Cihan University/ Sulaymaniya

College of Health Science

Medical Laboratory Analysis

4th Stage- 1st Semester

Pr. Clinical Immunology

Lab- 9: Flow Cytometry

2023- 2024

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What is a Flow Cytometer?

A purpose-built instrument:

A flow cytometer uses a combination of fluidic, optic, and electronic systems to report the properties of individual particles.

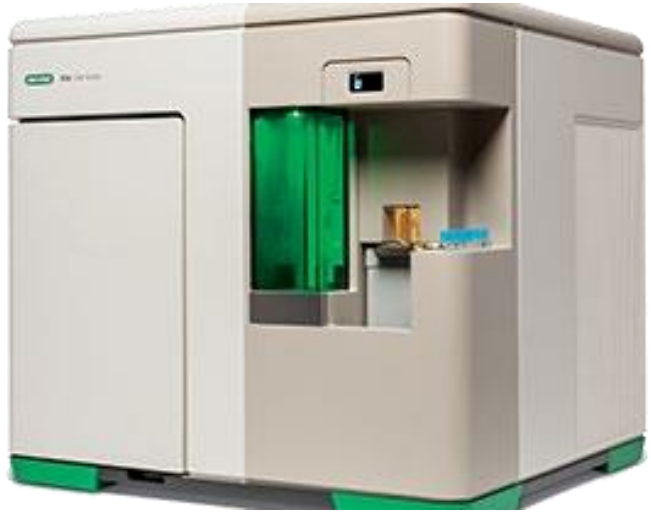
A well-established system:

A flow cytometer requires a suspension of single cells in a small volume of liquid.

An effective cell analyzer:

The scattered light detected is converted to electric signals by the electronic system. These are converted to data for analysis.

Examples of Flow Cytometers



CELL SORTERS	**Physically sort cells into populations with shared characteristics, which can be further analyzed after sorting.**
IMAGING FLOW CYTOMETERS	**Capture a picture of each cell as it is analyzed.**

Uses of Flow Cytometry

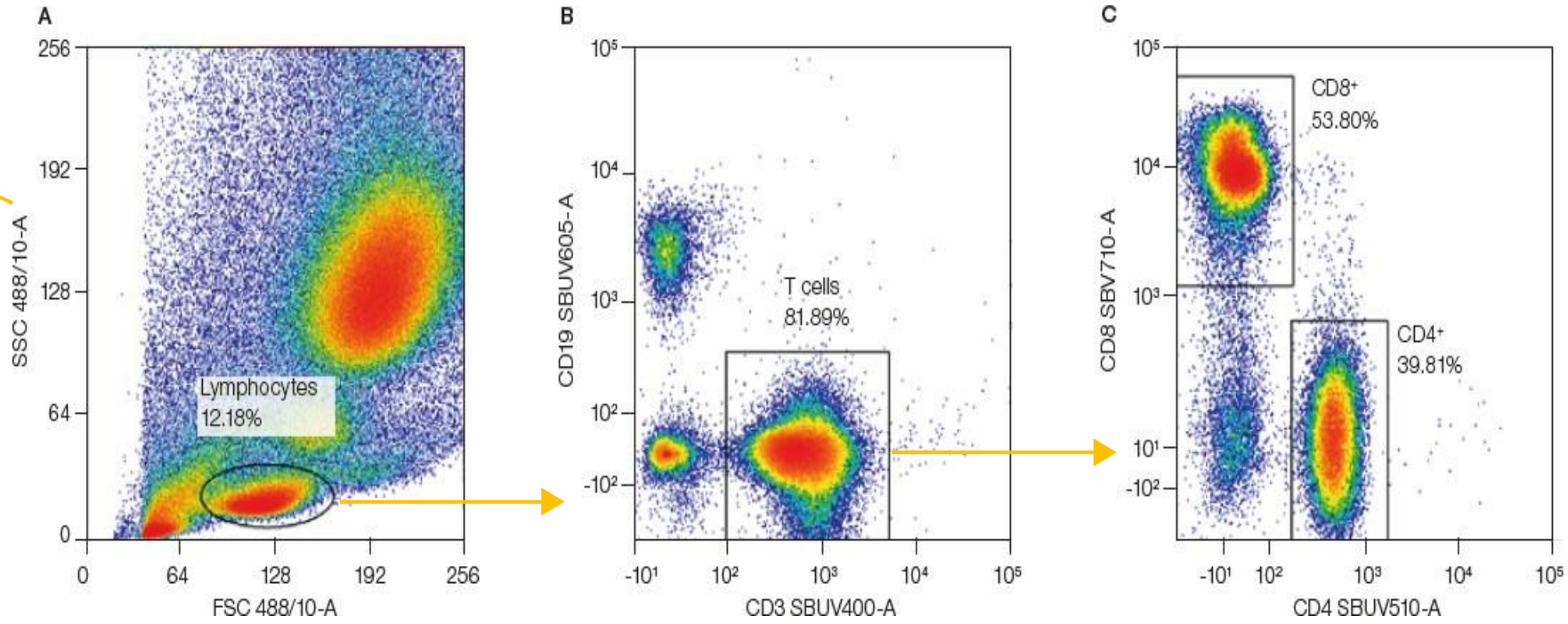
Some examples of things you can measure with flow cytometry include:

- Immunophenotyping
- Cell Cycle Analysis
- Regulated Cell Death Cell
- Proliferation

Immunophenotyping

PURPOSE | **MEASURABLE PROPERTY** | **READOUT**

Flow cytometry progresses the study of heterogenous cell populations to find the relative proportion of certain ones.



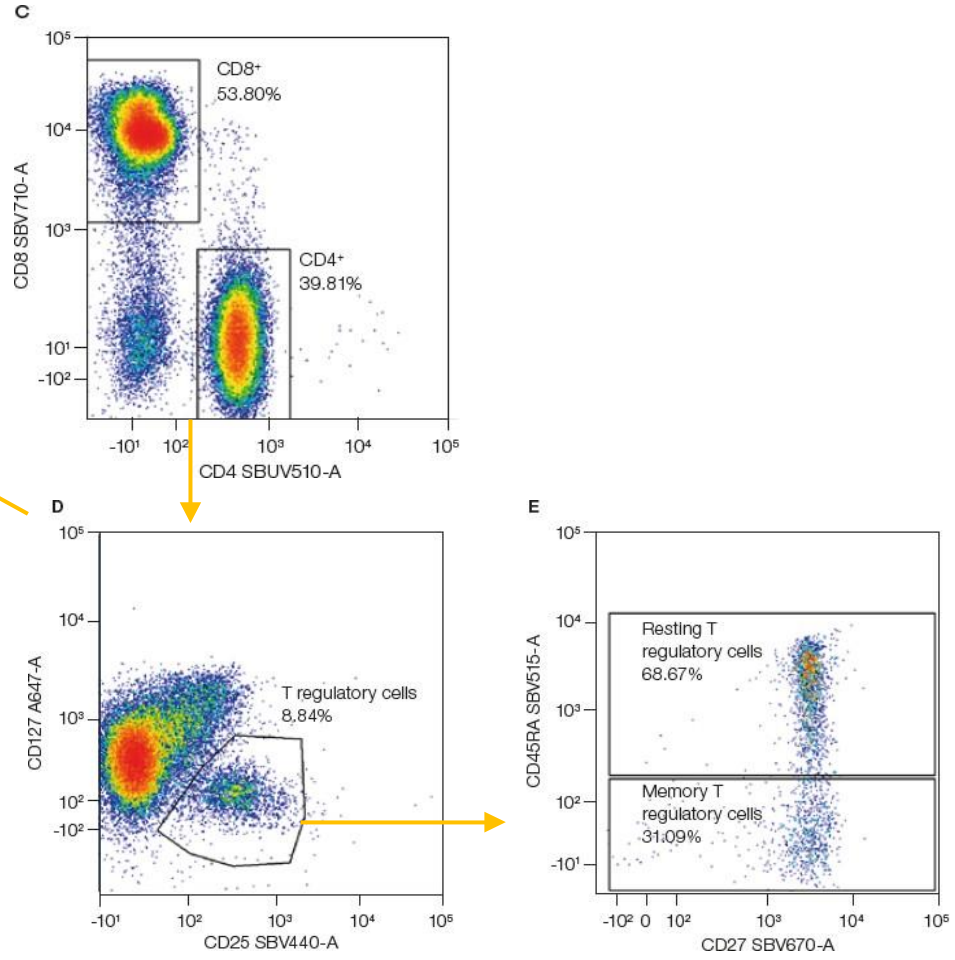
Immunophenotyping

PURPOSE

Fluorescent antibodies against cell surface markers can help differentiate cell types. Populations are gated and defined based on marker expression, which can be additionally gated to define subpopulations.

MEASURABLE PROPERTY

READOUT

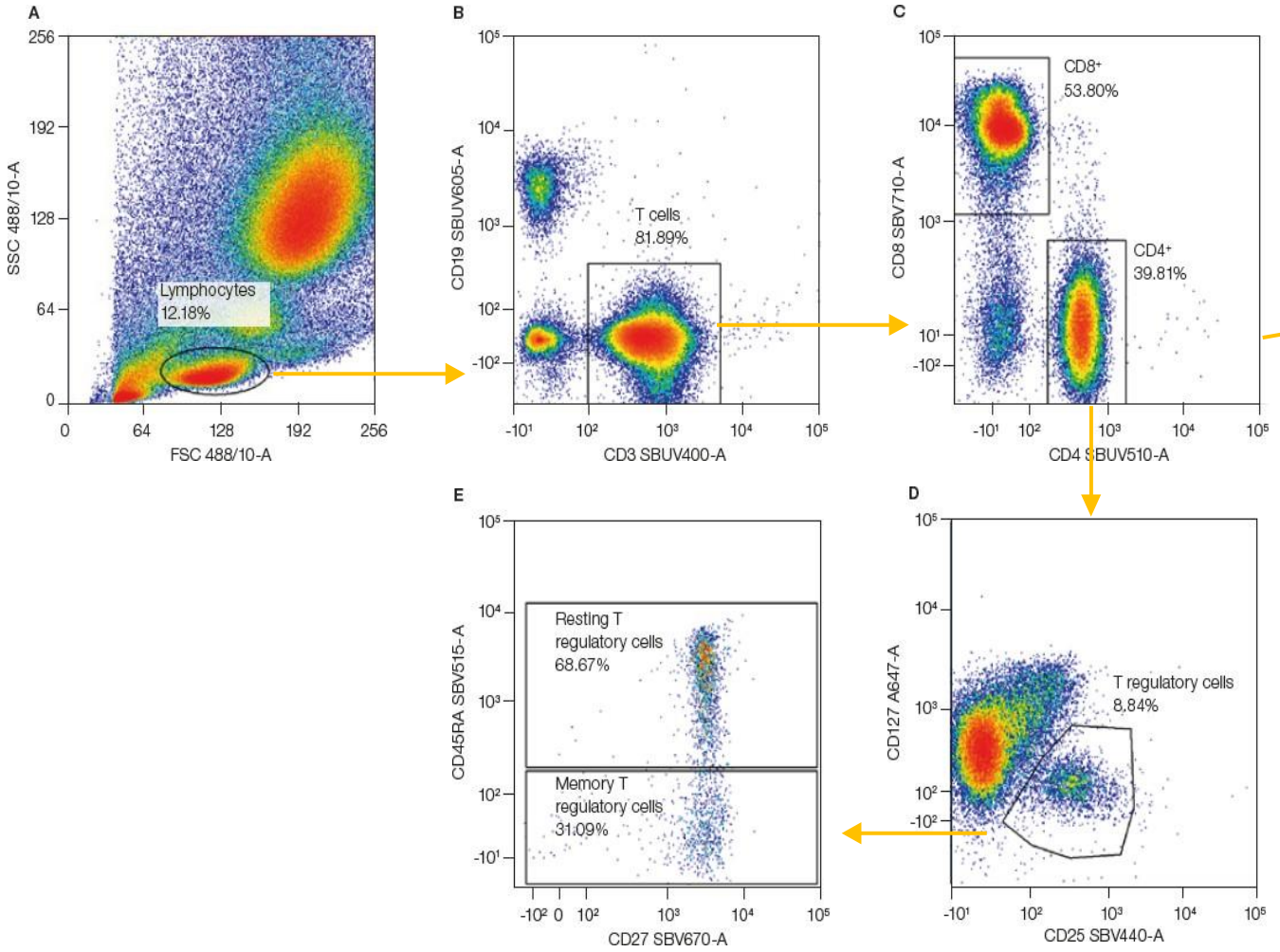


Immunophenotyping

PURPOSE

MEASURABLE PROPERTY

READOUT

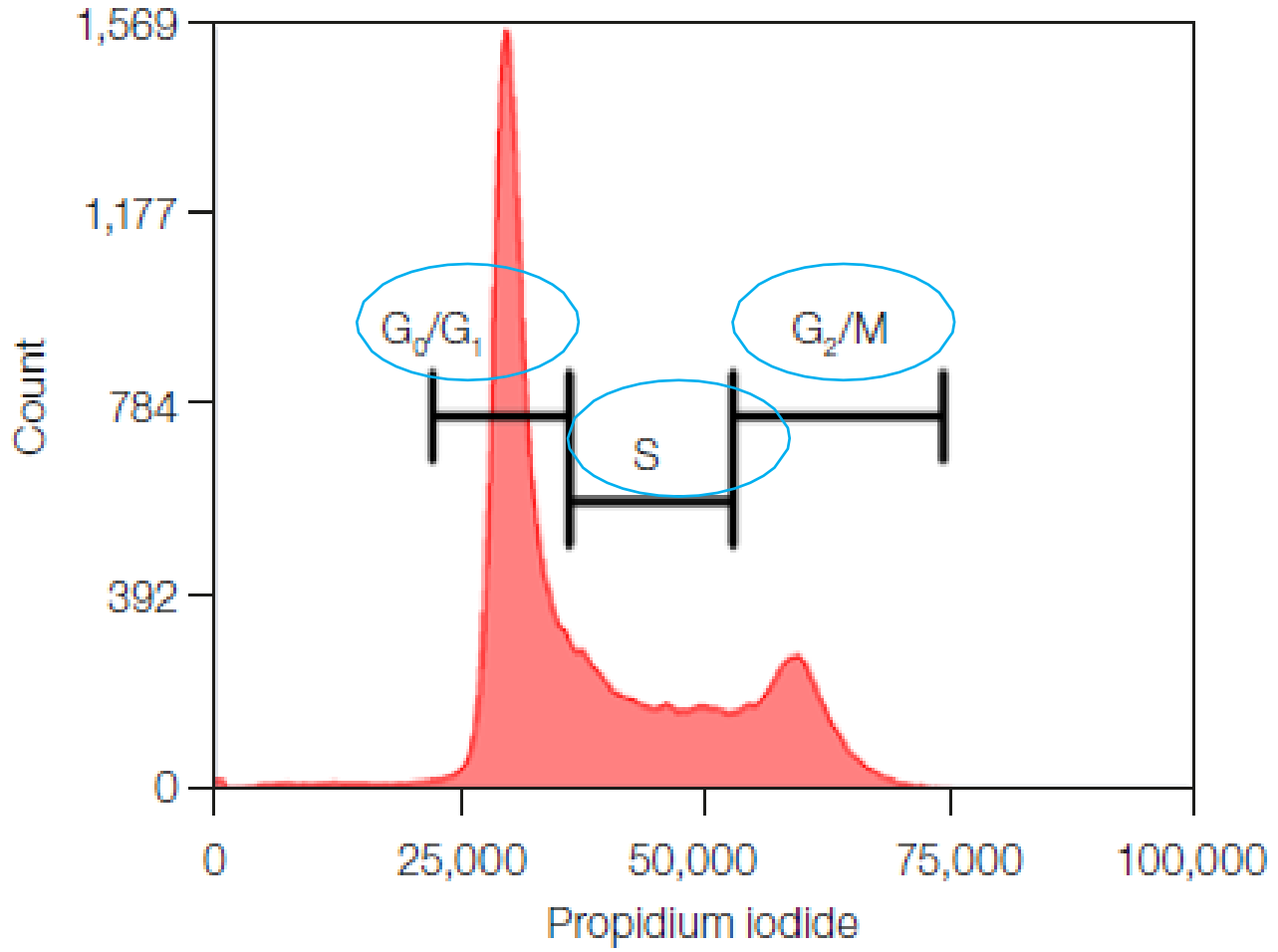


Dot plots (also known as scatterplots) are commonly used to analyze and display immunophenotyping data, with cell populations identified by fluorescence measurements.

Cell Cycle Analysis

PURPOSE	MEASURABLE PROPERTY	READOUT

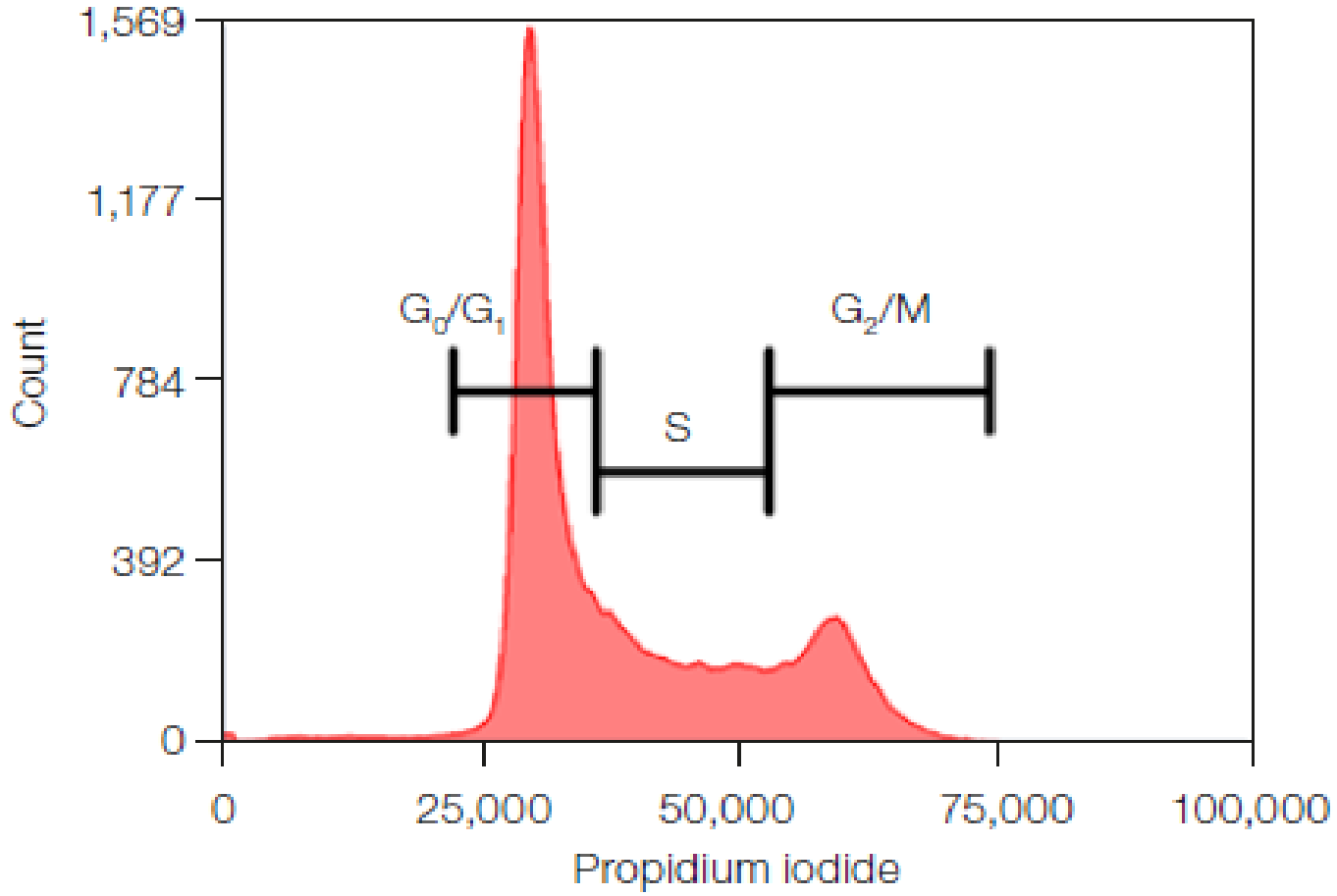
Flow cytometry determines which stage of the cell cycle an individual cell is in.



Cell Cycle Analysis

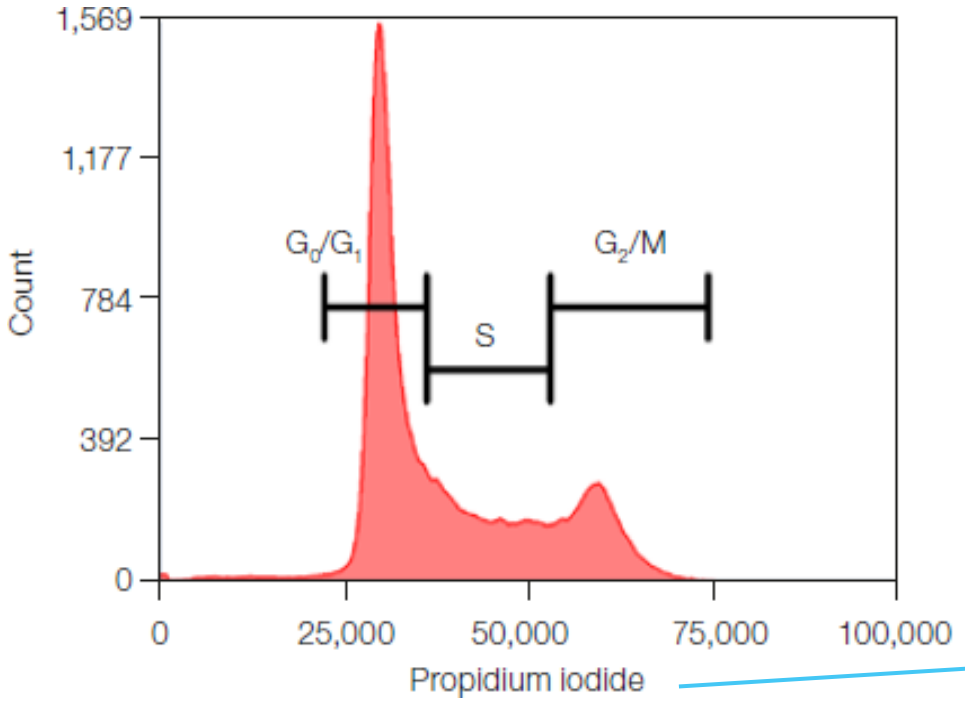
PURPOSE	MEASURABLE PROPERTY	READOUT

The quantity of DNA in each cell can be measured by flow cytometry. Cells at each stage of the cell cycle have different amounts of DNA.



Cell Cycle Analysis

PURPOSE | **MEASURABLE PROPERTY** | **READOUT**



DNA can be stained using a fluorescent dye, such as propidium iodide (PI), which is detected by shining a specific wavelength of laser light onto cells. The quantity of DNA in a cell can be read from the illumination of the fluorescent dye, where the fluorescent readout is proportional to the amount of DNA.

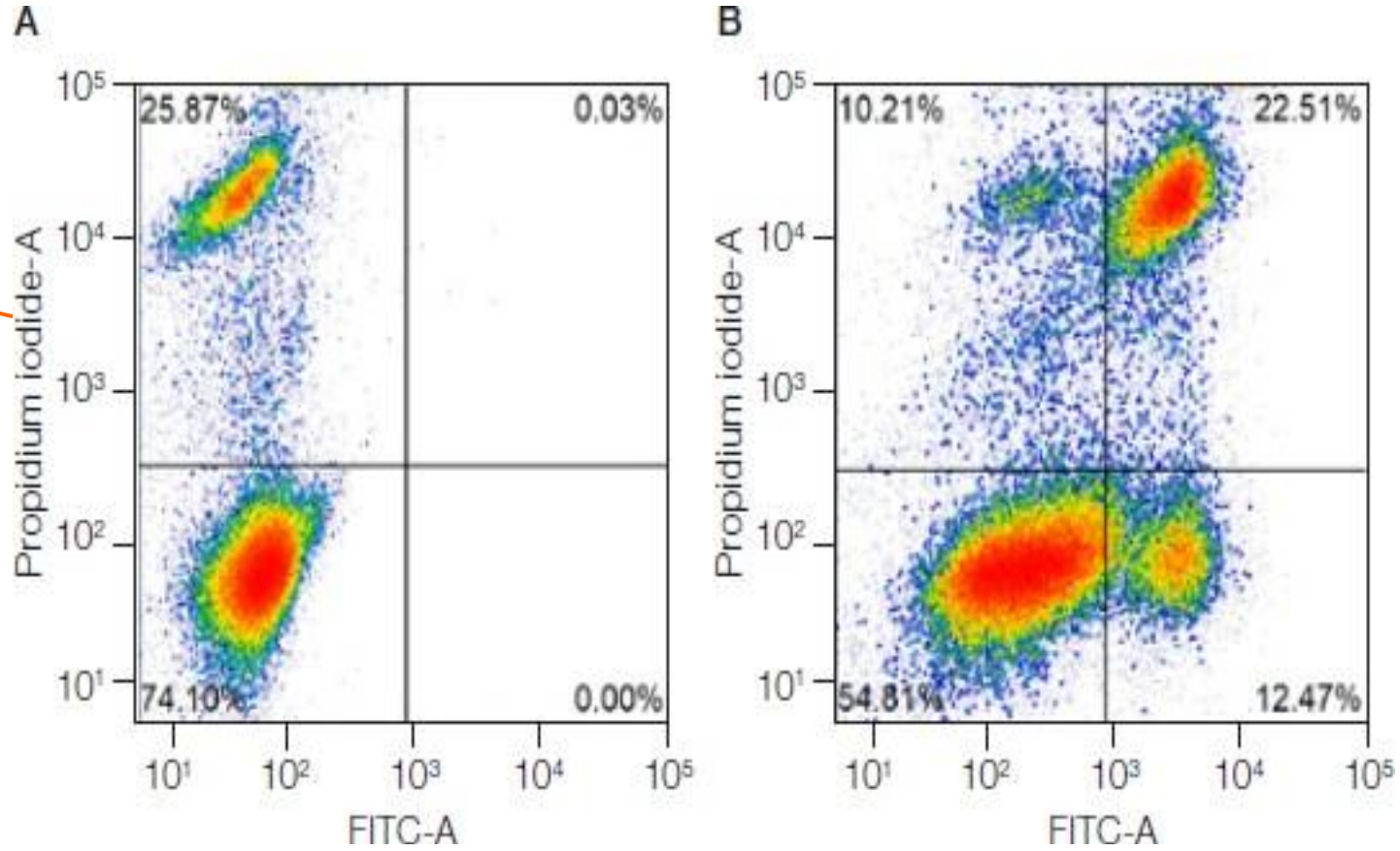
Regulated Cell Death

PURPOSE

MEASURABLE PROPERTY

READOUT

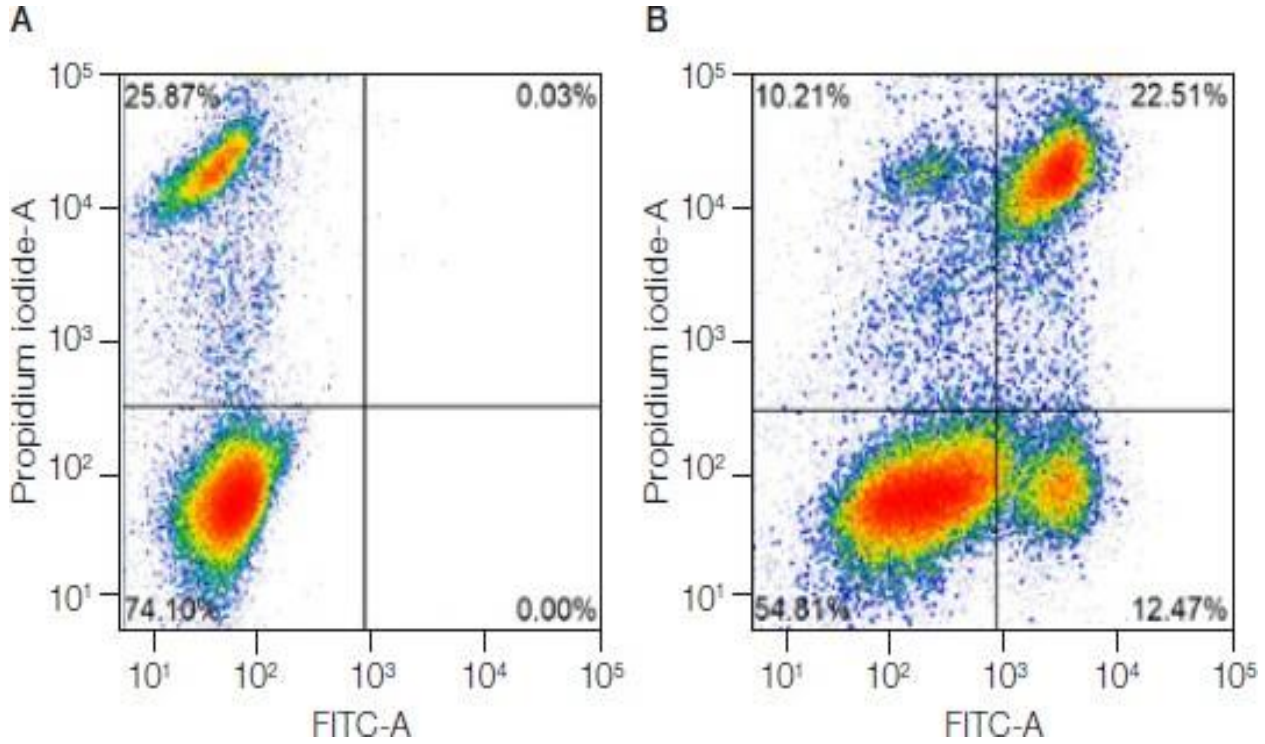
Flow cytometry can identify cells undergoing programmed cell death via apoptosis, pyroptosis, or autophagy.



Regulated Cell Death

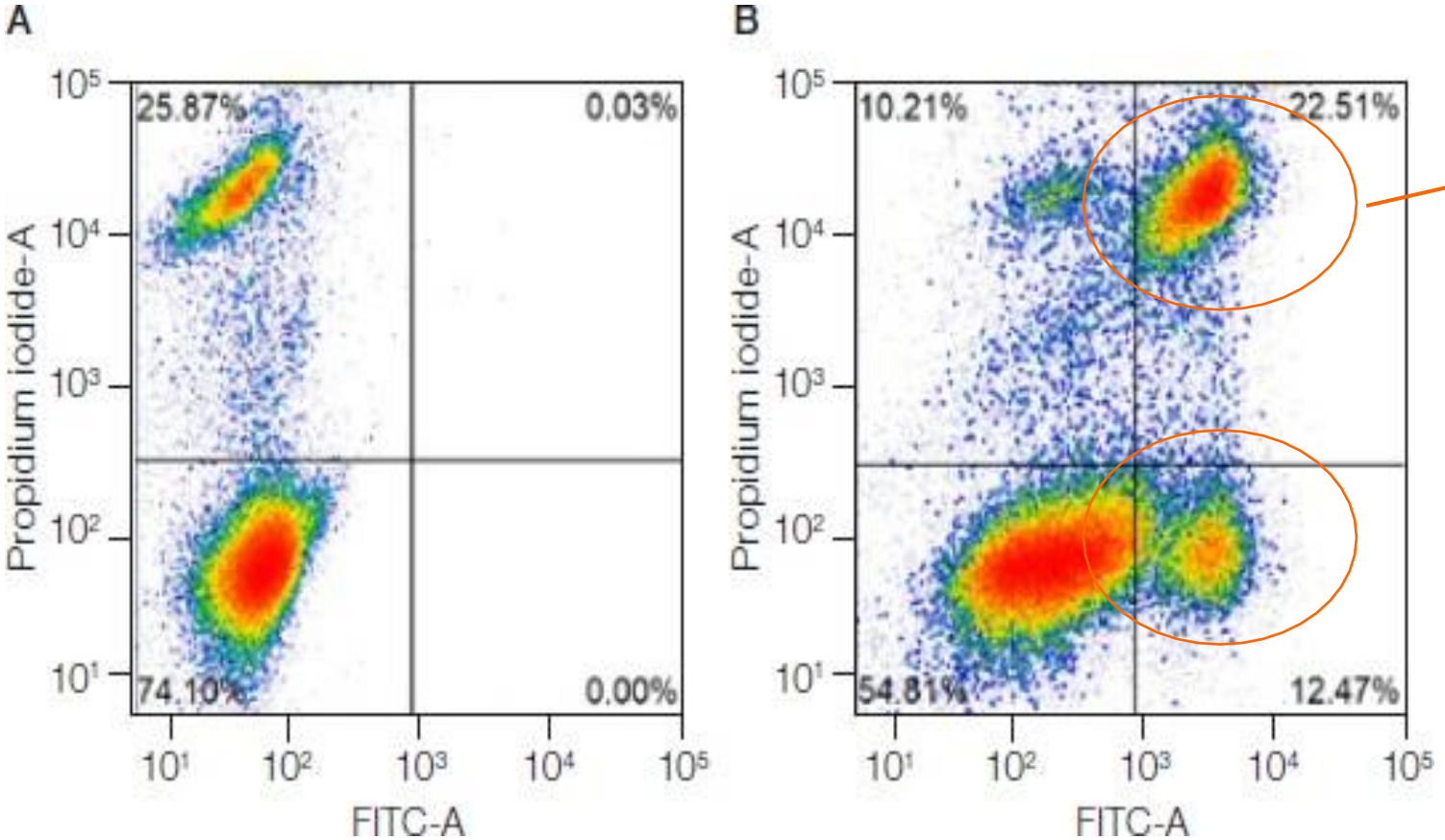
PURPOSE	MEASURABLE PROPERTY	READOUT

Annexin V assays or caspase activation, among other assays, can be used to identify apoptotic cells. Co-staining with PI can identify dead cells.



Regulated Cell Death

PURPOSE | **MEASURABLE PROPERTY** | **READOUT**



Flow cytometry helps reveal viable, apoptotic, and secondary necrotic cells when cells are co- stained with annexin V conjugated to fluorescein isothiocyanate (FITC) and PI.

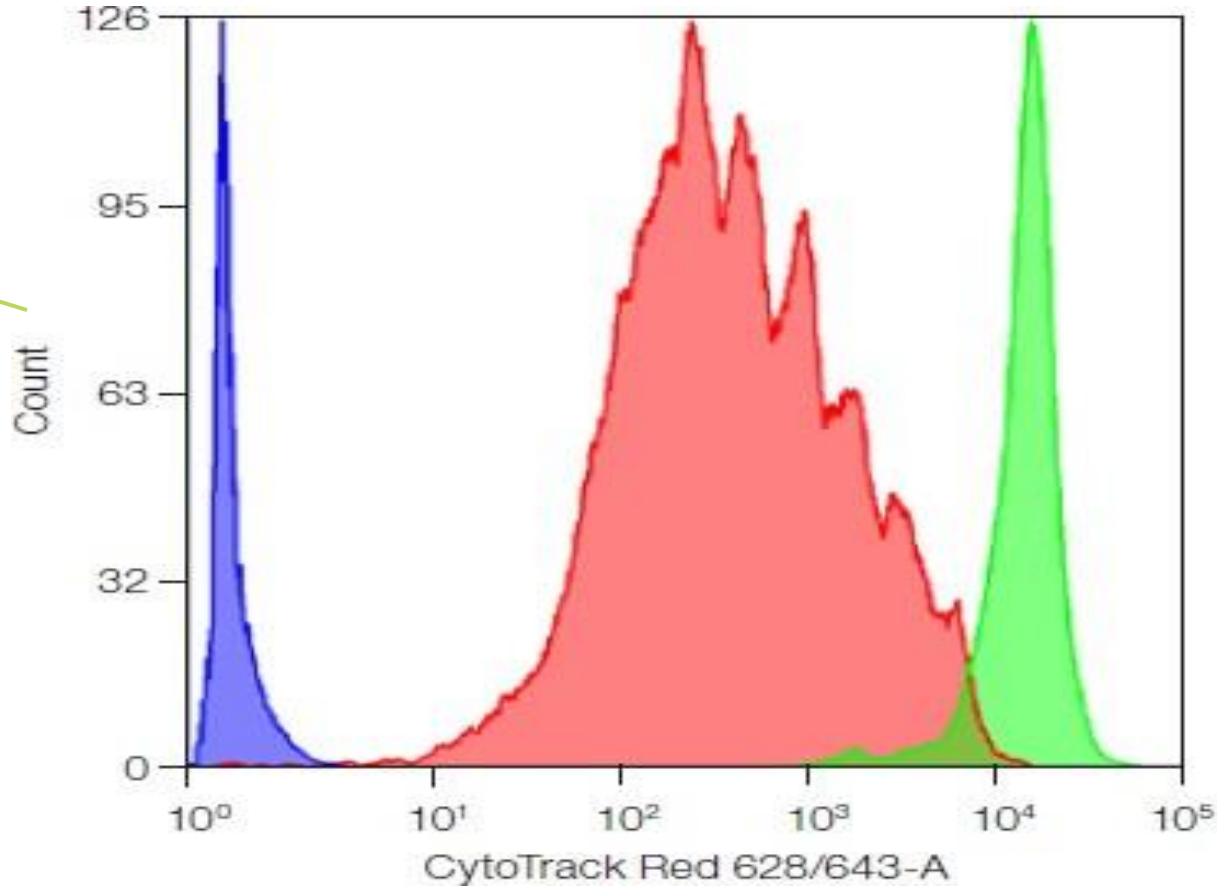
Cell Proliferation

PURPOSE

MEASURABLE PROPERTY

READOUT

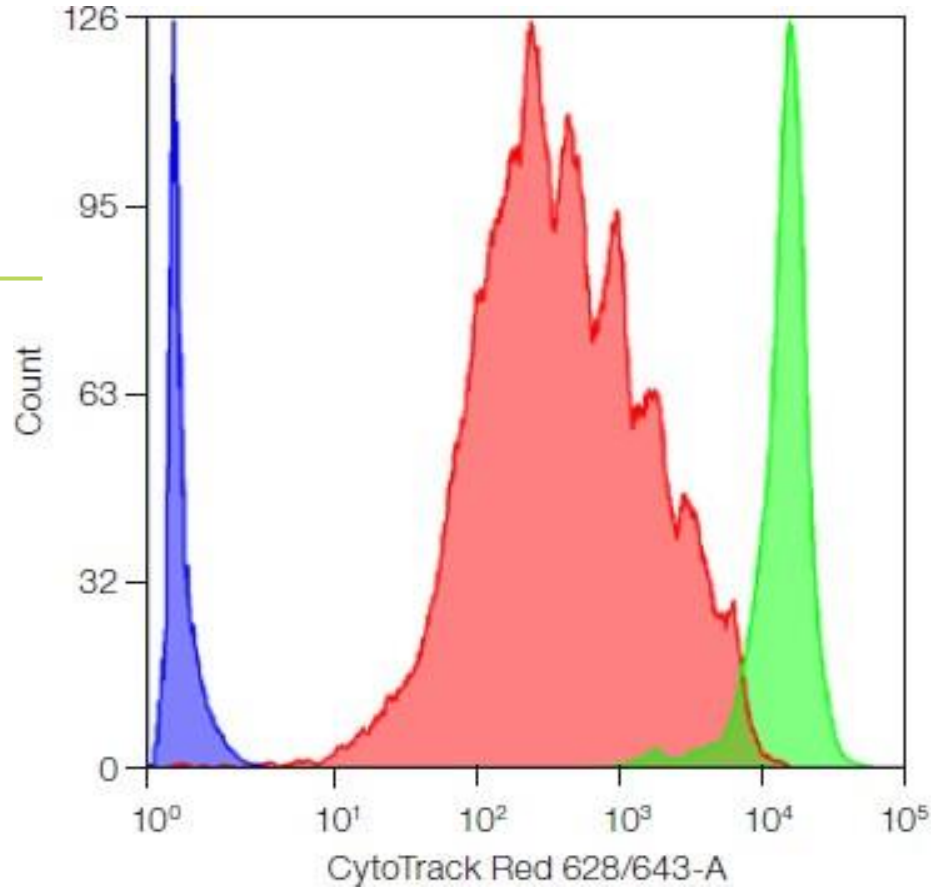
The rate of cell proliferation can be measured by flow cytometry using selected cell-permeable dyes such as CytoTrack Dyes.



Cell Proliferation

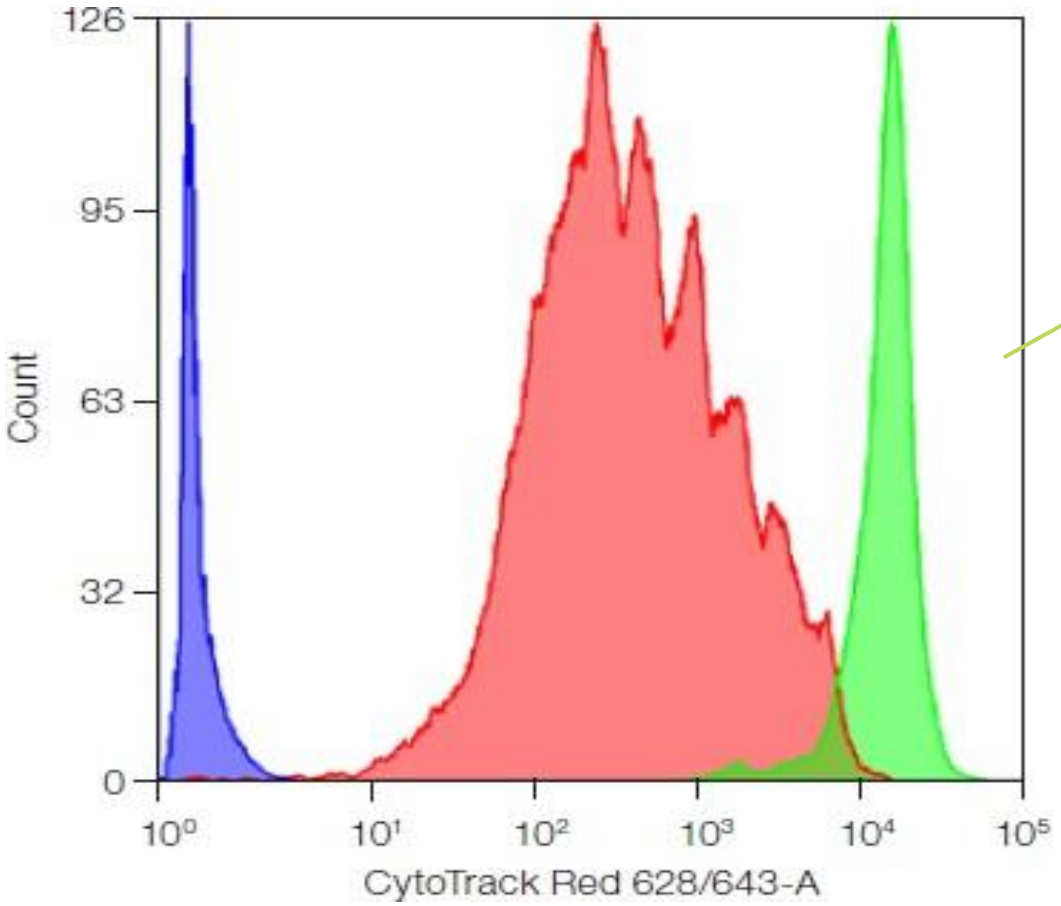
PURPOSE	MEASURABLE PROPERTY	READOUT

A dye can be composed of a fluorophore and a cell-retaining group. These form a covalent bond with intracellular cells as they interact with a live cell. Once cells begin to proliferate, they divide, and the fluorescence intensity is successively halved, allowing each cell division to be identified.



Cell Proliferation

PURPOSE | **MEASURABLE PROPERTY** | **READOUT**

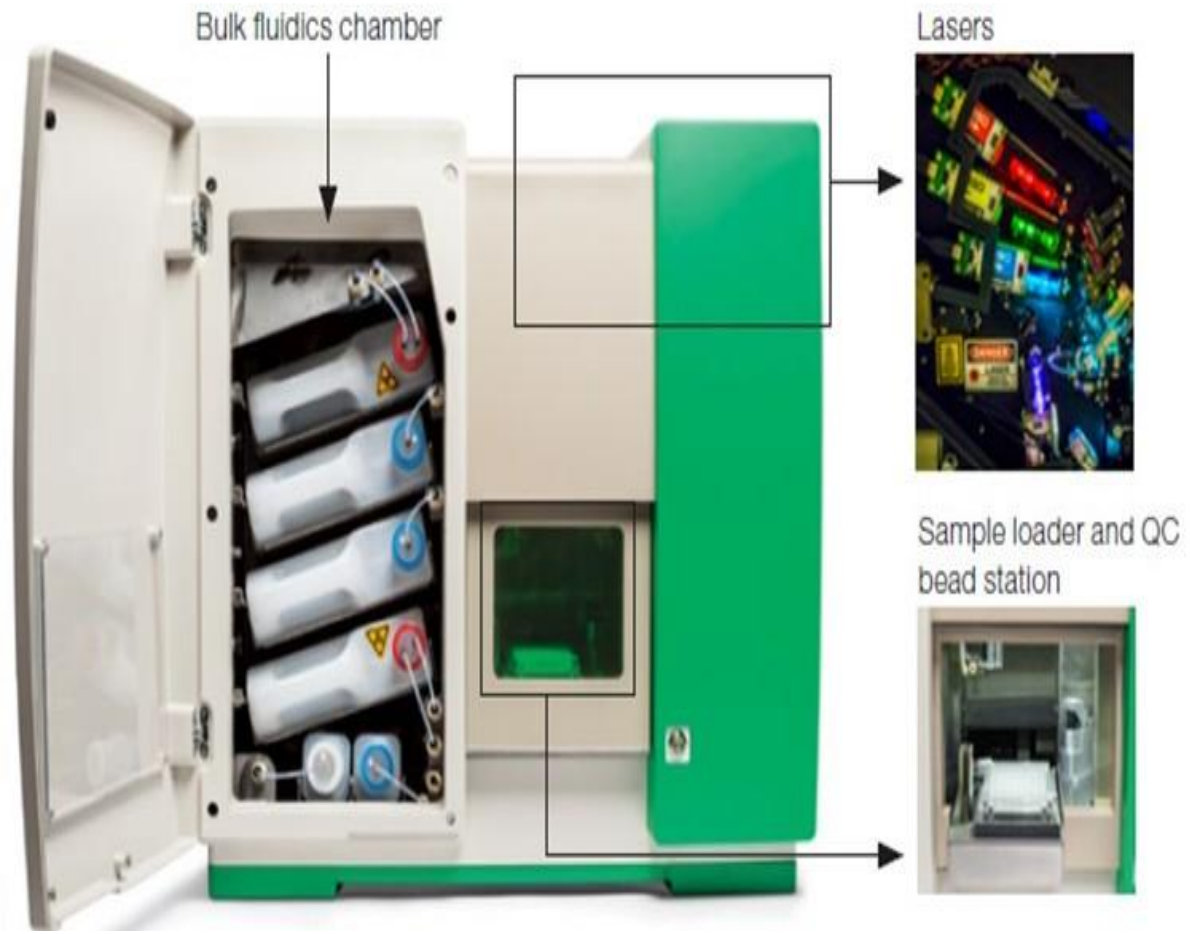


Using cells collected across several days, plotting dye intensity against cell count enables you to resolve each cell division cycle and therefore, the cell proliferation rate.

Inside a Flow Cytometer

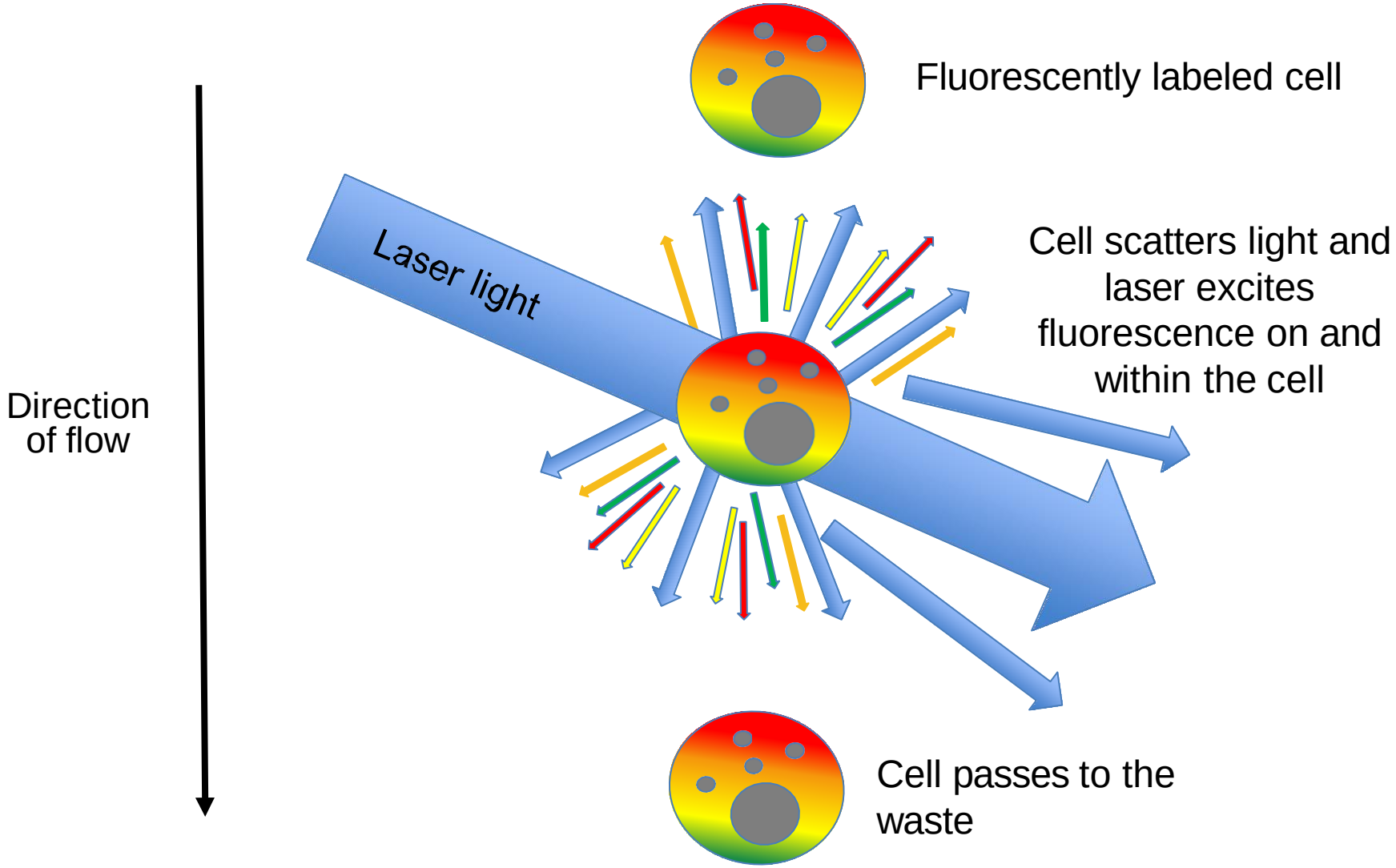
The Optics System

The Optics System



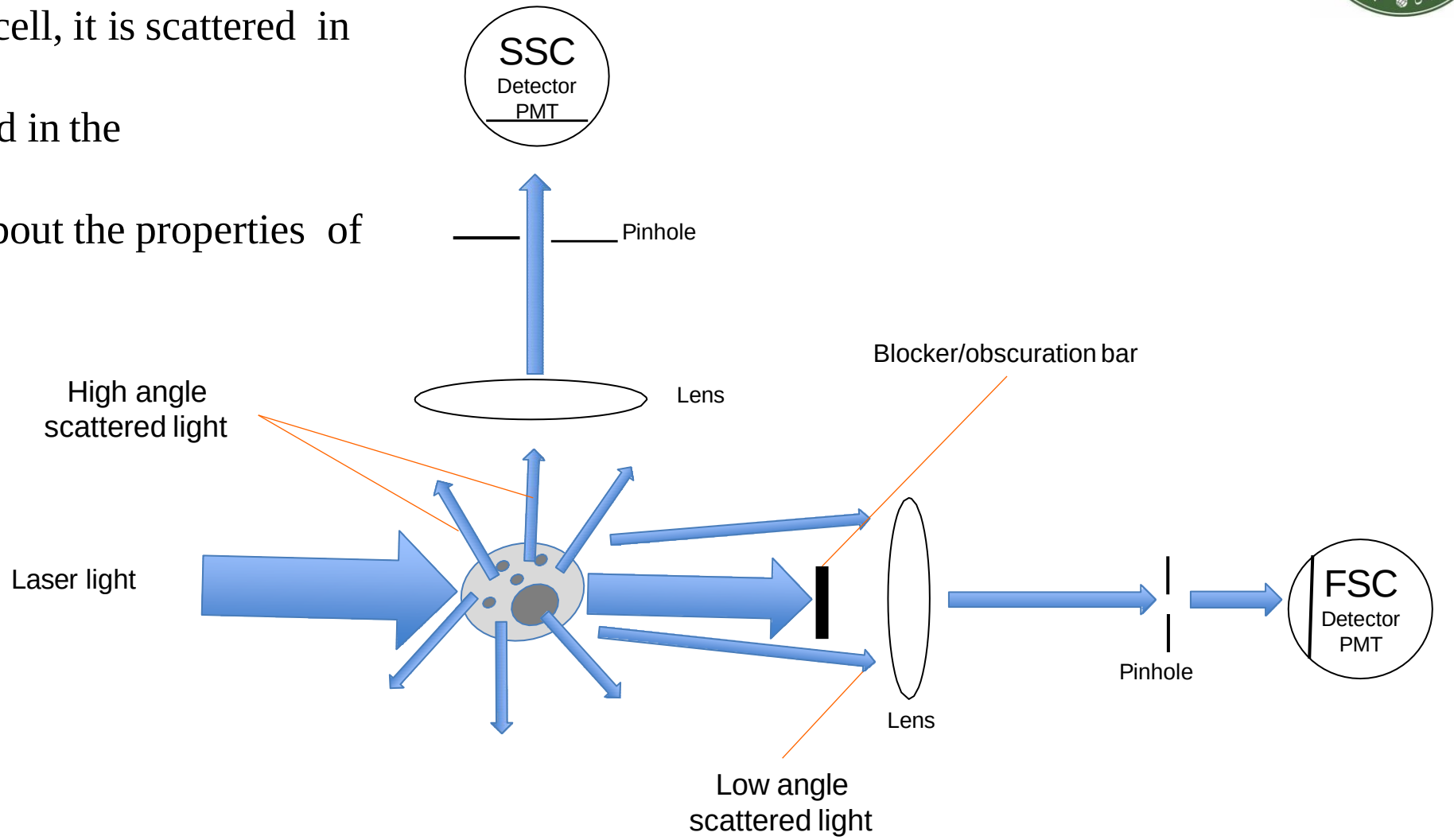
1. Fluidics system — delivers cells/particles to the optics
2. Optics system — directs light from the sample to the detectors
3. Electronics system — converts light into data

At the Interrogation Point



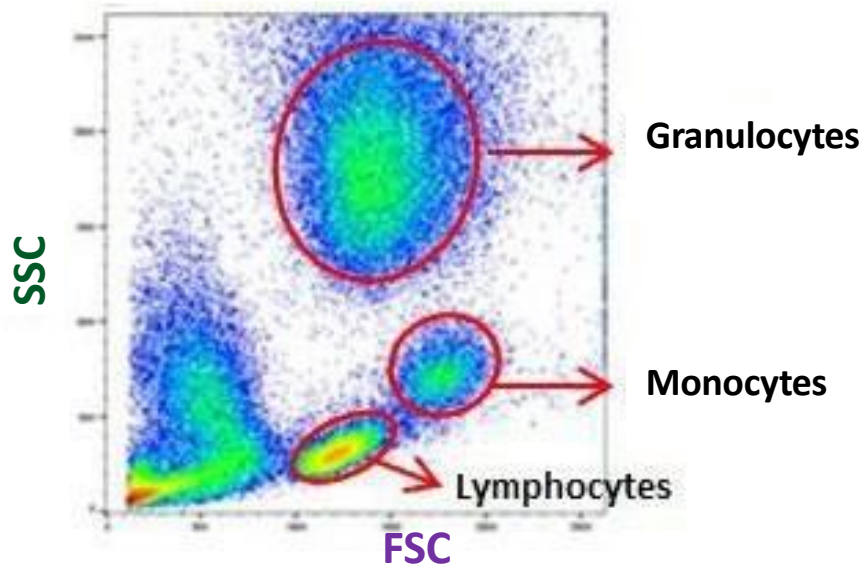
Scatter Signal

- After laser light hits the cell, it is scattered in all directions
- Scattered light is detected in the instrument's data output
- This gives information about the properties of your cell



Scatter Signal Continued

Whole blood scatter plot



Side Scatter:

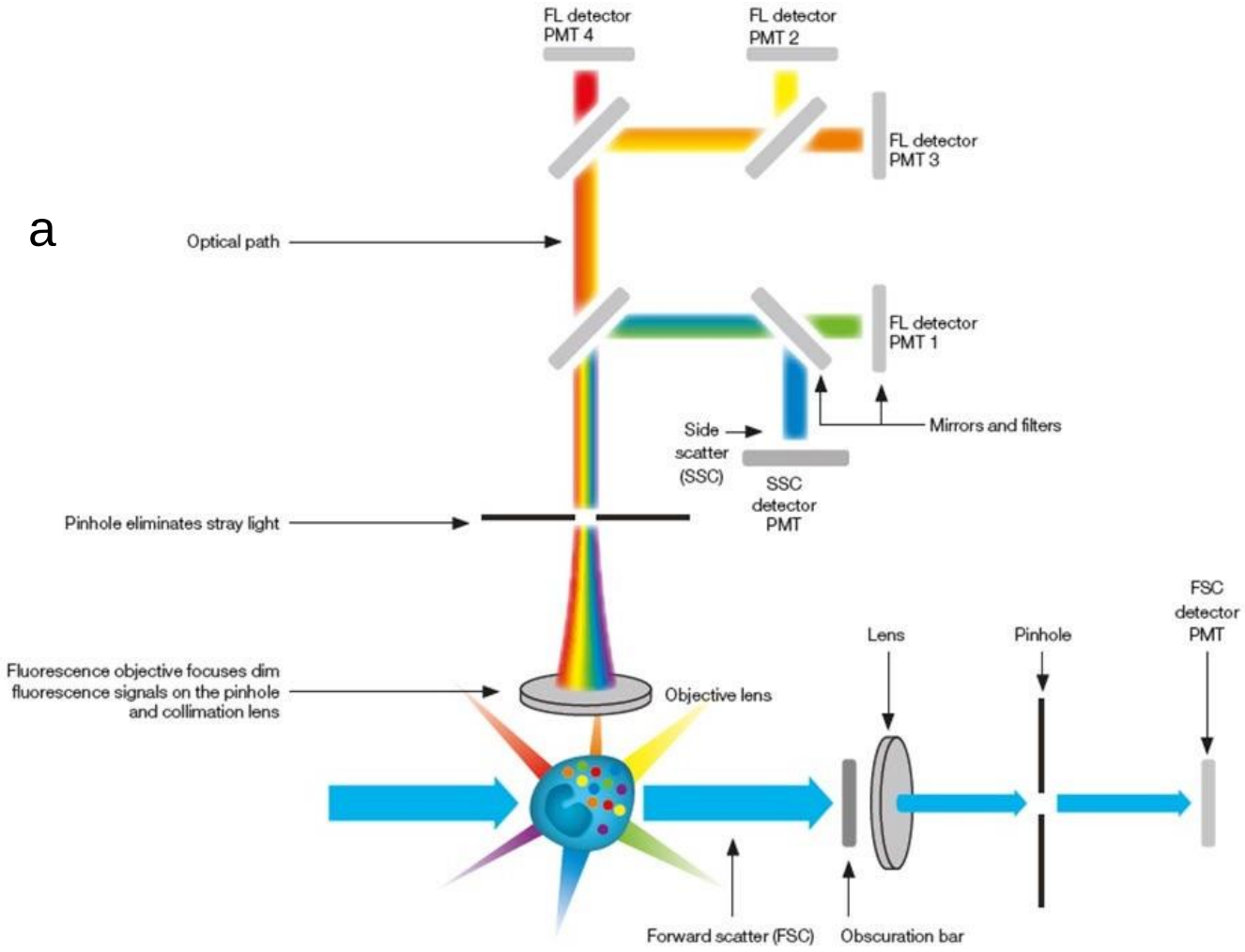
- Measured at 90° to the laser light
- Light bounces off internal structures
- More granular cells reflect more light
- Side scatter is an indication of cell "**granularity**"

Forward Scatter:

- Measured in line with the laser light
- Light diffracted around the cell
- Generally, bigger cells diffract more light than smaller cells
- Forward scatter is an indication of cell "**size**"

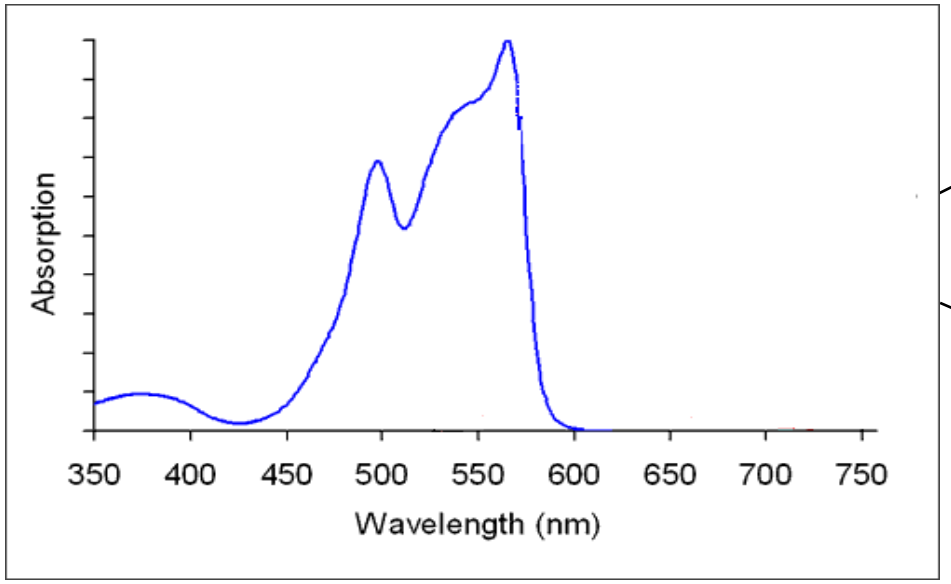
Fluorescence Signal

- Laser light excites fluorescent dyes or fluorophores on and within the cell
- Fluorescent light is emitted and passes through a series of filters and dichroic mirrors which split the light into its defined wavelengths
- Light is detected as individual photons by detectors called photomultiplier tubes (PMT)

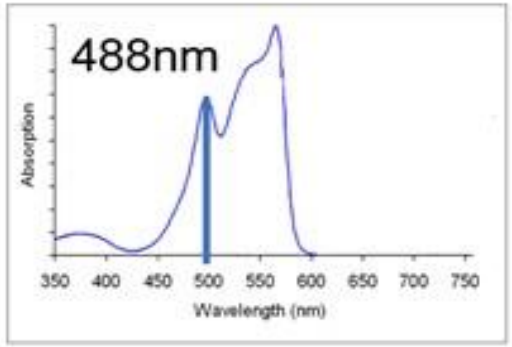


Excitation

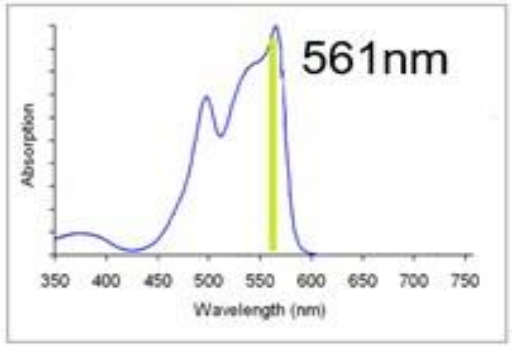
- Dyes can be excited over a wide range of wavelengths with greater or lesser efficiency,
  - This is the absorption or excitation spectrum
- In flow cytometry this is simplified to which available laser optimally excites the dye (e.g 488 nm or 561 nm)



Overall **excitation spectrum** of a dye



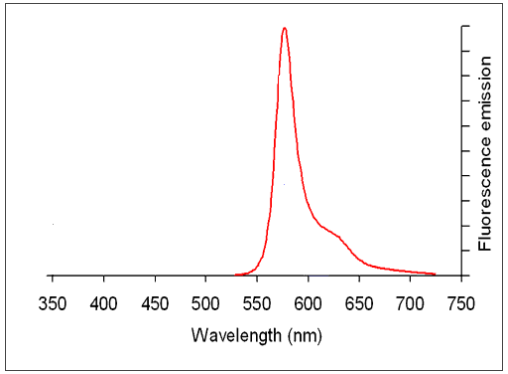
Excitation by the 488nm blue laser fails to excite the dye at its excitation maximum



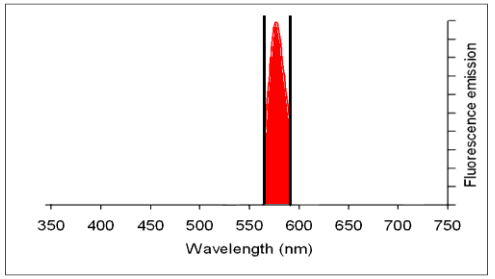
Excitation by the 561nm yellow/green laser optimally excites the dye at its excitation maximum

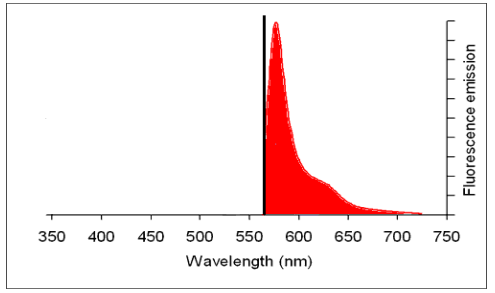
Emission

- Even when a dye is excited with a single wavelength of photons, it will emit photons with a wide range of wavelengths.
- These photons are filtered by wavelength to be collected by the detectors (PMTs)



Overall emission spectrum of the dye





Spectrum cut down by filter

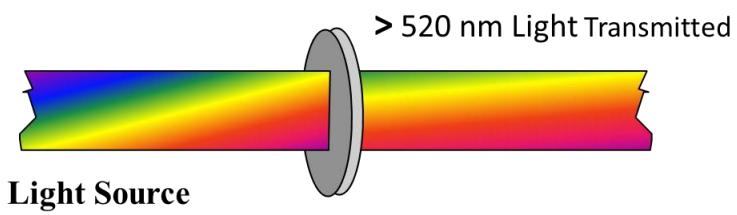
Optical filters can be used to present a small part

or

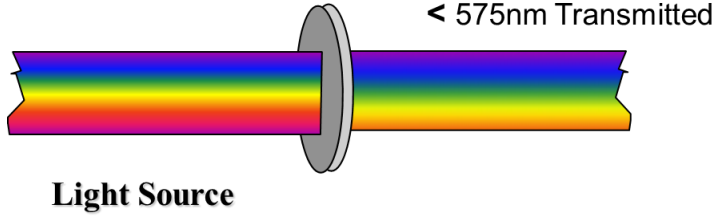
large part of the emission spectrum to the detector

Optical Filters

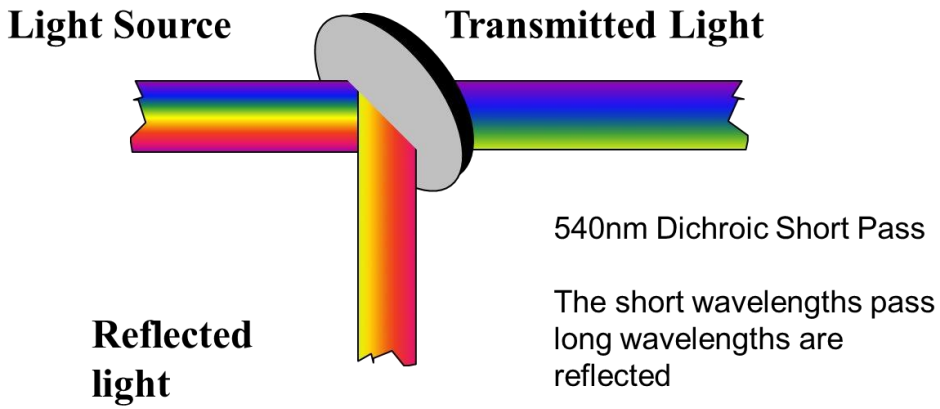
520 nm Long Pass Filter



575 nm Short Pass Filter



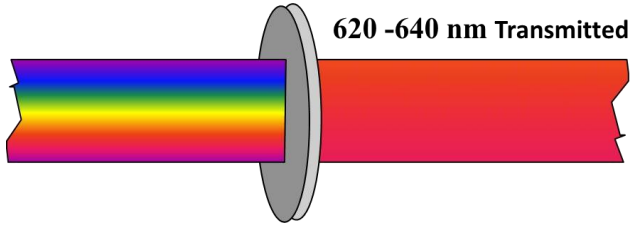
Filter placed at 45°



540nm Dichroic Short Pass

The short wavelengths pass long wavelengths are reflected

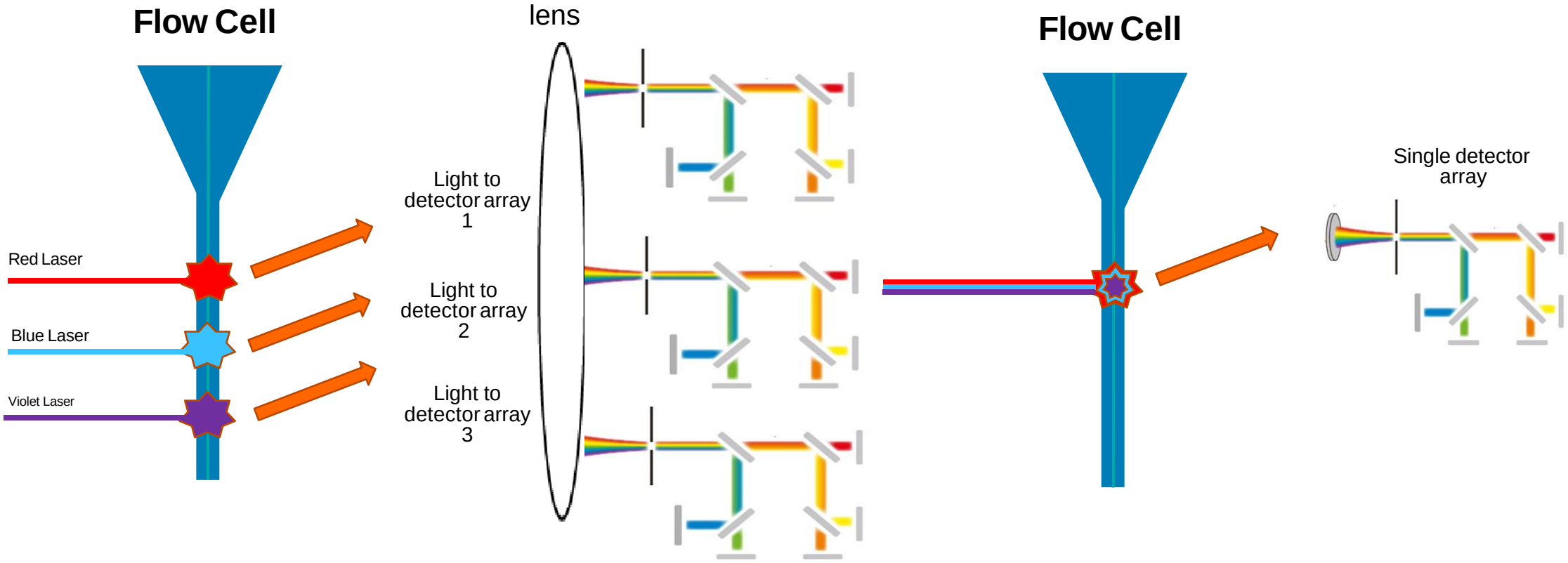
630/20 nm Band Pass Filter



Multi Laser Instruments

Spatially separated lasers

Co-linear lasers

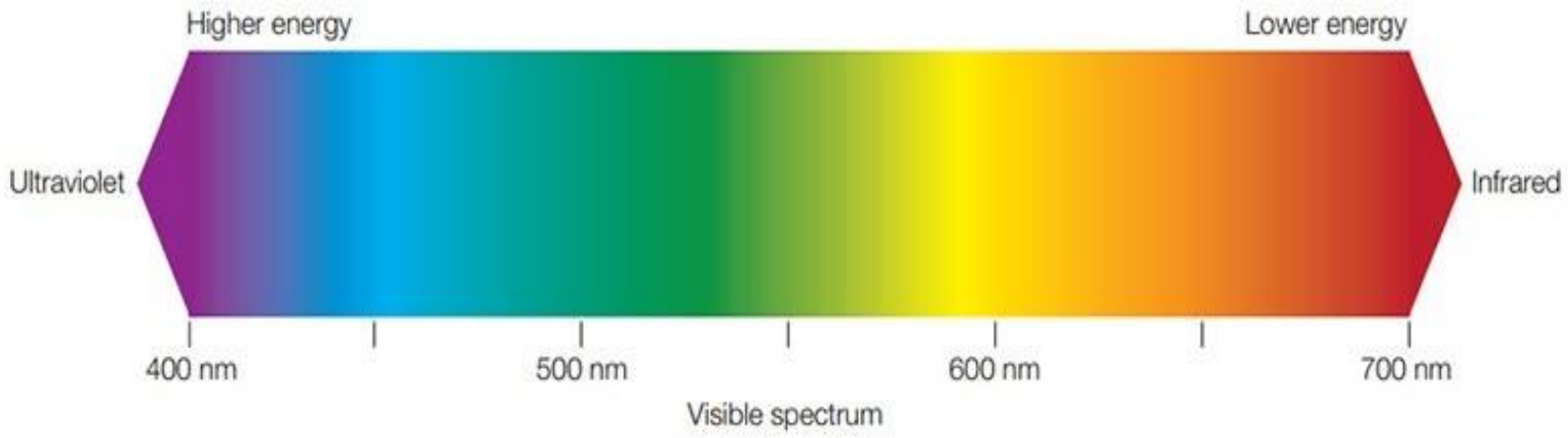


Optics System Summary

- Laser light strikes cells/particles
- Some laser light is scattered
- Low angle scattered laser light is detected by the forward scatter detector and can give an indication of relative size
- High angle scattered laser light is detected by the side scatter detector and can give an indication of granularity
- If a fluorescent dye is present it may be excited by one or more lasers to produce light which is detected by the fluorescence detectors
- The wavelength of light detected by the fluorescence detectors is determined by the filters present
- Multiple lasers can be used with multiple detector arrays

Fluorescence and Flow Cytometry Principles

The Light Spectrum



- Visible light is the portion of the electromagnetic spectrum that humans can detect
- Ultraviolet (UV) and far-red light are past detection of human photoreceptors and invisible to human eyes

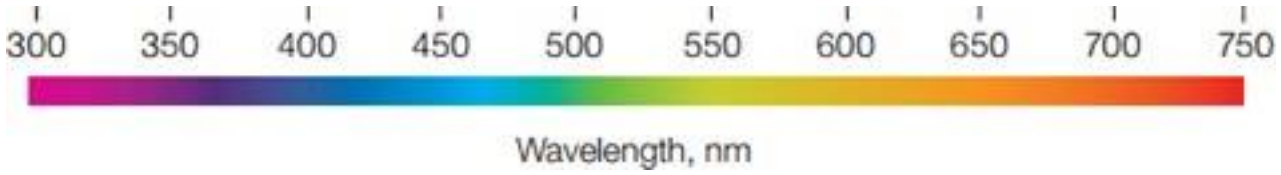
How are Fluorescent Dyes Used in Flow Cytometry?

- Antibodies linked to fluorescent dyes are used to detect proteins of interest
- A flow cytometer's lasers produce light of defined wavelengths to excite different dyes
- By detecting the fluorescence emitted by the dye you get a readout of antibody bound to its target (signal)
- You can also use unconjugated fluorescent stains such as 4,6-diamidino-2-phenylindole (DAPI)
- The range of an excitation histogram may span two laser excitation wavelengths
- When using multiple fluorescent dyes, choose dyes with nonoverlapping spectra
- When designing large multicolor panels, consider dye brightness

Each flow cytometer model will have a different configuration of lasers and differing ability to detect dyes.

Ensure experimental success by choosing fluorophores that are suitable for use with your flow cytometer.
Some dyes are brighter than others!

Common Fluorescent Dyes



Laser Line	Wavelength, nm	Fluorescent Dyes Used
Ultraviolet	355	SBUV400, SBUV445, SBUV510, SBUV575, SBUV605, SBUV665 SBUV740, SBUV795
Violet	405	Pacific Blue, SBV440, SBV475, SBV515, Amethyst Orange, SBV570, SBV610, SBV670, SBV710, SBV790
Blue	488	FITC, A488, SBB580, SBB615, PE, SBB675, SBB700, PE-A647, PE-Cy5, PE-Cy5.5, PE-A750, PE-Cy7, SBB765, SBB810, PerCP, PerCP-Cy5.5,
Yellow	561	SBY575, SBY605, PE, SBY665, PE-A647, PE-Cy5, SBY720, PE-Cy5.5, SBY775, PE-A750, PE-Cy7
Red	640	A647, APC, A700, APC-Cy7

APC, allophycocyanin; AXX, Alexa Fluor; Cy, cyanine, FITC, fluorescein isothiocyanate; PE, phycoerythrin;
SBB, StarBright Blue Dye; SBUV, StarBright Ultraviolet Dye; SBV, StarBright Violet Dye; SBY, StarBright Yellow Dye

References

- McKinnon, K. M. (2018). Flow cytometry: an overview. Current protocols in immunology, 120(1), 5-1.
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- https://youtu.be/B2zreF2dnWk?si=2s3iLaicljJf0kp9