

# MOD1 – DNA ENGINEERING

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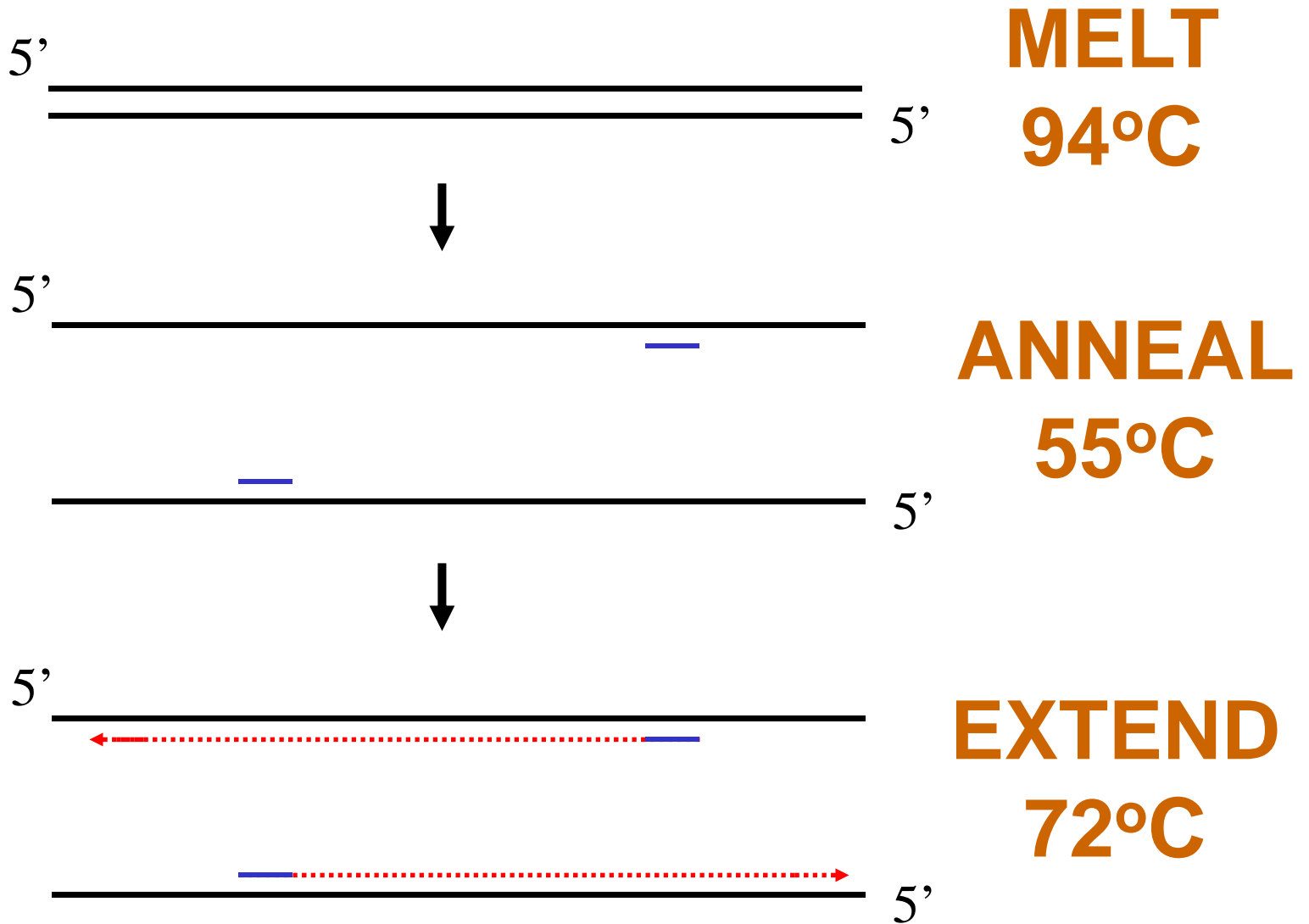
Spring 2008

**Day 8**

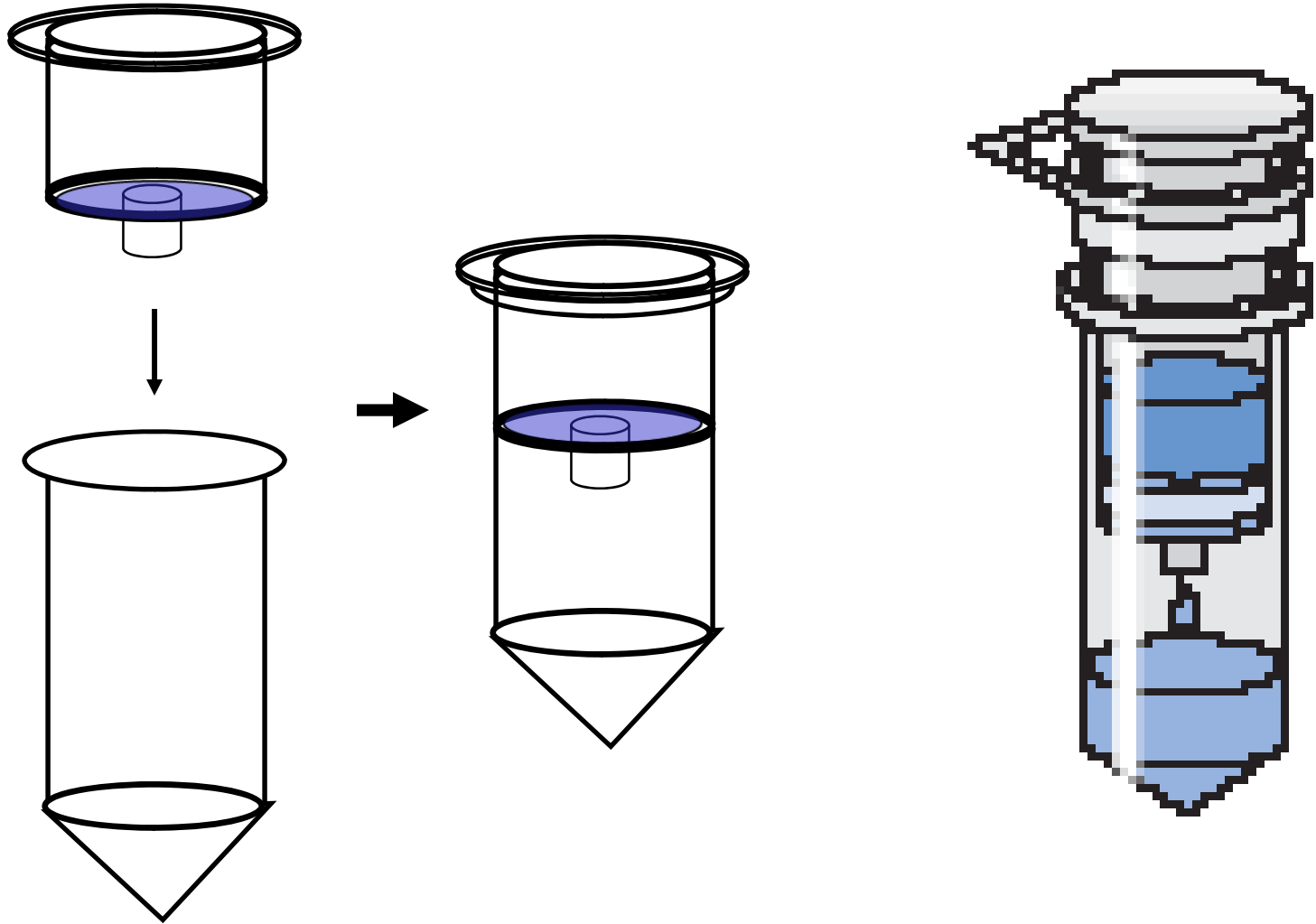
# Module in Review

# Experimental Approaches

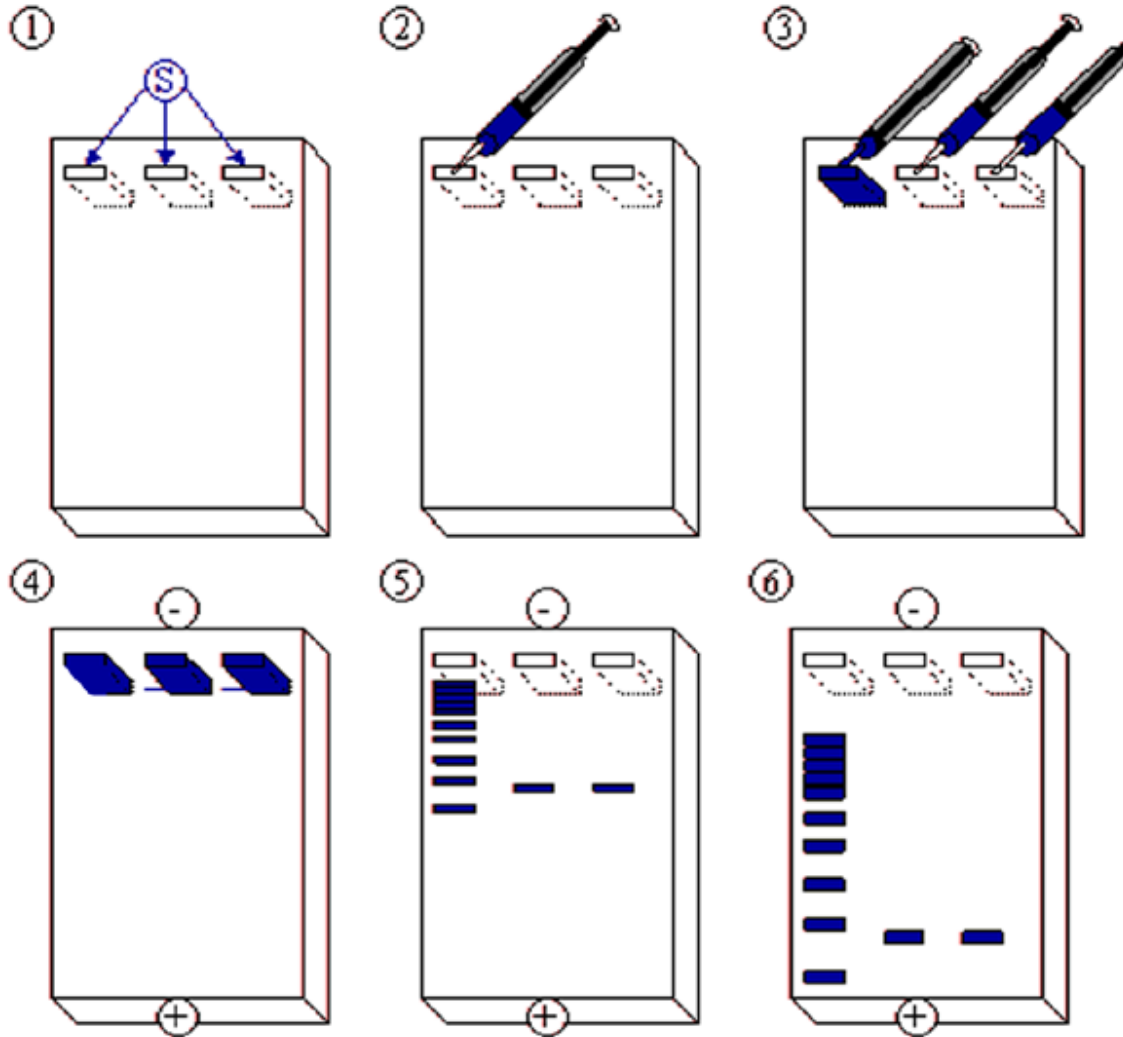
# PCR



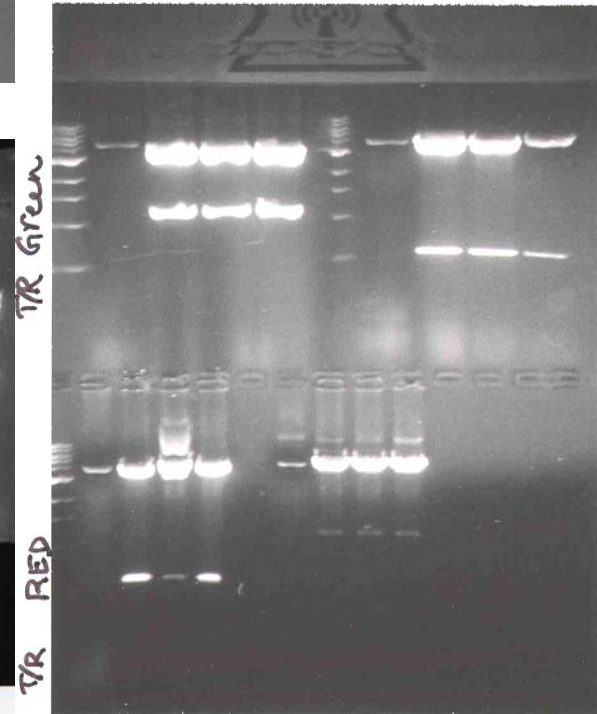
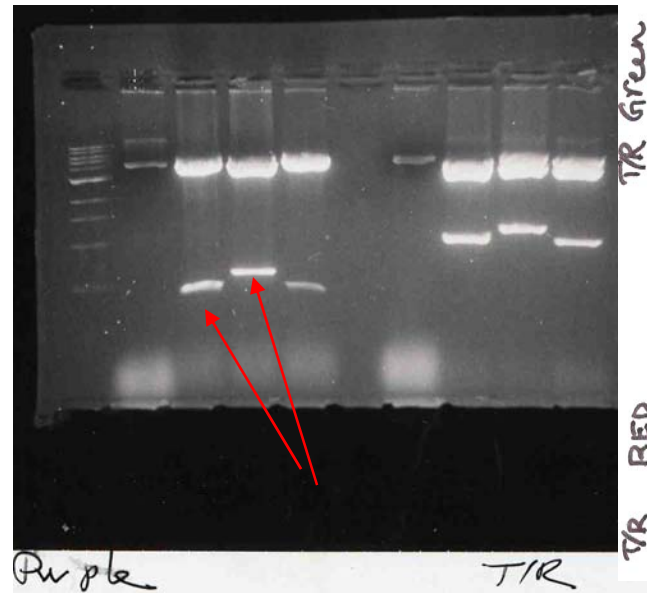
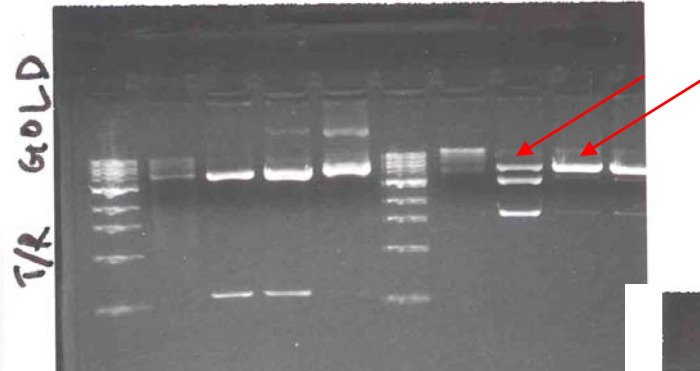
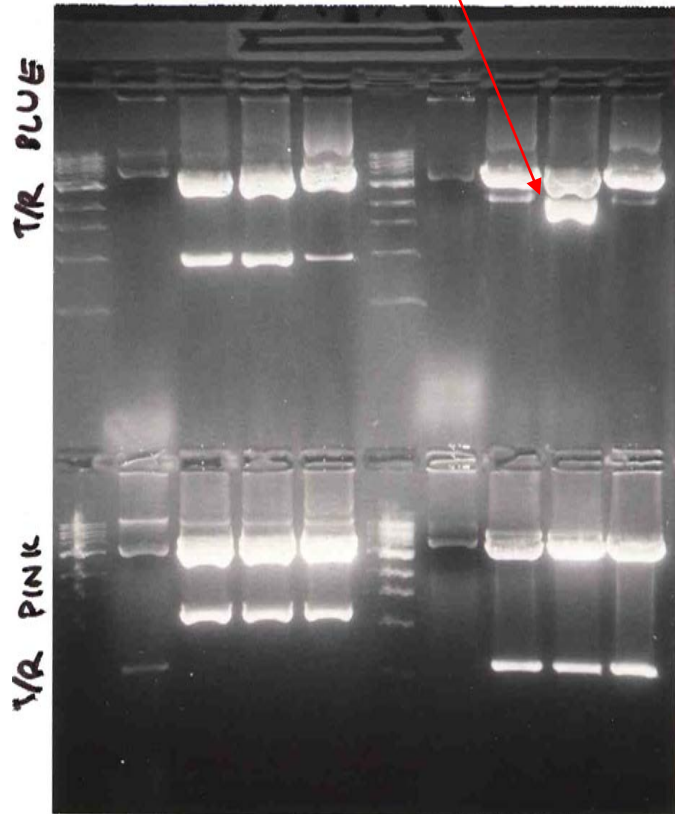
# DNA Purification



# Gel Electrophoresis



# Data Analysis



## Example of appropriate labeling.

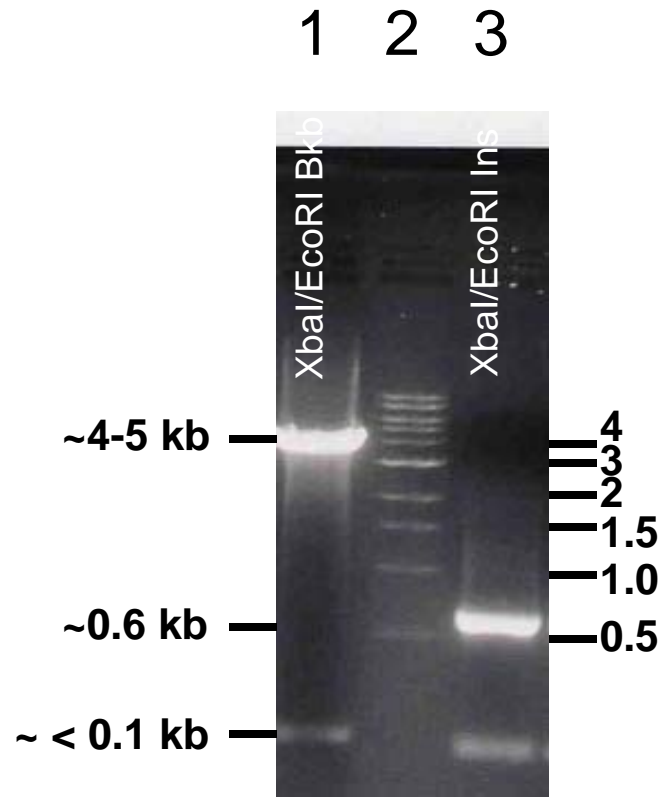
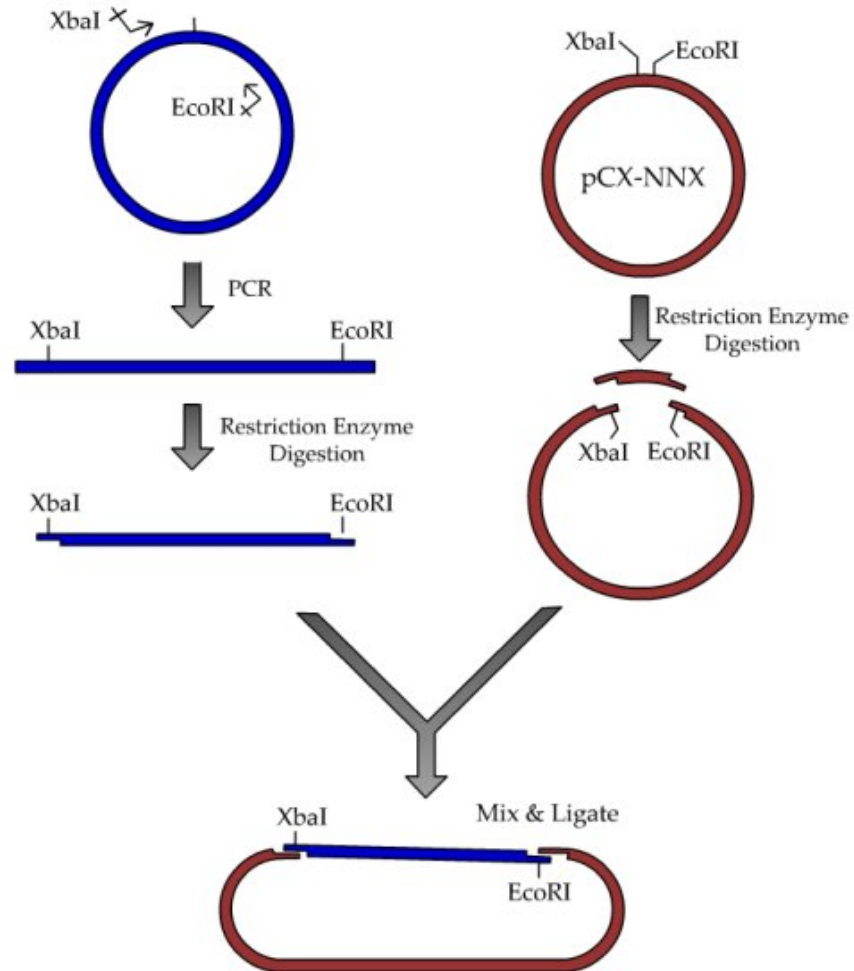


Figure 1. Agarose gel analysis of digested backbone and insert. Bkb, pCX-NNX. Ins, Insert (PCR product of truncated EGFP coding sequence). Restriction enzymes used to digest DNA are indicated.

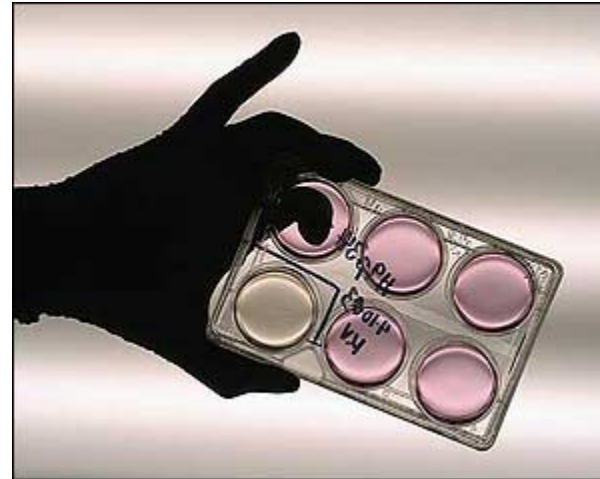
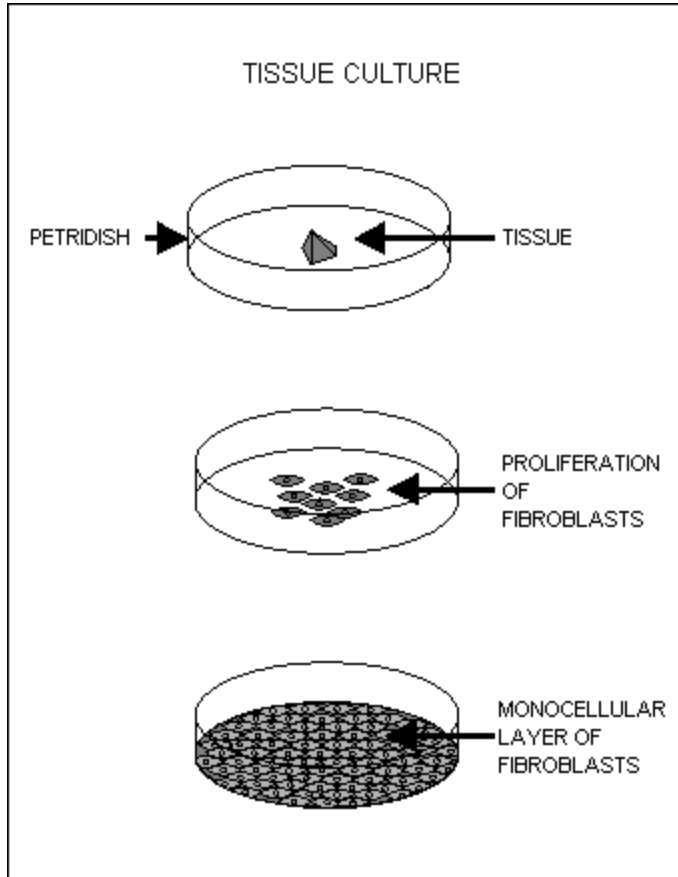


# Ligation

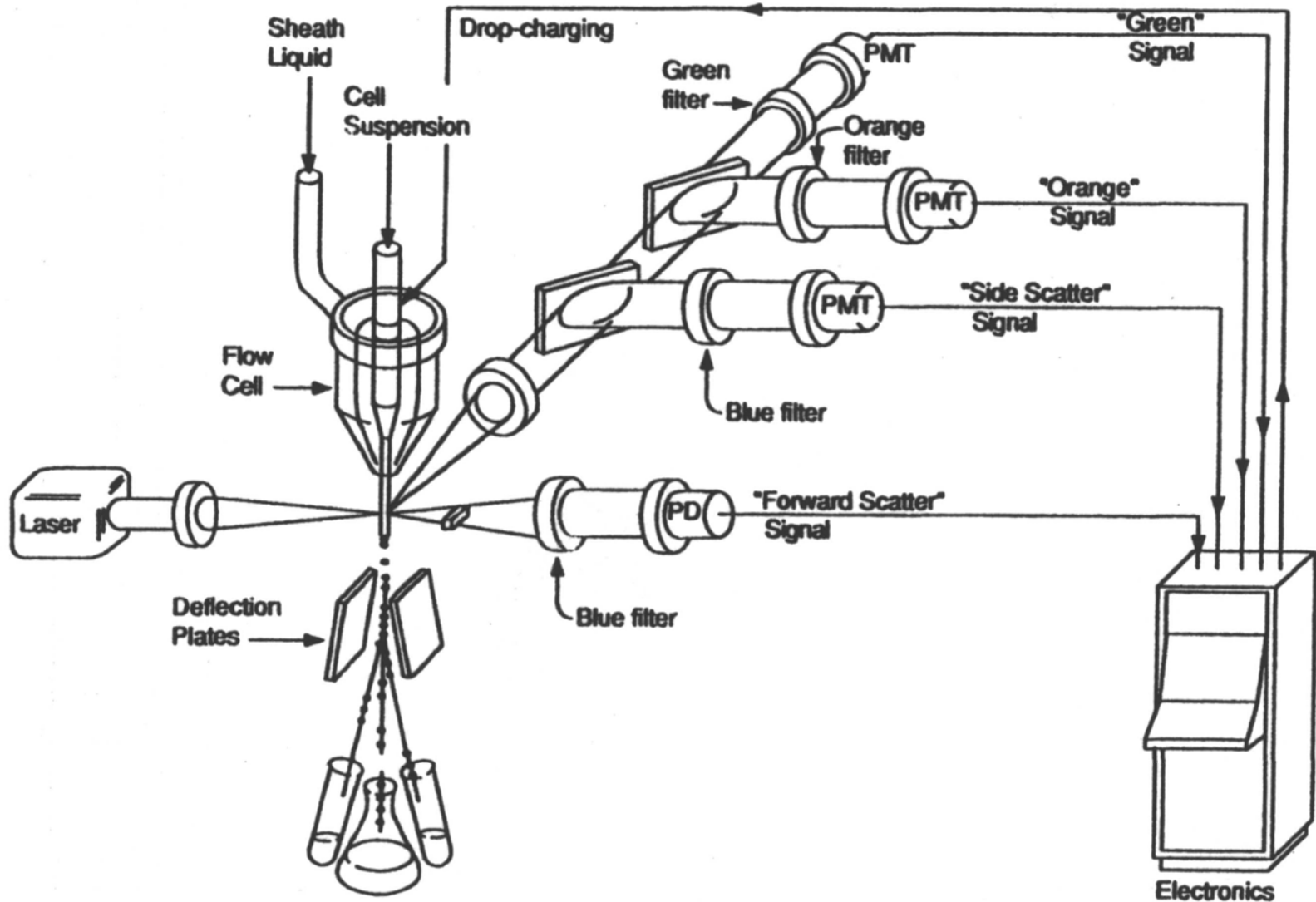
## *Roadmap for Plasmid Construction*



# Mammalian Cell Culture



# Flow Cytometry



# Key Concepts:

**Nothing is 100%**

**Ask 'What else might be happening'?**

**Avoid Assumptions (Controls!)**

**Double Check at Every Opportunity**

**Ask the same question in several ways**

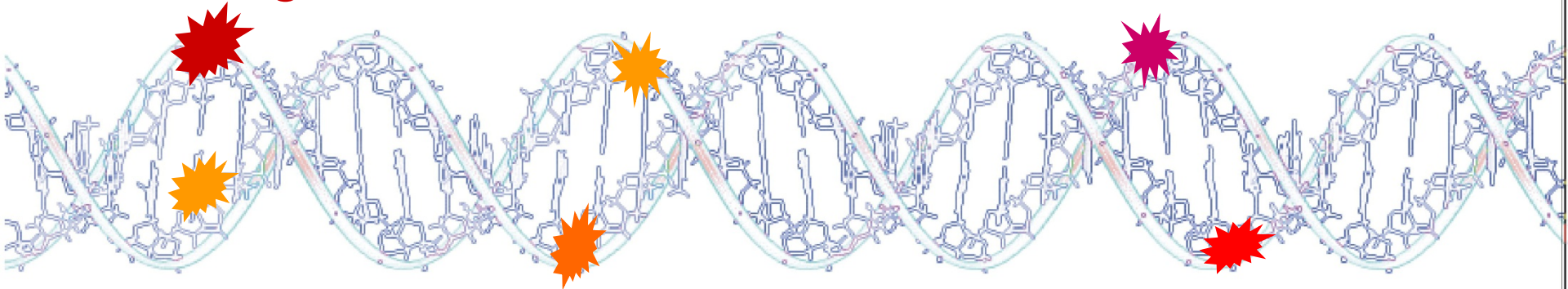
# Biological Principles

# **DNA Damage & Repair via Homologous Recombination**

Sunlight

Radiation

Oxidative  
Radicals



Cigarette  
Smoke

Pollution &  
Food

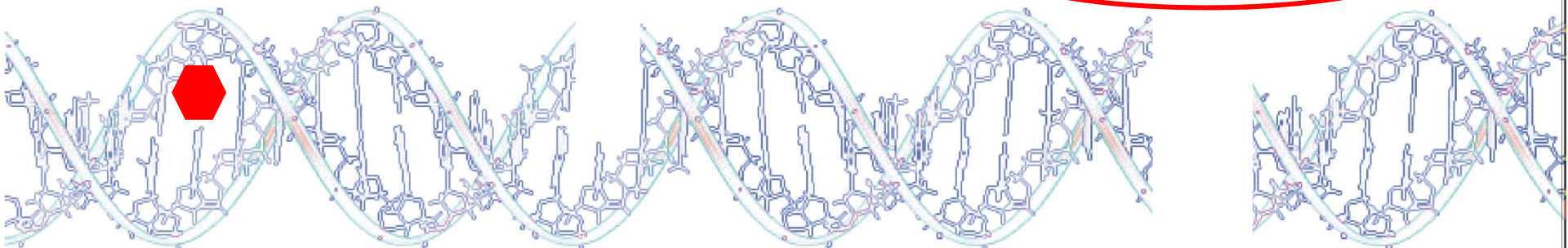
Nitric Oxide



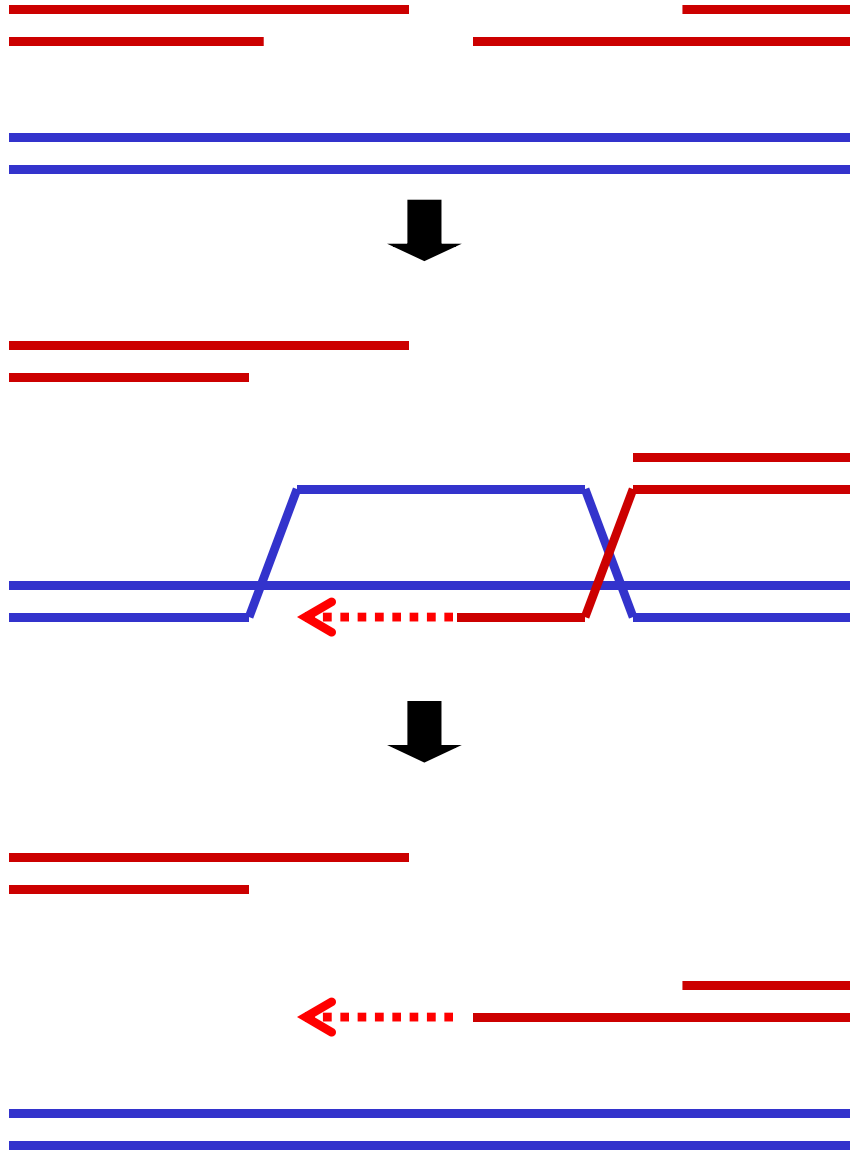
Base Lesions

Single Strand Breaks

Double Strand Breaks

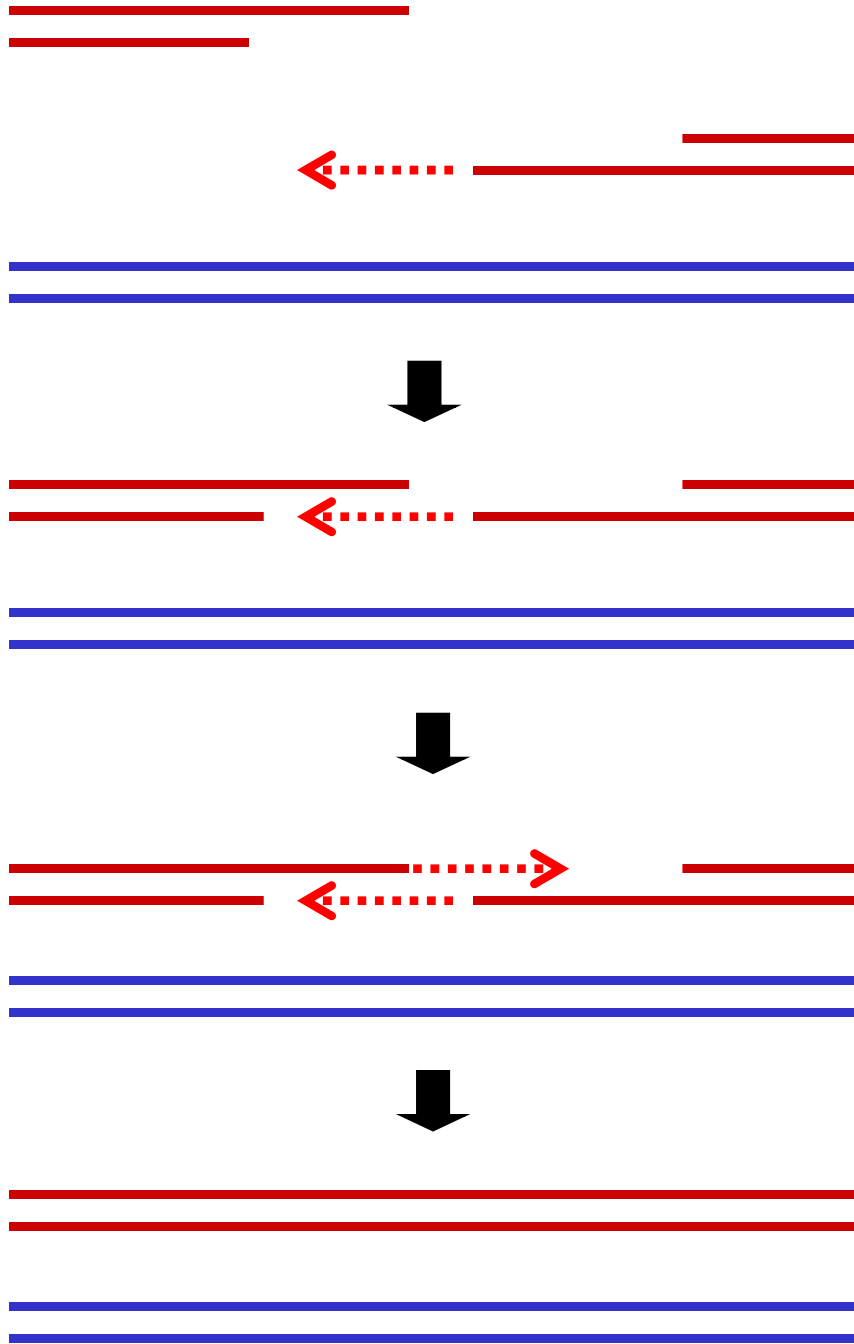


SDSA



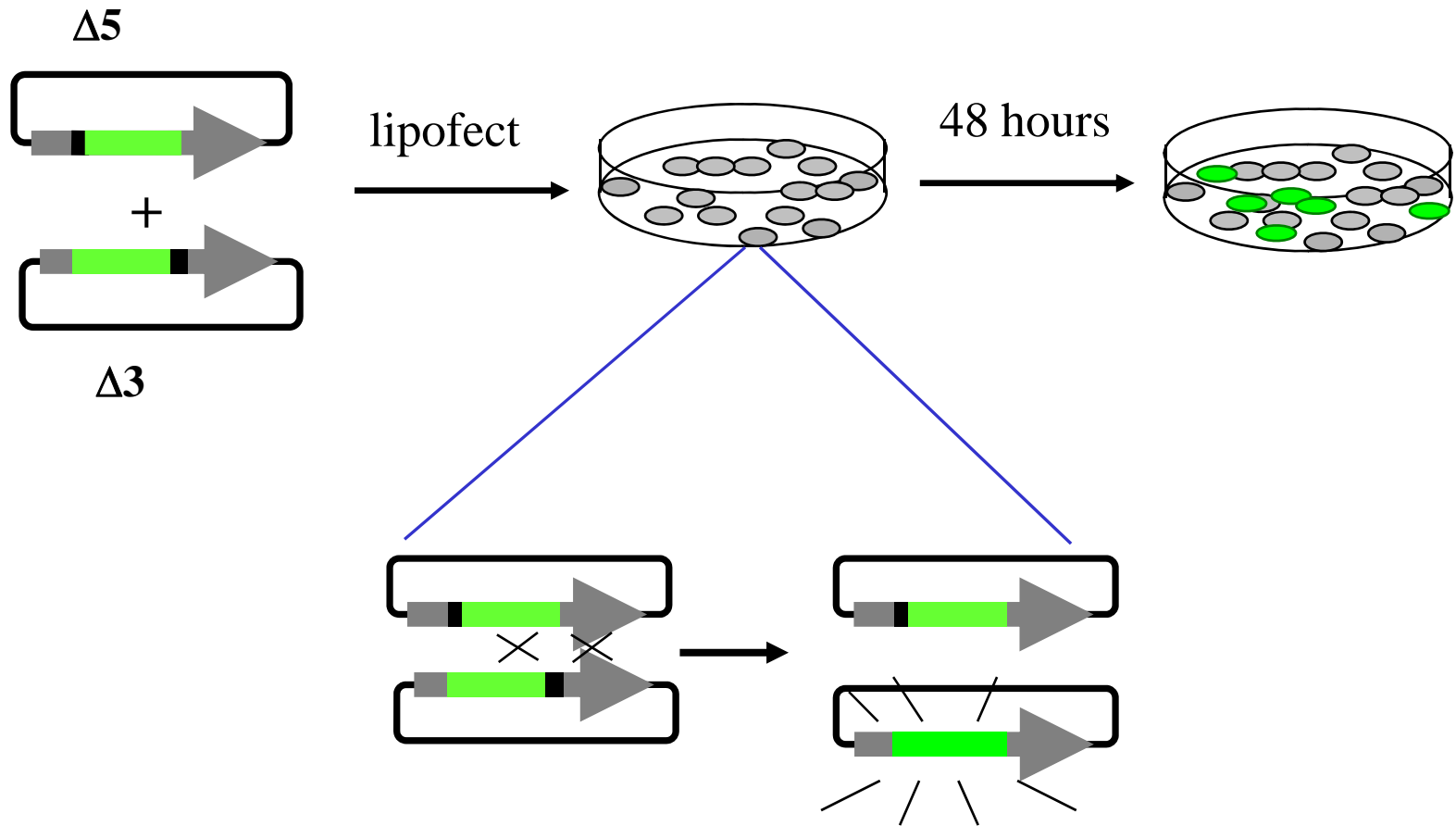


SDSA



# **Engineering an Assay for Homologous Recombination**

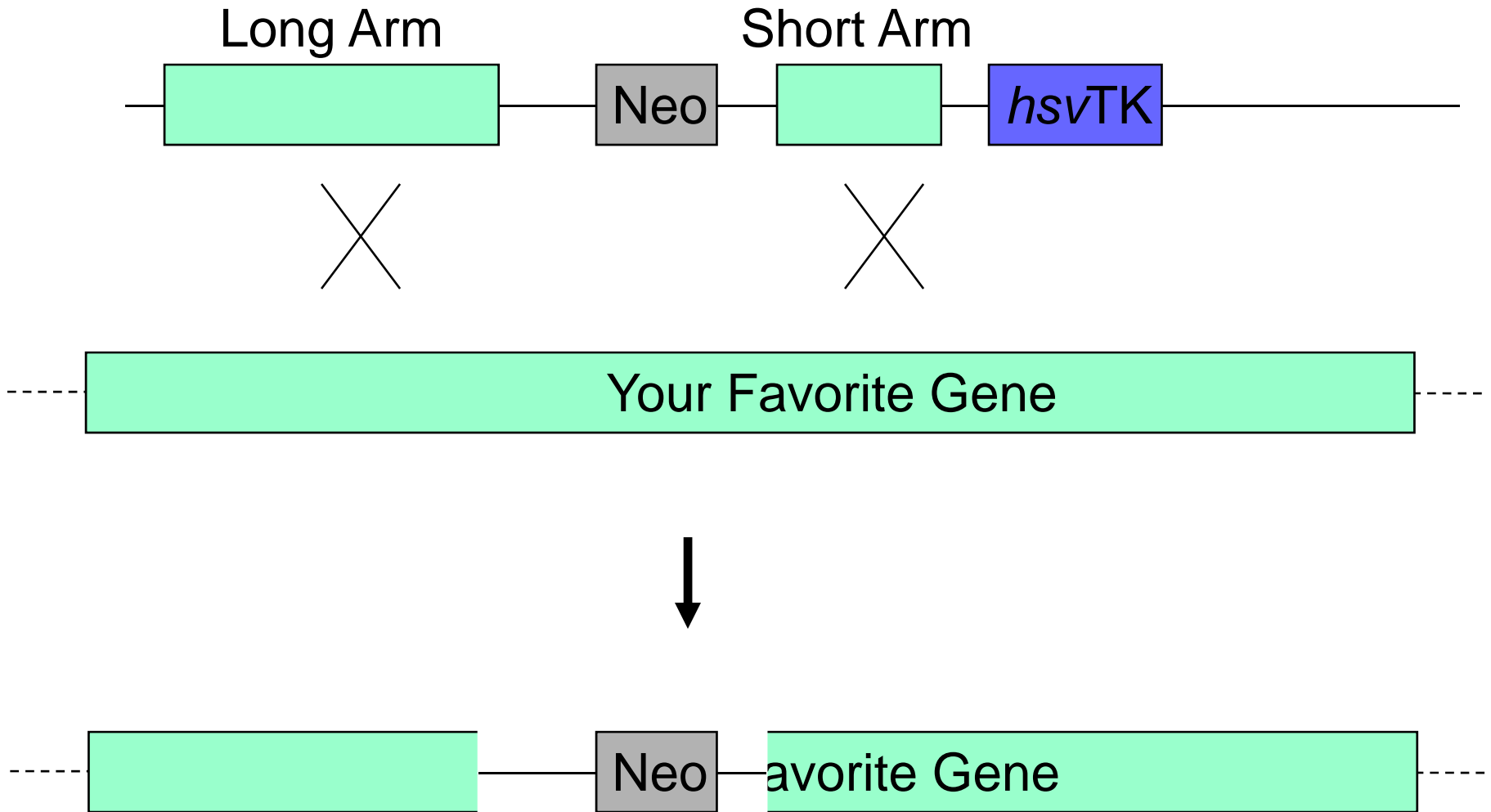
# A Plasmid-Based Assay for Homologous Recombination in Mammalian Cells



# **Exploiting Homologous Recombination for Gene Targeting**

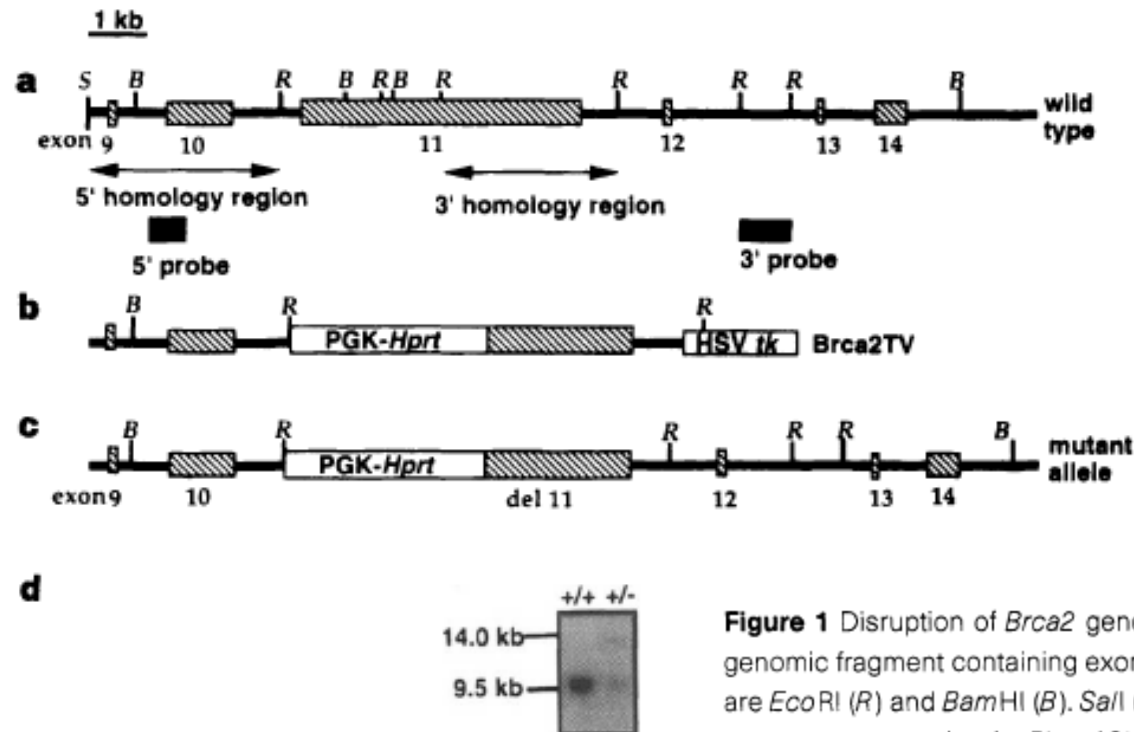
# Traditional ES Knock-Out Technology

## Targeted Homologous Recombination



# Embryonic lethality and radiation hypersensitivity mediated by Rad51 in mice lacking *Brca2*

Shyam K. Sharan<sup>†</sup>, Masami Morimatsu<sup>‡§||</sup>, Urs Albrecht<sup>†</sup>, Dae-Sik Lim<sup>‡☆</sup>, Eva Regel<sup>†</sup>, Christopher Dinh<sup>†</sup>, Arthur Sands<sup>‡</sup>, Gregor Eichele<sup>†</sup>, Paul Hasty<sup>‡</sup> & Allan Bradley<sup>††</sup>

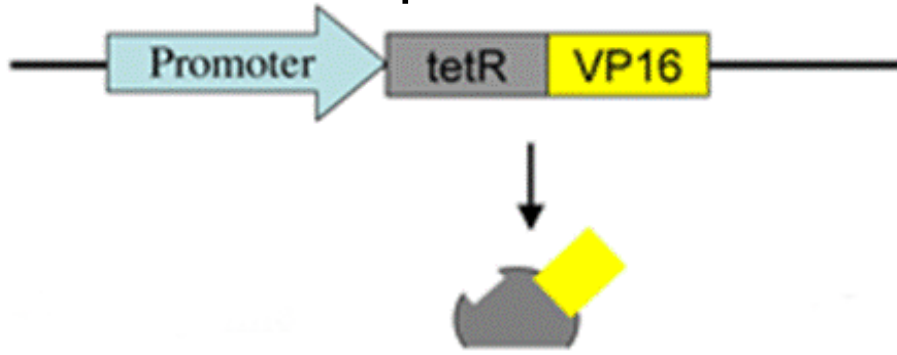


**Figure 1** Disruption of *Brca2* gene in ES cells. **a**, Restriction map of the *Brca2* genomic fragment containing exon 9–15 is shown. Restriction sites shown here are *Eco*RI (*R*) and *Bam*HI (*B*). *Sal*I (*S*) is from the cloning vector. Double-headed arrows correspond to the 5' and 3' homology regions and the dark shaded boxes show the probes used. **b**, Restriction map of the targeting vector p*Brca2*TV. **c**, Expected restriction map of the mutated *Brca2* locus. A 2.8-kb genomic region is deleted and replaced by the 3.6-kb *Hprt* gene. **d**, Southern analysis to identify heterozygous ES cells by digesting genomic DNA with *Bam*HI. The 3' probe detects a 9.5-kb wild-type band and a 14.0-kb mutant band. **e**, Restriction map of

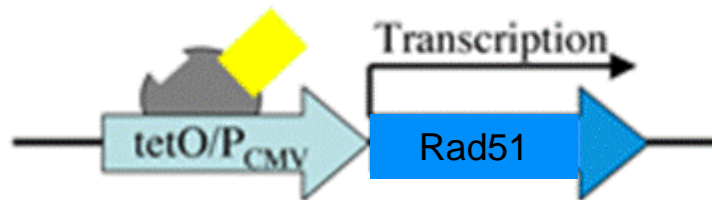
**Engineering  
Inducible  
Gene Expression**

# Tet-Repressible Expression

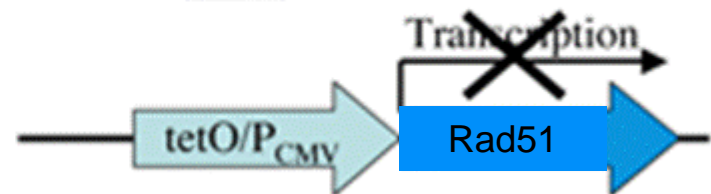
Vector for Expression of Transcription Factor



Add Doxycycline  
(= a tetracycline analog)



Expression On



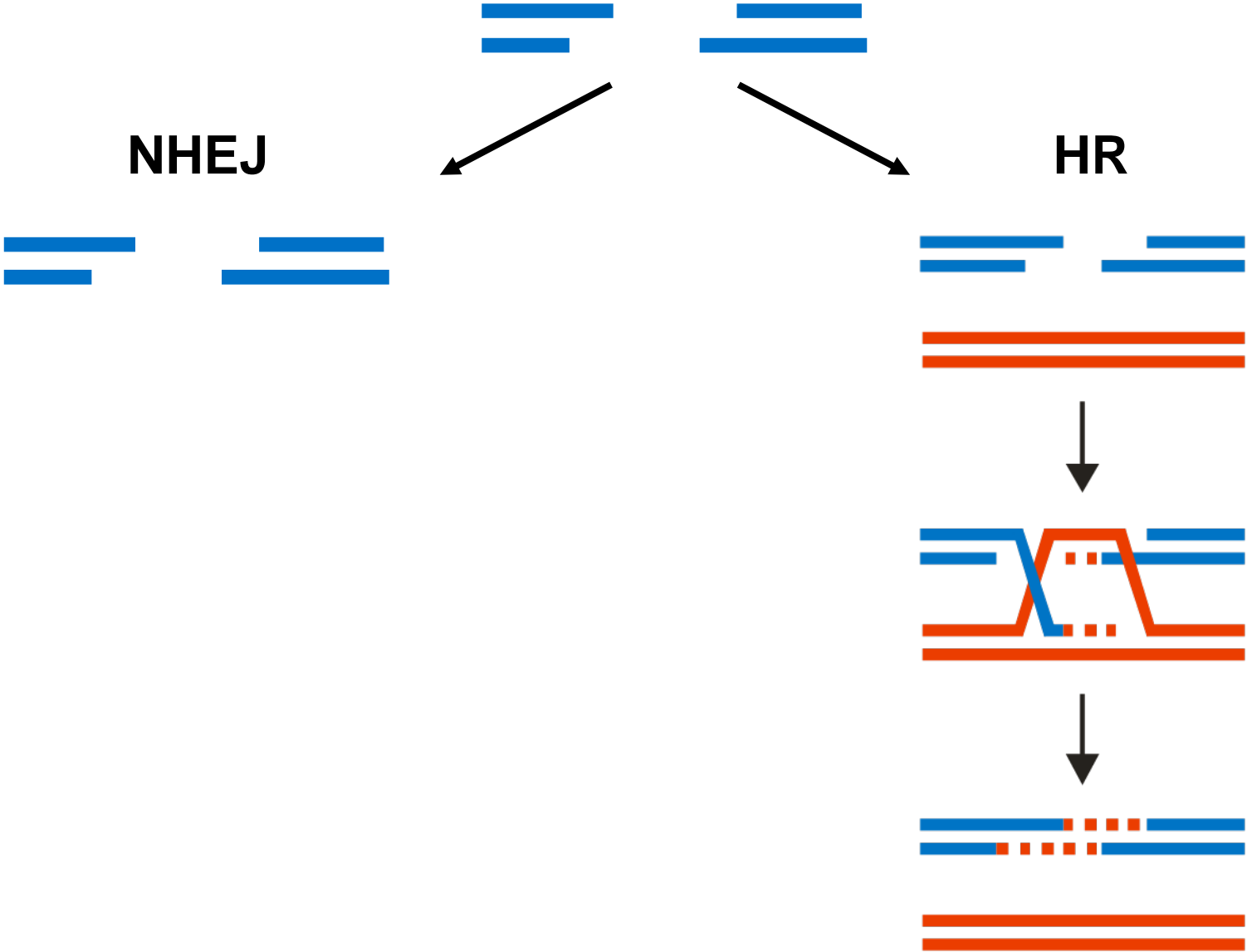
Expression Off



**Maintaining the Genome:**

**Using Homologous  
Recombination to Fix Broken  
Replication Forks**

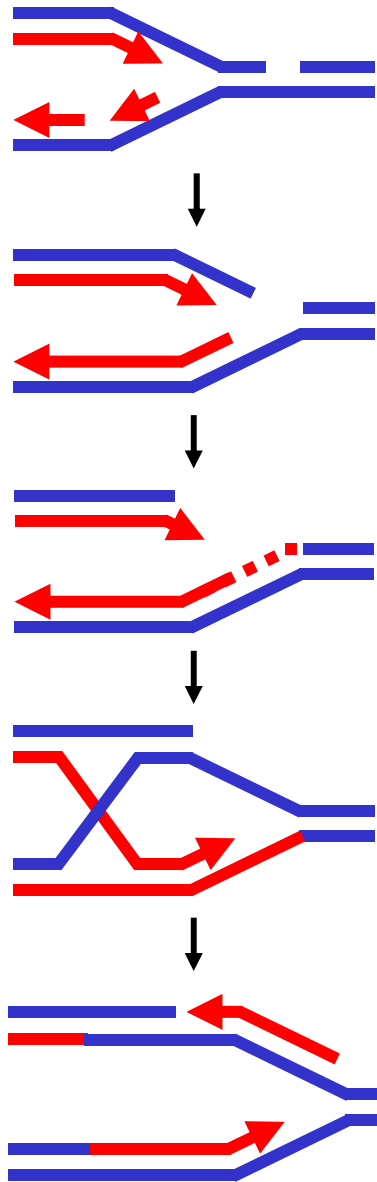
# Double Strand Breaks



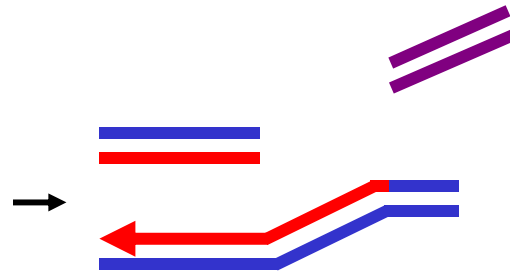
# **Genomic Instability:**

**BRCA2: Without homologous recombination, cells suffer genomic instability**

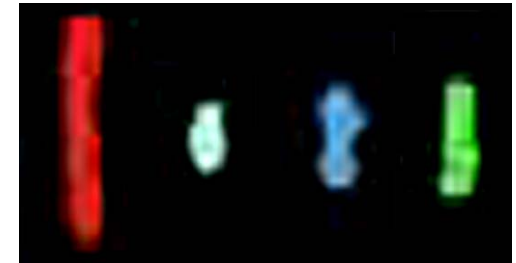
# Too Little HR: Mis-Repair of Broken Forks



HR provides the only pathway to accurately repair broken replication forks



Normal



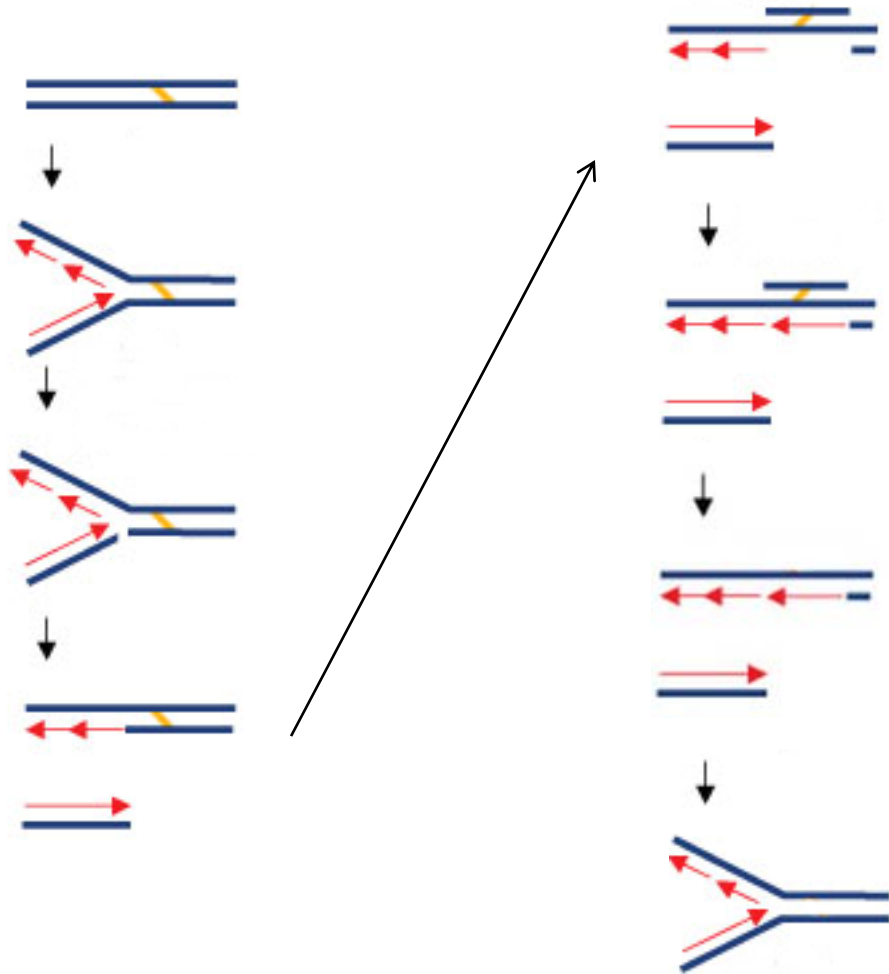
BRCA2 Null

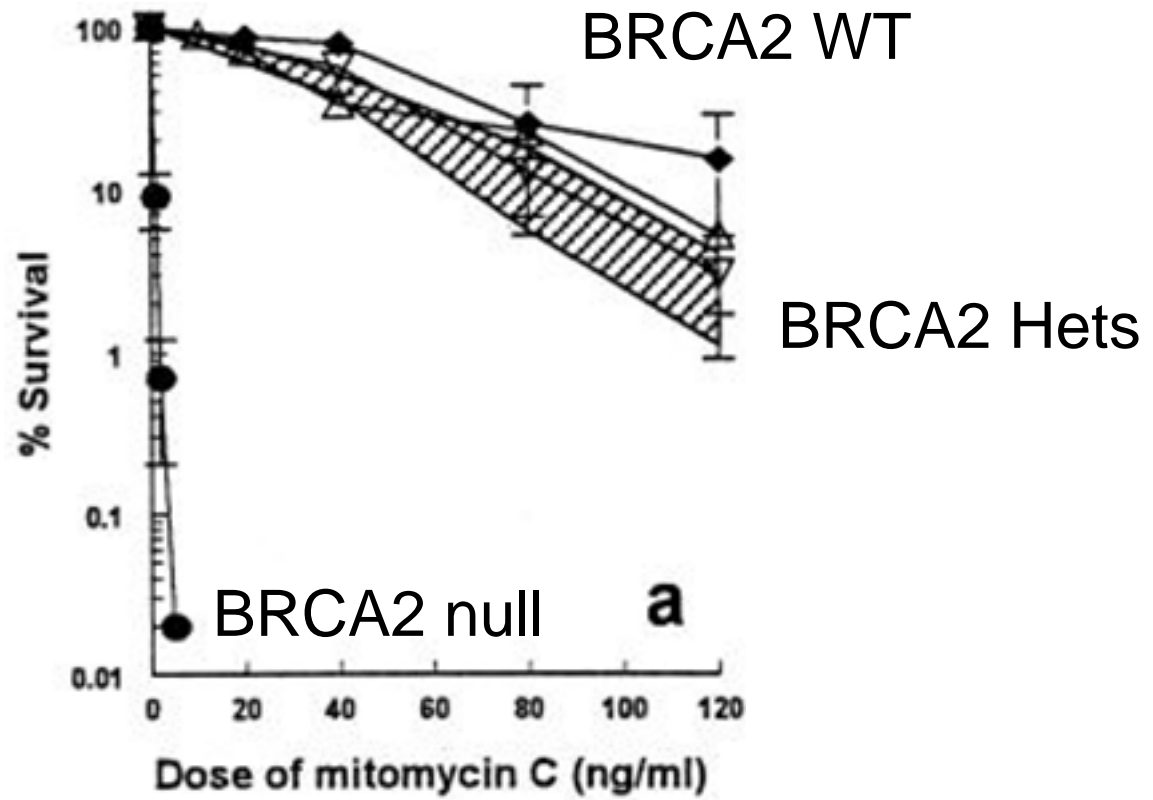


Data from Grigorova *et al.*,  
Cytogen. Gen. Res. 104:333 (2004)

# **Genomic Instability:**

**BRCA2: Without homologous recombination, cells become sensitive to chemo (xlinks)**

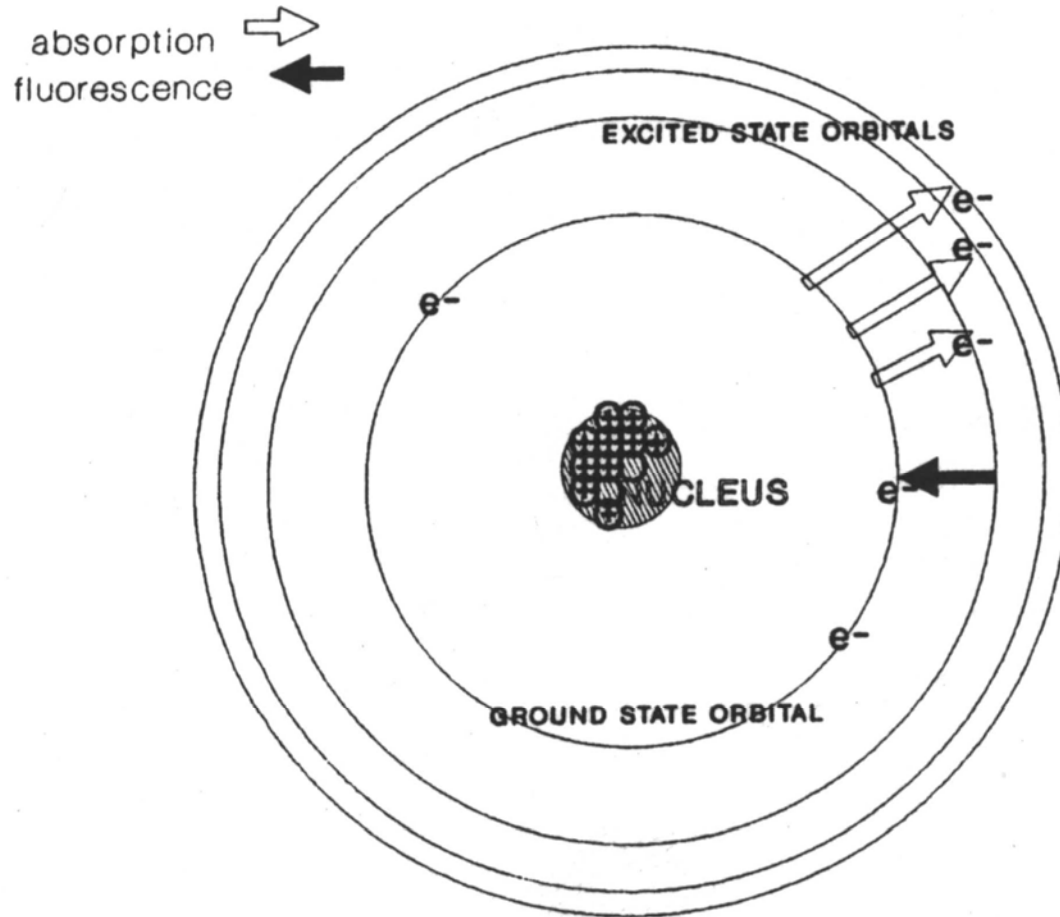




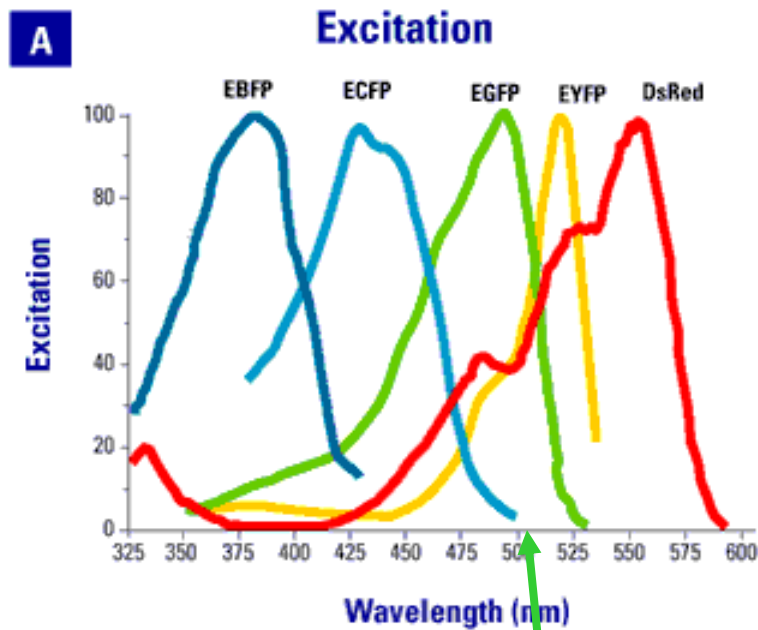
# Flow Cytometry



# Principle of Fluorescence Activation

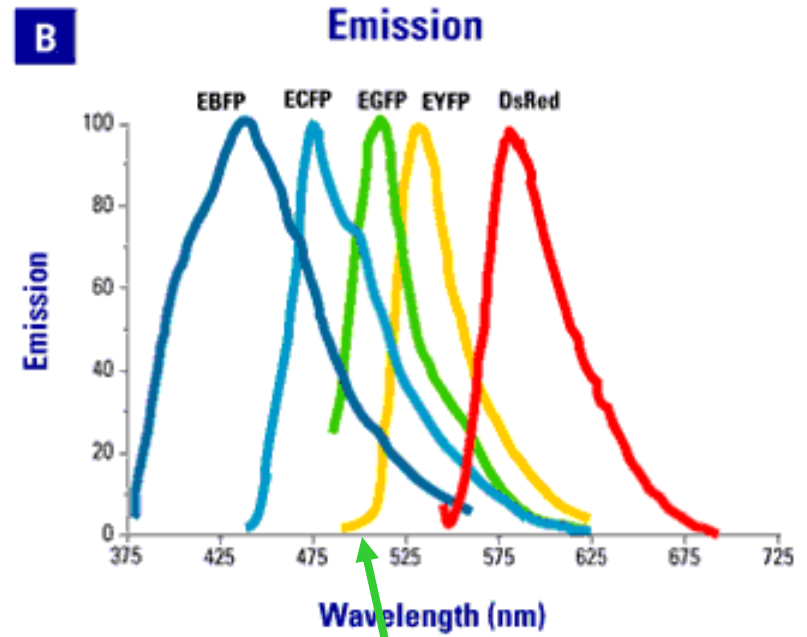


# See Specific Colors By Restricting Ex and Em:



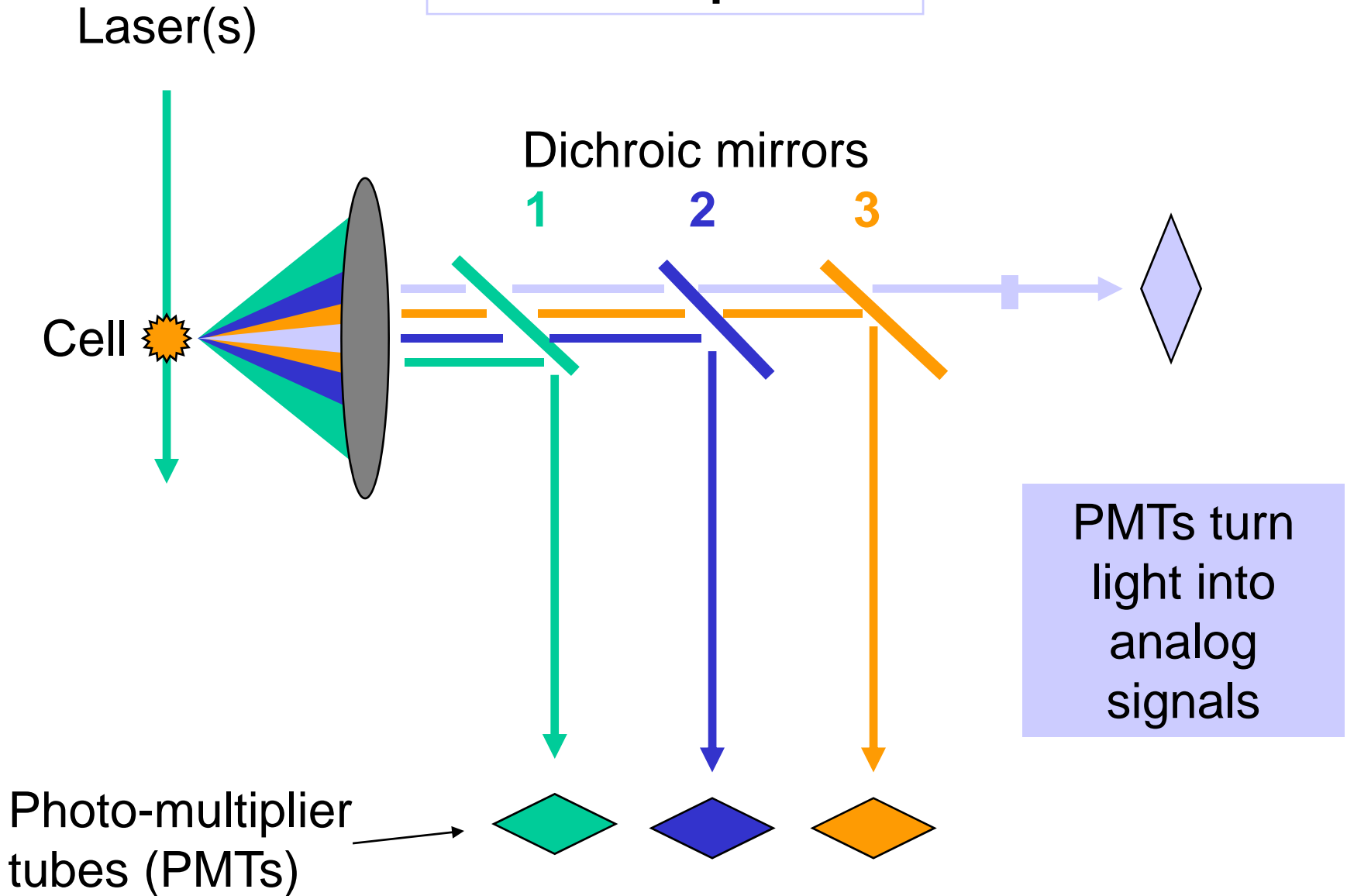
Your laser  
is 488 nm

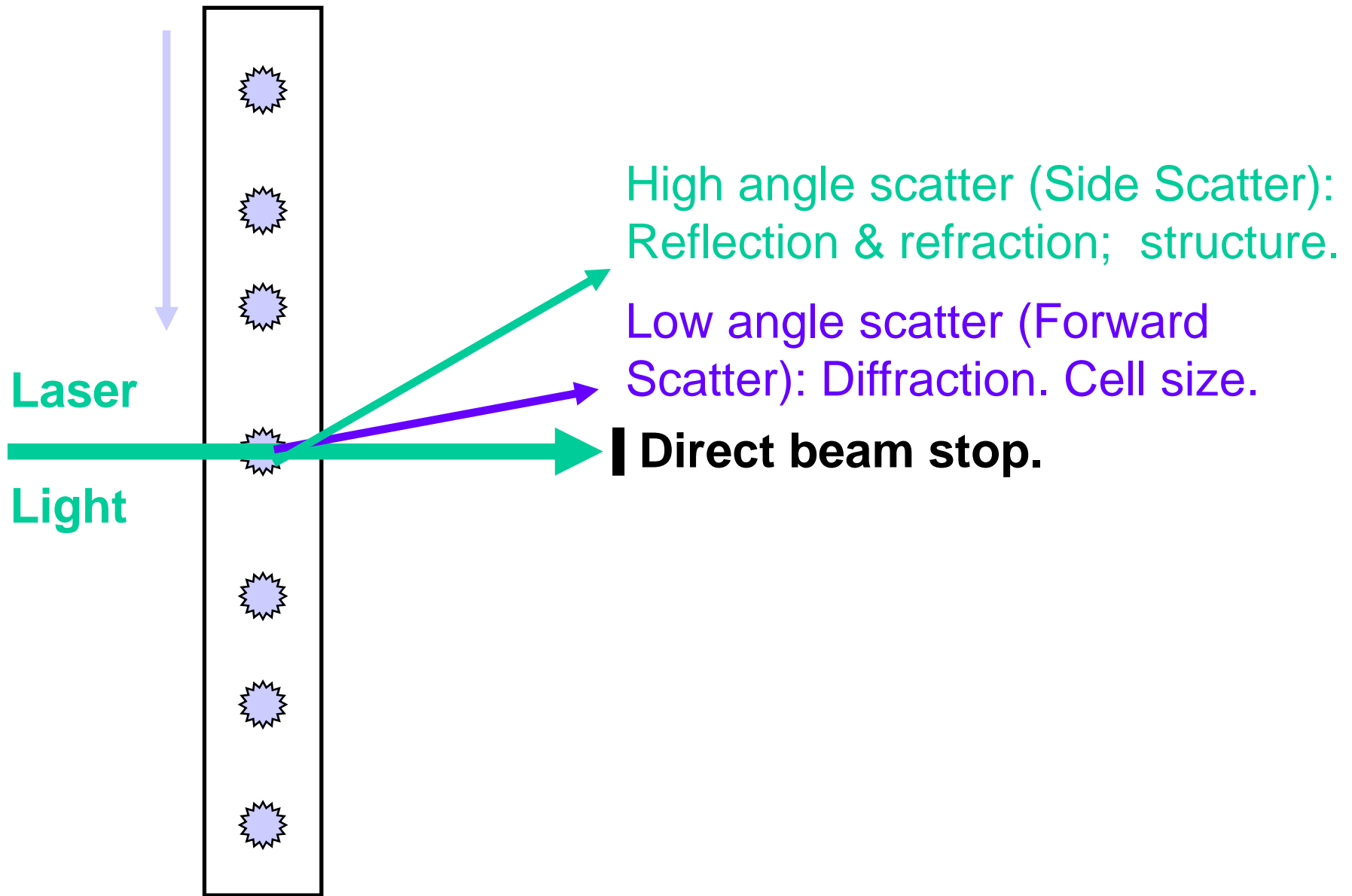
For Green,  
Can excite using <510 nm

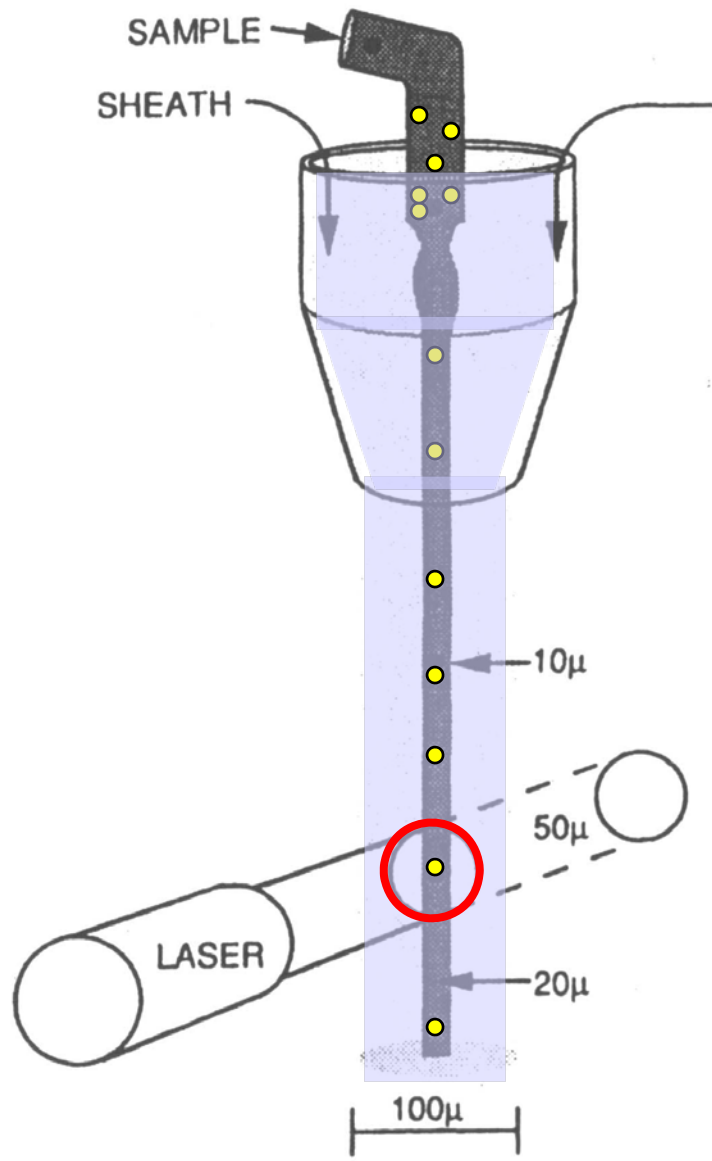


For Green,  
Can capture emission  
when >510 nm

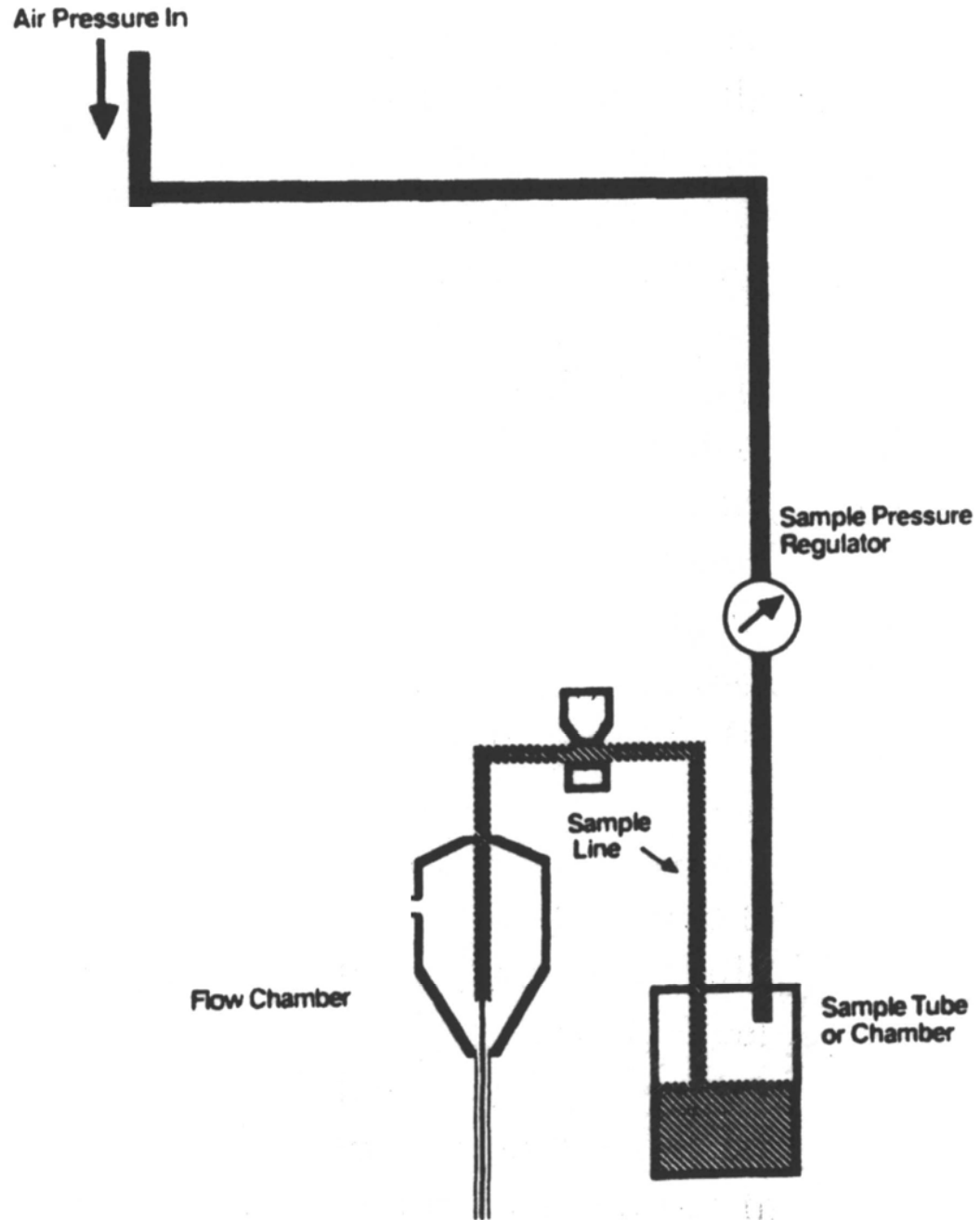
# Basic Optics





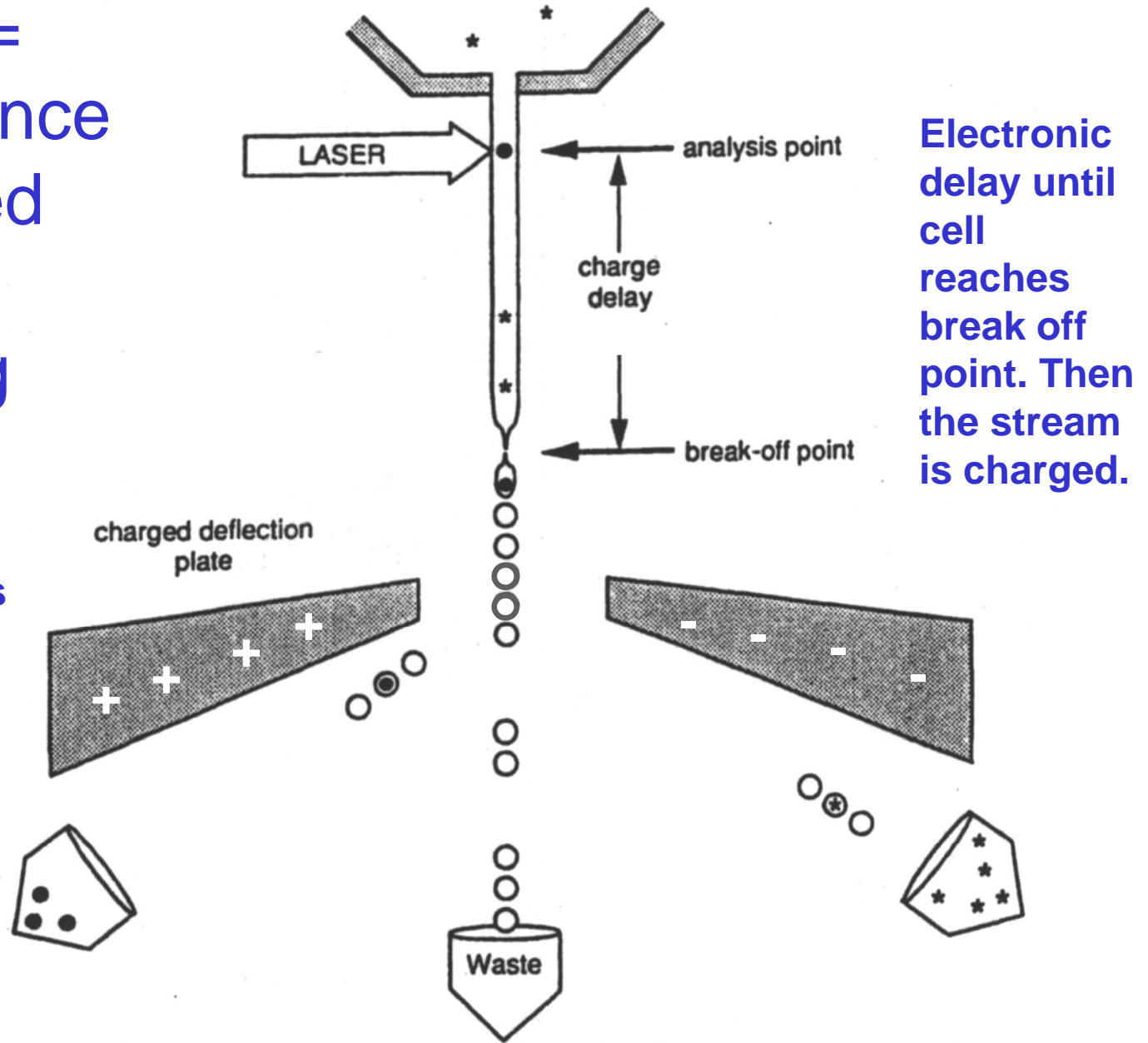


# Sample Flow

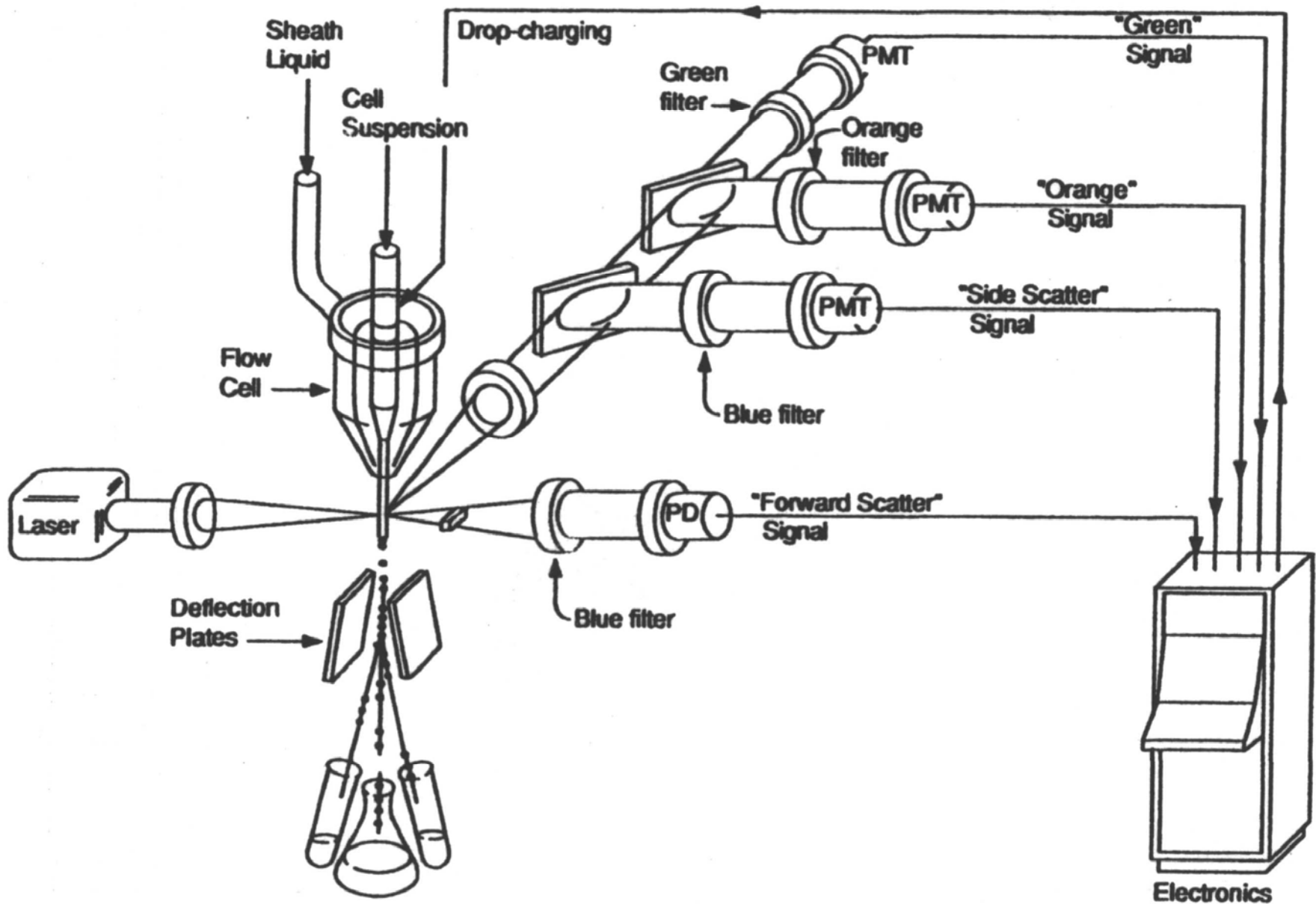


# FACS = Fluorescence Activated Cell Sorting

Vibrating the nozzle produces a stream that breaks into droplets.



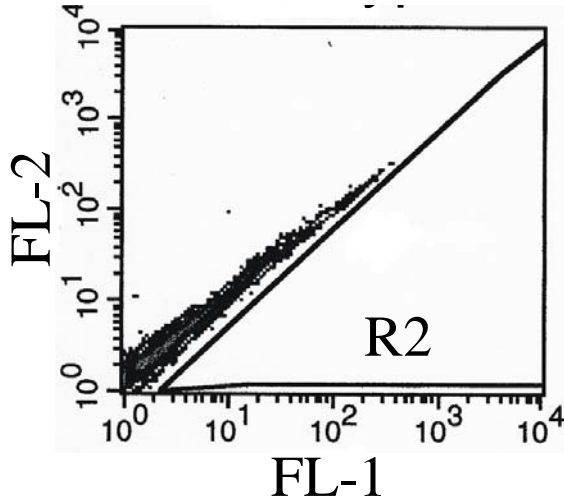
Electronic delay until cell reaches break off point. Then the stream is charged.



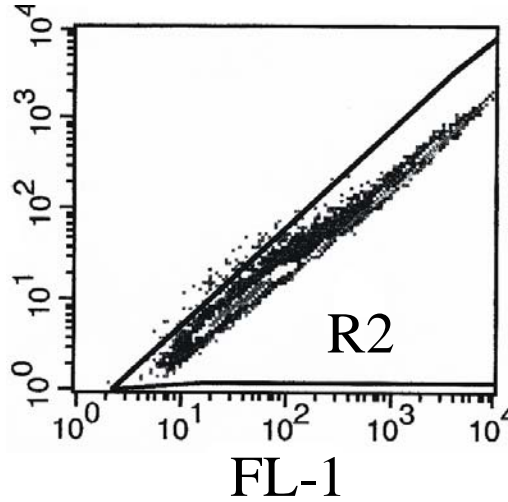


562-588 nm (FL-2)

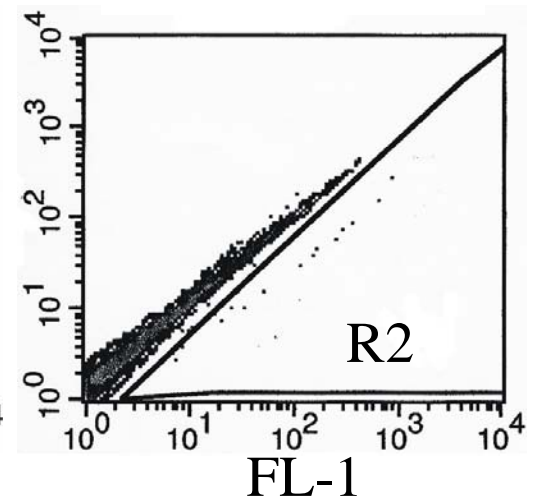
Normal Cells



EGFP  
Expressing  
Cells

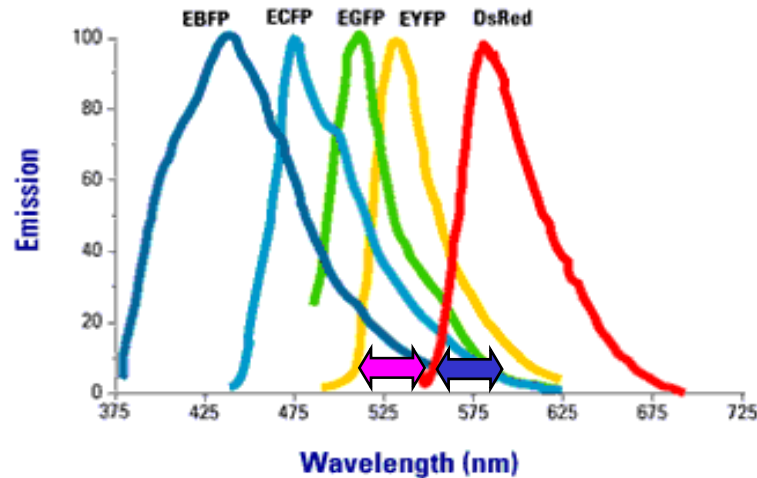


Rare Cells  
Expressing  
EGFP



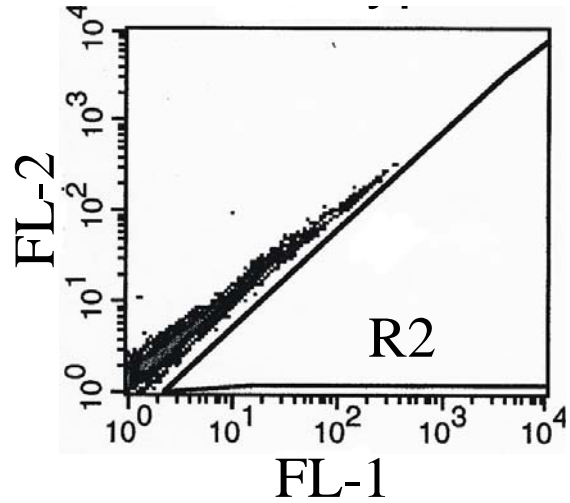
515-545 nm (FL-1)

Emission



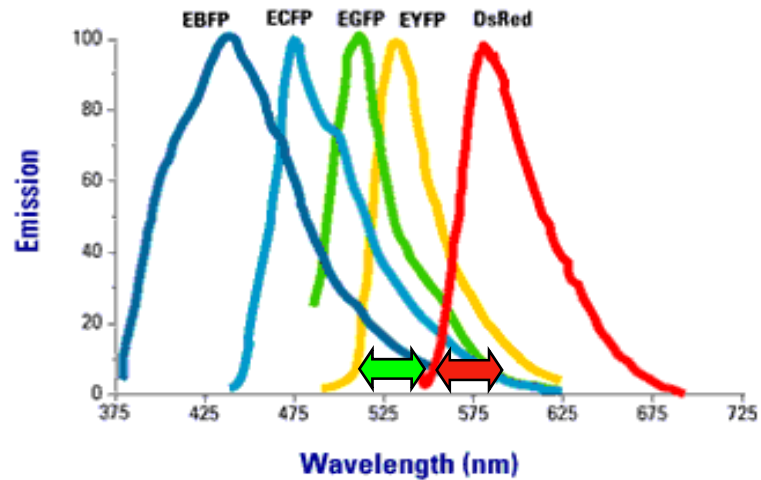
562-588 nm (FL-2)

## Normal Cells



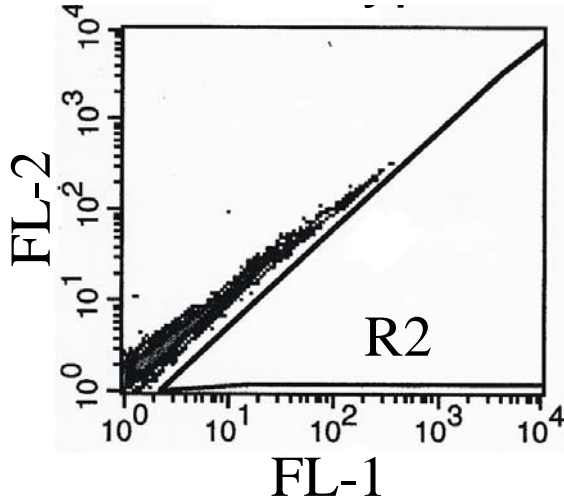
515-545 nm (FL-1)

## Emission

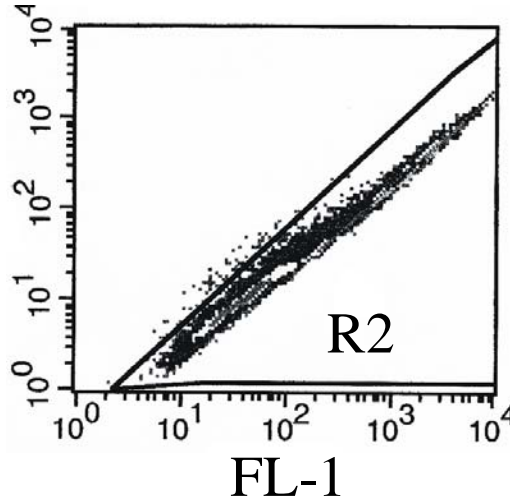


562-588 nm (FL-2)

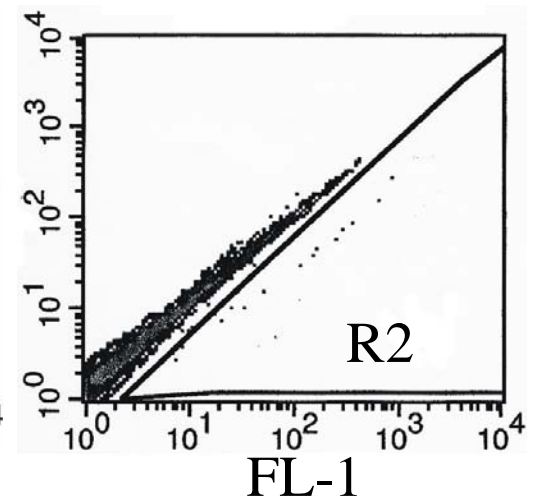
Normal Cells



EGFP Expressing Cells

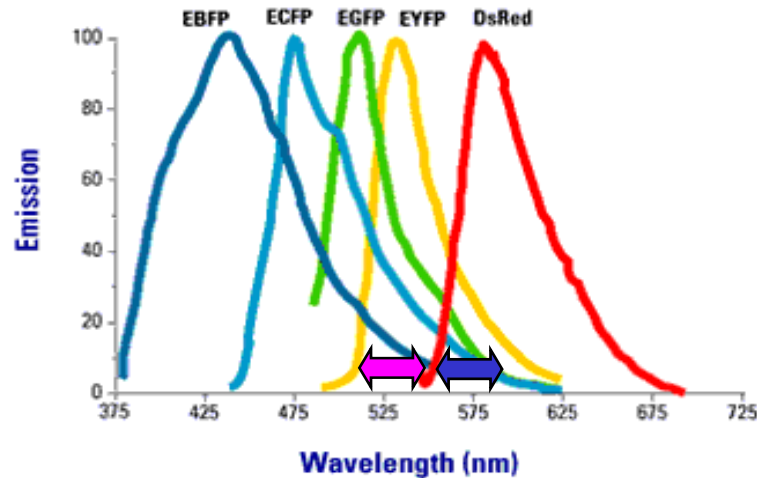


Rare Cells Expressing EGFP

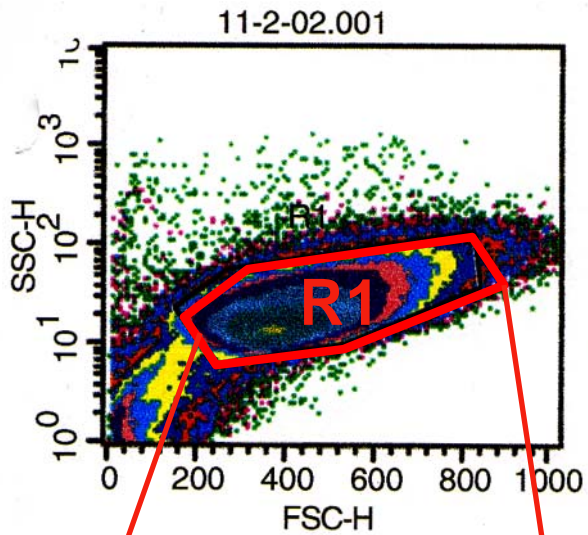


515-545 nm (FL-1)

Emission



562-588 nm (FL-2)



File: 11-2-02.001

Log Data Units: Linear Values

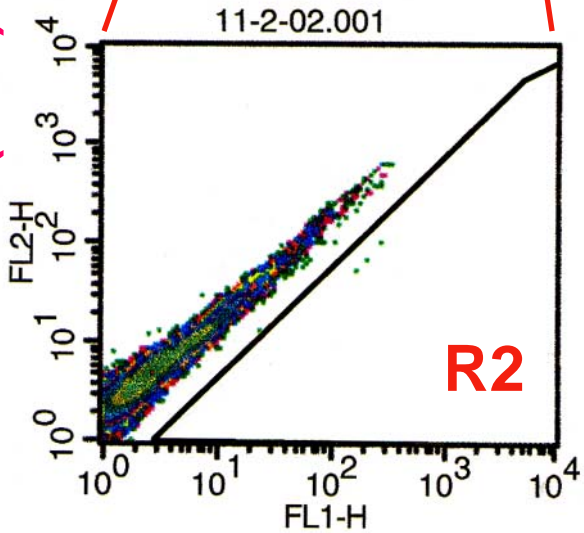
Sample ID: YI ears 0 MMC

Gate: No Gate

Gated Events: 1261613

Total Events: 1261613

Region	Events	% Gated	% Total	X Mean	Y Geo Mean
R1	1088917	86.31	86.31	418.15	21.33
R2	3	0.00	0.00	412.33	18.71



File: 11-2-02.001

Log Data Units: Linear Values

Sample ID: YI ears 0 MMC

Gate: G1

Gated Events: 1088917

Total Events: 1261613

Region	Events	% Gated	% Total	X Geo Mean	Y Geo Mean
R1	1088917	100.00	86.31	3.08	5.68
R2	3	0.00	0.00	197.50	75.21

515-545 nm (FL-1)

# Flow Cytometry

Flow cytometry analyzes cells one by one

Fluorescence, diffracted, and reflected light can be measured for each cell

Multiple lasers and multiple colors can be analyzed at millions of cells per minute

Resulting plots show the relative level of fluorescence of each cell for specific wave lengths (a dot is a single cell)

Flow cytometry is an analysis method, where as FACS actually sorts cells

**Module in Review:**

**Experimental Approaches &  
Biological Concepts**

**Flow Cytometry**