University of Zurich[UZH]

Flow Cytometry Facility

Fundamentals and Applications of Fluorescence-activated Cell Sorting

Flow Cytometry Seminar Series

March 2017

José Duarte

Topics covered in this seminar:

1. Understand the basics of flow cytometry
2. Understand how a flow cytometry sorter works
3. Understand how cells are sorted
4. Description of different cell sorting possibilities
5. Tips on sample preparation
6. Overview of sorting services provided by the FCF

Which techniques are appropriate for my cell separation?

- Sample
  - Fluid (Particles in suspension)
    - Fluorescence activated cell sorting **FACS**
    - Microfluidics (Tyto / DEP Array)
    - Large Particle Sorting (COPAS)
  - Tissue
    - Lasermicrodissection microscopy

What is Flow Cytometry?

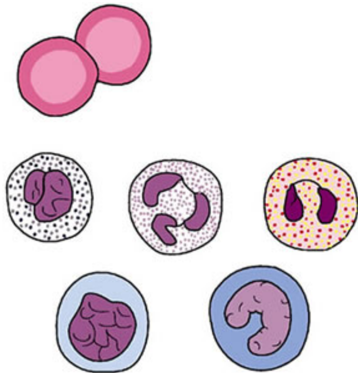
Flow = fluid

Cyto = cells

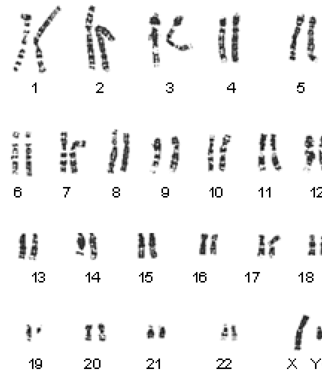
metry = measurement

What type of particles can be analysed?

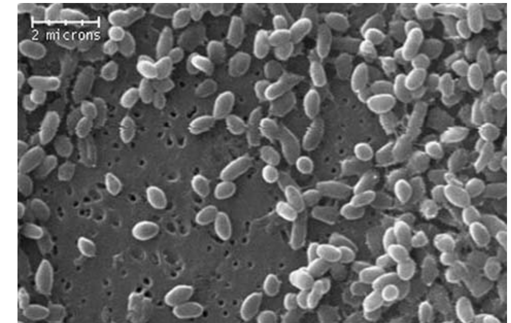
Cells



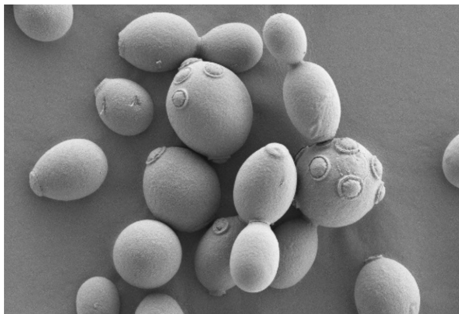
Chromosomes



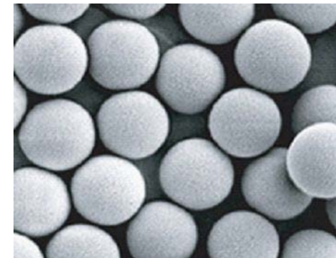
Bacteria



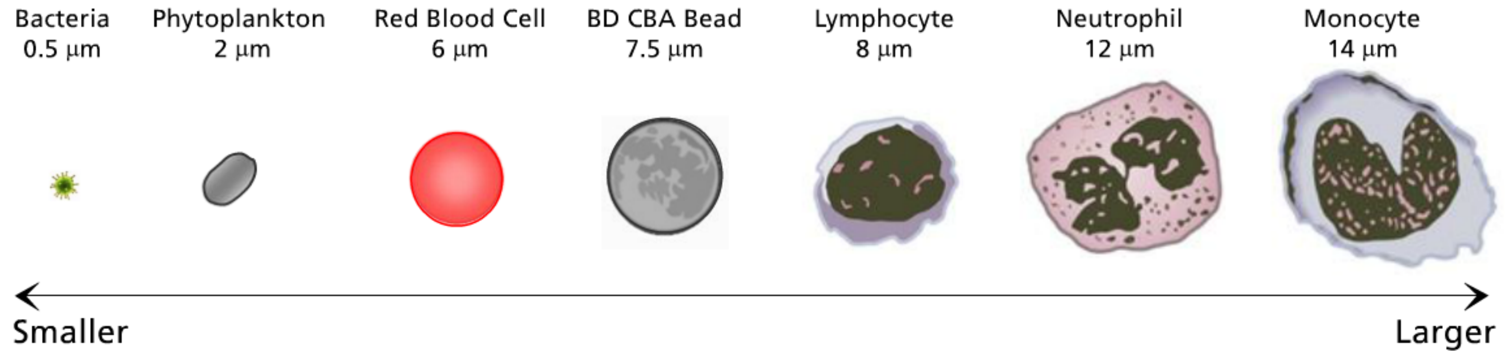
Yeast



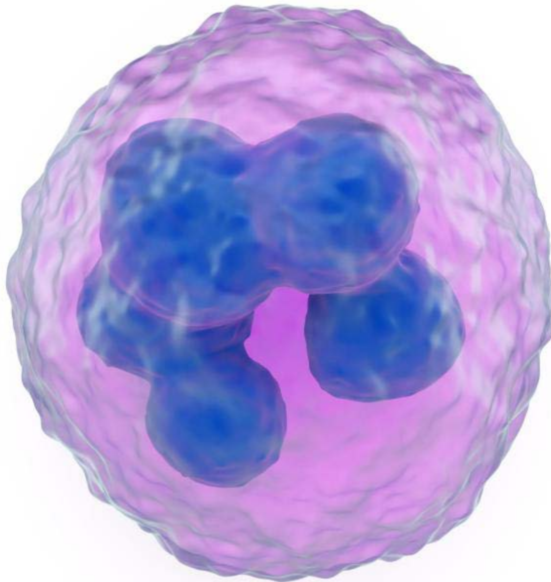
Microparticles (beads)



What particle sizes can be analysed?



What does it measure?



Three key parameter are measured:

- Relative size
- Relative internal complexity
- Relative fluorescence intensity

Where are samples run?

Analyser

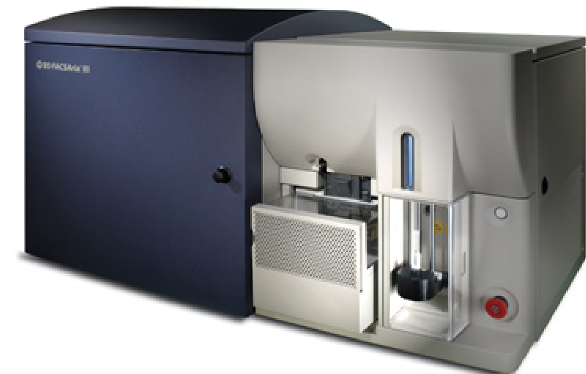
Analyser

Analyser

Analyser

Analyser

Sorter



A sorter can be divided into 3 main subsystems:

Fluidics

To introduce and focus the cells for interrogation and create a stable breakoff for sorting

Optics

To generate and collect the light signals

Electronics

To convert the optical signals to proportional digital signals, process the signals and communicate with the computer

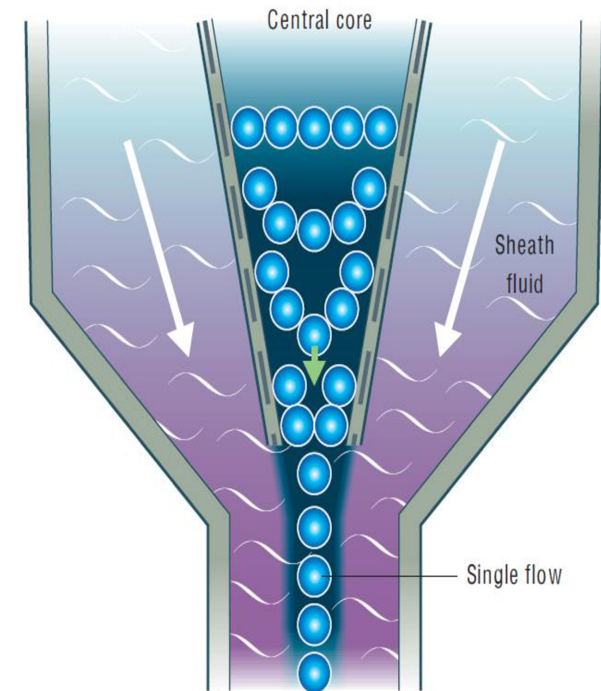
How can we record individual cells?

- The sample is introduced into the running sheath fluid in the **flow cell**
- Sample is hydrodynamically focused in the core stream
- Sheath fluid and sample do not mix
- Reduction of cross-section causes an accelaration of the central core stream that leads to laminar fluidics
- Sample flow rate adjustable

10 µl/min - Low

35 µl/min - Medium

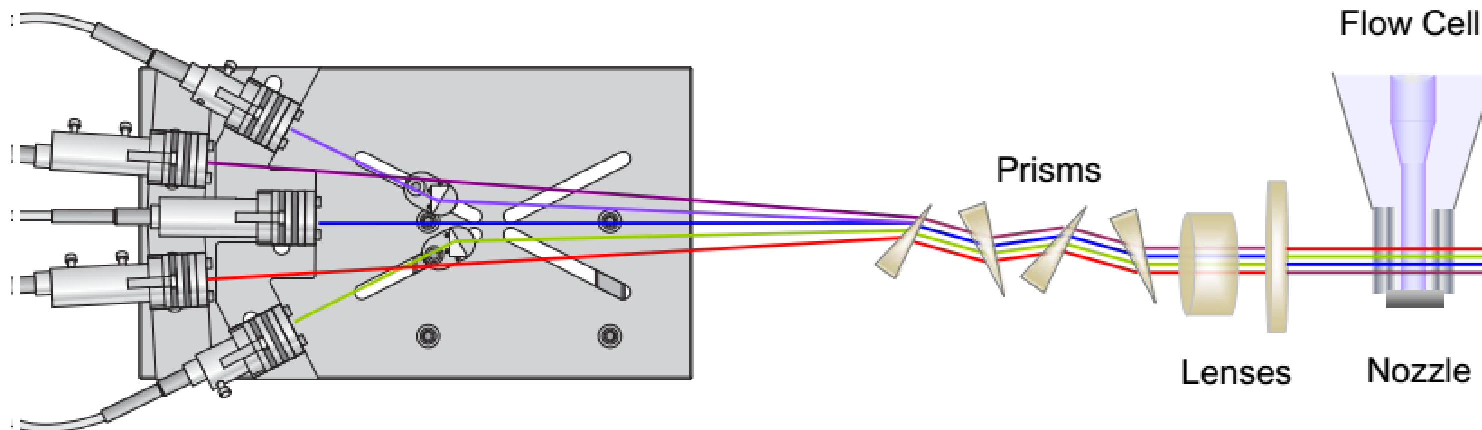
60 µl/min - High



There are two different optics' systems in a sorter:

Excitation optics consisting of:

- Lasers
- Fibre optic cables that carry beams to steering prisms, which then direct laser beams to the stream

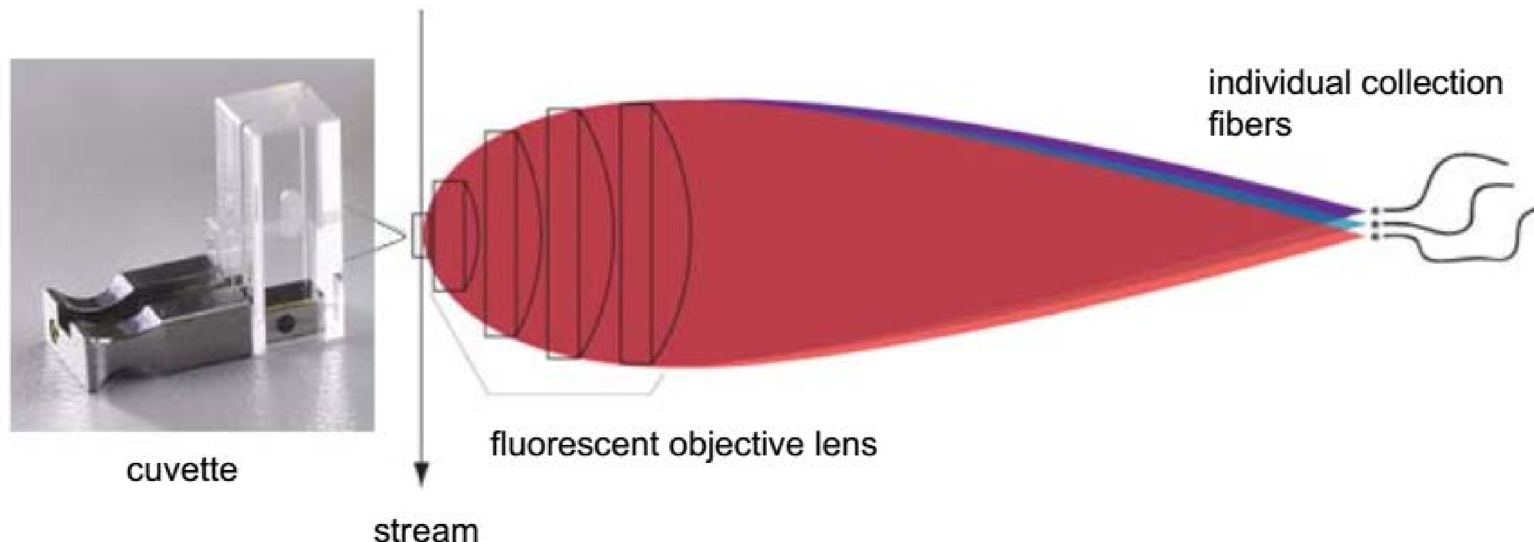


http://www.bdbiosciences.com

There are two different optics' systems in a sorter:

Collection optics consisting of:

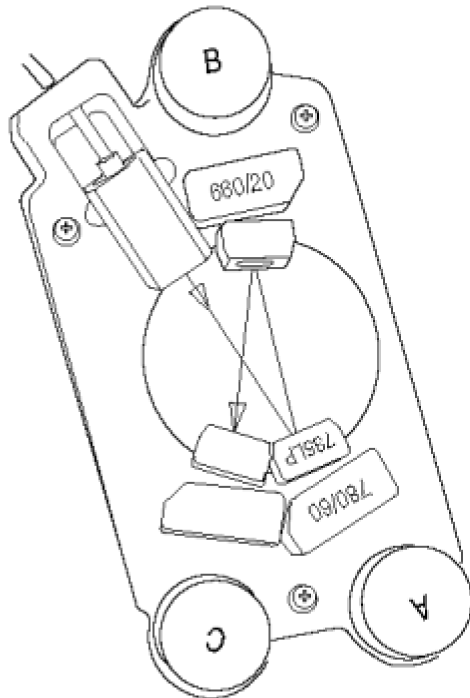
- Fibre optic cables that direct the emitted light to the appropriate emission filter block
- Filters that direct the signals in the emission block to the appropriate photon multiplier tube (PMT)



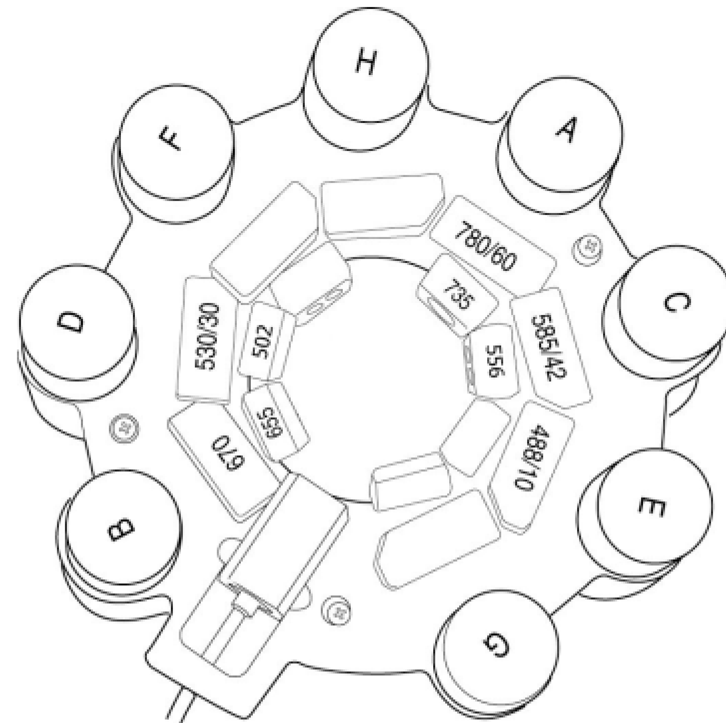
http://www.bdbiosciences.com

Filters are arranged in different arrangements

Trigon



Octagon



Photon multiplier tube (PMT)

cathode
photon of light
vacuum tube
dynodes
e$^-$
current
focusing electrodes
anode

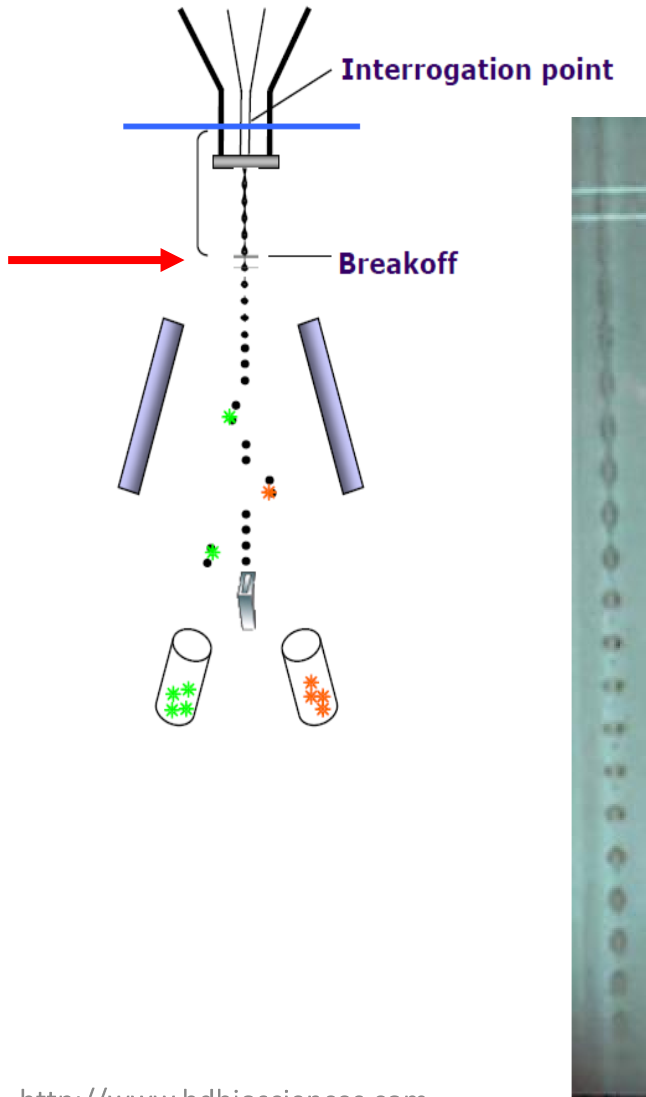
How are cells actually sorted?

Cell sorting

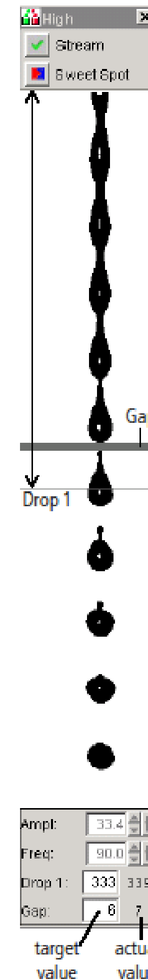
1. Sample generates light scatter and fluorescent signals; signals are analyzed.
2. Charge is applied via the stream-charging wire.
3. Charged droplets break off.
4. Deflection plates attract or repel charged droplet.
5. Uncharged droplets pass to waste.
6. Charged drops containing particles of interest are collected.

http://www.bdbiosciences.com

Cell sorting



- „Live“ image of the stream taken from the video monitor.
- Drops are visible because the stream is illuminated by a stroboscope lamp which is driven by the drop drive frequency.



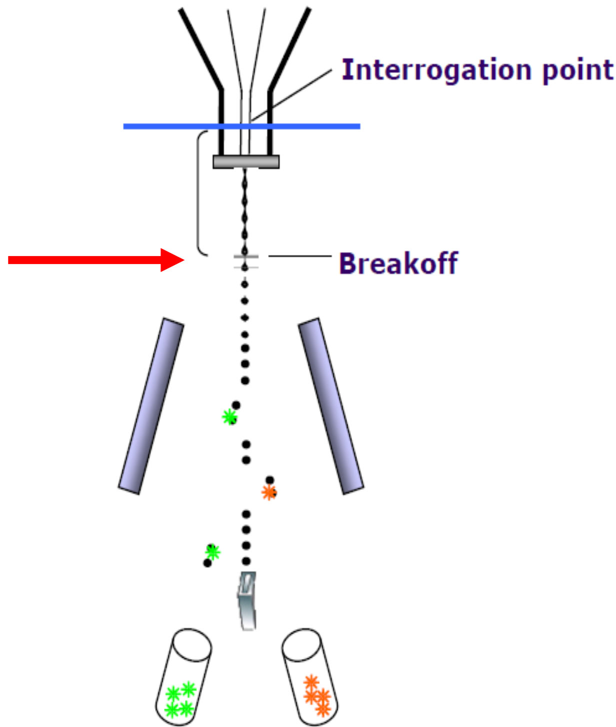
- Processed image from the video capture card.
- Notice the ligament extending from the last connected drop and the minor „fast“ satellite drops which will „melt“ with the main drops on their way down.

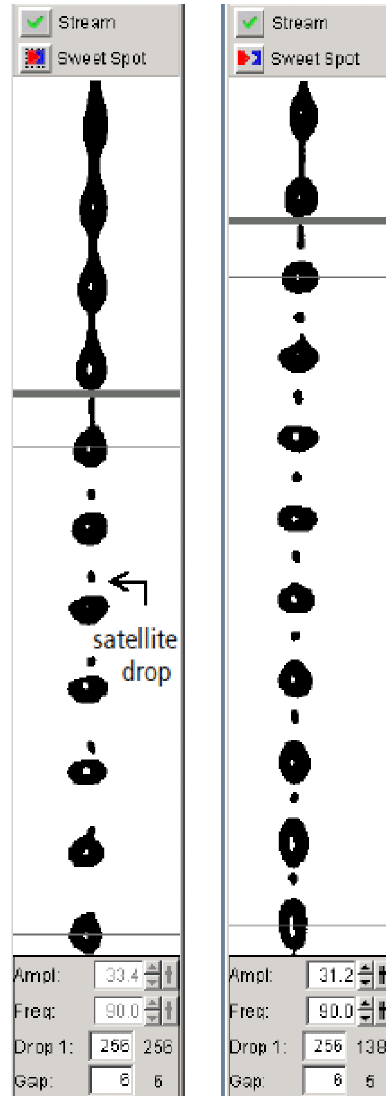
http://www.bdbiosciences.com

University of Zurich[UZH]

Flow Cytometry Facility

Cell sorting

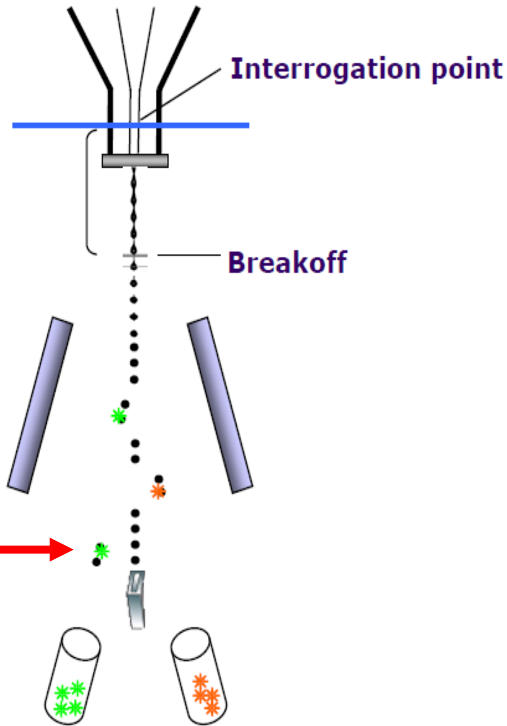




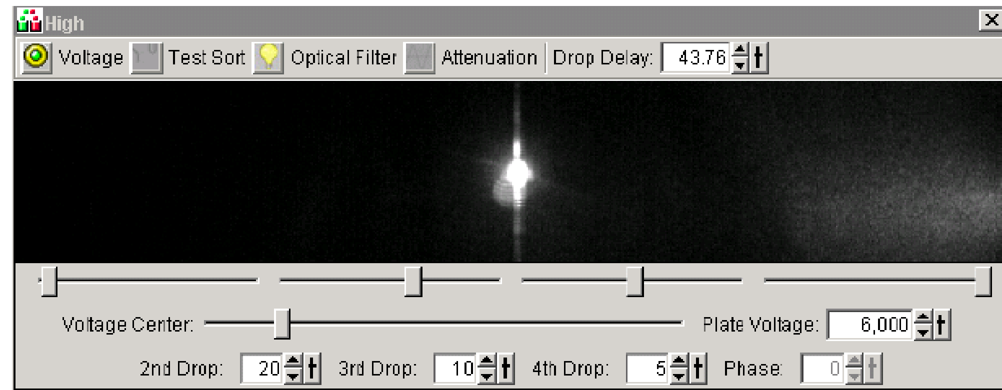
- This is a bad example: Slow satellite drops will never merge and always give bad side streams.
- It is of no use trying to sort with this kind of stream!
- Remember to check the satellite drops. There should be only few of them, and they should be of the „fast“ type.

http://www.bdbiosciences.com

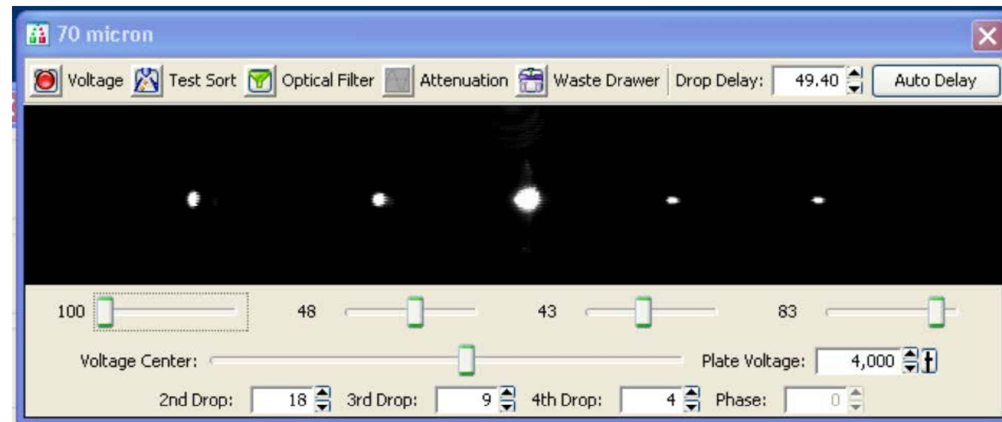
Cell sorting



Core stream undeflected



Core stream and deflected side streams

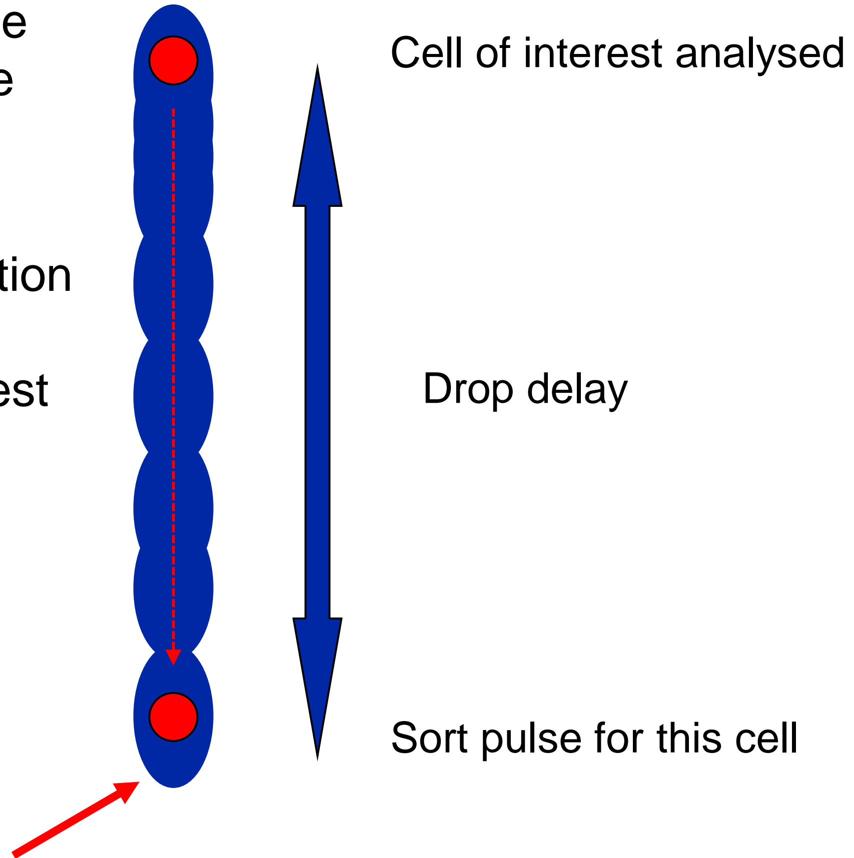


http://www.bdbiosciences.com

Cell sorting

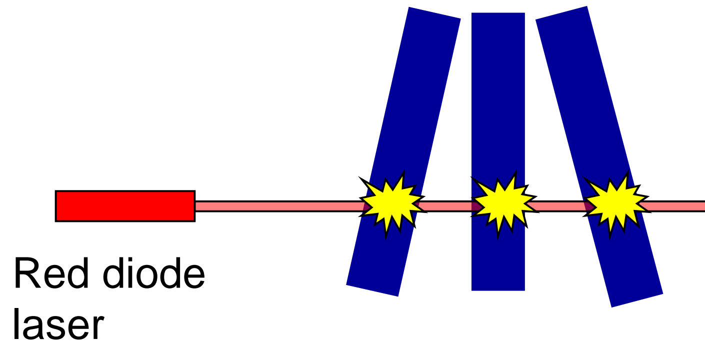
At the very moment when the cell is analysed, it is still quite far away from the droplet formation point.

Thus, somehow the information to charge a drop must be delayed until the cell of interest has reached this „breakoff“ point.

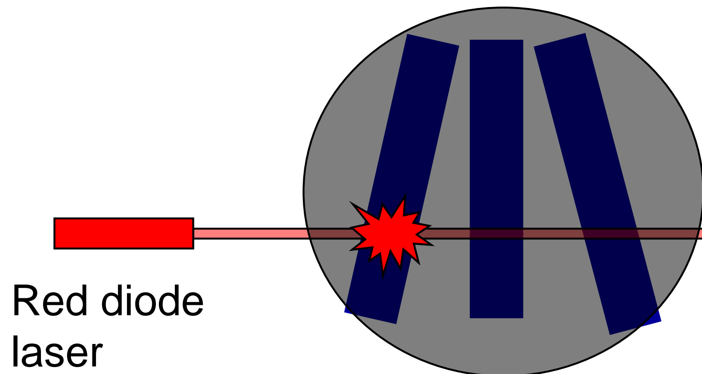


The stream has to be charged when the cell of interest arrives at the last droplet. This „delay“ in charging the stream is called the drop delay.

Cell sorting

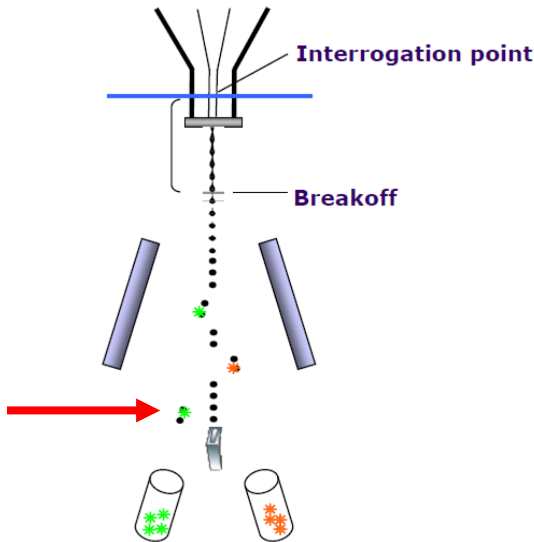


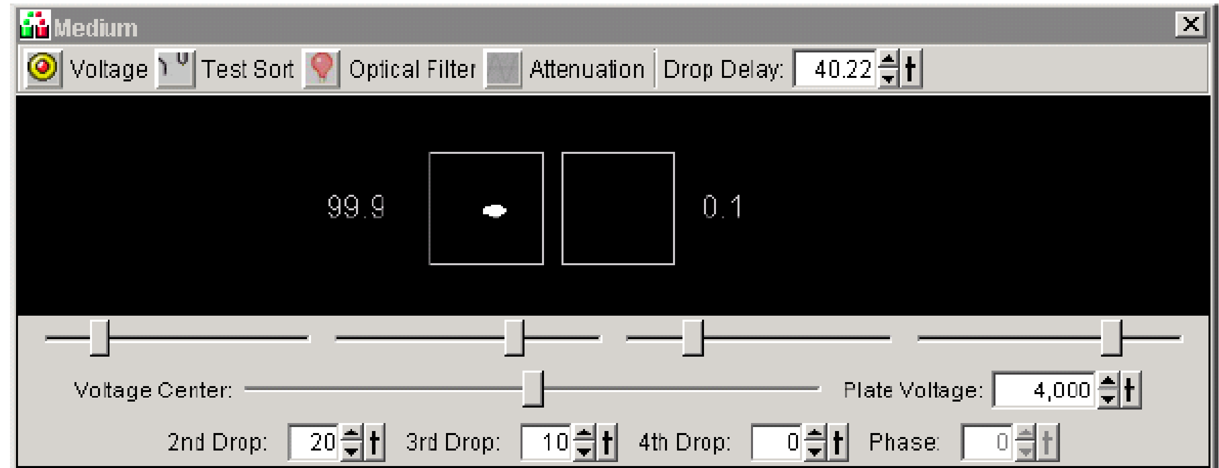
Without filter, the AccuDrop camera shows a picture of the streams with the intercept points of the laser.



With the filter in place, only the fluorescence signals of the AccuDrop beads can be seen on the screen.

Cell sorting



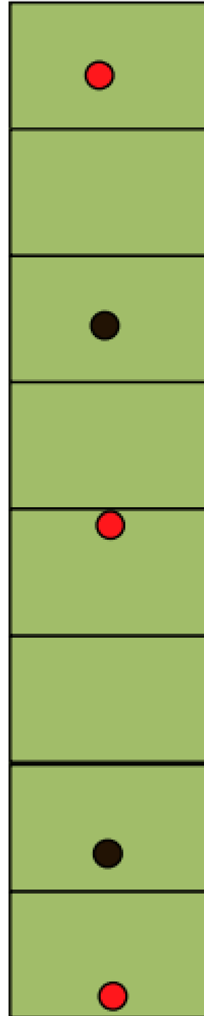


Determining the correct and robust drop delay is one of the most important steps in the machine setup

With a wrong drop delay, no cells or wrong cells reach your collection tube

http://www.bdbiosciences.com

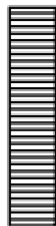
Cell sorting - Masks



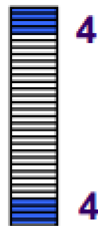
Cells are randomly distributed throughout the stream

Sorting mask

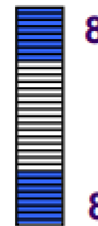
- A region of the stream monitored for the presence of cells
- Determines how droplets will be deflected if a sorting conflict occurs
- Measured in 1/32 drop increments



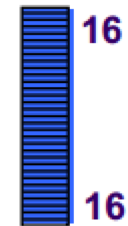
Mask = 0



Mask = 8



Mask = 16



Mask = 32

Cell sorting - Masks

http://www.bdbiosciences.com

Into what collection devices can I sort my samples?

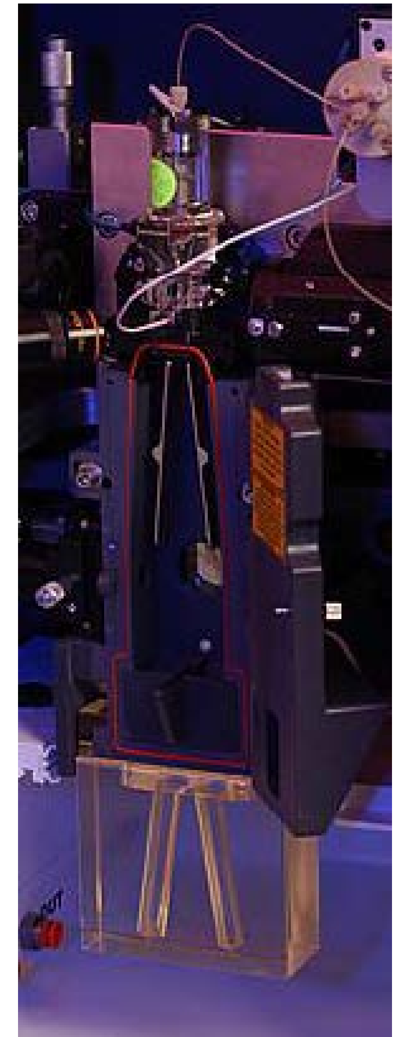
Cell sorting - Bulk

<u>Bulk sorts</u> are done with the purpose of enrichment for cell culture / DNA + RNA Analysis / adoptive transfer / purification of cells expressing a fluorescent protein, etc…

Cells can be sorted into:

- 1.5 ml / tubes (**4 populations in parallel**)
- 5 ml FACS tubes (**4 populations in parallel**)
- 15 ml tubes (**2 populations in parallel**)
- Beaker (**1 population**)
- Samples can be cooled / uncooled

<u>Collection medium is adjusted to the experiment (bring it along)</u>



Cell sorting - Plates

<u>Plate sorts</u> are done with the objective of sorting directly into multiwell plates for the purpose of tissue culturing/ T and B cell clonal expansion/ single cell DNA/RNA analysis/ etc…

Cells can be sorted into:

- 6 to 384-well plates

<u>Only one population can be sorted</u>

Cell sorting - Slides

<u>Slide sorts</u> are done with the purpose of sorting directly into slides that can be used for *in vitro* assays (e.g. Seahorse assay) or microscopy

Cells can be sorted into:

- Microscopy slides
- Ibidi-type chamber slides

<u>Only one population can be sorted</u>

Quick tips on sample preparation

Tips on sample preparation

- **Plan your experiment well** and choose your fluorescent antibodies in agreement with the optical configuration of the intended sorter. Note that different sorters may slightly vary in the optical configuration

- Bring along the following **controls**:

  - ✓ Unstained/non-transfected cells to estimate background signal
  - ✓ Bring single stained samples (cells and/or beads) to set compensation
  - ✓ Bring FMOs if needed to set gates

Tips on sample preparation

- A maximum of 2% FCS / BSA is recommended in the suspension medium of cells to be sorted because otherwise cells become sticky
- Avoid phenol-red media due to autofluorescence; most cell types will do well in just PBS+FCS/BSA
- If your cells have a tendency to for aggregates consider adding one of the following:
    - use calcium/magnesium free sorting buffer or add EDTA (< 5 mM)
    - if the cell preparation induces increased cell lysis use 25 µg/ml DNAse I + 5 mM $MgCl_2$ (no EDTA!)
    - use 1% Accutase in sorting buffer

All samples need to be filtered, preferably directly before sorting

Cell sorting

For a **bulk sort** provide cells with following concentrations depending on the used nozzle:

- ✓ 70 µm nozzle ⇒ 40 x 10^6 cells / ml
- ✓ 85 µm nozzle ⇒ 20 x 10^6 cells / ml
- ✓ 100 µm nozzle ⇒ 10 x 10^6 cells / ml

For a **single cell sort** provide cells with a concentration 5 x 10^6 cells / ml

The nozzle size should be 3 to 4x bigger than your sorted cells

Cell sorting

- The minimum sample volume for sorting is 200 uL
- Samples can be loaded from 1.5 mL Eppendorfs, 5 mL FACS tubes and 15 mL Falcon tubes
- Prepare **collection tubes** and add sufficient collection medium with regard to the expected yield / target cell number. You can calculate with following drop volumes/sort event:

  70 µm nozzle ⇒ ~1 nl
  85 µm nozzle ⇒ ~1.5 nl
  100 µm nozzle ⇒ ~2.5 nl
  130 µm nozzle ⇒ ~8 nl

- The choice of **collection medium** depends on the downstream applications and is therefore variable

Volatile harmful substances such as 2-Mercaptoethanol or Trizol are not allowed

[use instead lysis buffer from a kit (e.g.Rneasy) or sort into RNAlater buffer]

What sorting services do we provide?

Operator-based sorting Service

Available at Irchel and Schlieren campus for all projects registered with the facility:

- Users bring their samples, controls, collection media and collection tubes/plates/slides
- Operator sets up the sorter and experiment and runs the samples to be sorted
- Very important that users prepare their samples according to our guidelines
- Operator-based sorts can be booked via facs@cytometry.uzh.ch

Only possible during weekdays

Power User Training

For specific approved projects (at all FCF locations) that demand:

- high number of sorts (1-2x per week)
- work location where only power-users can use the sorter (e.g. USZ)
- Irregular hours for sample availability (patient material, weekend sorts)

Training Curriculum consists of:

- 3 to 4 3h group sessions on sorting theory (1st session max. 4 users, following sessions split into groups of 2)
- 1 to 2 sorting slots on a 1:1 basis with an operator
- Followed by a power user in training period lasting app. 6 months (at Irchel/Schlieren locations)
- Training finishes with a troubleshooting session followed by an exam

After a successful training a power user should feel confident enough to sort and solve the most common problems independently

Summary

- If you can measure it in our analyzers we can sort it in our sorters
- Possibility to sort from the smallest bacteria to the largest adipocyte
- Plan your fluorochrome panel according to the optical configuration of the sorter to be used
- Prepare your samples following our guidelines (bulk vs single cell sort)
- Do not forget to use controls (unstained, single stains, FMOs)
- Bring appropriate collection media for your downstream technique (culturing, RNA/DNA analysis)

Useful links:

FCFacility website:

http://www.cytometry.uzh.ch/en/index-fcf.html

Cell sorting sample preparation guidelines:

http://www.cytometry.uzh.ch/en/index-fcf/sorters/Sample-prep.html

Info on operator-based sorts:

http://www.cytometry.uzh.ch/en/index-fcf/sorters/Operator-based-service.html

Info on power-user training:

http://www.cytometry.uzh.ch/en/index-fcf/teaching/Sorter-Training.html

BD Biosciences Tools:

http://www.bdbiosciences.com/us/s/tools

Invitrogen SpectraViewer:

https://www.thermofisher.com/ch/en/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html

On behalf of the FCF team

THANK YOU