Optical Measurement Principles

Cytometer Components

Reagents

Cytometer Setup

Sample Preparation

Automation

Networked Components

Quality Assurance

Data Management

Limitations

Standardisation

Clinical Laboratory Applications

Data Analysis Software

Cases

Relative size

Relative internal complexity

Relative fluorescence intensity
Optical Measurement Principles

Forward Scatter (FSC)

Optical Measurement Principles

Side Scatter (SSC)

Cell Markers and Monoclonal Antibodies

Fluorescence Measurement Principles

Identifying Cells By Scatter Light Analysis
Fluidics
Moves cells to the interrogation point for interaction with one or more lasers and discards to waste

Fluidics
Sample travels through the sample injection tube
Hydrodynamic focusing within the flow cell forces particles to flow in a single-file stream through the centre of the flow cell
Laser light intercepts the stream at the sample interrogation point
Increasing the sample pressure increases the core diameter and the flow rate

Fluidics
A lower flow rate : optimal resolution and sensitivity
A high flow rate : data is less resolved but is acquired more quickly
Optics

(BD FACSCanto™ II)

Laser excitation and collection optics

- Illuminate cells passing through the flow cell
- Designed to reduce excitation losses

Excitation source 2 to 3 lasers:
- Blue (488-nm, air-cooled, 20-mW solid state)
- Red (633-nm, 17-mW HeNe)
- Violet (405-nm, 30-mW solid state)

Collection optics direct light scatter and fluorescence signals through spectral filters to the detectors

Key features of Excitation Optics

1. Spatially separates beam spots in the flow cell
   - Accommodates multiple fixed-wavelength lasers
   - Fiber optics pass light up to the beam-shaping prisms
   - Achromatic focusing lenses

2. Each lens focuses the laser light into the gel-coupled cuvette flow cell.

3. Fixed optical pathway and sample core stream
   - No need for user intervention
Excitation Optics

Menu of lasers

Working Selection
Blue laser 488nm 4 / Red laser 633nm 2 / Violet laser 405nm 2

Optics

Fluorochrome Excitation / Emission

Collection Optics

The emission signals are transmitted from the flow cell to the detector arrays:

- an Octagon for the blue laser
  - the octagon contains five PMTs and detects light from the 488-nm blue laser
  - a PMT in the octagon collects side scatter signals
- a Trigon each for the red and the violet lasers
  - each trigon contains two PMTs and detect light from the 633-nm (red) and the 405-nm (violet) lasers
The Octagon and Trigon detector arrays (BD-patented) use serial light reflections to guide signals to their target detectors:

- Resulting in efficient light collection and signal retention at the detector level.
- Enhanced instrument sensitivity by collecting the dimmest emission signals first.

PMTs

Labelled filters

Long pass filter directs highest $\lambda$ to first detector (PMT) and progressively lower $\lambda$ to successive detectors.

Lasers

Collection Optics

Fibre optic cables

Detector Arrays

- Labelled filters
- Long pass filter directs highest $\lambda$ to first detector (PMT) and progressively lower $\lambda$ to successive detectors
- PMTs
Electronic system

Main functions:

- Digitise light signals
- Electronically remove debris
- Correctly assign different data collected from multiple lasers for each cell

Digitise light signals:

Correctly assign different data collected from multiple lasers for each cell.

Electronic system

Laser Delay

Converts optical signals to electronic signals and digitizes:

Signal processing: amplification, current, and voltage, followed by a digital output.
Electronic System

Reagents

- RBC lysis buffer
- Monoclonal & Polyclonal Antibodies
- DNA dye
- Propidium iodide / 7-AAD
- Fixative
- Balanced Salt Solution

Instrument

- Sheath Fluid
- Instrument Tracking Beads
- DI Water / Bleach

Monoclonal Antibody Management

Expensive $300 at 500ul – 1000ul
- Titrated
- Aliquotted
- Light & temperature sensitive
- Mixed into ‘Cocktails’
- Cocktails verified before use

Cytometer Setup

Immediately Post Installation → Almost useless
- Exceptions include Predefined Programming
  - Lymphocyte Subset

Useful After:
- Setting Instrument Baseline (One baseline per configuration)

Cytometer Setup & Tracking Beads (CST)

Dilute suspension of beads analysed on the cytometer
- Consist of fluorescence dim (2um), mid and bright (3um)
polyethylene beads dyed with a mixture of fluorochromes which
emit fluorescence measured in the detectors
- Establish baseline reference values
  - optical / electrical noise
  - detector efficiency
  - resolution of fluorescent populations

Cytometer Setup

Definition and linkage
- Experiment = Test with
  - Sample panel e.g. Acute Leukaemia Panel
- Application settings
  - Include PMT voltages / Threshold (Debris Cutoff)
  - Adjust (optimise) FSC / SSC / PMT (fluorescence)
  - voltages while acquiring positively stained control
cells
- saved for subsequent use
- Compensation settings
  - spillover corrections determined using stained cells
  - mean MFI of positive and negative populations
  - aligned
Compensation calculation corrects inter-detector “Spillover”

Compensation enables simultaneous use of multiple fluorochromes with overlapping emission spectra.
**Flow Cytometer Daily Setup & Quality Control**

**Daily Setup**
- CS&T Beads run daily
  - monitor variation from baseline measurements and flag trends which indicate fault with cytometer and action
  - measurements plotted on Levey-Jennings charts
  - PMT voltages and laser delay adjusted
  - User-defined application settings are corrected for drift

**Daily QC Troubleshooting**
- Trend: Falling detector efficiency
  - Laser power: Alignment or failing laser
  - Dirty flow cell or degraded sheath filter P
  - PMT performance

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**Clinical Laboratory Samples & Sample Preparation**

**Peripheral Blood**
- Leucocytes
- Erythrocytes
  - %Fetal RBC Semi-quantitation (FMH)

**Bone Marrow**

**Bone Marrow Trephine (Dry Tap)**

**Tissue**
- Any site involved with Haematopoietic Malignancy

**Fluid**
- Peritoneal
- Bronchoalveolar Lavage
- CSF

**Paraffin Sections for DNA Ploidy**

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**Prepare sufficient cells at concentration 2x10^7/ml**

**Blood / Bone Marrow**
- Cell Count
- Lyse RBCs
- Centrifuge / Pellet
- Resuspend to volume

**Tissue**
- Deaggregate
- Lyse RBCs
- Centrifuge / Pellet
- Resuspend to volume

**Fluid**
- Centrifuge / Pellet
- Cell Count
- Resuspend to volume
Automation

Lyse Wash Assistant
Automates sample preparation
Lyses, mixes, washes, and fixes cells
Processes up to 40 samples per batch
Eliminates the need to transfer samples to a centrifuge
Programmable

Sample Prep Assistant
Walkaway automation
Sample tube cap piercing, blood and reagent aliquoting, incubations, lysing and mixing
Predefined & customizable protocols

Automation

FACS Loader
Replaces manual tube handling
Removable Carousel holds 1-40 tubes
Automatically loads & unloads tubes
Compatible with other automation
• Lyse Wash Assistant
• Sample Prep Assistant

High Throughput Sampler
Rapid sample acquisition
96 & 384 well plates

Networked Components

2 x BD FACS Canto
2 x Printer
2 x Sample Prep Assistant
3 x PC Workstation
(1 x Lyse Wash Assistant)

External Quality Assurance

RCPA Immunology QAP: Immunophenotyping
Lymphocyte Subsets %
• CD3+ lymphocytes
• CD3+, CD4+ Lymphocytes
• CD3+, CD8+ Lymphocytes
• CD19+ Lymphocytes
• CD3-, CD16+, CD56+ Lymphocytes

Monthly

RCPA Haematology QAP: Oncology Immunophenotyping

UK NEQAS - Minimal Residual Disease Quantitation (Acute Lymphoblastic Leukaemia)
Six monthly
Data Management
Quarterly backup to DVD
  • FCS files
    • Immunophenotyping
    • Lymphocyte Subset
  Electronic storage of Immunophenotyping Report Sheets

Standardisation
CLSI
Enumeration of Immunologically Defined Cell Populations by Flow Cytometry; Approved Guideline - Second Edition
Lymphocyte subsets and CD34+ (hematopoietic) stem cells
Clinical Flow Cytometric Analysis of Neoplastic Hematolymphoid Cells; Approved Guideline - Second Edition
Performance guidelines for the immunophenotypic analysis of neoplastic hematolymphoid cells
AFCG
International Clinical Cytometry Society
July/August
Flow cytometry immunophenotyping; rapid qualitative evaluation of bone marrow status
Optimising antibody panels in haemiaurology and effective flow cytometric interpretation of bone marrow aspirates

Clinical Laboratory Applications
Diagnostic Haematology
Rapid diagnosis & subclassification of Acute Leukaemia & Lymphoma by expression
  • surface markers (B, T or NK-cell and myeloid markers),
  • cytoplasmic markers (MPD, CD3, CD22, CD79a etc)
  • nuclear markers (TdT)
  • forward & side light scatter
  • WHO Classification "Tumors of Haematopoietic & Lymphoid Tissues"
Assessment of Minimal Residual Disease
  • Detected low levels of cells with aberrant immunophenotype
    • B and T-ALL and AML
Diagnosis of PNH
Primary Immunodeficiency
Enumeration CD34+ Stem Cells
DNA Ploidy
  • Triploidy in partial hydatidiform mole
  • Aneuploidy in paediatric B-ALL
**Limitations of Flow Cytometry in the Clinical Laboratory**

**Limited role in diagnosis / follow up:**
- **Classical Hodgkins Lymphoma & variants**
  - Useful to exclude B or T cell disorder
  - False negative findings
  - Neoplastic cells too large / scarce

**High Grade Lymphoma**
- Large B-cell lymphoma & anaplastic large cell lymphoma
  - Selective dropout of neoplastic cells

**Subset of T-cell LPD**
- Lack of aberrant expression of pan-T cell markers
- Normal CD4:CD8 ratio
- Deaggregated sample loses disturbed 'architecture'
- Failure to aspirate clonal B-cells

**Data Analysis Software**
- Transform FCS Files into interpretable data
- Purchased with the instrument
  - BD FACS Diva (Becton Dickinson)
  - Kaluza (Beckman Coulter)
- Third Party
  - FCS Express (De Novo Software)
  - FlowJo (Tree Star, Inc)

**Cases**
- **Case 1:** Chronic Lymphocytic Leukaemia
Case 1: Chronic Lymphocytic Leukaemia
Approximately 64% of total cells express CD5 weak, CD19, CD20, CD23 and kappa surface light chains.

Case 2: Chronic Lymphocytic Leukaemia
Approximately 19% of total cells express CD5 weak, CD19, CD20, CD23 and indeterminate surface light chains.
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