Cellular Research Techniques in Medicine and Research: An Overview

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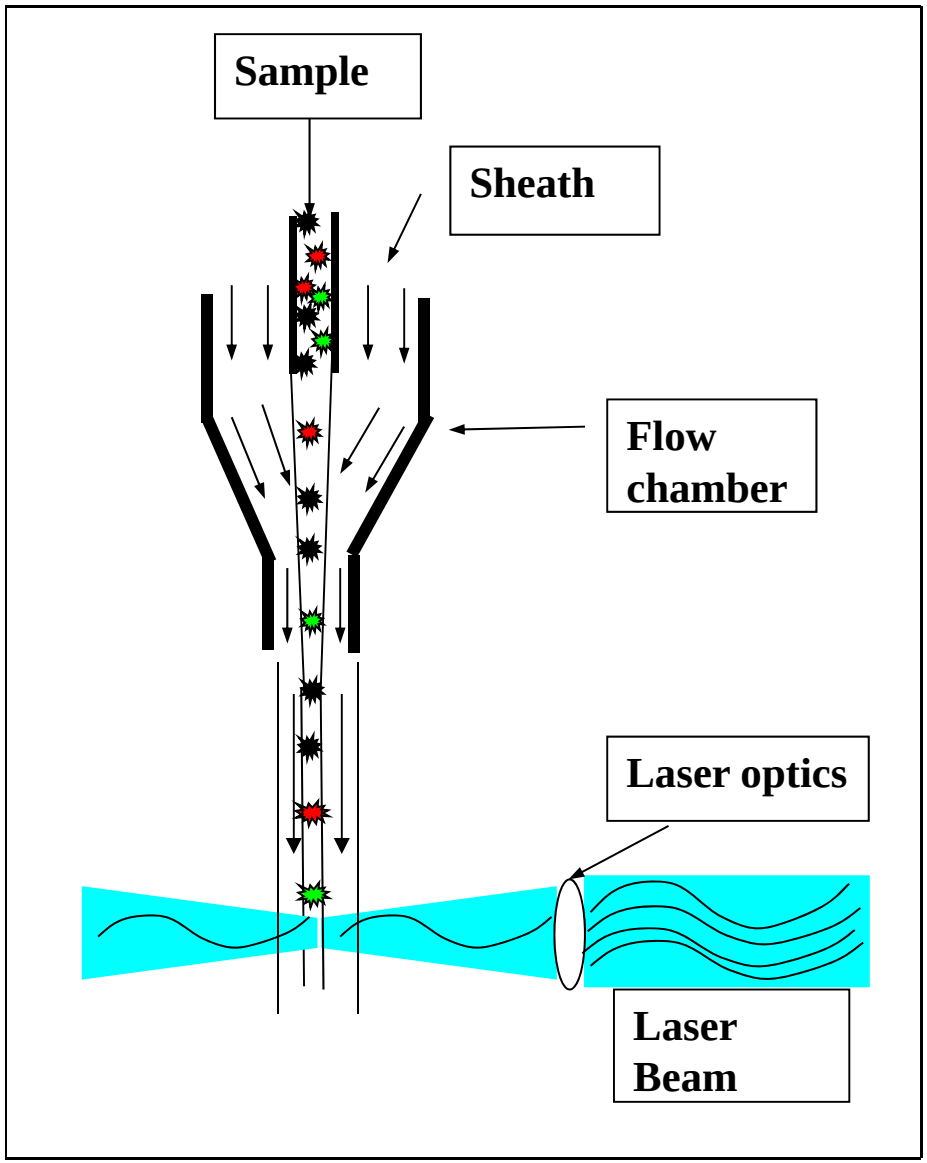
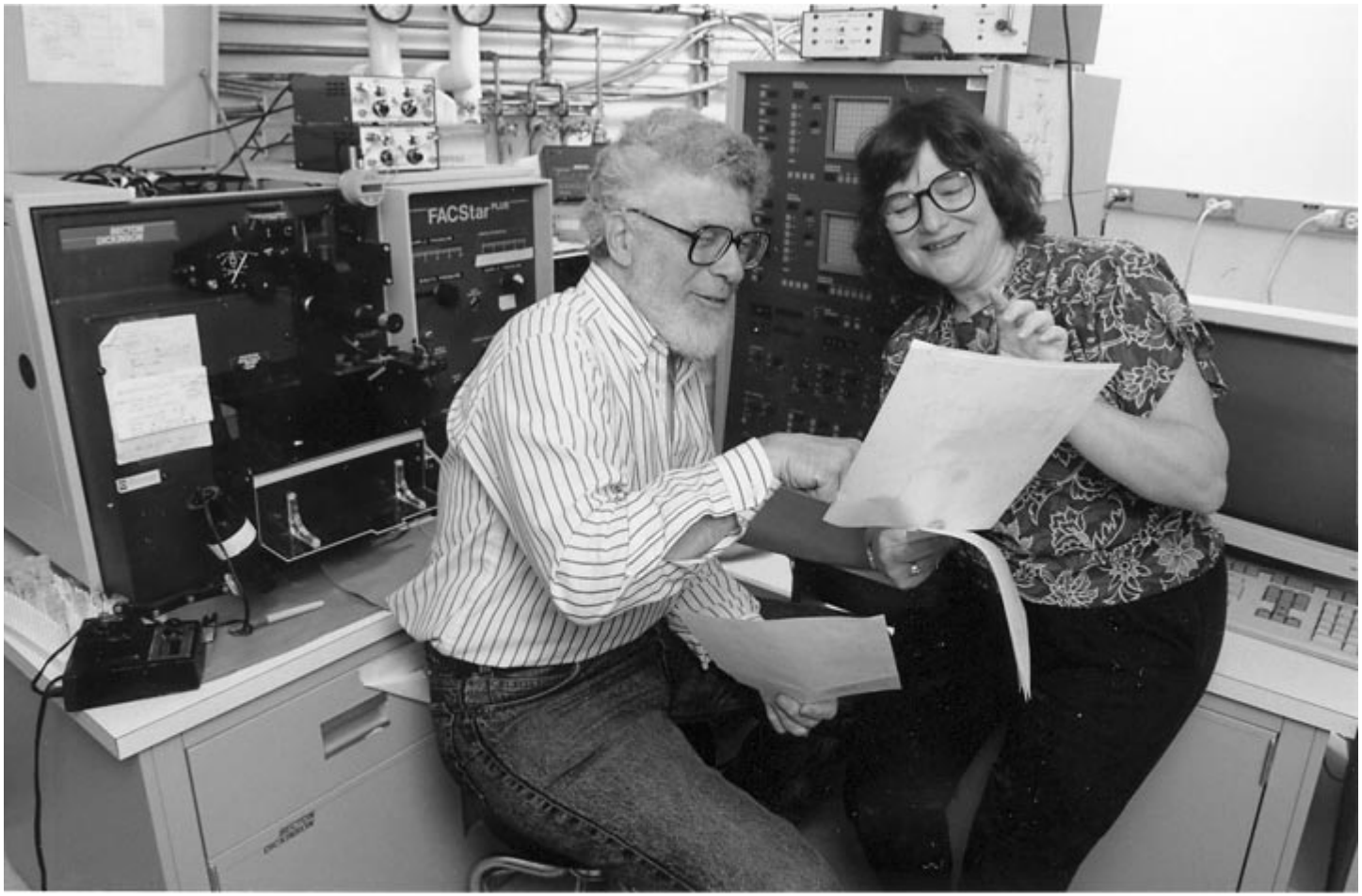
Flow Cytometry

- A rapid, sensitive, and quantitative method to identify populations of cells based on protein expression patterns.
  - PBMCs
  - BAL cells
  - Lung tissue
- Antibodies labeled with fluorescent dyes are used to identify cells.
  - Proteins can be on the cell surface or intracellular
  - We are able to determine up to 12 different fluorescence tags simultaneously.

Instrumentation



Cells are presented to the laser using principles of hydrodynamic focusing



Len Herzenberg

Leonore Herzenberg

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GENETICS, FACS, IMMUNOLOGY, AND REDOX:
A Tale of Two Lives Intertwined

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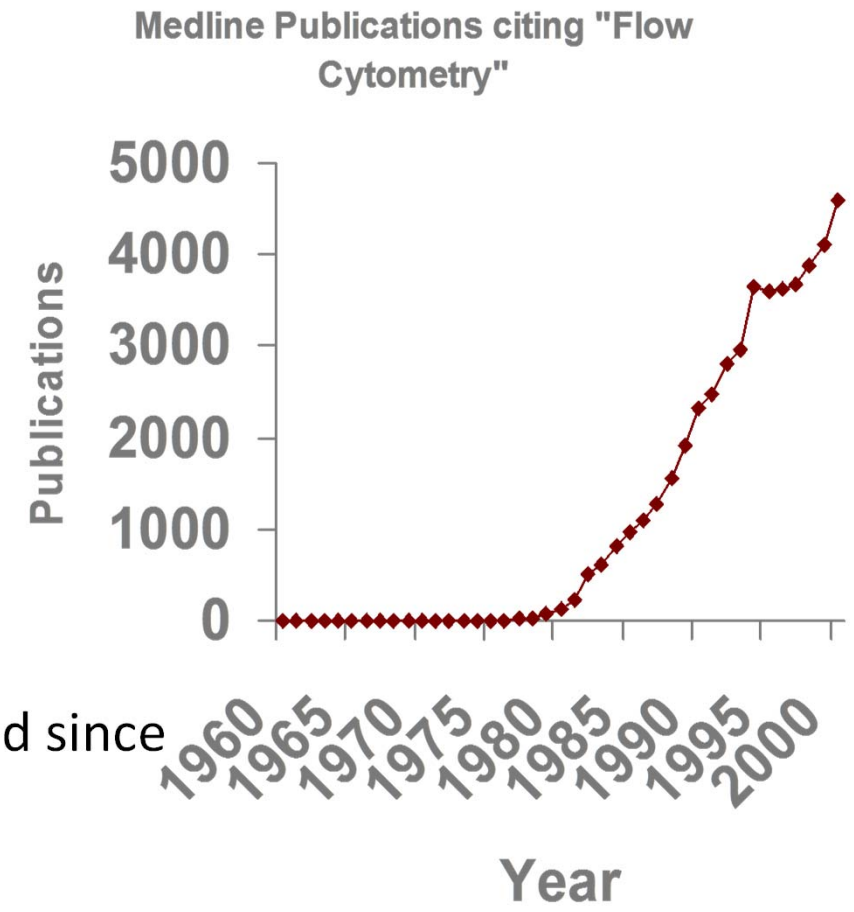
Key Words glutathione, B cells, flow cytometry, allotype, HIV

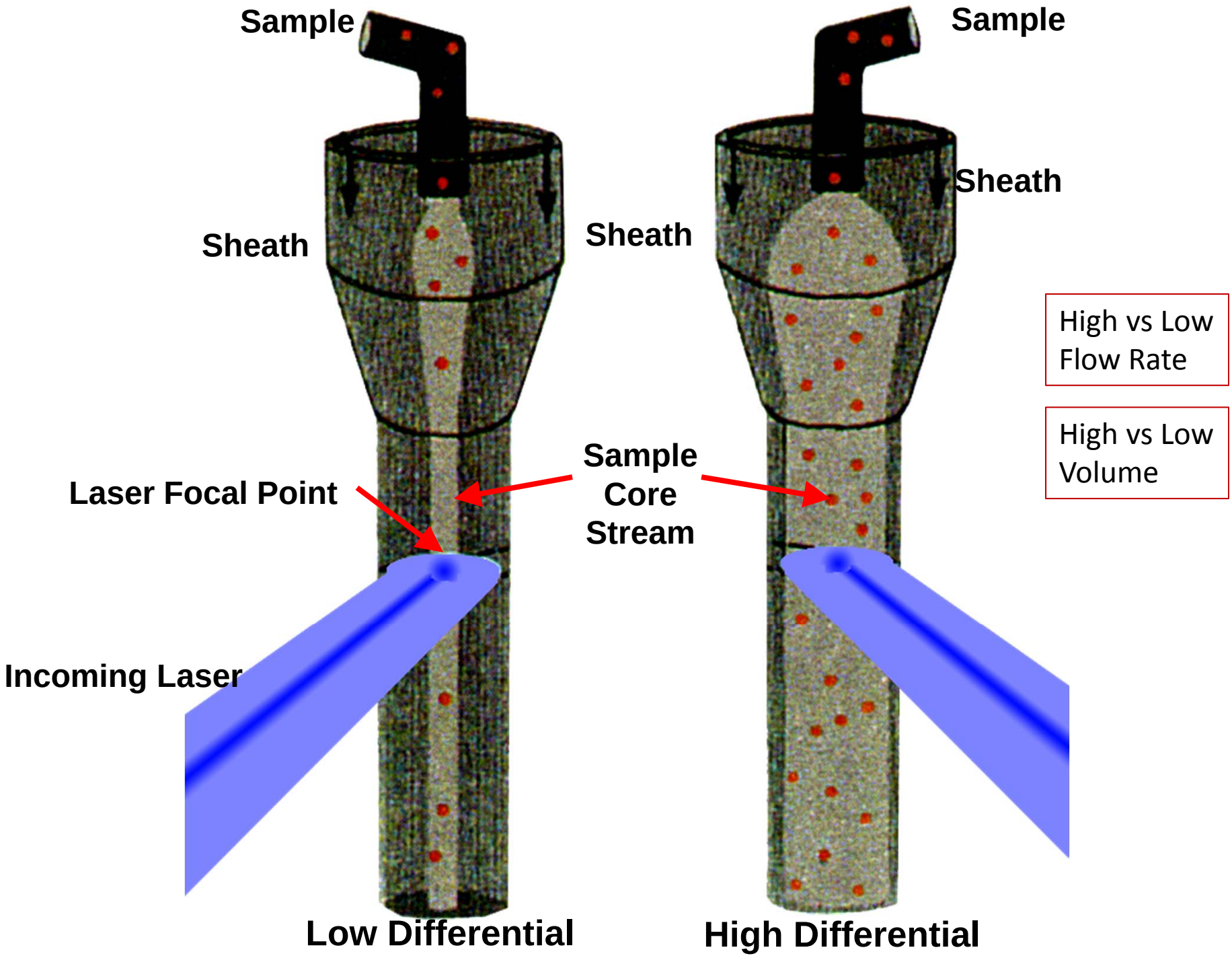
INTRODUCTION

We (Len and Lee Herzenberg) have worked separately and together for more than 50 years. This blending of independence and mutual reliance is reflected here as we shift back and forth in telling the story of the laboratory we have led and the life we have lived. The space provided for this chapter is very generous. Yet, calculated out, it amounts to roughly 100 words per year for each of us. To make the most of this, we have written an autobiography rather than a history. In many instances, we have referred only briefly, or not at all, to work that had major influences on our thinking. In addition, we have adopted a policy of naming the many students, fellows, and collaborators with whom we have worked only by referring to our joint work with them. We hope the reader realizes there would be no biography worth writing were it not for the contributions made by these and all of our other colleagues.

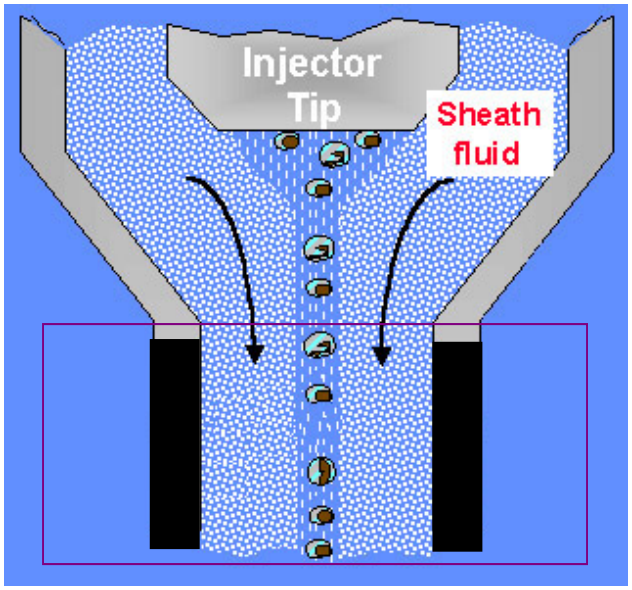
Uses of Flow Cytometry

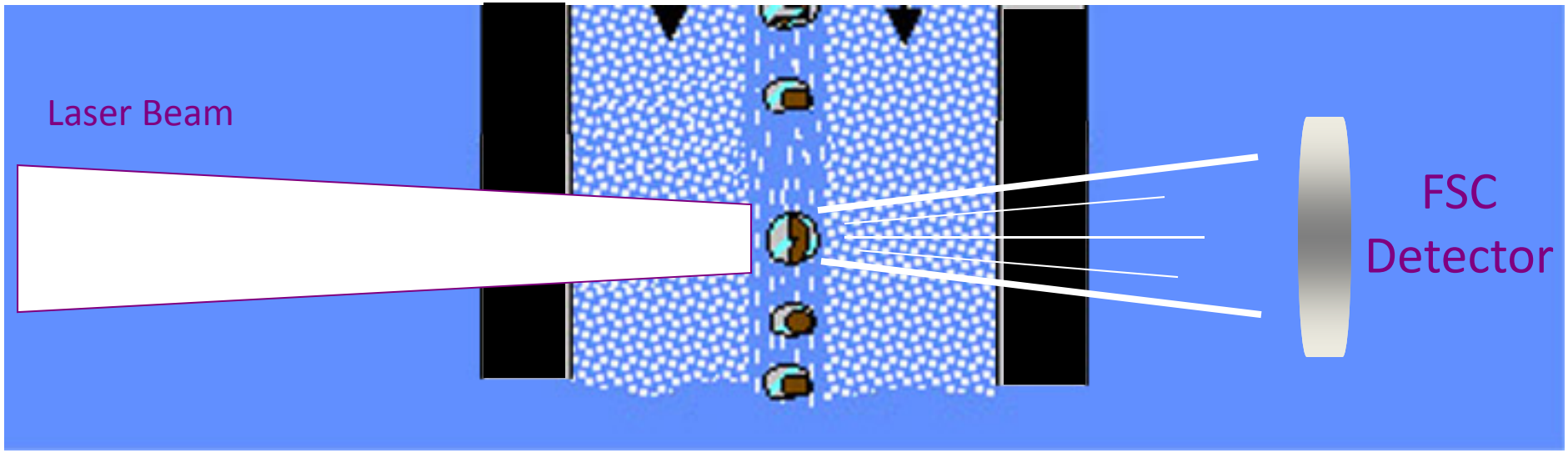
- It can be used for…
  - Immunophenotyping
  - DNA cell cycle/tumor ploidy
  - Membrane potential
  - Ion flux
  - Cell viability
  - Intracellular protein staining
  - pH changes
  - Cell tracking and proliferation
  - Sorting
  - Redox state
  - Chromatin structure
  - Total protein
  - Lipids
  - Surface charge
  - Membrane fusion/runover
  - Enzyme activity
  - Oxidative metabolism
  - Sulfhydryl groups/glutathione
  - DNA synthesis
  - DNA degradation
  - Gene expression
- The use of flow in research has boomed since the mid-1980s




Sample
Sheath
Laser Focal Point
Incoming Laser
Low Differential
Sample
Sheath
Sample
Core
Stream
Sample
Sheath
High Differential
High vs Low
Flow Rate
High vs Low
Volume

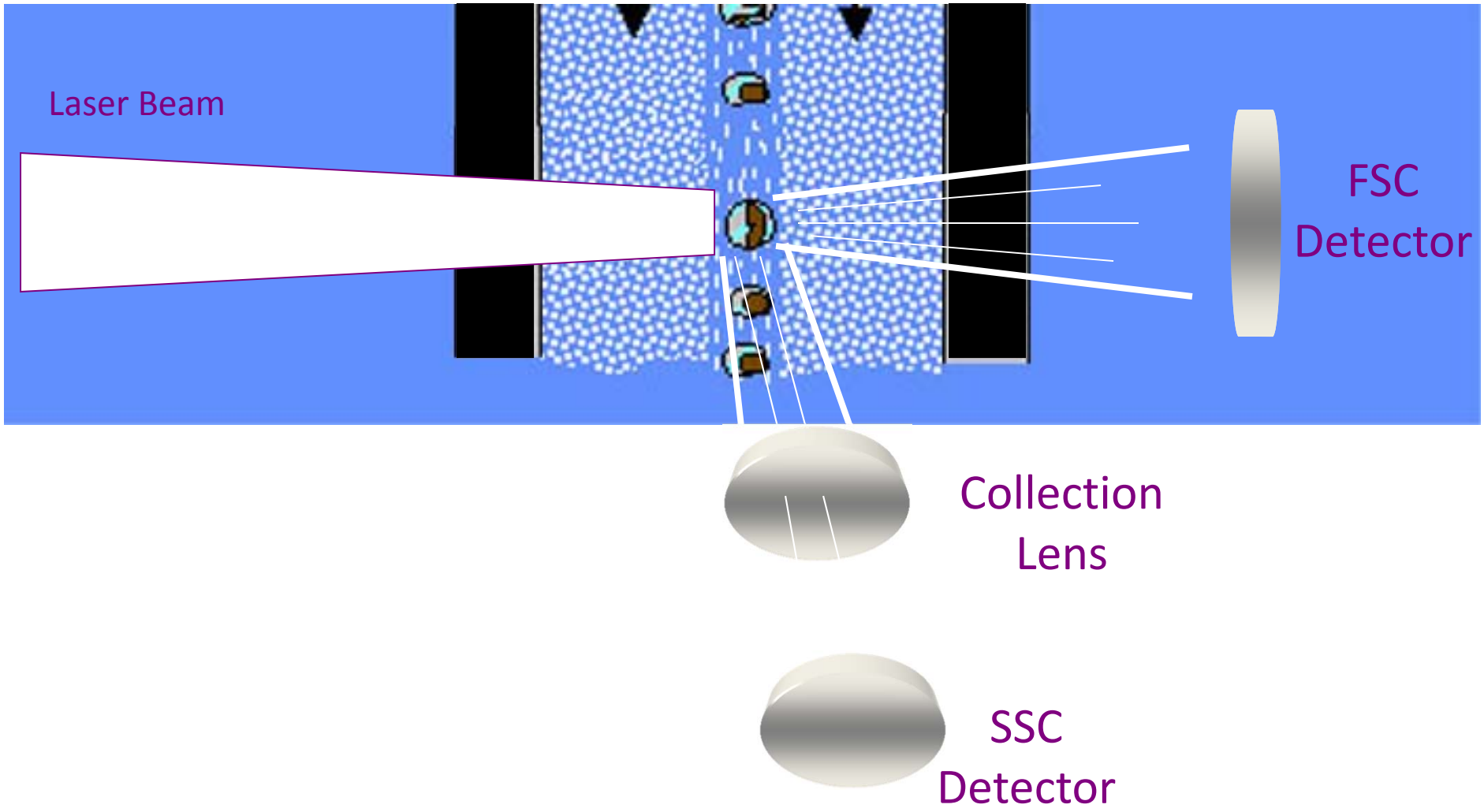
Forward Scatter





Original from Purdue University Cytometry Laboratories

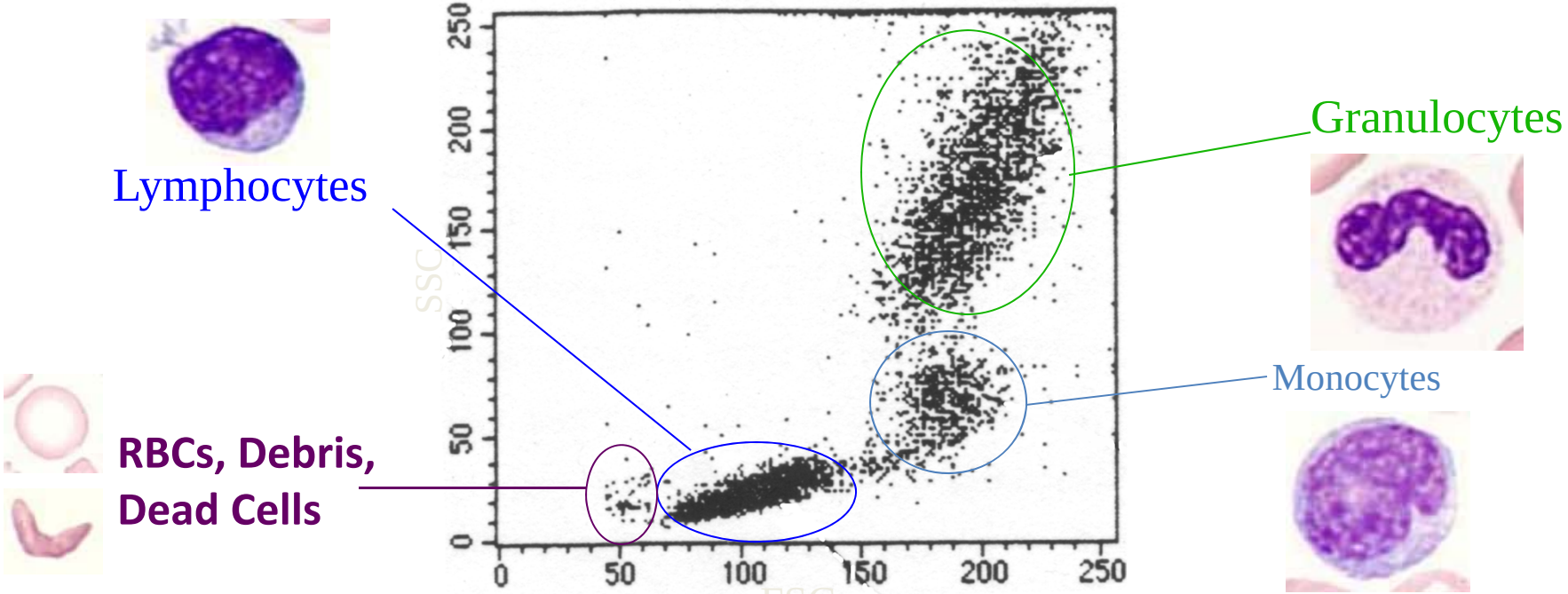
Side Scatter



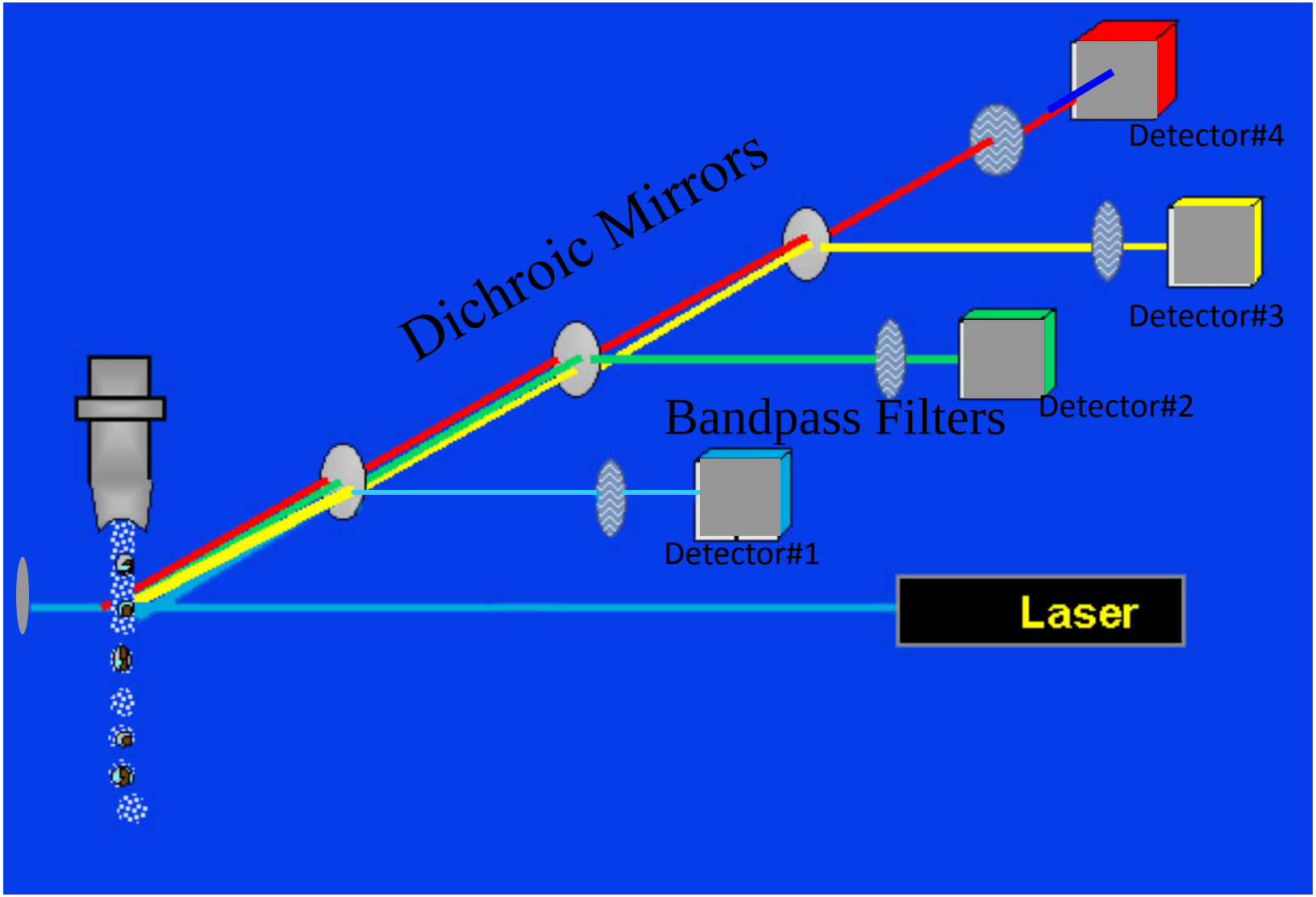
Original from Purdue University Cytometry Laboratories

Why Look at FSC v. SSC

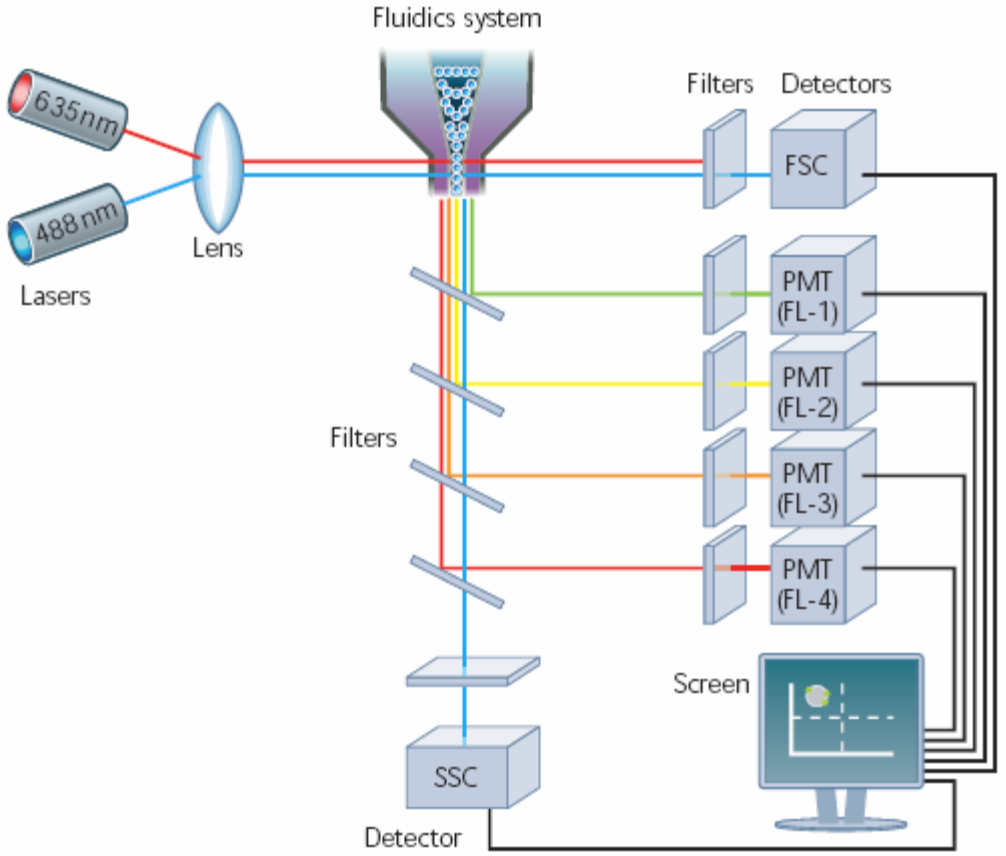
- Since FSC ~ size and SSC ~ internal structure, a correlated measurement between them can allow for differentiation of cell types in a heterogenous cell population



Example Channel Layout

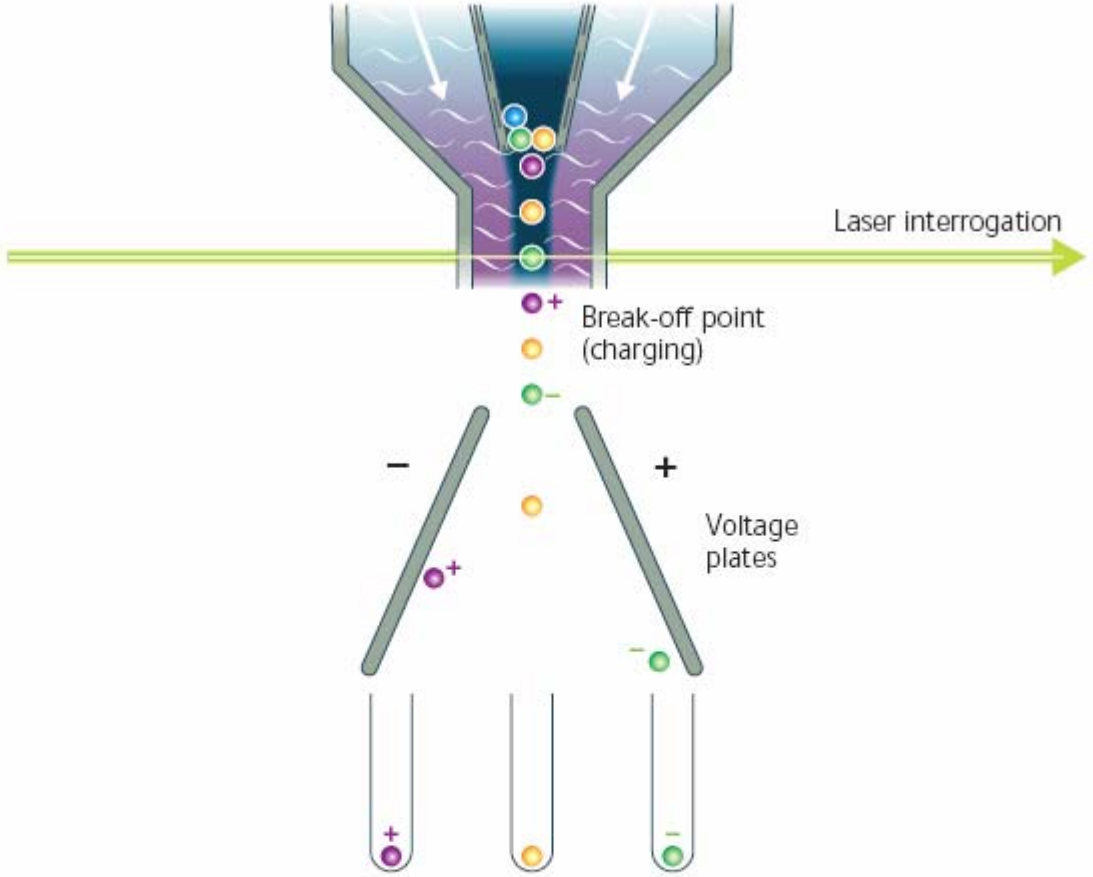


Original from Purdue University Cytometry Laboratories



Schematic overview of a typical flow cytometer setup

parameter are known as the 'events' and refer to the number of cells displaying the physical feature or marker of interest.



Electrostatic flow sorting

The speed of flow sorting depends on several factors including particle size and the rate of droplet formation. A typical nozzle is between 50–70 μM in diameter and, depending on the jet velocity from it, can produce 30,000–100,000 droplets per second, which is ideal for accurate sorting. Higher jet velocities risk the nozzle becoming blocked and will also decrease the purity of the preparation.

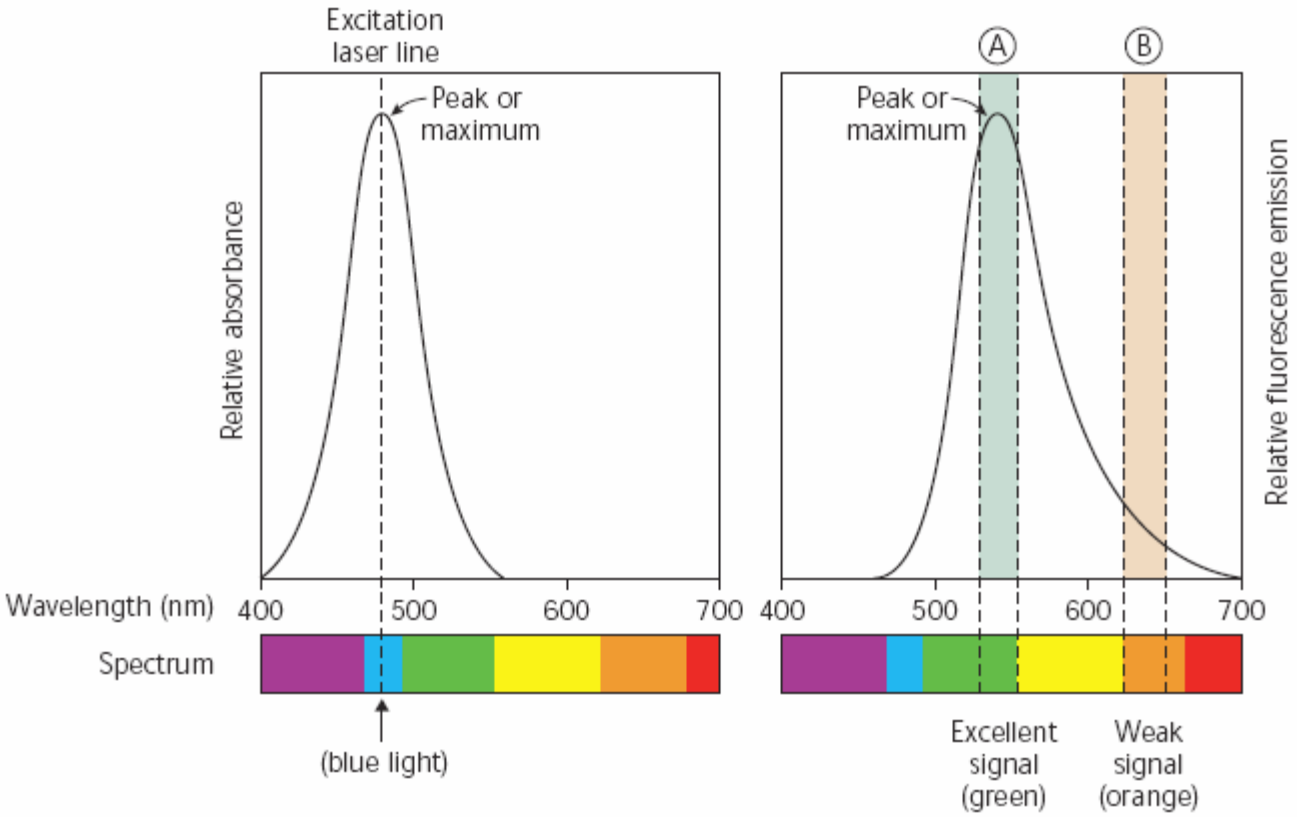


FIGURE 7 **Light absorbance (LEFT) and light emission (RIGHT) of FITC**

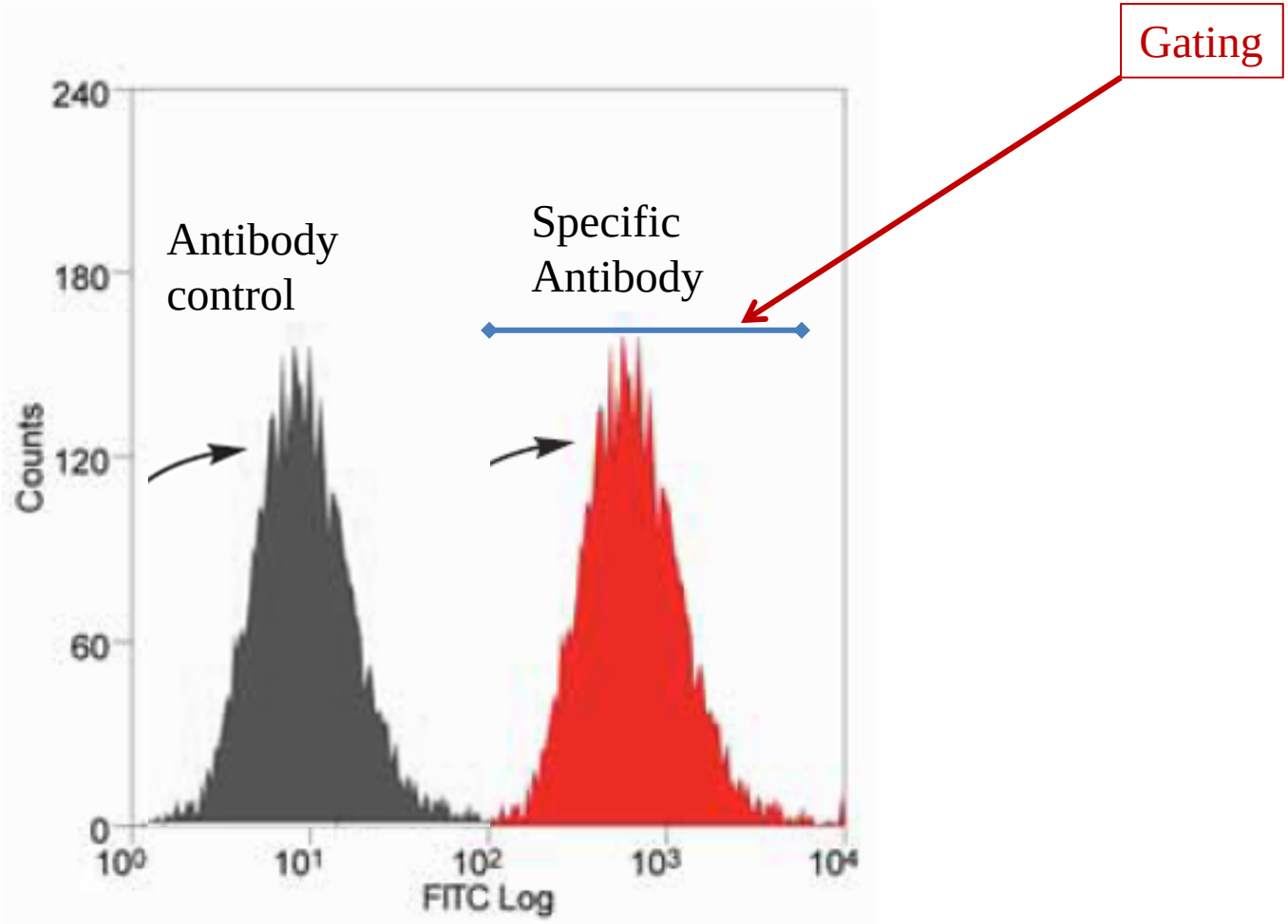
These optimal conditions are termed maximal absorbance and maximal emission wavelengths.

Maximal absorbance usually defines the laser spectral line that is used for excitation. In the case of FITC, its maximum falls within the blue spectrum. Therefore, the blue Argon-ion laser is commonly used for this fluorochrome, as it excites at 488 nm, close to FITC's absorbance peak of 490 nm.

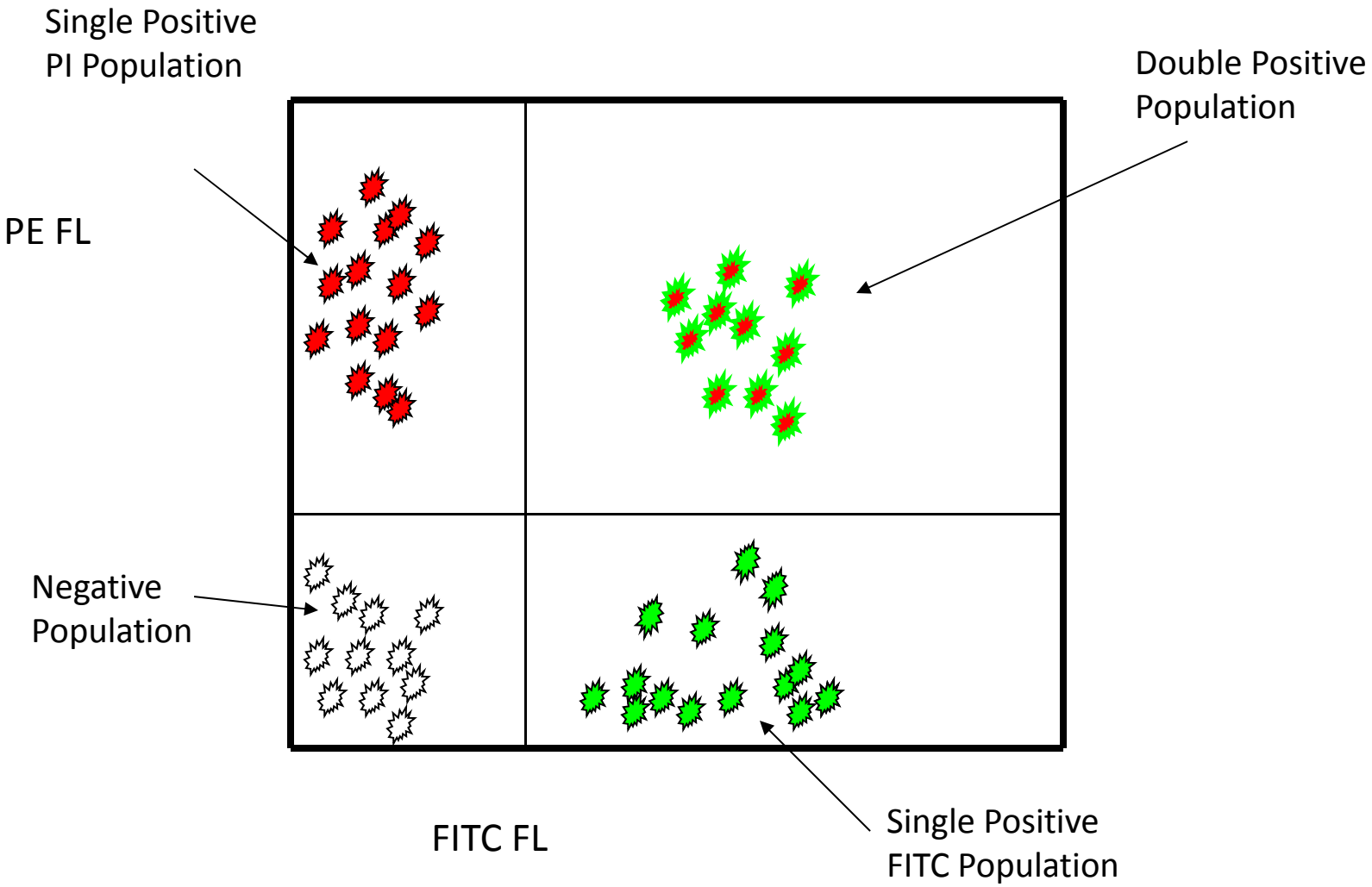
FITC emits fluorescence over the range 475–700 nm peaking at 525 nm, which falls in the green spectrum. If filters are used to screen out all light other than that measured at the maximum via channel A (see Figure 7), FITC will appear green. Hence, 'fluorescence color' usually refers to the color of light a fluorochrome emits at its highest stable excited state. However, if FITC fluorescence is detected only via channel B (see Figure 7), it will appear orange and be much weaker in intensity. How the flow cytometer is set up to measure fluorescence will ultimately determine the color of a fluorochrome.

Single-parameter histograms

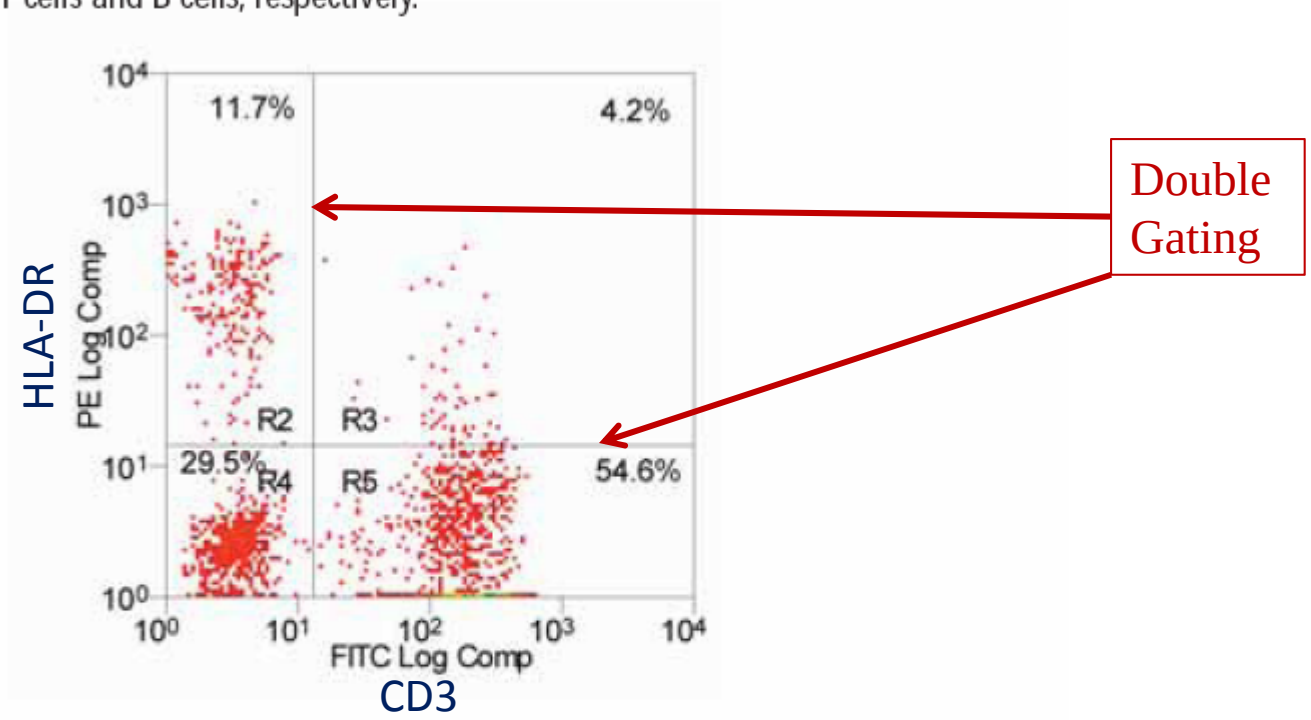
These are graphs that display a single measurement parameter (relative fluorescence or light scatter intensity) on the x-axis and the number of events (cell count) on the y-axis.



2 Parameter Histogram



Another example is the dual-color fluorescence histogram presented below. Lymphocytes were stained with anti-CD3 in the FITC channel (x-axis) and anti-HLA-DR in the PE channel (y-axis). CD3 and HLA-DR are markers for T cells and B cells, respectively.



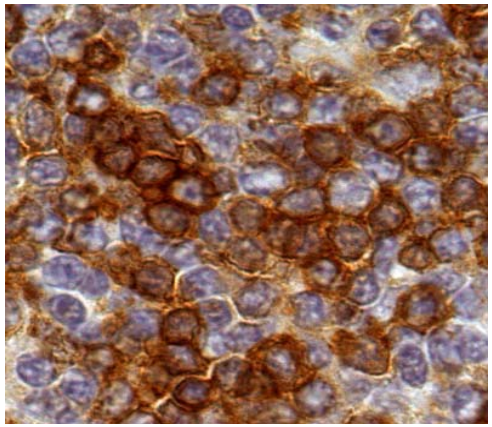
Two-parameter (dual-color fluorescence) histogram

In Figure 14, R2 encompasses the PE-labeled B cells – note their positive shift along the PE axis. R5 contains the FITC-labeled T cells (positively shifted along the FITC axis). The top right quadrant contains a few 'activated T cells' (about 4% in this sample) that possess some HLA-DR expression also. As these stain with both antibody markers they are grouped in their own region (R3). R4 contains cells negative for both FITC and PE (no shift).

Cytometry vs. Flow Cytometry

Cytometry

- Localization of antigen is possible
- Poor enumeration of cell subtypes
- Limiting number of simultaneous measurements



Flow Cytometry.

- Cannot tell you where antigen is.
- Can analyze many cells in a short time frame.
- Can look at numerous parameters at once.

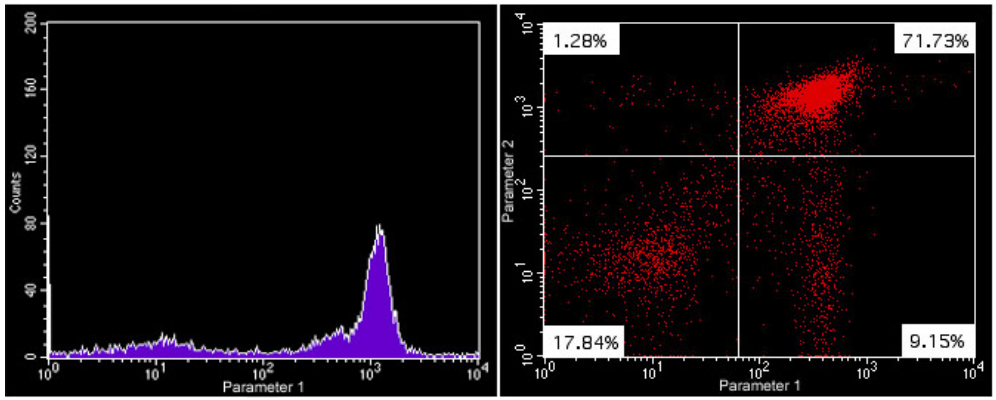
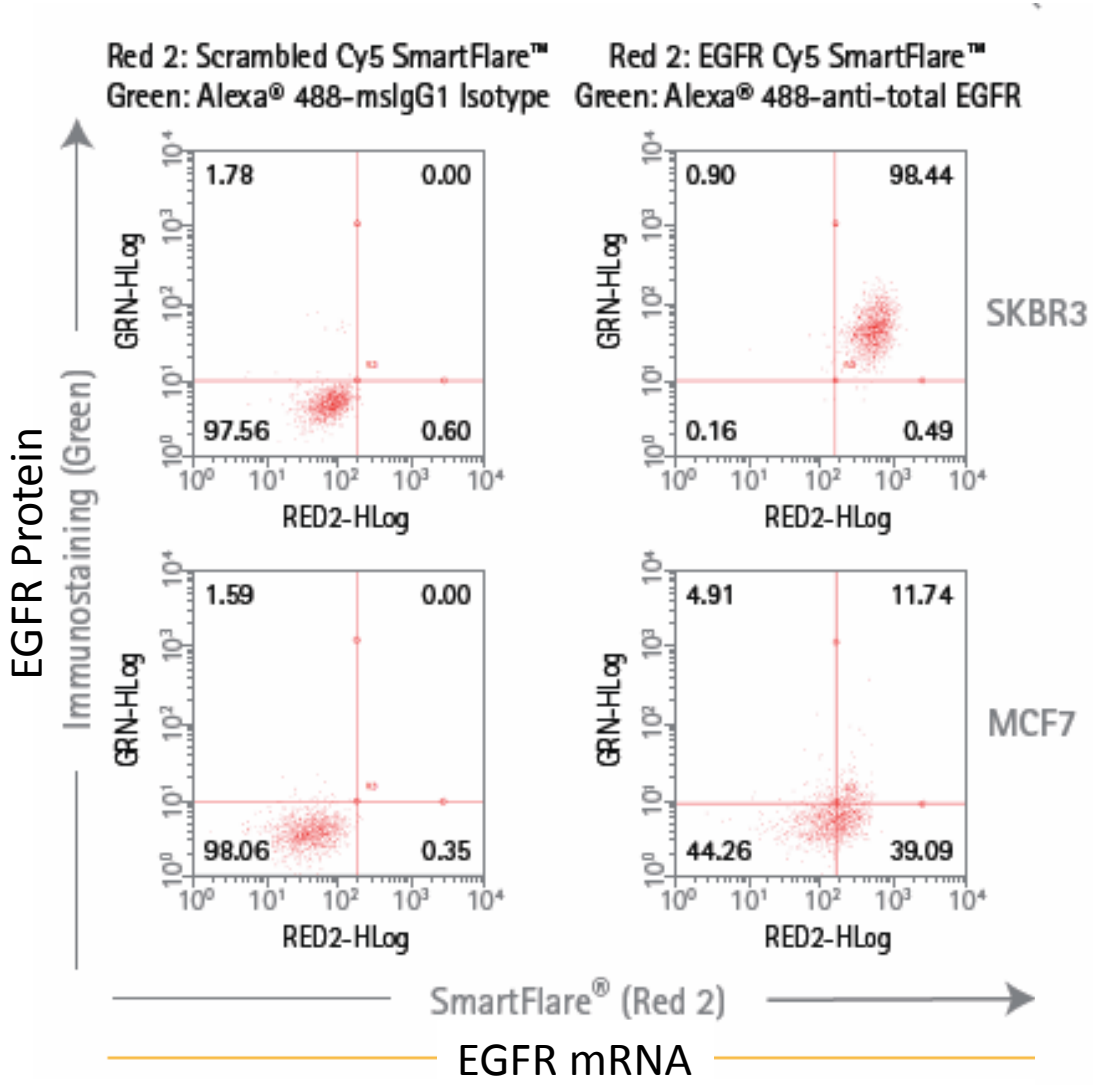


Table 1. Common clinical uses of flow cytometry.

Field	Clinical application
Immunology	Histocompatibility cross-matching
	Transplantation rejection
	HLA-B27 detection
	Immunodeficiency studies
Oncology	DNA content and S phase of tumors
	Measurement of proliferation markers
Hematology	Leukemia and lymphoma phenotyping
	Identification of prognostically important subgroups
	Hematopoietic progenitor cell enumeration
	Diagnosis of systemic mastocytosis
	Reticulocyte enumeration
	Autoimmune and alloimmune disorders
	Anti-platelet antibodies
	Anti-neutrophil antibodies
	Immune complexes
	Feto-maternal hemorrhage quantification
Blood banking	Immunohematology
	Assessment of leukocyte contamination of blood products
Genetic disorders	PNH
	Leukocyte adhesion deficiency

[a] PCNA, proliferating cell nuclear antigen; TdT, terminal deoxynucleotidyltransferase; MPO, myeloperoxidase.



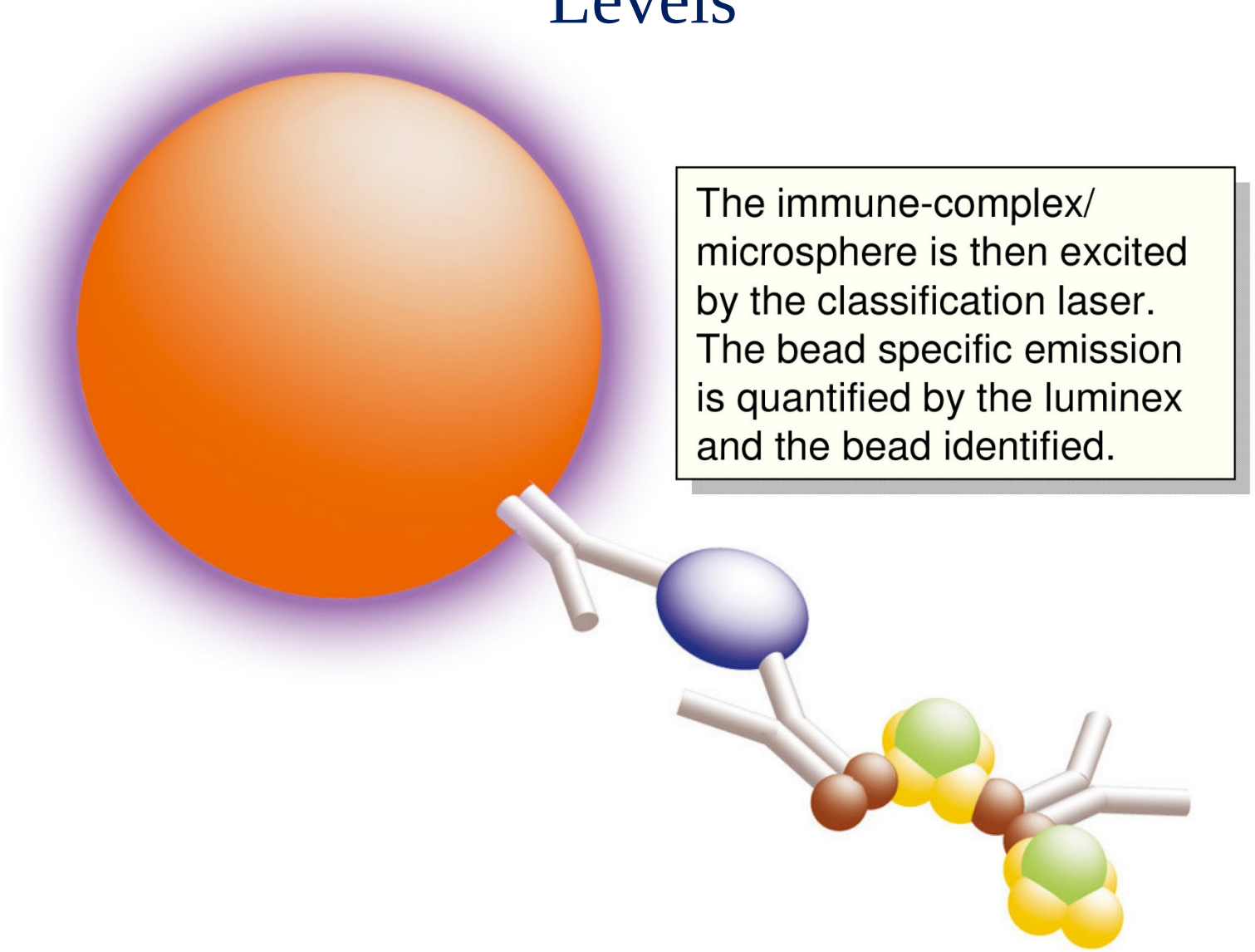
SmartFlare RNA Detection:

- Oligonucleotide gold nanoparticle
- Detects mRNA following endocytosis
- Binding to target initiates a signal (flare)
- Live cells
- Can be combined with conventional surface staining
- Live cells can be physically sorted

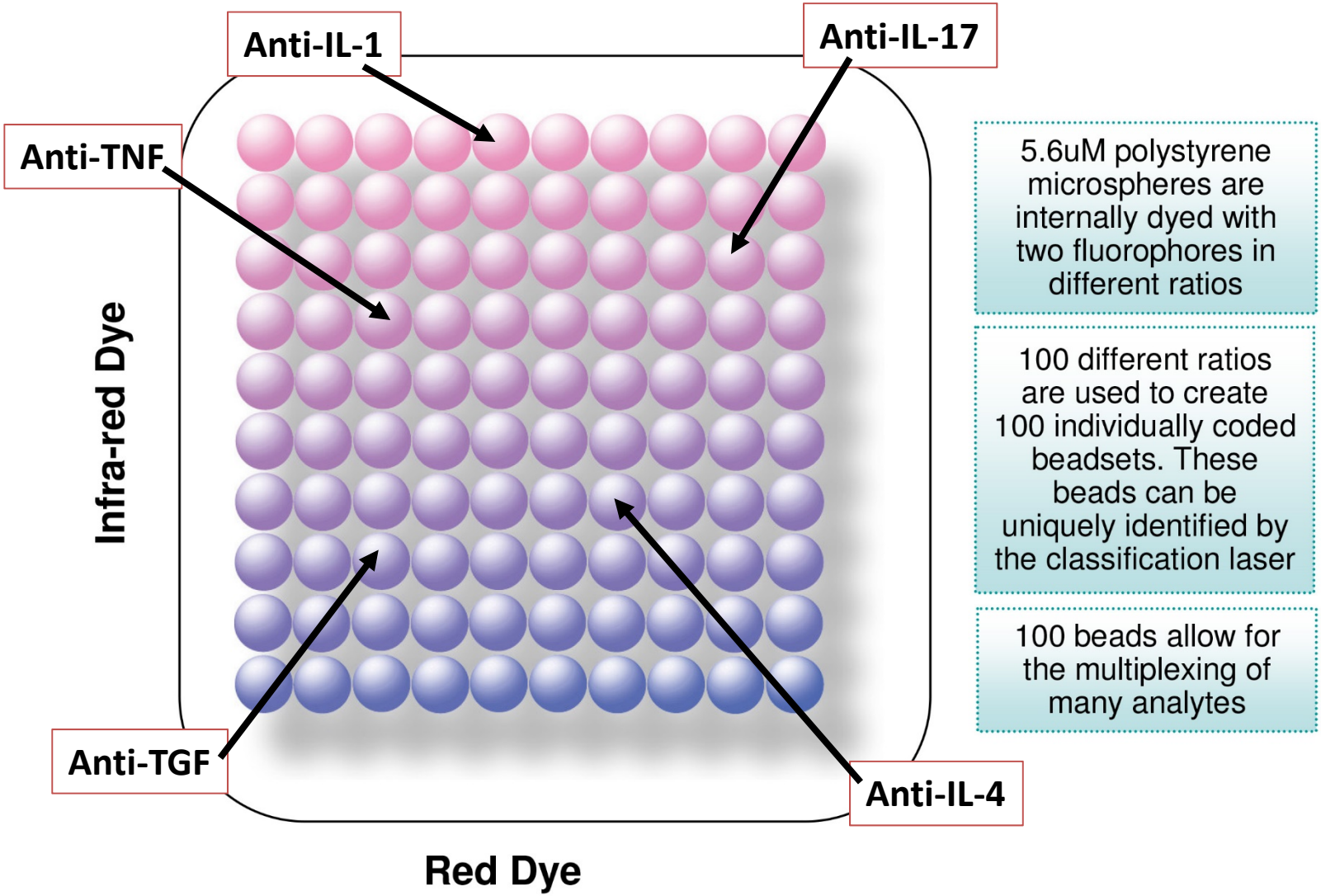
Figure 2.

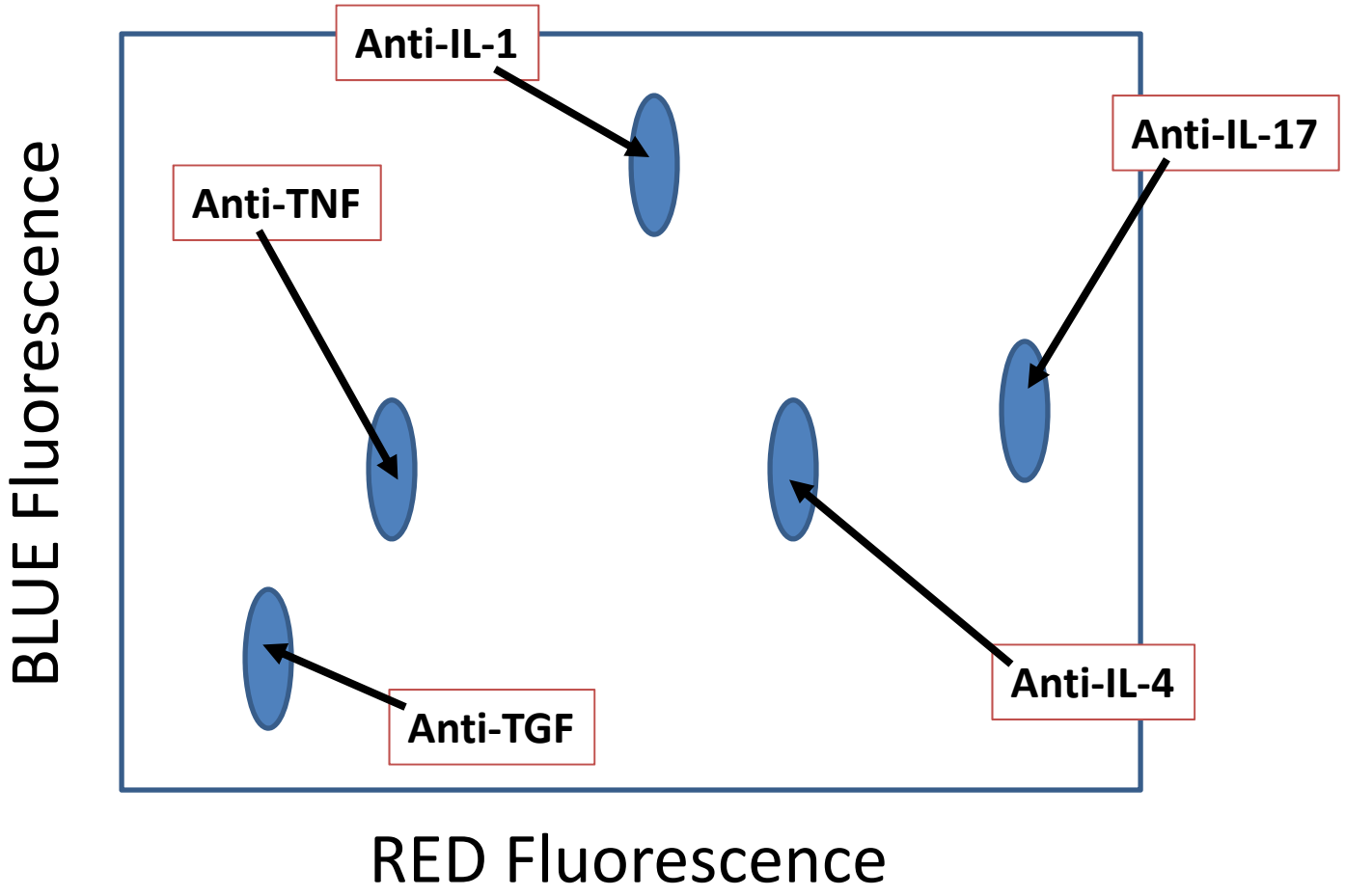
Single cell, multiplexed detection of cell surface EGFR and EGFR mRNA by flow cytometry. SKBR3 (top panels) and MCF7 (bottom panels) cells were loaded overnight with Cy5-labeled SmartFlare™ probes for scrambled control or EGFR. Cells were then stained with Alexa Fluor® 488-conjugated isotype control (left panels) or anti-EGFR extracellular domain (right panels) antibodies, and analyzed on the guava easyCyte™ 8HT flow cytometer. Strong correlation was observed between EGFR mRNA levels and cell surface EGFR levels.

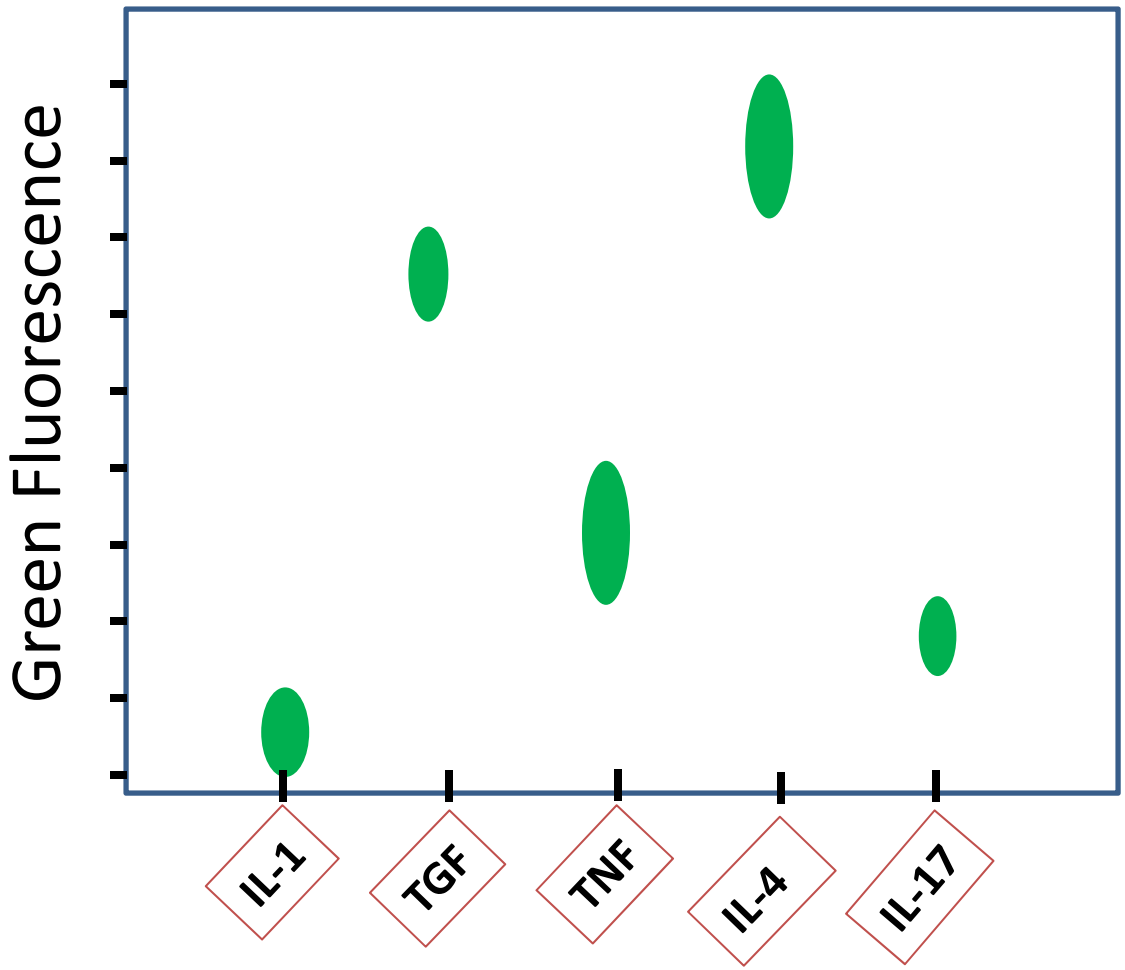
Luminex Analysis to determine Biomarker Levels



Luminex Analysis to determine Biomarker Levels




Anti-IL-1
Anti-IL-17
Anti-TNF
Anti-IL-4
Anti-TGF
BLUE Fluorescence
RED Fluorescence


Green Fluorescence
IL-1
TGF
TNF
IL-4
IL-17

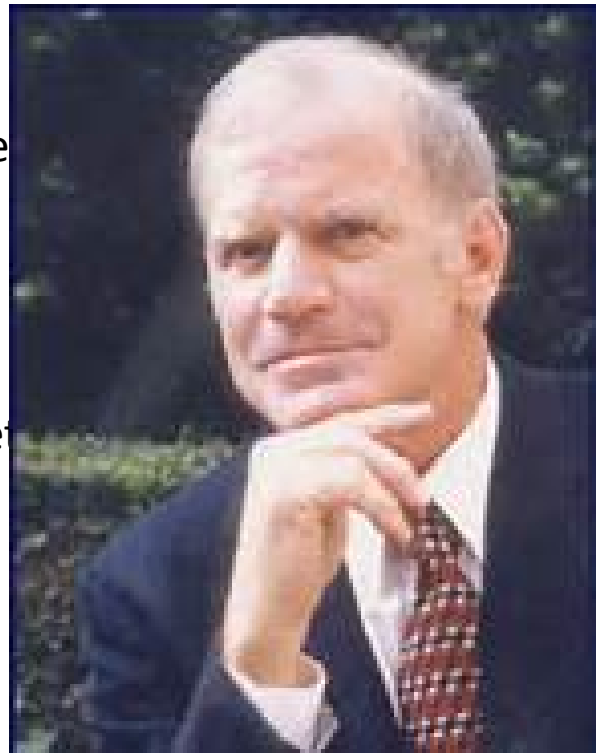
Molecular Techniques in Common Use

1. Polymerase Chain Reaction (PCR)
2. RT-PCR
3. DNA Sequencing
4. Microarray Analysis

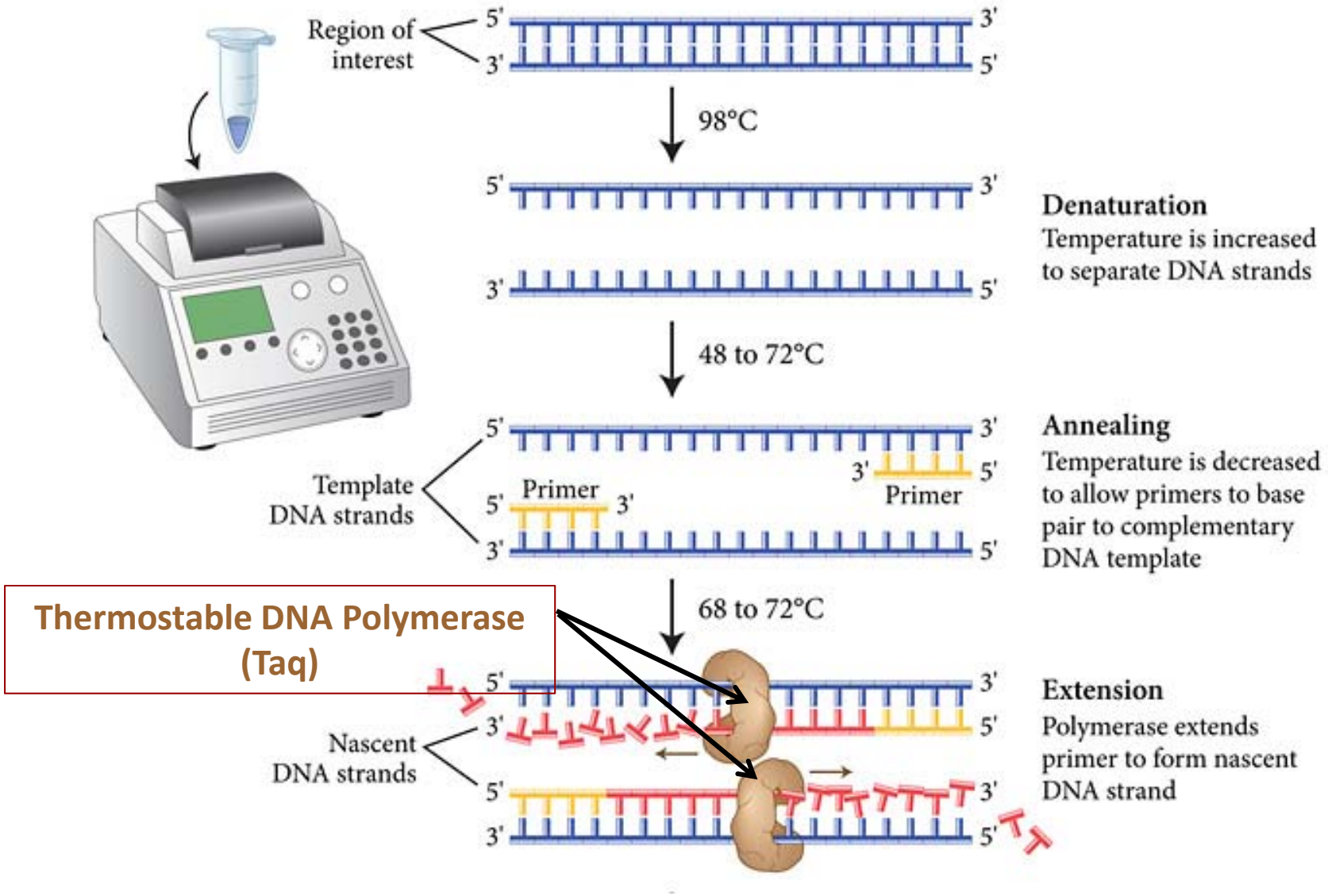
Molecular Techniques in Common Use: Why do we care?

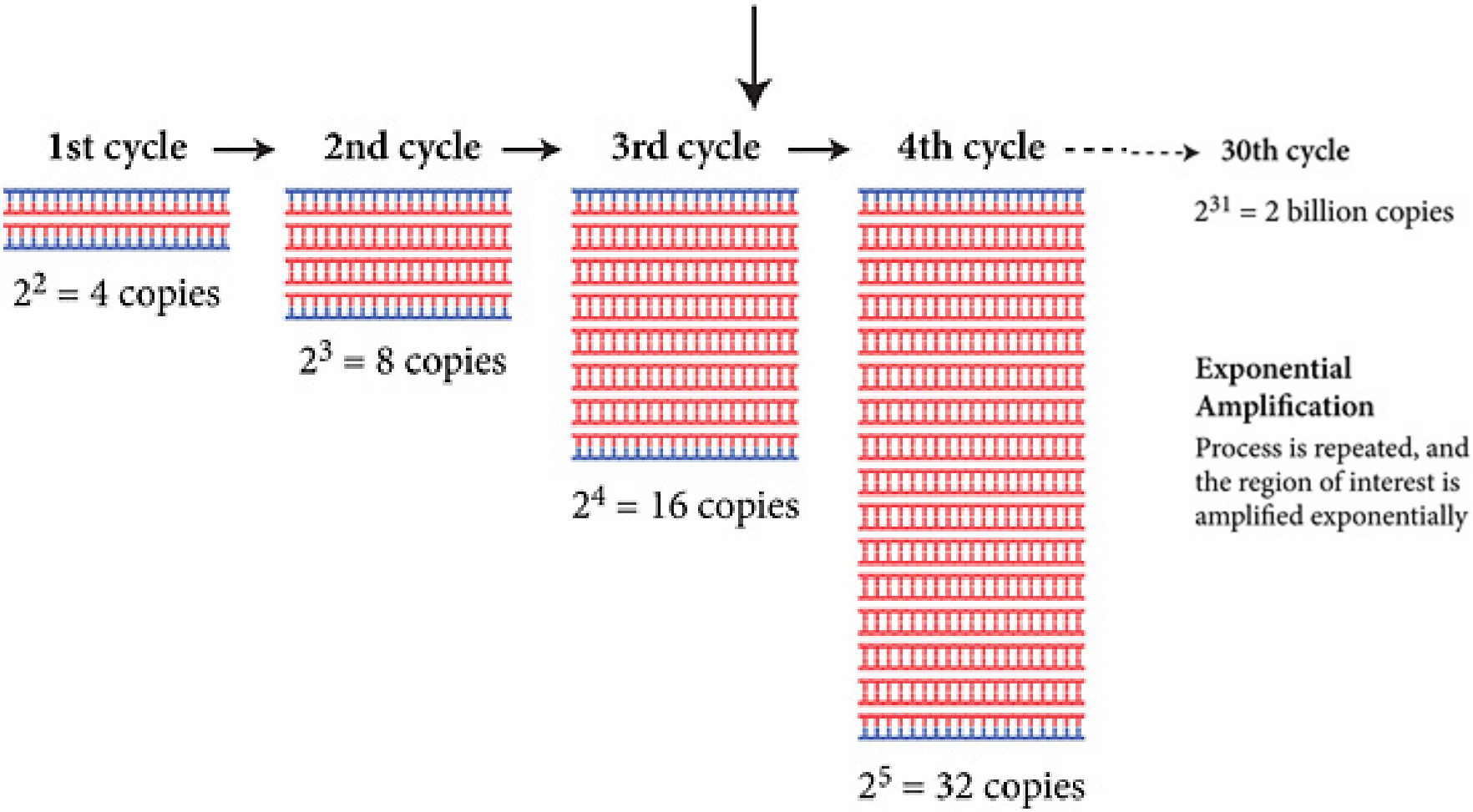
Applications: PCR: Amplify DNA (Kary Mullis; Nobel 1993)

1. Analysis/diagnosis of microbial infe
    1. Simple diagnosis (HIV, others)
    2. Polymorphisms
2. Genotyping
    1. Allele determinations
    2. Polymorphisms (oncogenes, et
    3. Forensic Applications
3. Other techniques
    1. Sequencing
    2. RT-PCR



PCR Overview




1st cycle
2nd cycle
3rd cycle
4th cycle
30th cycle
2^2 = 4 copies
2^3 = 8 copies
2^4 = 16 copies
2^5 = 32 copies
2^{31} = 2 billion copies
Exponential Amplification
Process is repeated, and the region of interest is amplified exponentially

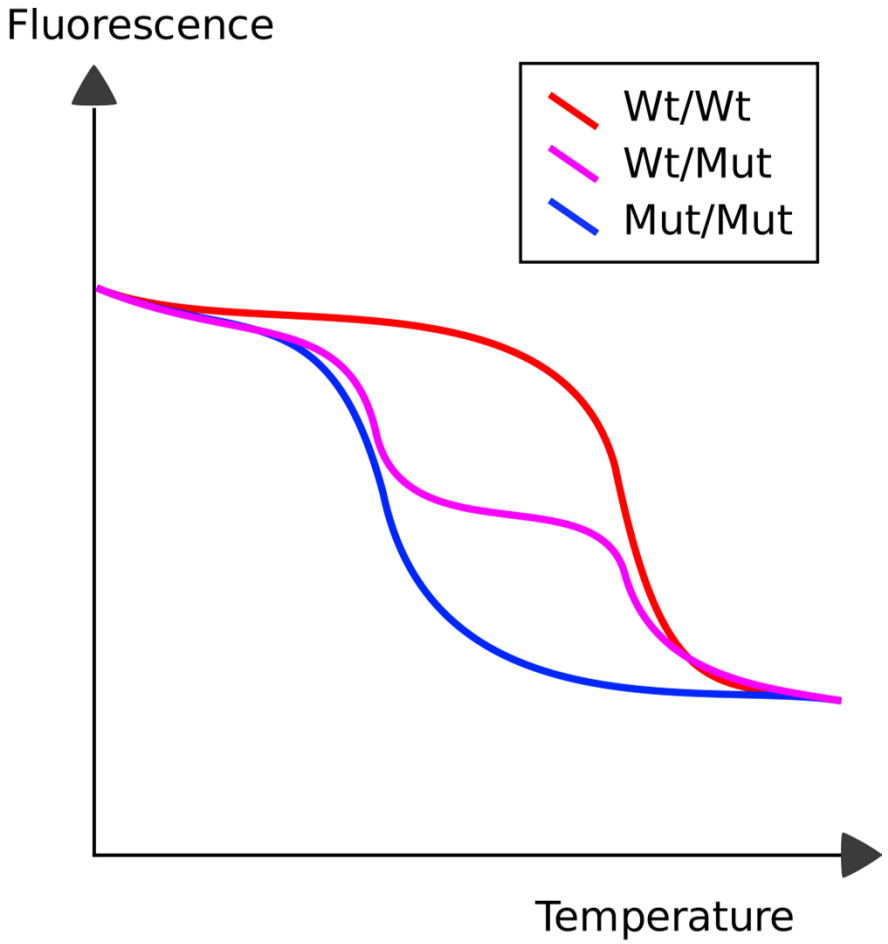
Reverse Transcriptase (RT)-PCR

(RT: Howard Temin and David Baltimore; Nobel 1975)

1. Detect and quantitate mRN
2. Examine the functional acti
3. Can be a substitute for pro
   when protein measurem
4. Diagnostic applications
   a. cancer
   b. microbiology
   c. immunopathology

Detection of Single Nucleotide Polymorphisms (SNP) by RT-PCR

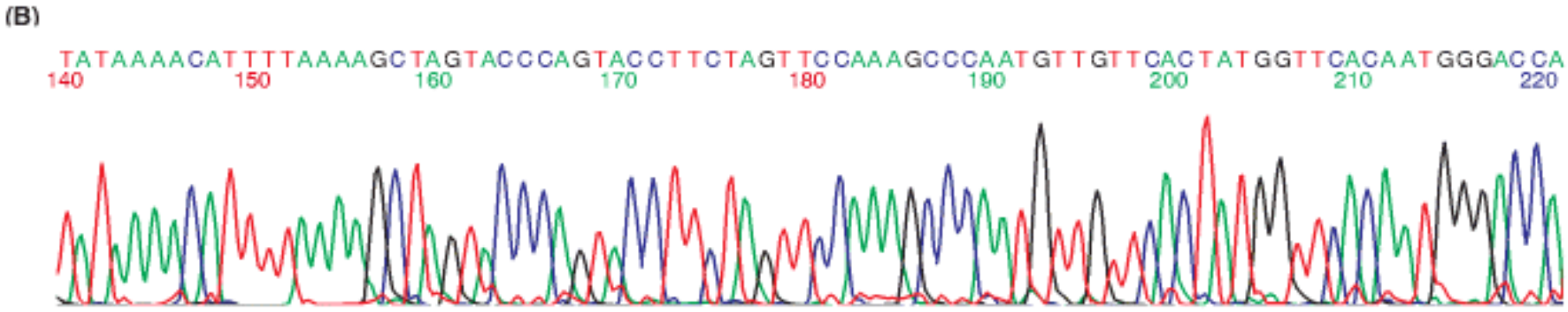
Melting curves for PCR products



Sequencing of DNA by PCR

1. Many techniques utilized, including PCR-based method
2. Provides exhaustive data regarding gene sequences
3. Provides information regarding polymorphisms
4. Has diagnostic value
5. Limitations are rapidly declining with advancing technology

Sequencing of DNA by PCR

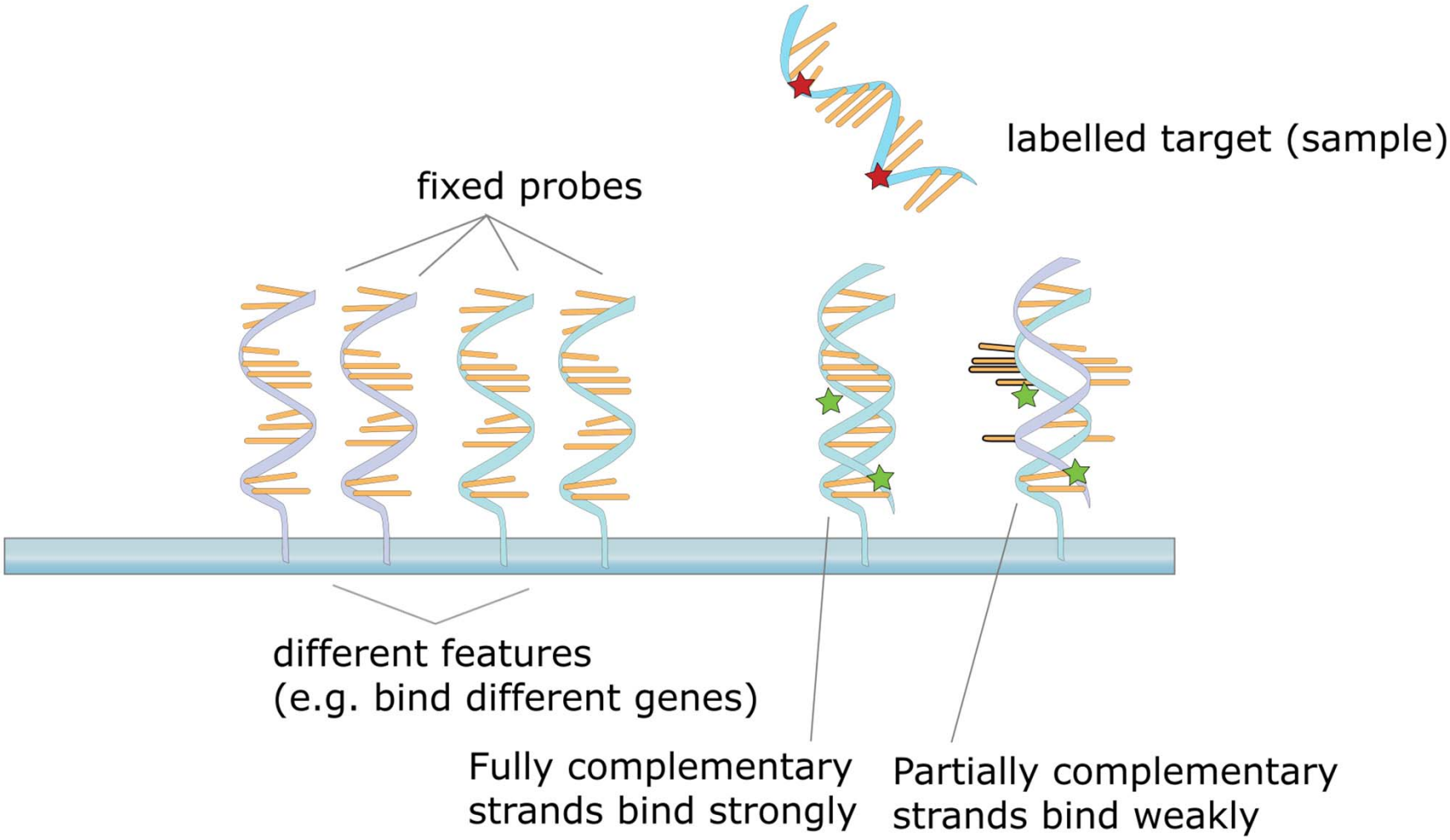


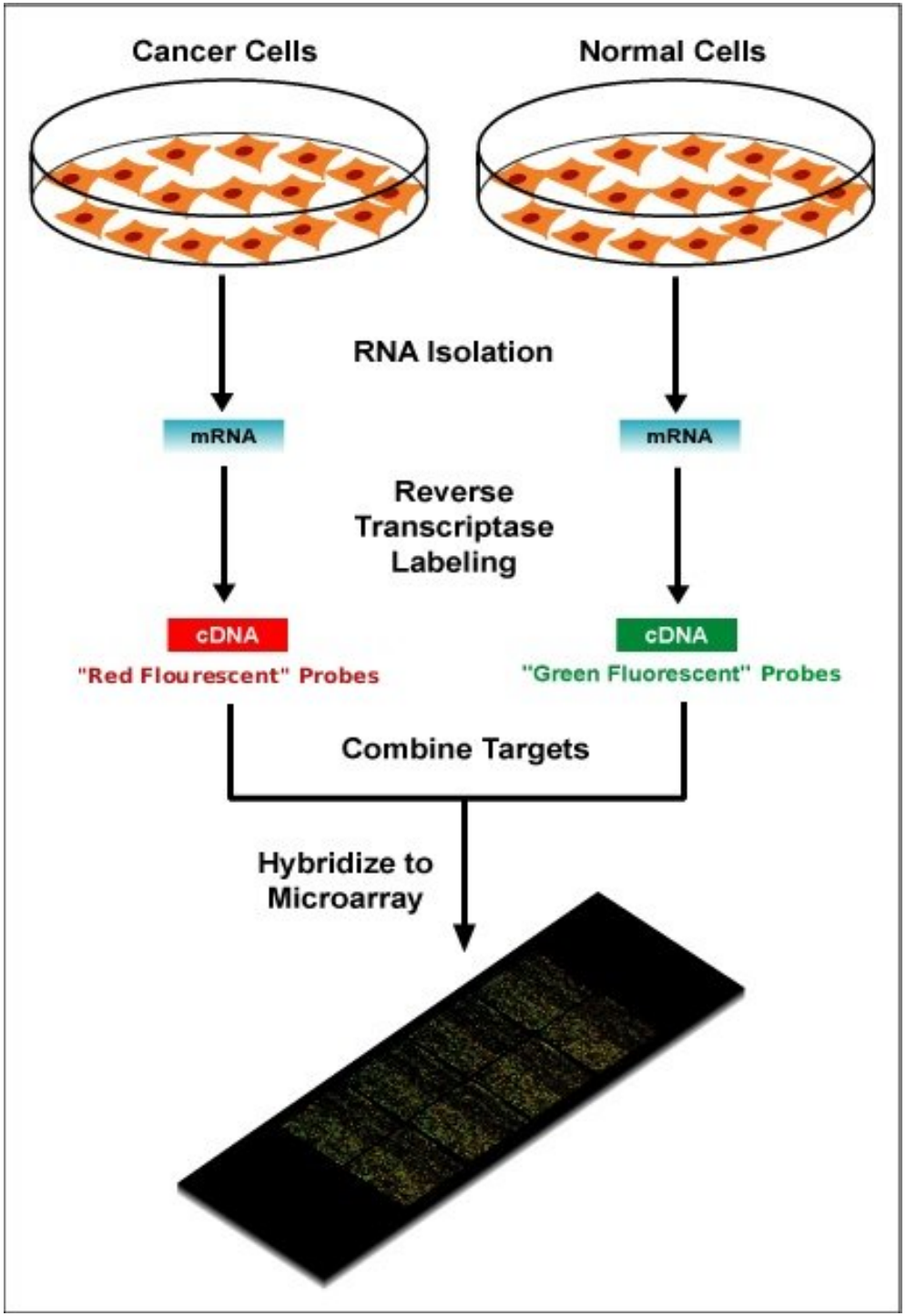
Large Scale mRNA Analysis: cDNA Microarray Analysis

(Howard Temin and David Baltimore; Nobel 1975)

1. Detect and quantitate mRNA – *FOR ALL GENES*
2. Examine the functional activity of cells
3. Can be a substitute for protein methods
   when protein measurements are not practical
4. Diagnostic applications
   a. cancer
   b. microbiology
   c. immunopathology

cDNA Microarray Analysis




Cancer Cells
Normal Cells
RNA Isolation
mRNA
mRNA
Reverse
Transcriptase
Labeling
cDNA
cDNA
"Red Flourescent" Probes
"Green Fluorescent" Probes
Combine Targets
Hybridize to
Microarray

cDNA Microarray Analysis