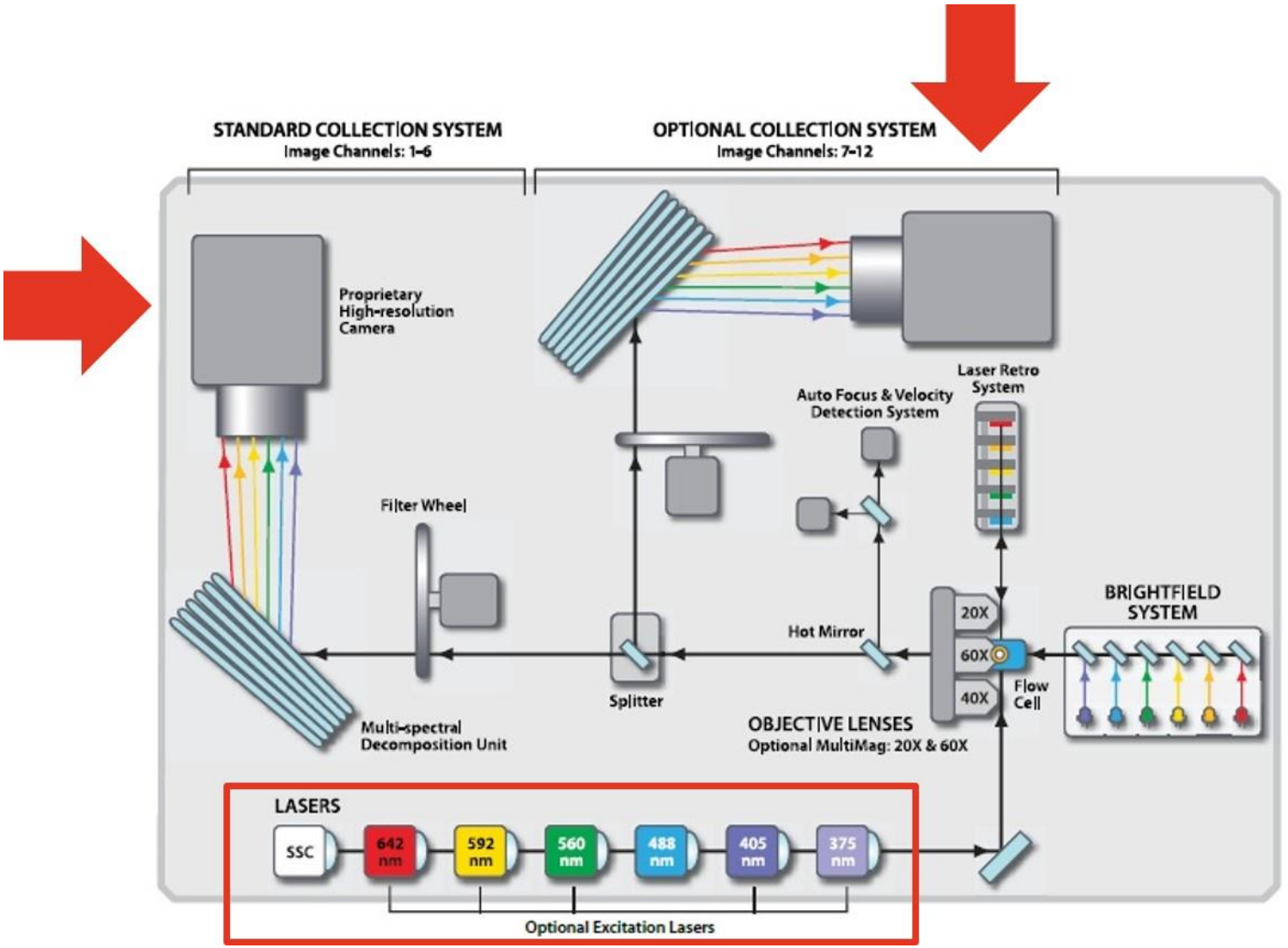
Inside the Box



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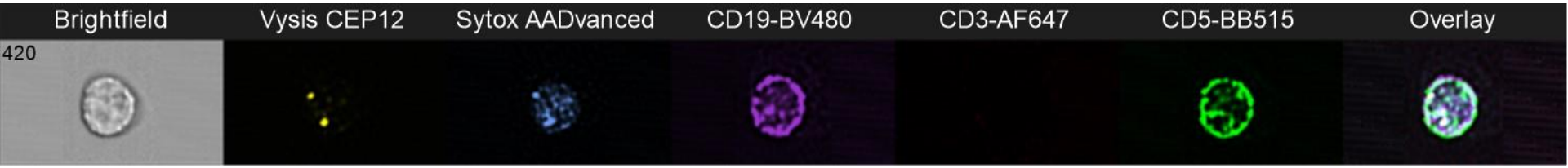
2. Applications for Leukaemia

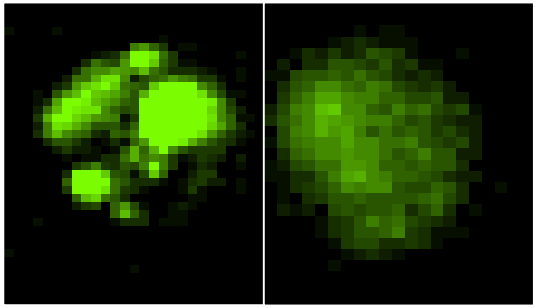
1. Surrogate markers of chromosome abnormalities in leukaemia:
   a) Pattern of expression of a molecule: PML
   b) Cellular localisation of a molecule: NPM
2. Fluorescent *in situ* hybridisation
3. Immunophenotyping and fluorescent *in situ* hybridisation ("Immuno-flowFISH")



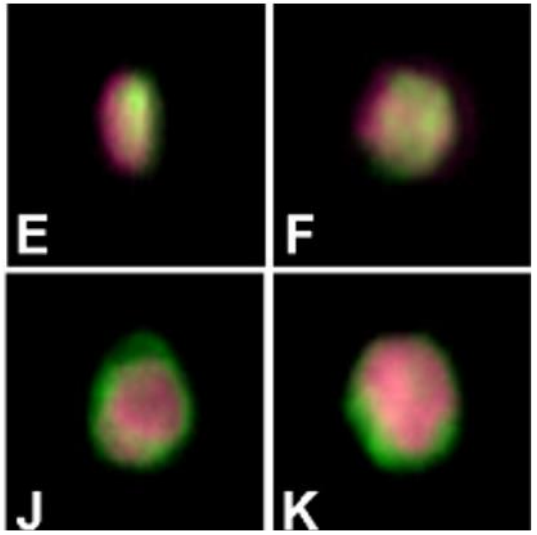
Localisation of Molecules (AML)

- Localise cellular molecules:
  - Membrane; cytoplasmic; nuclear
- Normal vs abnormal pattern
  - E.g. PML bodies
  - E.g. cytoplasmic vs nuclear NPM
- Analysis:
  - Images give visual assessment
  - Numerical data
  - IDEAS software
  - Count, pattern, percent etc

PML bodies

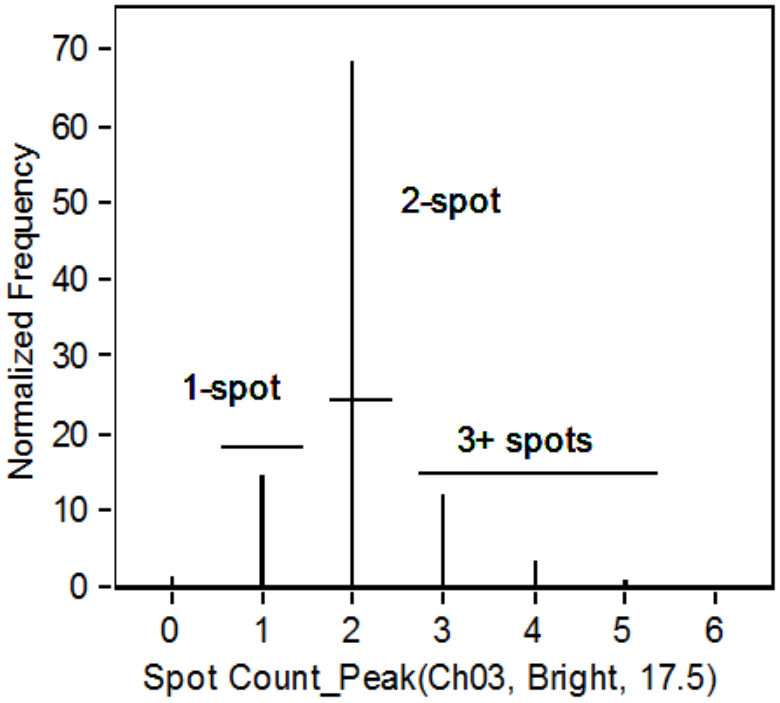


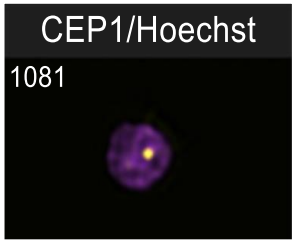
NPM: nuclear vs cytoplasmic

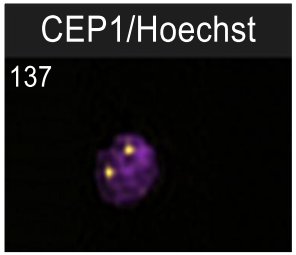


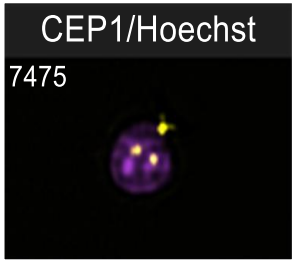
Chromosomal Analysis of Leukaemia

- Flow cytometry for fluorescent *in situ* hybridisation (FISH)
- FISH of cells in suspension; +8 AML Minderman et al. 2012; Maguire et al. 2016
- CEP1-SpectrumOrange probe and Hoechst nuclear stain
- "Spot" recognition using EDF (with nuclear stain)









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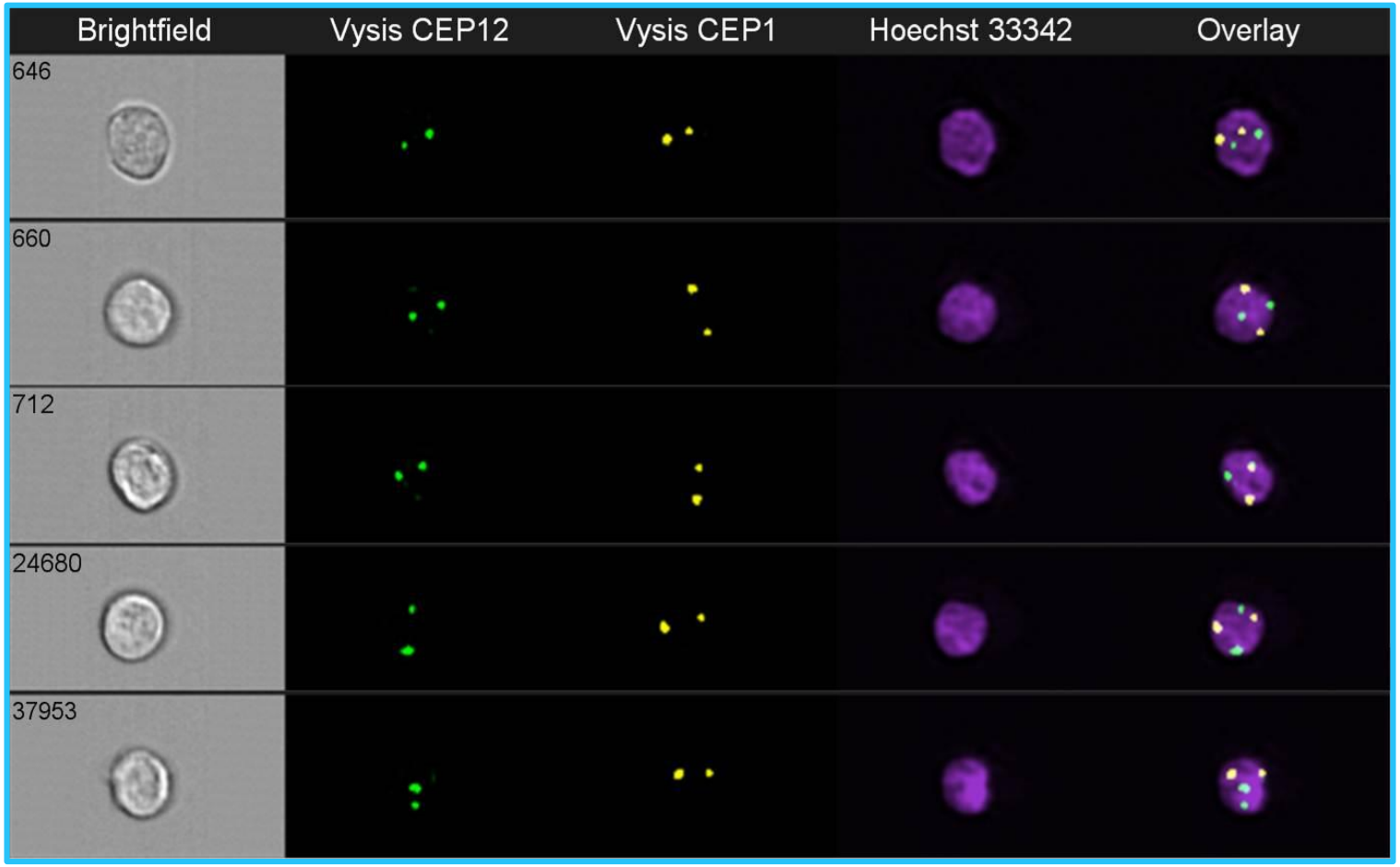
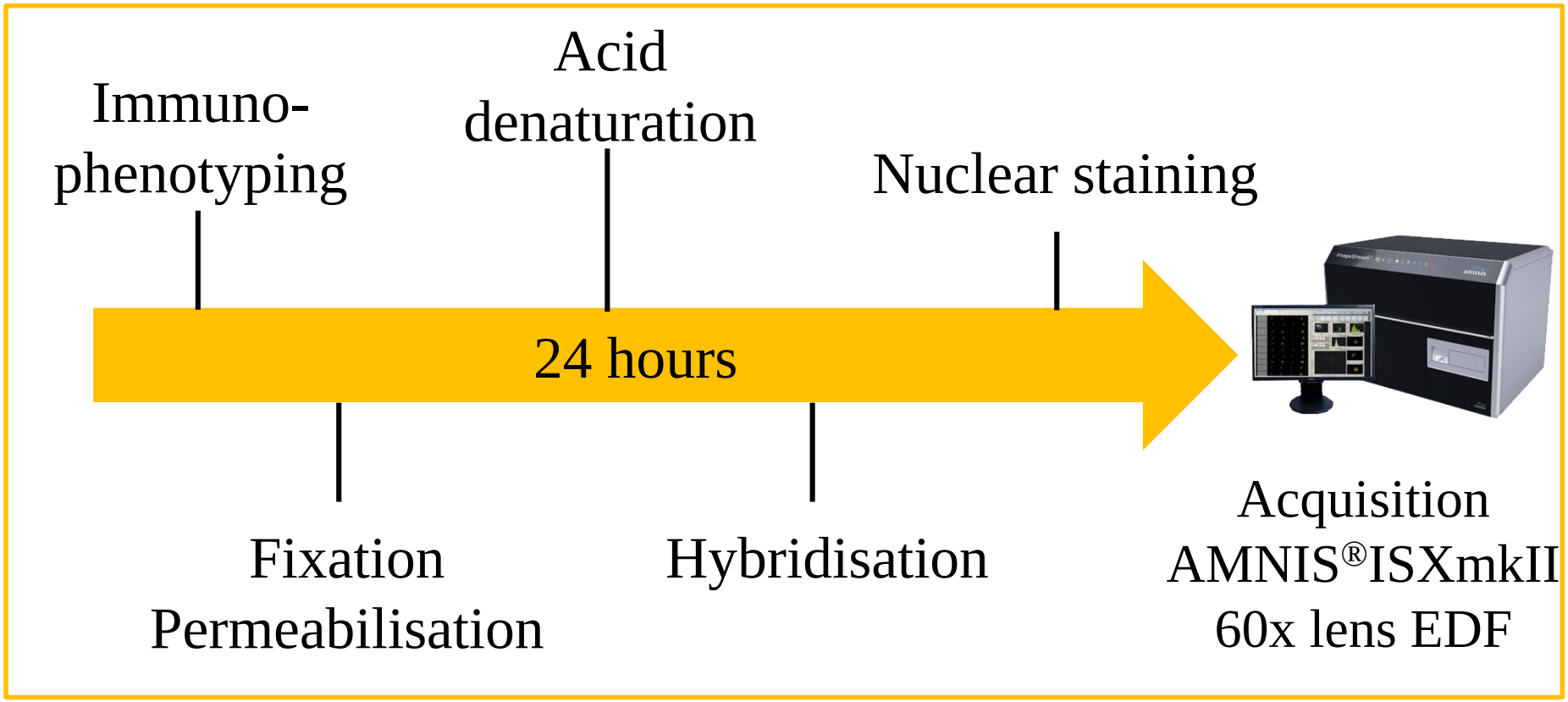
Dual FISH probe analysis

Normal blood: **CEP1** / **CEP12** + Hoechst nuclear stain



3. “Immuno-flowFISH”

Imaging flow cytometry for FISH chromosomal analysis on immunophenotyped cells in suspension



Hui et al. *Methods* 2018
Hui et al. *Cytometry A* 2019

Australian patent filed. October 2017
International patent filed, April 2020

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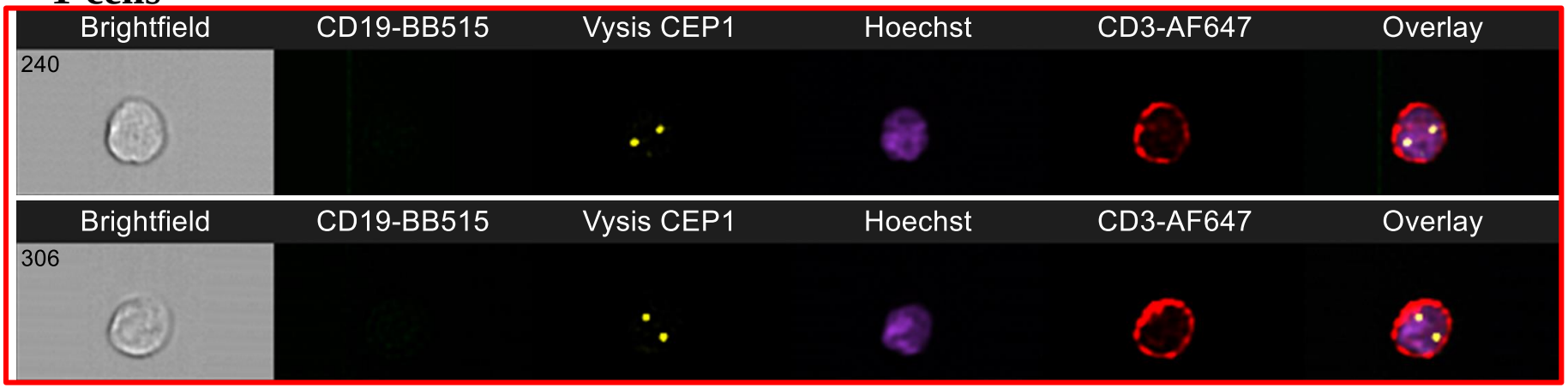
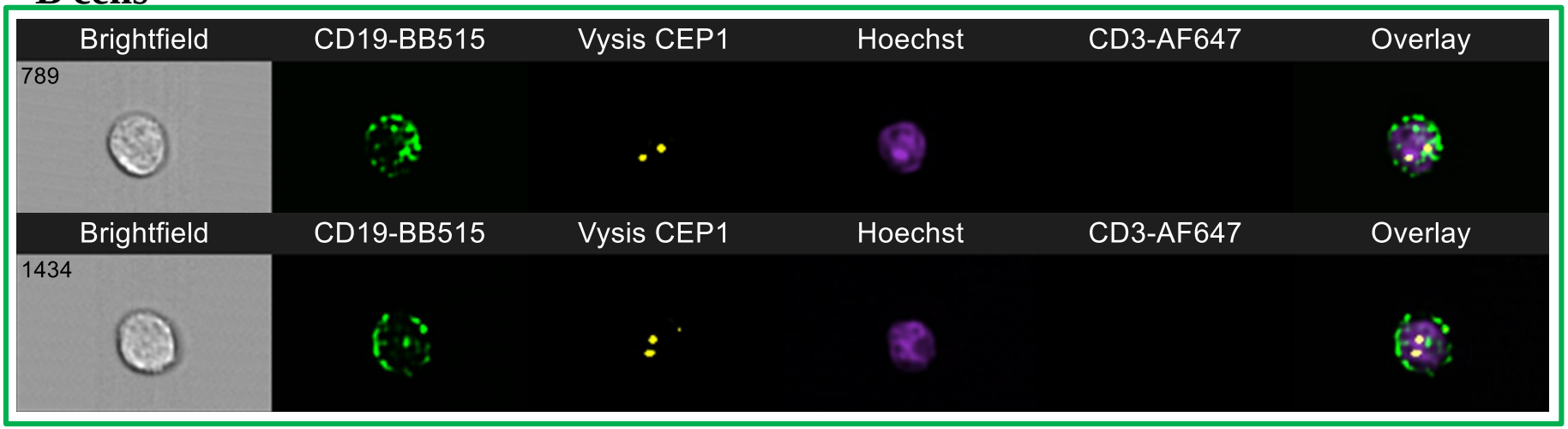
Normal Blood: T and B cells

Two (CD3 CD19) antibodies, one probe (CEP1)

T cells



B cells



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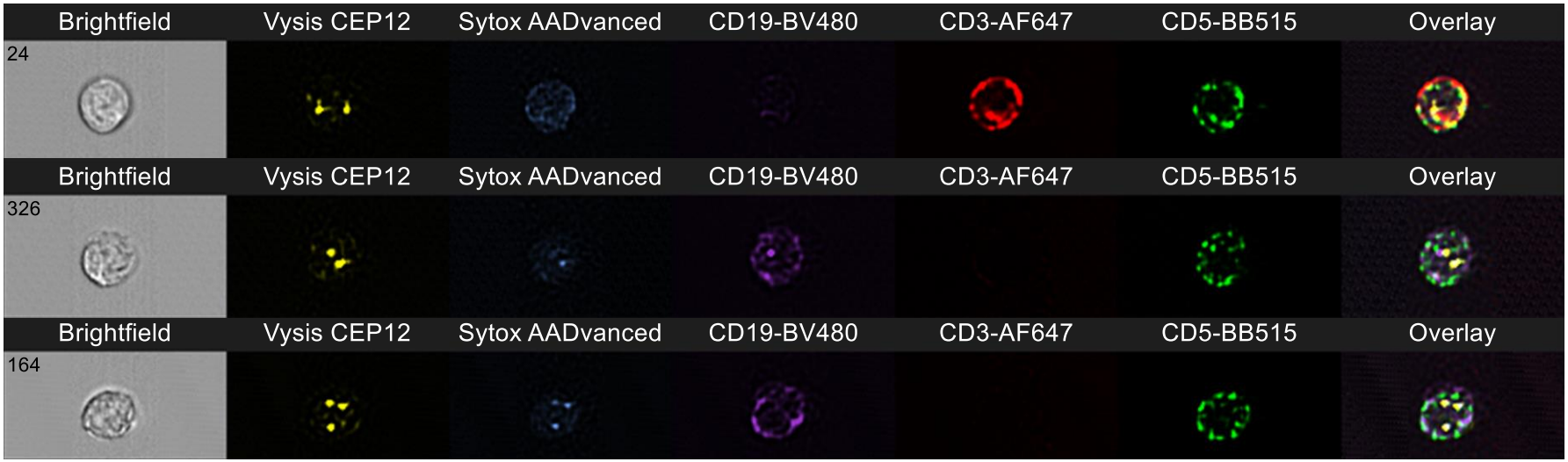
4. Technical Aspects

- Sample type: fresh cells or biobanked?
- Immunophenotyping: antibody clone and fluorophore
- FISH probe: size (CEP, LSI) and fluorophore
- FISH probe specificity: nuclear localisation
- IDEAS® software analysis: “spot” detection, count accuracy



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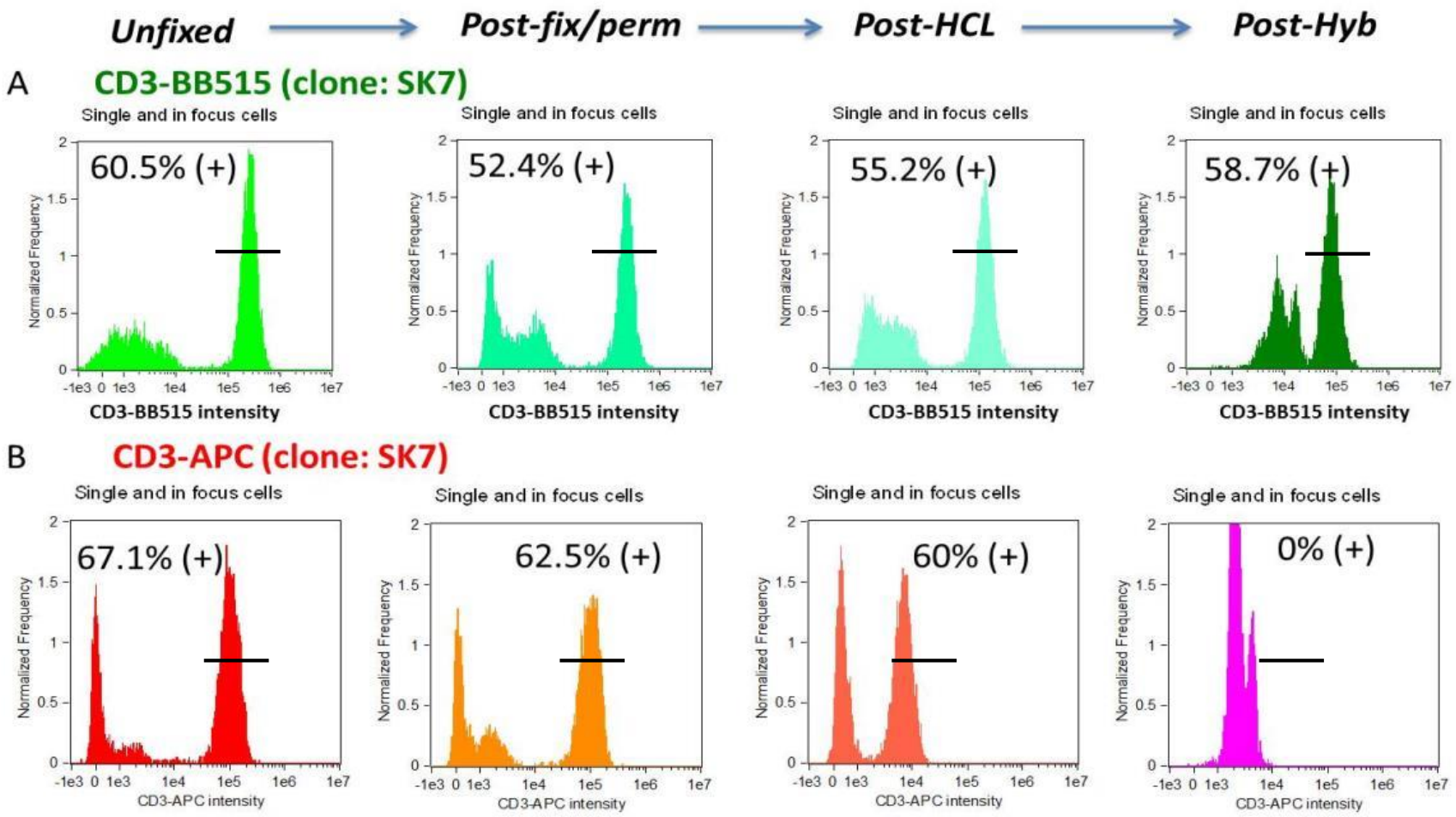
Immunophenotyping

CD3 (SK7 clone)



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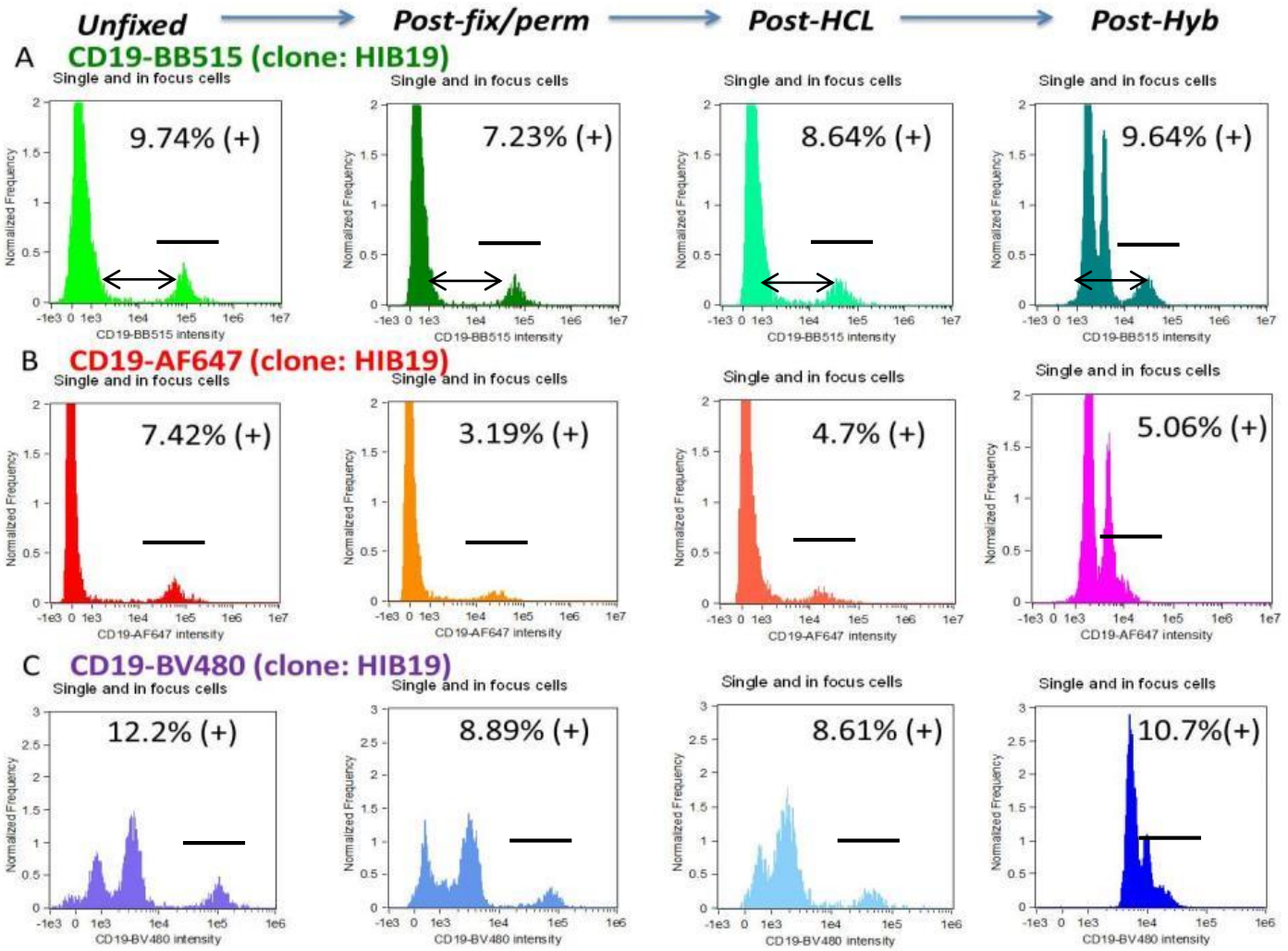
Immunophenotyping

CD19 (HIB19 clone)



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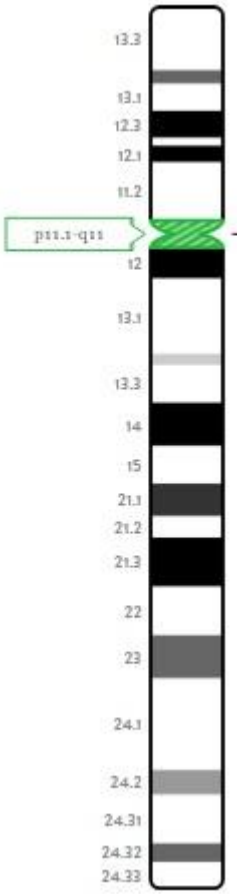
Fluorophore Overview

Fluorophore	Post stain	Post fix	Post acid denaturation	Post hybridisation
FITC	Yes	Yes	Yes	No
BB515	Yes	Yes	Yes	Yes
PE	Yes	Yes	No	No
PerCP-Cy5.5	Yes	Yes	No	No
PE-Cy7	Yes	Yes	No	No
BV421	Yes	Yes	Yes	Yes
BV480	Yes	Yes	Yes	Yes
BV510	Yes	Yes	Yes	Yes
BV605	Yes	Yes	Yes	Yes
APC	Yes	Yes	Yes	No
AF647	Yes	Yes	Yes	Yes
APC-Cy7	Yes	Yes	Yes	No

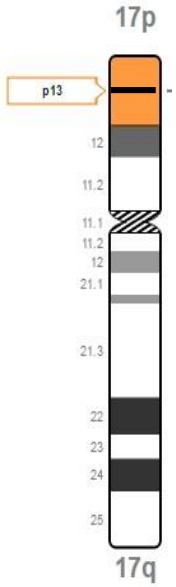
Human CD4 Fluorochrome Evaluation Kit (SK3 clone aka Leu3a) BD Biosciences

FISH Probes

Chromosome Enumeration (CEP) and Locus Specific (LSI)

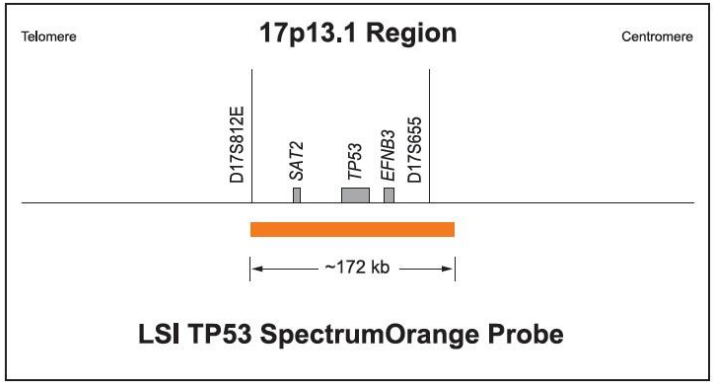


Vysis CEP12-SpectrumGreen

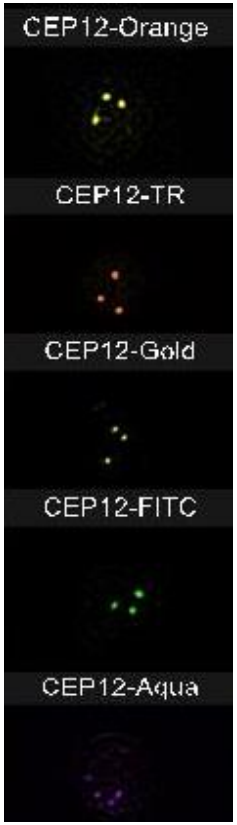


Vysis 17p13.1-SpectrumOrange

- Probe size
- Binding site
- Numerical; deletion, fusions
- Fluorophore (SO, SG)



Vysis FISH probe maps courtesy Abbott Molecular



Cytocell CEP12 FISH probes with CLL+12

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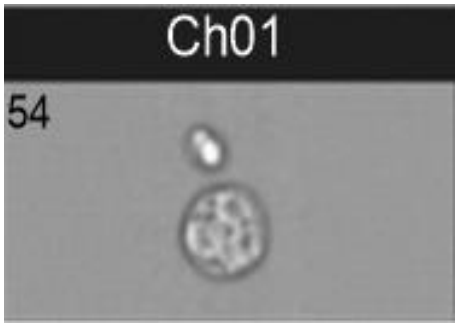
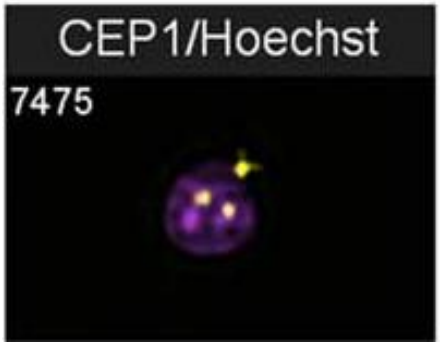
FISH Probe Specificity

Probe binding is influenced by

- Hybridisation temperature
- Hybridisation time
- Hybridisation buffer

➢ Assess co-localisation with nuclear marker



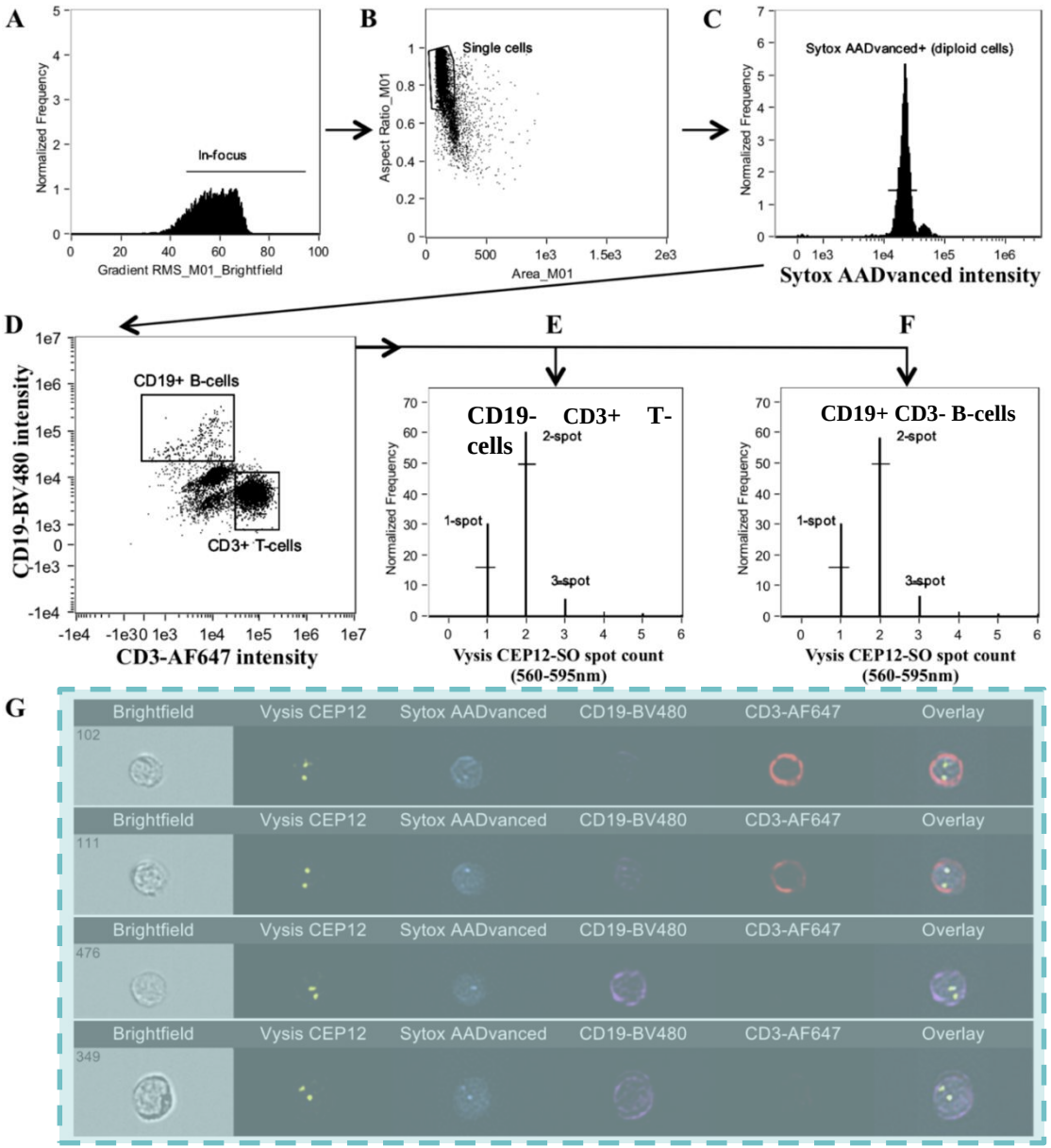


IDEAS® Analysis

Image analysis using masks

Flow cytometry gate strategy

Feature calculations



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IDEAS® Analysis

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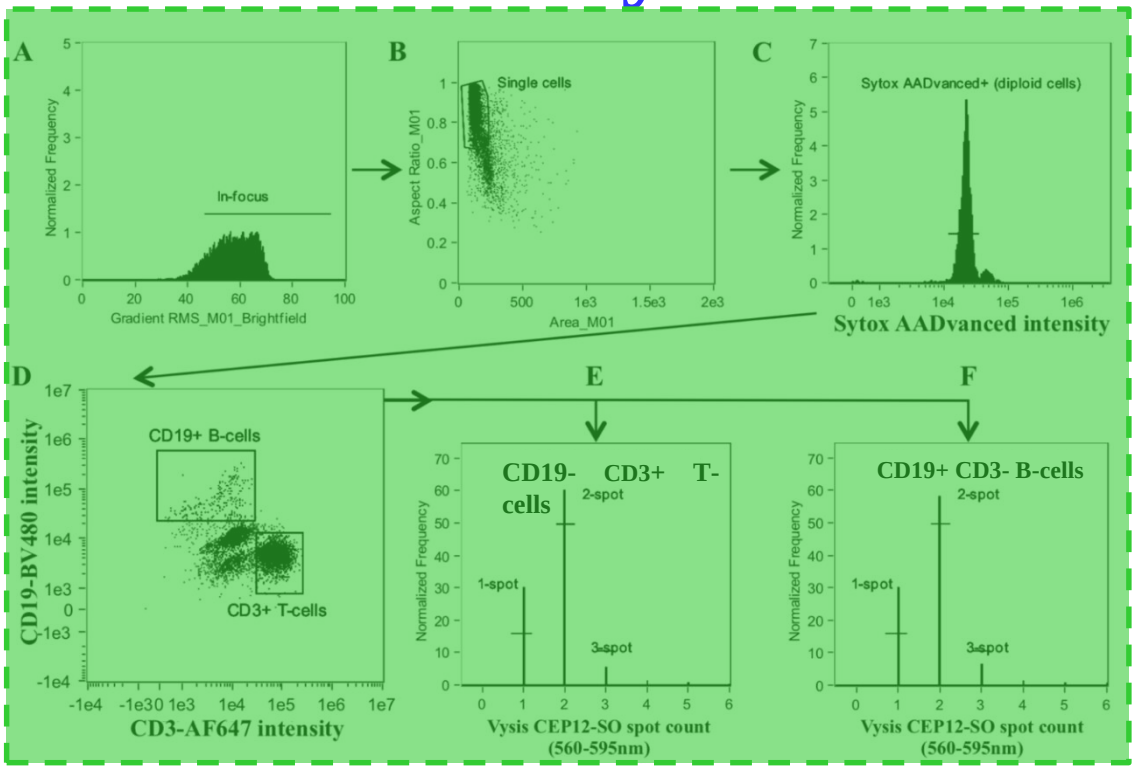

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IDEAS® Analysis

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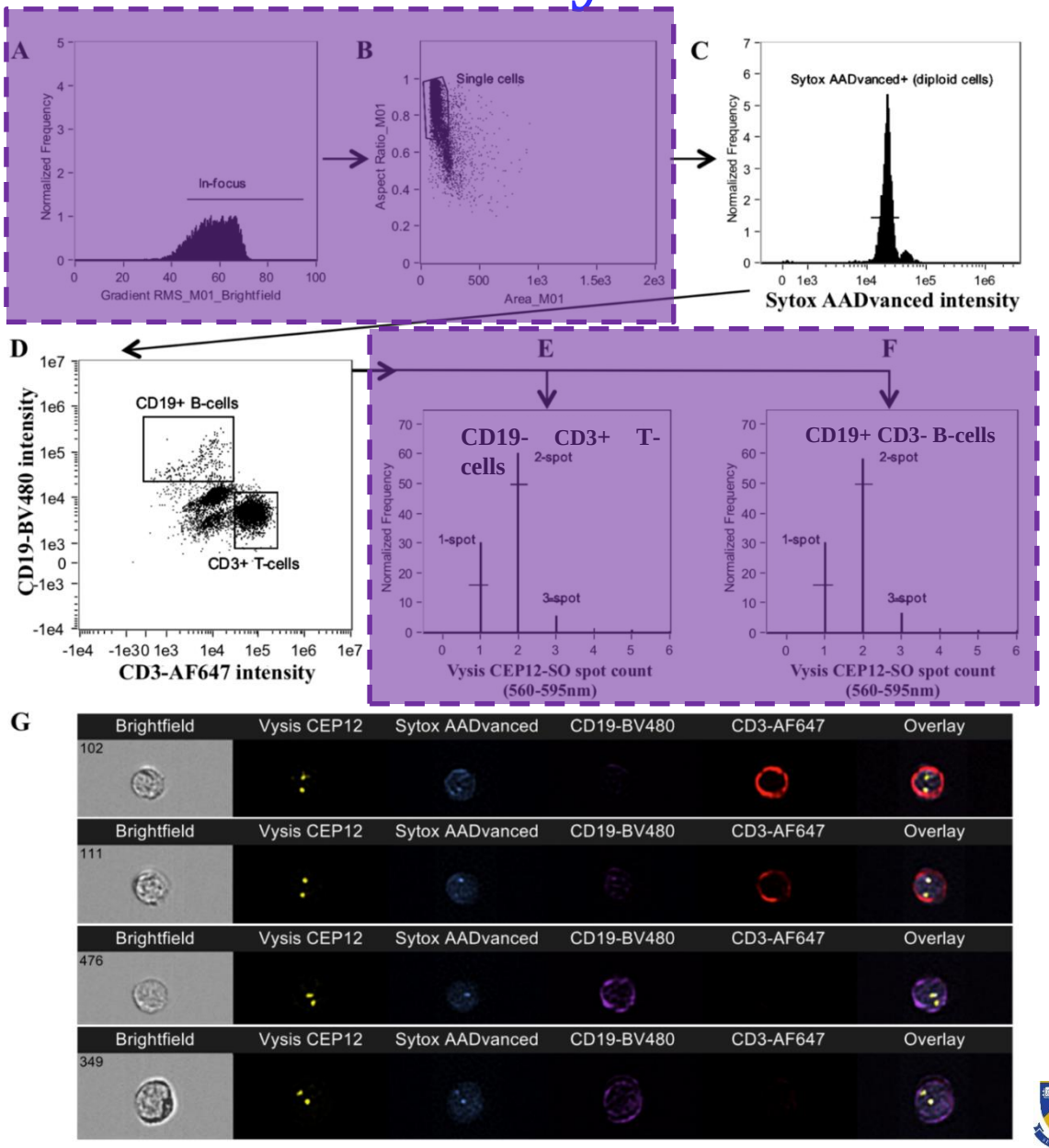

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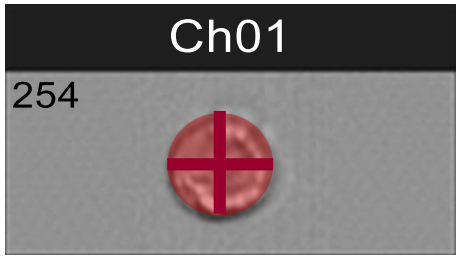
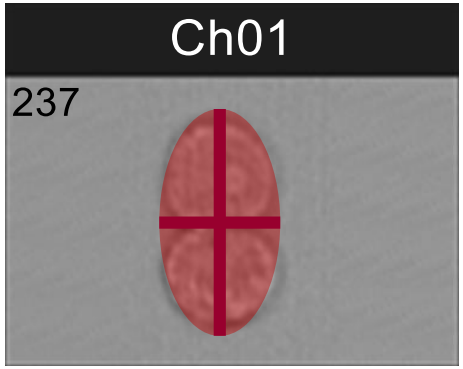
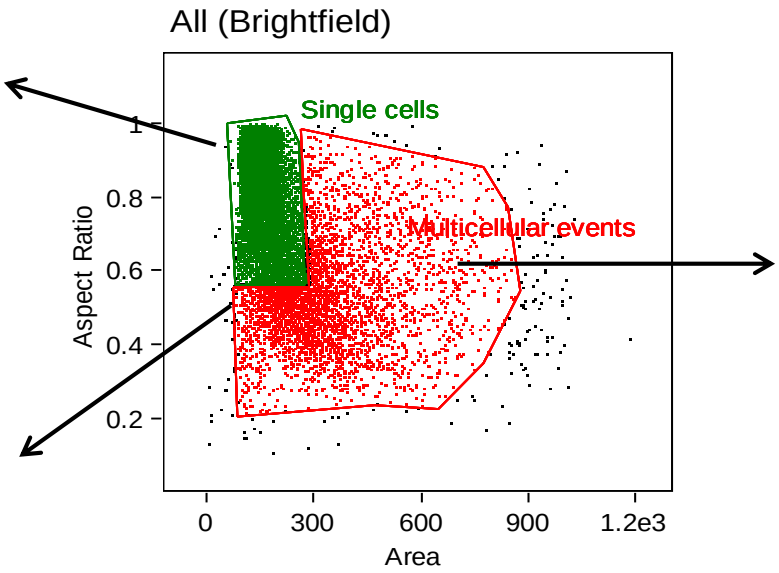
Image Analysis: excluding doublets and debri

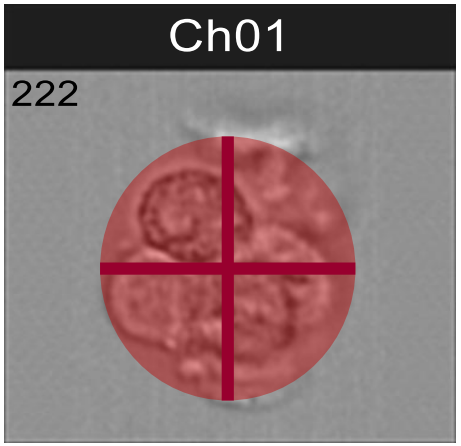


Aspect ratio = 1
Area = small



Aspect ratio = 0.5
Area = small





Aspect ratio = 1
Area = large

Area, Aspect Ratio

Population	Count	%Gated
All	25021	100
Single cells	20692	82.7
Multicellular events	3972	15.9

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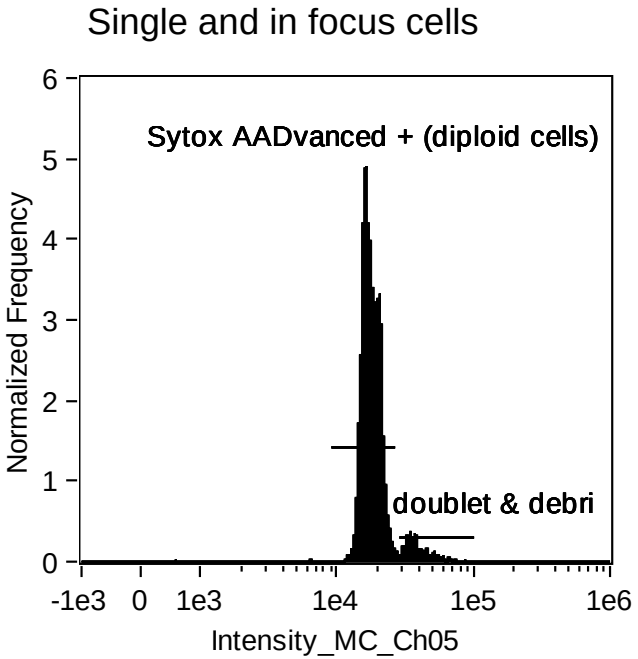
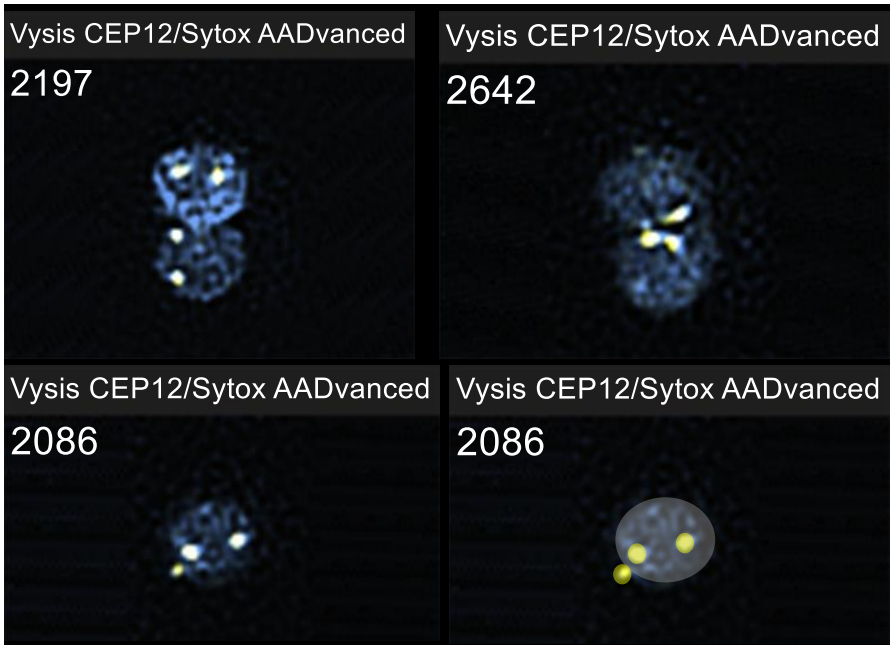
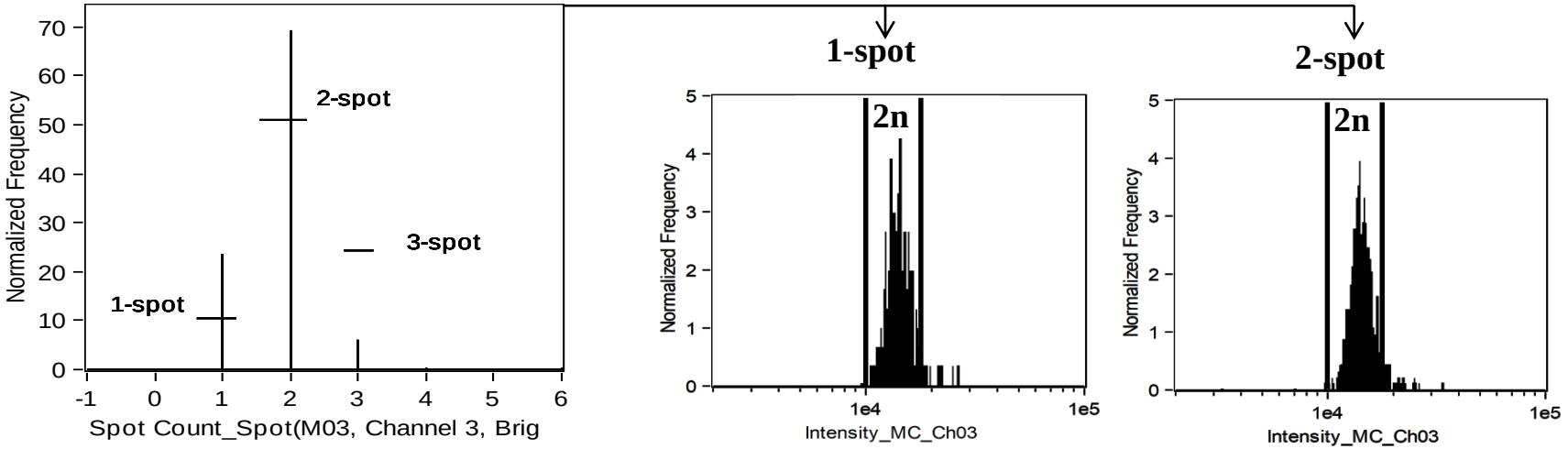
Nuclear Marker

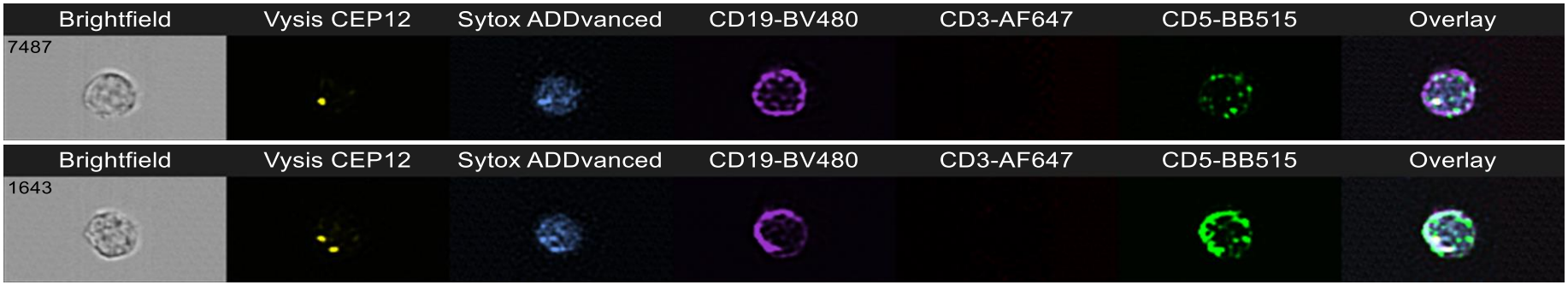
DNA fluorescence: one fluor two functions…





“Spot Count” Verification





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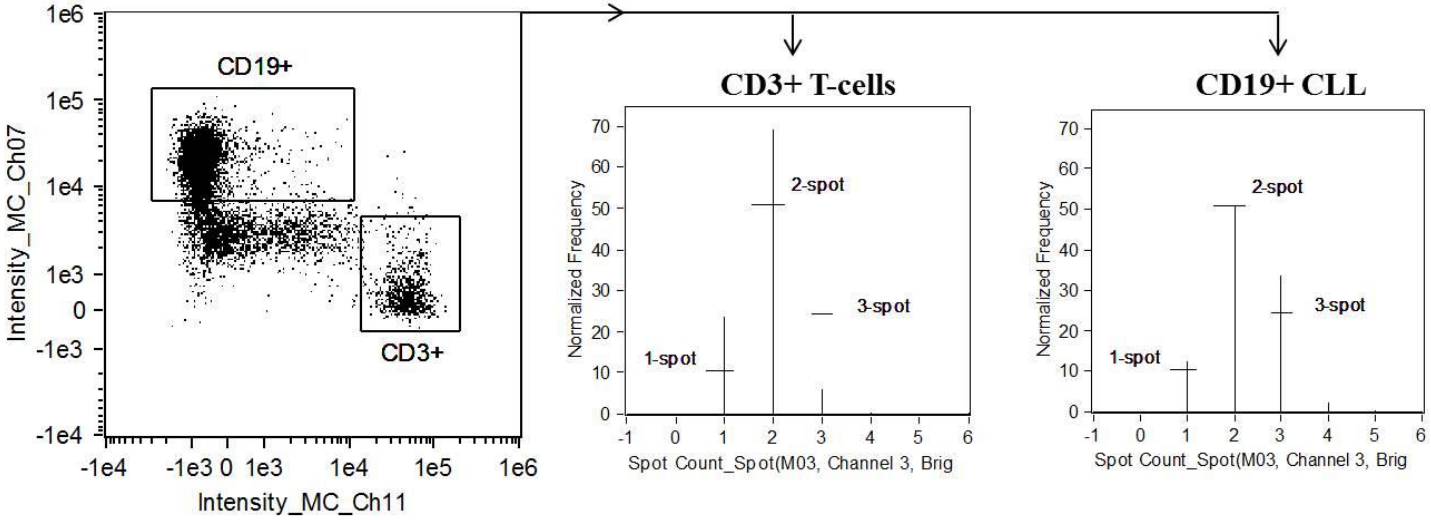
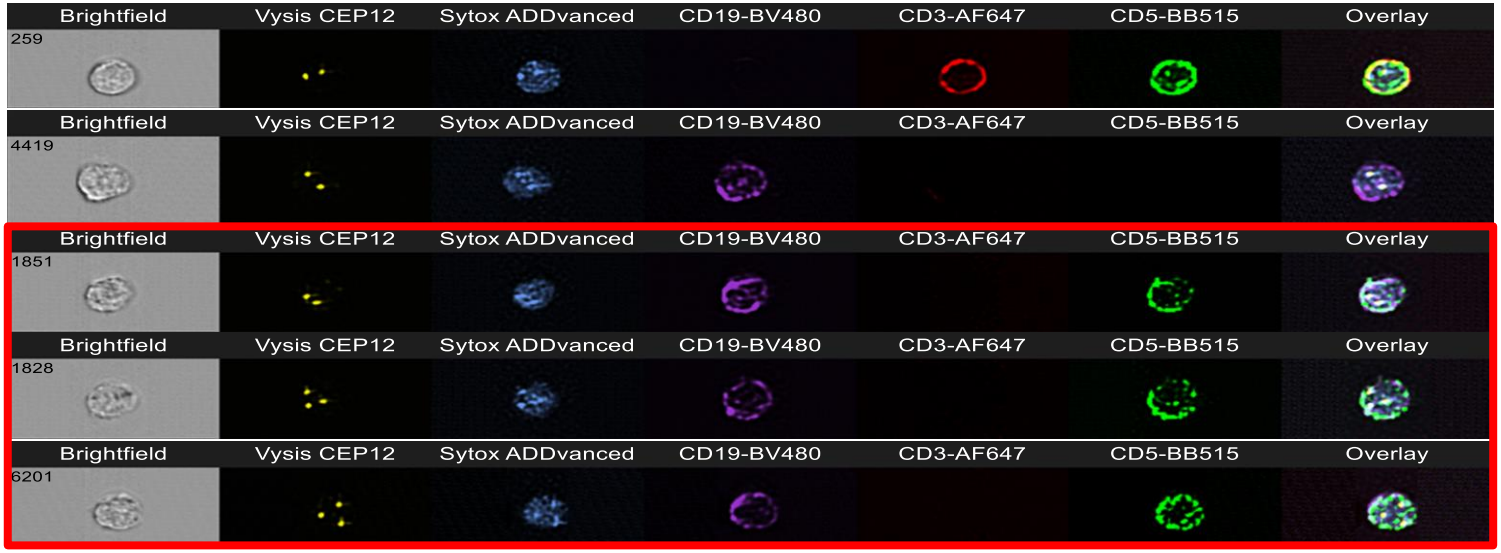
Trisomy Verification





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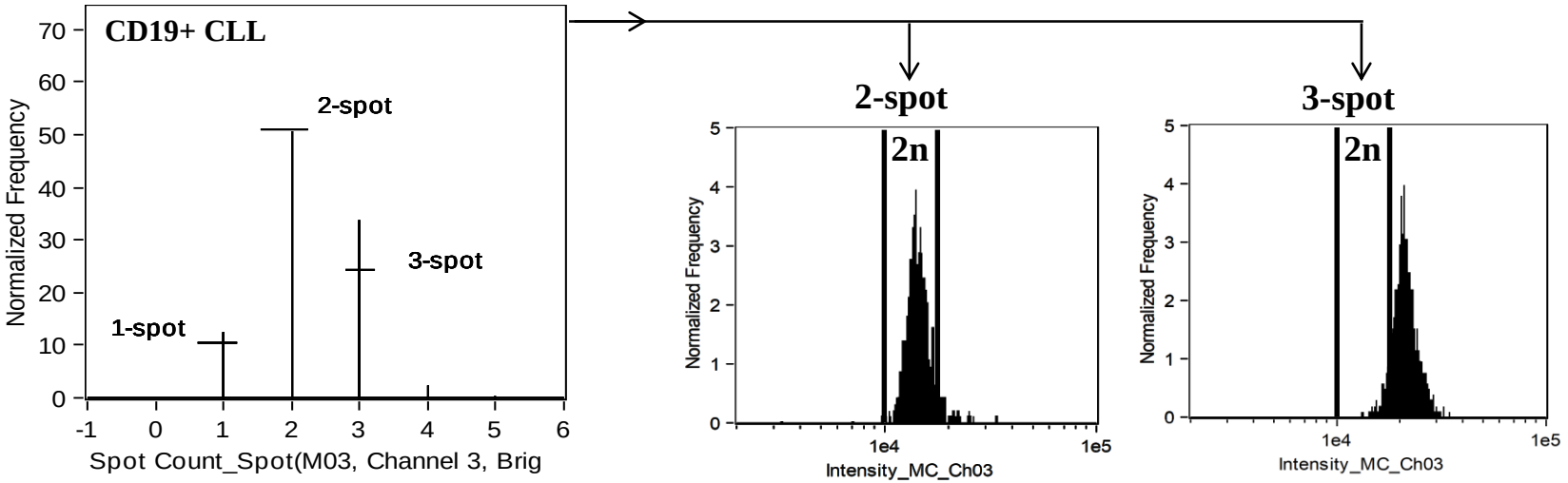
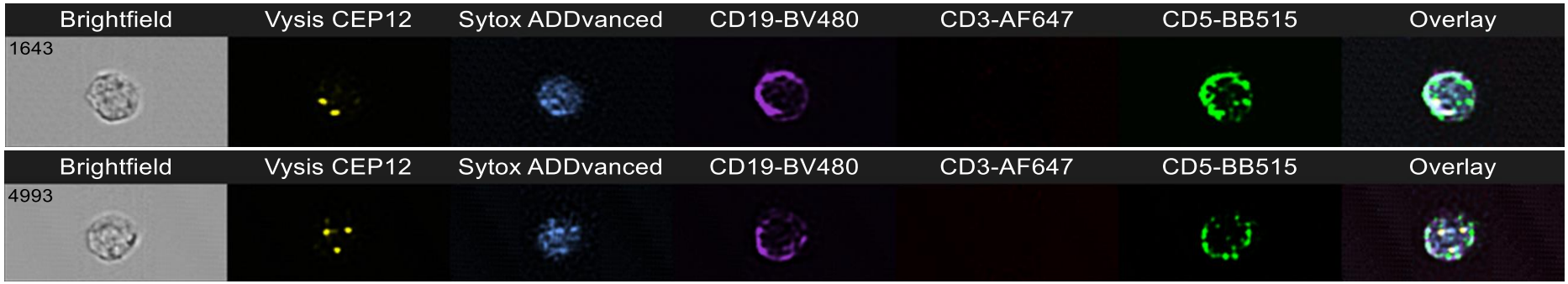
CLL+12 “Spot Count” Accuracy





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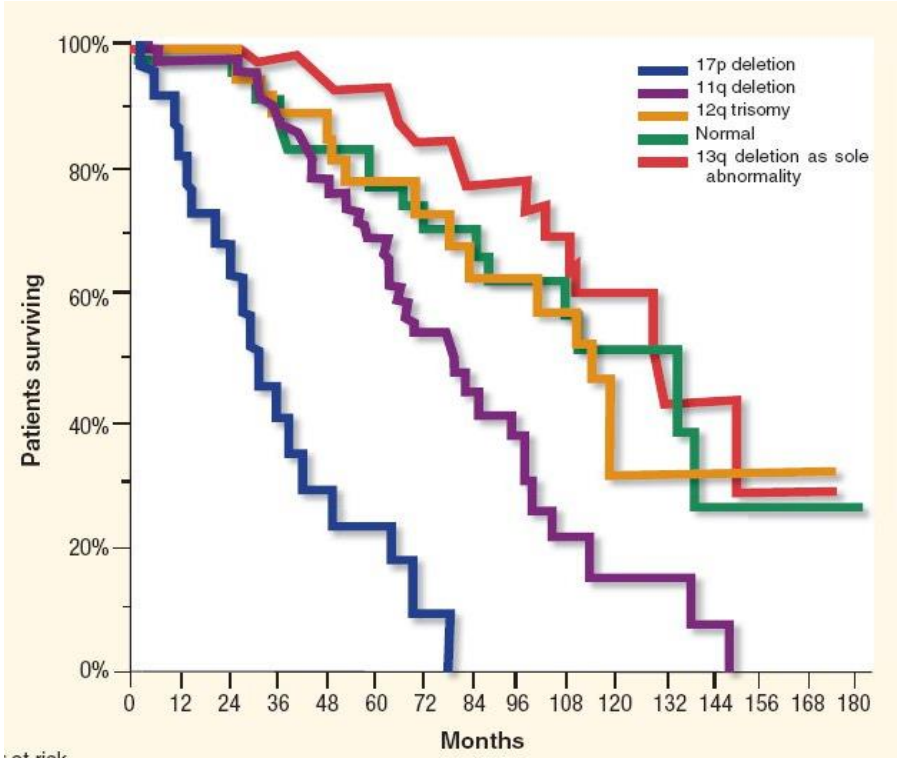
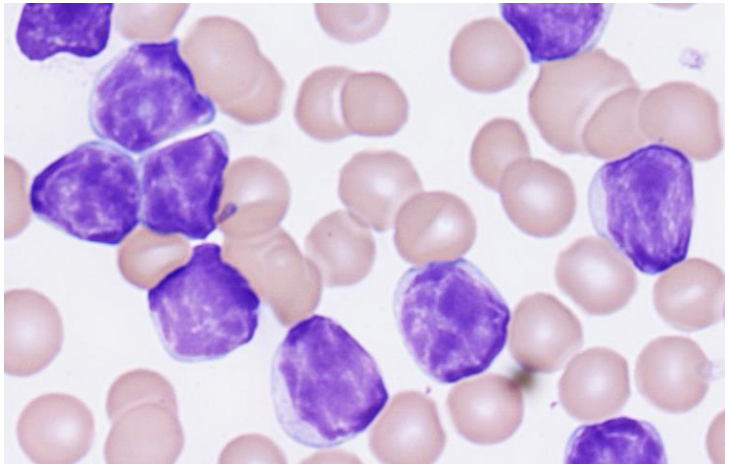
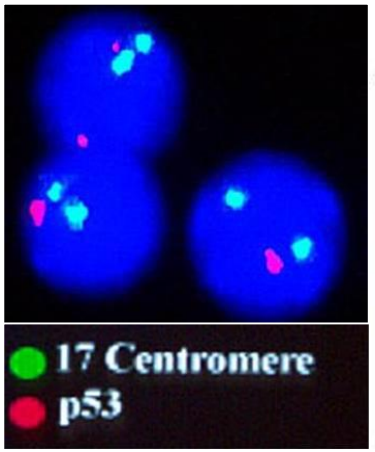
5. Chronic Lymphocytic Leukaemia (CLL)

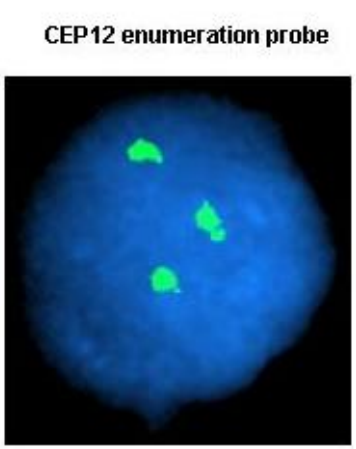
Chromosomes and Prognosis

- FISH on a slide: 100 – 200 nuclei analysed
- Nuclei only: what cell is that?
- Percent positivity cut-off: 5-7%
- Critical: del(17p)









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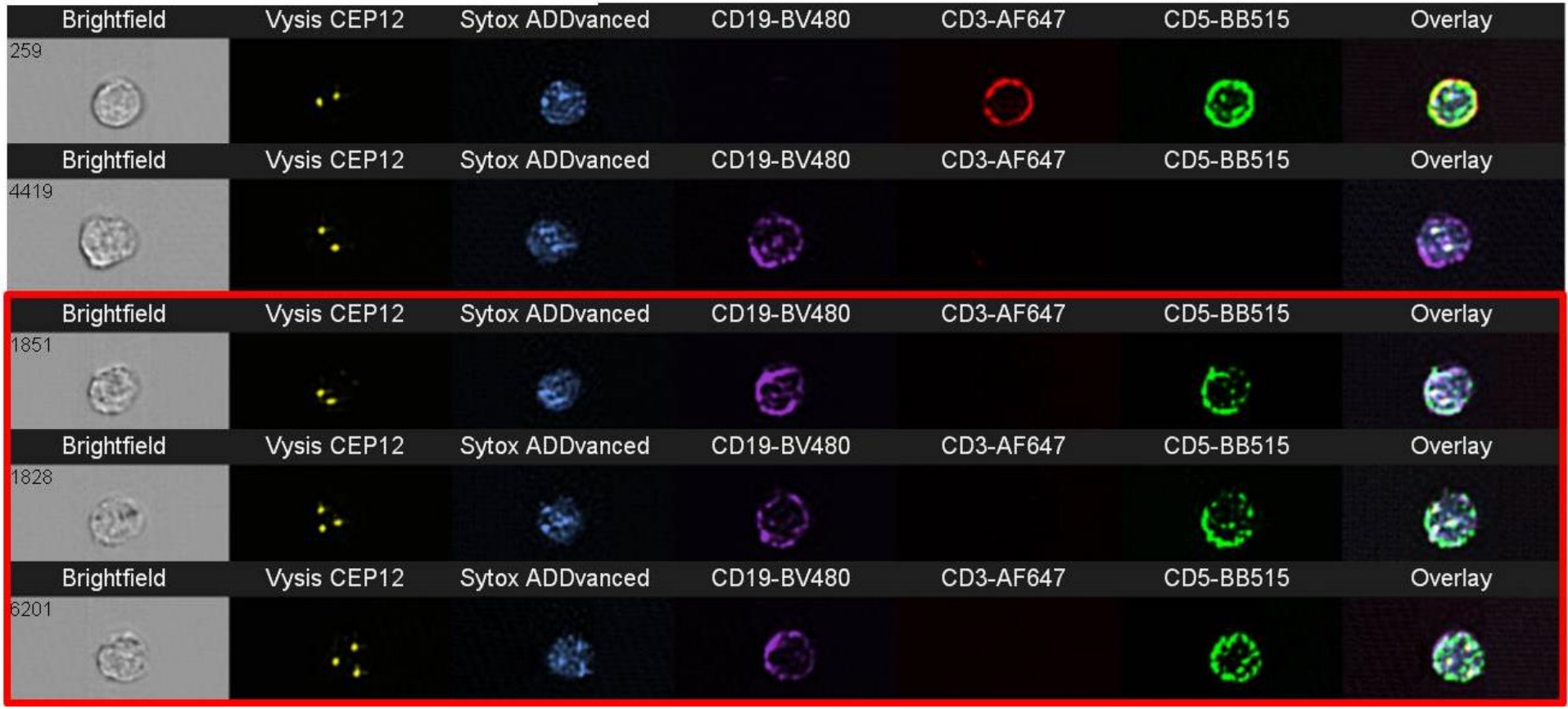
CLL, +12



Row 3: CD19+, CD5+ CLL

Row 4, 5: CD19+, CD5+ CLL, +12

45% CD5/CD19-positive B cells with +12

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CLL, del(17p12)

CD3 CD5 CD19 antibodies, FISH (17p12) probe

Row 1:	CD3, CD5:	T-cell
Row 2:	CD5, CD19:	CLL
Row 3,4:	CD5, CD19:	CLL, del(17p12)

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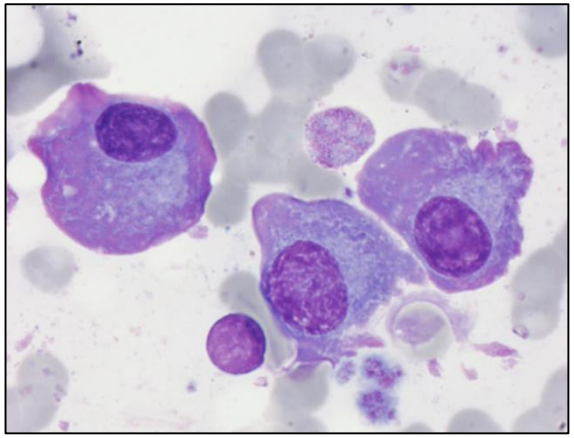
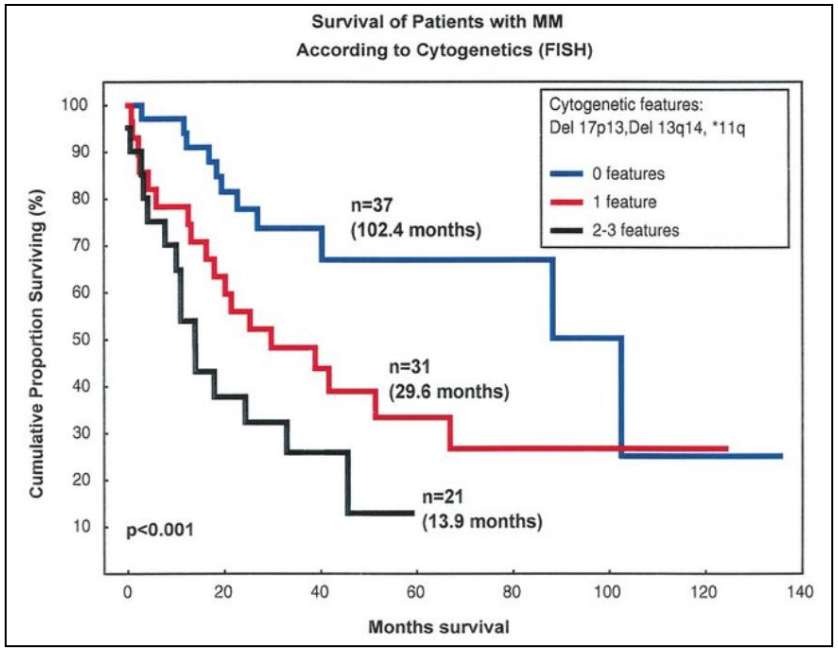
6. Plasma Cell Myeloma

- Marrow neoplasm of plasma cells
- CD38, CD138 positive cells
- Cytogenetics and prognosis
  a) Ploidy
  b) Structural: e.g. del(17p)
  c) Translocations: e.g. t(4;14)
- Treatment prediction: t(11;14)
- Circulating plasma cells





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7. Summary

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Methods 2018; 134-135: 32-40

Imaging flow cytometry to assess chromosomal abnormalities in chronic lymphocytic leukaemia

Henry Hui [a,1], Kathryn A. Fuller [a,b,1], Hun Chuah [a,c], James Liang [a,d], Hasib Sidiqi [a,d], Dejan Radeski [a,b,d], Wendy N. Erber [a,b,*]

Cytometry Part A 2019; 95A; 521–533

"Immuno-flowFISH" for the Assessment of Cytogenetic Abnormalities in Chronic Lymphocytic Leukemia

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Detection of Del(17p) in Hematological Malignancies By Imaging Flow Cytometry

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Australian Invention with Global Recognition

"Immuno-flowFISH and the improved sensitivity of detection, has potential to lead to discoveries of new prognosticators of disease and response." (*Hui H, 2019*)

Editorial. *Cytometry.* 2019

"*An unexplored frontier is the molecular testing of cytological samples. One of the most exciting opportunities is new technological capability of FISH using probes with specificity for mutations and other genetic abnormalities." (Hui H, 2019)*

Weissleder & Lee. *Nature Reviews Materials*. 2020

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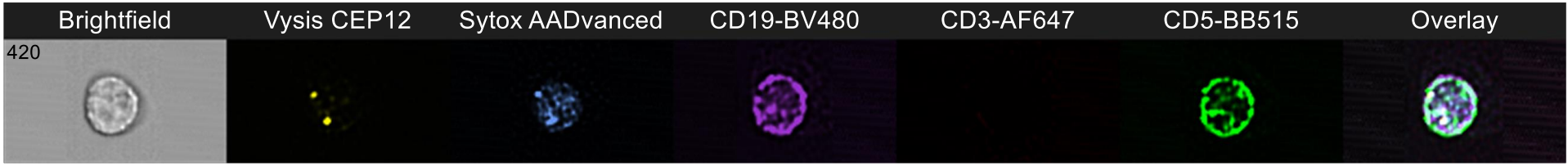
Attributes of Immuno-flowFISH



- Number of cells (100,000 cf standard FISH n = 100-200)
- Cell identification (membrane phenotype and FISH probe) increases sensitivity and specificity
- Structural abnormalities: +12, del(17p)
- Numerical quantitative data: ratio; % cells positive
- Limit of detection (to date): 1/10,000
- Automated “flow”: fast, high throughput
- Reproducible: can replicate results (100% accuracy)

Australian patent filed. October 2017
International patent filed. April 2020

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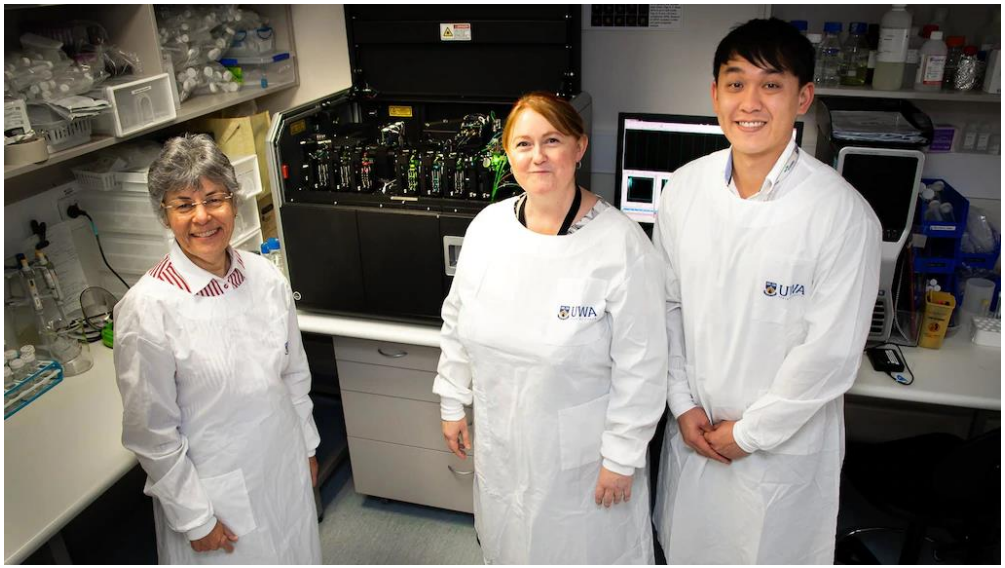

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Haematologists at Sir Charles Gairdner Hospital, Royal Perth Hospital, Hollywood Private Hospital and PathWest Laboratory Medicine