Flow Cytometry Training Course

Overview

- Flow cytometry is a technique for counting, examining and sorting microscopic particles- cells, bacteria, yeast- suspended in a stream of fluid. It allows simultaneous analysis of multiple parameters of the physical and/or chemical characteristics of single cells flowing through an optical and/or electronic detection apparatus. By combining this with fluorescent tagging, large amounts of data can be collected.

Instrument Components

1. Fluidics:

The heart of the fluidics system on all flow cytometers is the "nozzle". This is the component where cells are injected into the sheath fluid stream, are hydrodynamically focused, and in cuvette-nozzle designs, where the cells are illuminated by the laser beams.

- Hydrodynamic focusing
    - To achieve a single line of cells, the sample stream is focused using a speed differential between the sheath fluid and the sample stream. This allows the laser to intercept a single event in a thin stream as opposed to several events in thicker sample stream
- Sample Injection Port (SIP)
    - This tube draws up the sample and injects it into the sheath stream
- Flow Cell
    - Carries and aligns the cells so they can be excited by the laser

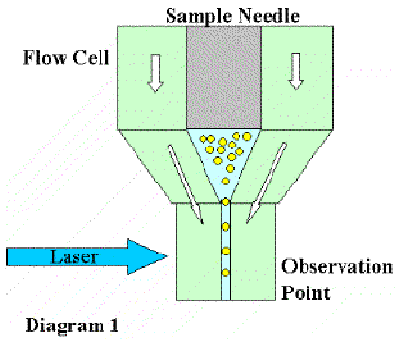


Diagram 1

- Waste Lines and Tank
    - Once the cells have been past the laser, they are carried to the waste tank.

2. Optics

Lasers

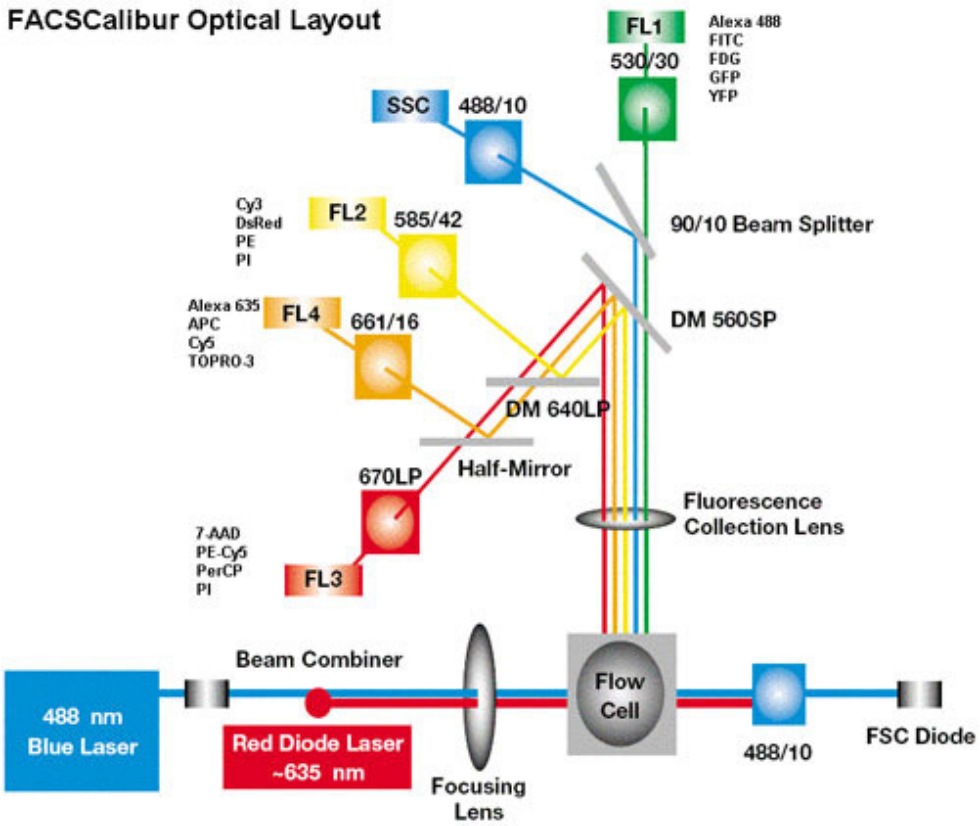
Blue (Argon) laser

- Excites at the 488 nm range.
- Standard on all cytometers and is used for forward scatter (size, cell volume) and side scatter (complexity: shape of the nucleus, granularity, or the membrane roughness) detection

Red (Helium Neon) laser

- Excites at the 633 nm range

Filters & Detectors



Filters and Detectors work together to collect light scatter and the light emitted from fluorescent probes. In order to measure and resolve data we must collect data in proper places and colors so that we know which parameter we are measuring.

- Forward scatter signal is measured in the same direction as the path of the laser beam.
- Side Scatter and all fluorescent measurements in typical flow cytometers are made at some large angle (e.g. 90° relative to the primary laser beam) and use photomultiplier tubes (PMTs).

The light is collected through sophisticated optical elements. In most instruments these are essentially microscope objective lens assemblies. The color of the light collected determines which probe is being measured. To isolate the color for each probe, interference filters are used. Filters are placed in front of each detector (PMT). A particular color of light is split off from the incoming mixture and directed to the detectors using interference filters designed to work at other angles (e.g. 45°), and these are called dichroic mirrors.

Types of Filters:

Band-Pass Filter: allows signals between two specific frequencies to pass, but discriminates against signals at other frequencies. Example: 530/30 bandpass: the filter allows light frequencies of 515-545 nm pass to the detector.

Short Pass Filter: allows signals below a specific frequency to pass. Example 500 SP

Long Pass Filter: allows signals above a specific frequency to pass. Example 670 LP

- **Blue laser filters examples:**
    1. Forward Scatter (FSC)
        - Identifies relative size of particles
    2. Side Scatter (SSC)
        - Identifies relative internal complexity (granularity) of particles
    3. FL1 (530/30 nm) bandpass filter
        - Common Fluorochromes: FITC, GFP, Alexa Fluor 488
    4. FL2 (585/42 nm) bandpass filter
        - Common Fluorochrome: PE, PI
    5. FL3 (670 nm) longpass filter
        - Common Fluorochromes: PE-TxR, 7AAD

- **Red laser filters example:**
    1. FL4 (661/16 nm) bandpass filter
        - Common Fluorochromes: APC, Alexa Fluor 633

**Note: The FACSCalibur and CyAn ADP have blue AND red lasers. The FACScan only has the Blue laser

3. Electronics

Electrical pulses originating from light detected by PMTs are converted into signals which allow for events to be plotted on a graphical scale such as a dot plot or histogram.

Fluorochrome Excitation & Emission

1. **Fluorochrome Absorption**
    a. Each fluorochrome has a unique excitation spectra which shows the percentage of absorption of light at various wavelengths
        i. For each fluorochrome you wish to use, you must ensure that it is reasonably excited by one of the lasers we have (currently 488 or 633 nm)

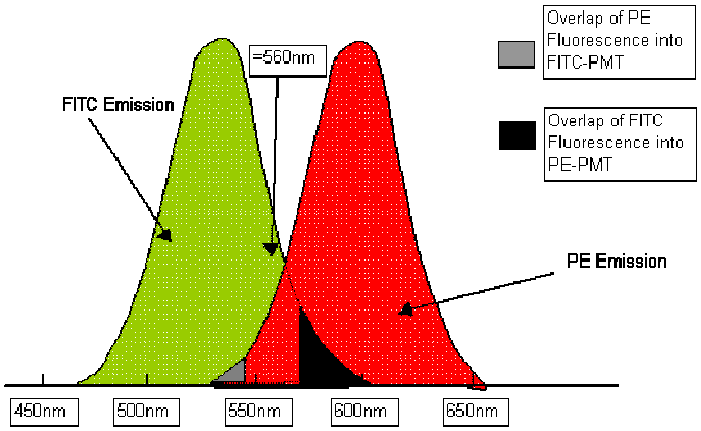
2. **Fluorochrome Emission**
    a. Each fluorochrome also has a unique emission spectra which shows the wavelengths of light that are emitted after it has been excited
        i. Check the fluorochromes emission spectra to ensure our filters will detect the emitted light (See Fluorescent Parameters Sheets)
        ii. Also be sure that any other fluorochromes you are using are not primarily detected by the same filters (see filters & detectors under instrument component section)

3. **Spectra Viewers** are available on either Invitrogen's or BD's websites, and are great tools for setting up experiments:
    - http://www.bdbiosciences.com/external_files/media/spectrumviewer/index.jsp
    - http://www.invitrogen.com/site/us/en/home/support/Research-Tools/Fluorescence-SpectraViewer.html

Spectral Overlap & Compensation

1. **Overlap of emission spectra**

- Most fluorochromes will have at least a small amount of overlap into a filter that is not being used to detect the signal. This overlap causes the fluorochrome to appear as positive in another filter, giving a false signal



2. **Compensation of overlap**

- Compensation is the correction of this overlap by the software. **To correctly compensate, single-color controls are needed to determine the amount of overlap that is occurring**
- Some software (i.e. FlowJo and Summit) allows you to compensate after acquisition of the sample. In most cases, compensation is not performed until after you have saved the file.
- If using CellQuest for analysis, you have to compensate using the voltage settings BEFORE collecting data.
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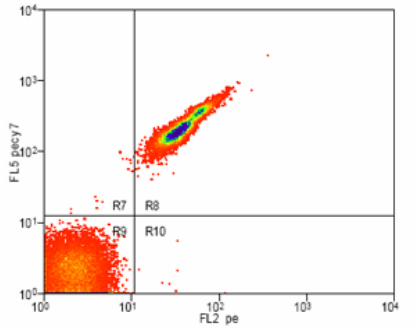
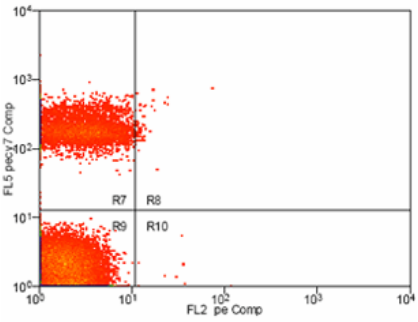
Before Compensation:



After Compensation:



Data Analysis

The CIC offers help with data analysis as well as training for independent use of the three software programs used in the facility.

- **CellQuest:** This program is used for Acquisition of data on the FACScan and FACSCalibur, as well as data analysis. We have two workstations available free of charge for post acquisition data analysis

- **Summit:** This program is used for Acquisition of data on the CyAn ADP, as well as data analysis. It is a free software, and customers can download Summit to their PC for personal use.

- **FlowJo:** This program is a third party Analysis software. It is available for use at any of the workstations in the CIC.

Practical Training

Sample Prep

1. Cell suspensions need to have a minimum amount of aggregates to decrease the chance of clogs
   - Resuspend cells in PBS without Ca2+, Mg2+ as the ions will induce clumping

- Have a minimum amount of additives (FBS or BSA, tween-20, etc)
- Filter the sample through a 40 um filter immediately before running on the instrument

2. Choose fluorochrome combinations carefully
   - Make sure your fluorochromes are in separate channels
   - Tandem dyes (such as PE-Cy5, PE-Texas Red, etc) tend to degrade faster than other dyes and can make compensation difficult or even impossible (i.e. using PE and PE-Cy5)

3. Bring proper controls for proper instrument set-up
   - In addition to negative controls, you need single positive controls for every color you are using and, if possible, controls to show non-specific staining (such as an isotype control)

Biosafety Considerations

- Since some of our users run BSL-2 experiments on our equipment, all users must comply with certain BSL-2 regulations

1. Room Requirements
   - Lab door must be kept closed at all times
   - Only persons informed of BSL-2 requirements are allowed access to the room

2. Personal Protective Equipment (PPE)
   - Lab coats must be worn (and kept in the room at all times)
   - Gloves must be worn when handling potentially infectious material
   - Goggles must be worn for potential aerosol or sprays of infectious material

3. Disinfection and Decontamination
   - Hands must be washed after removing gloves and before leaving the room
   - All spills must be decontaminated with a 10% bleach solution (such as the Decontamination solution)
   - Samples must be disposed of in the red biohazard boxes
   - Waste tanks must be filled with bleach to kill any potentially hazardous materials

ALL HUMAN SAMPLES MUST BE FIXED