

20.109 Spring 2016 Module 2 – Lecture 3

System Engineering (March 15th, 2016)



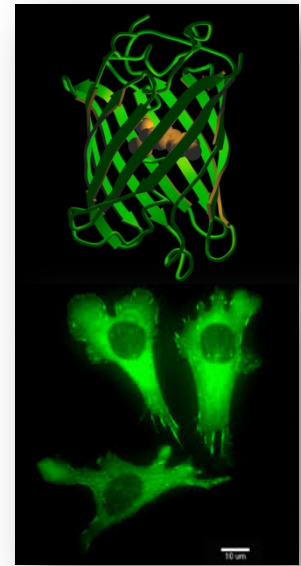
Noreen Lyell
Leslie McLain
Maxine Jonas
Jing Zhang(TA)

Leona Samson (Lectures)

Zachary Nagel (help with development) Alex Chaim

What experimental question will you ask in Module 2?

How efficiently does DNA repair by the Non Homologous End Joining (NHEJ) pathway act on DNA damage with different topologies?



This raises the following questions

- How does DNA get damaged?
- What is DNA repair?
- Why does DNA repair exist?
- Why do we care about how efficient DNA repair is?
- How does one actually measure DNA repair efficiency?

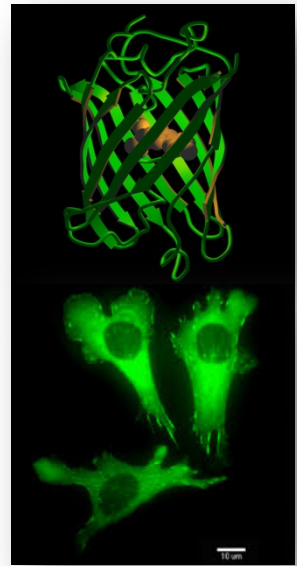
Key Experimental Methods for Module 1

- Mammalian tissue cell culture
- Monitoring protein level by Western blot
- Generating plasmids with DNA damage
- Transfecting plasmids into mammalian cells
- Using fluorescent proteins as reporters of biological processes
- Flow cytometry to measure DNA repair
- Statistical analysis of biological data



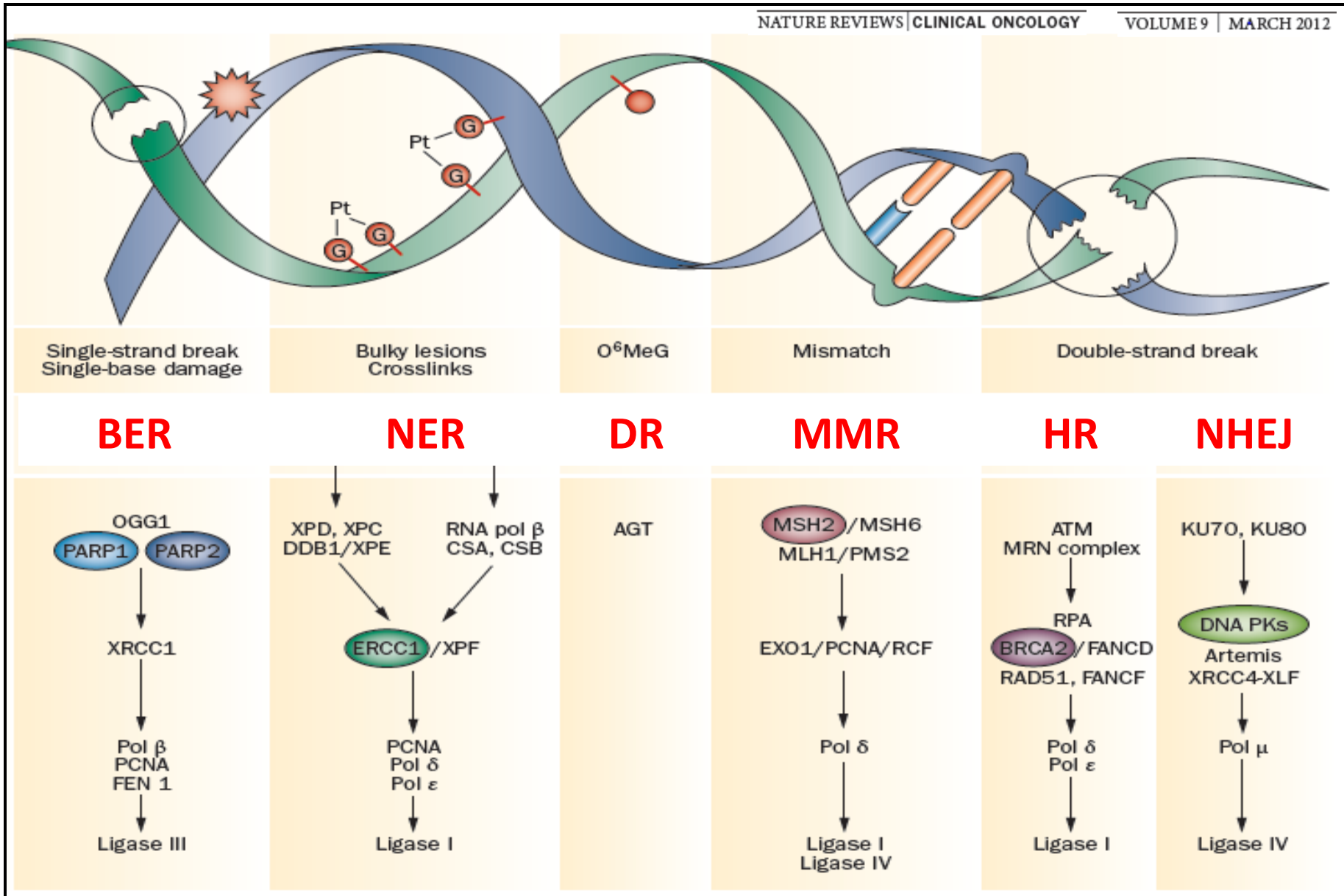
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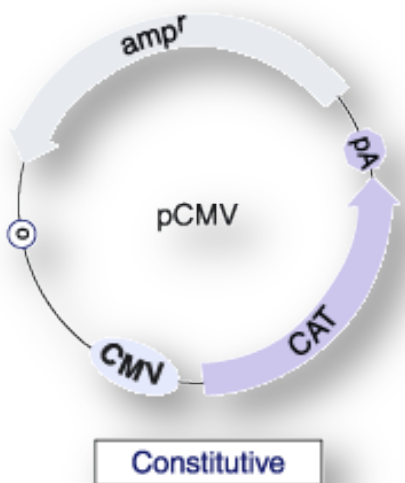
Six Major DNA Repair Pathways

NATURE REVIEWS | CLINICAL ONCOLOGY | VOLUME 9 | MARCH 2012

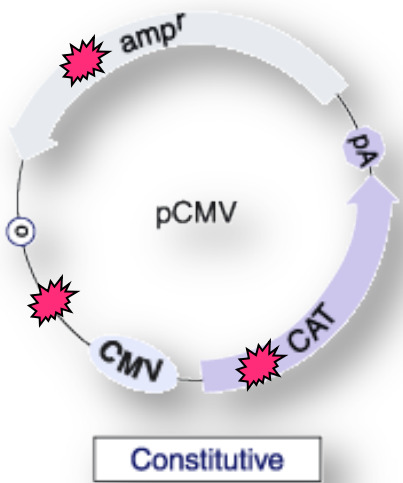


Reactivation of UV damaged DNA by Host cell Reactivation (HCR)

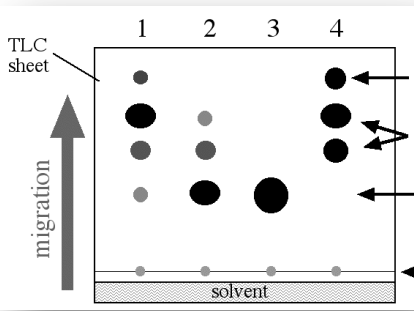
Athas & GROSSMAN
Cancer Res. 1991



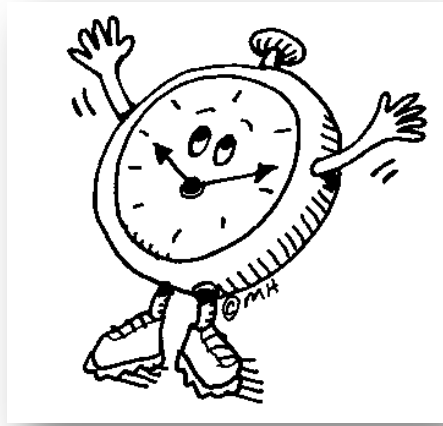
+ UV
light



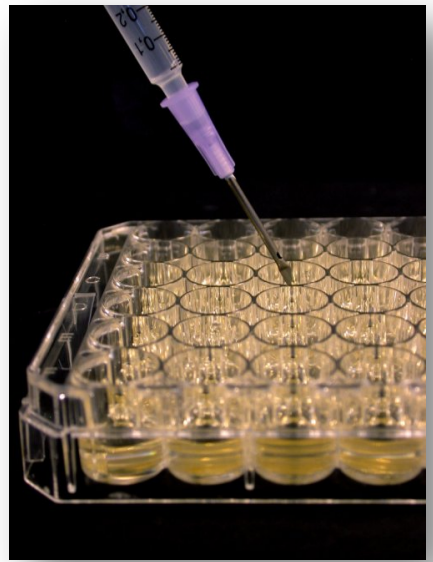
Transient
transfection
peripheral
blood
lymphocytes



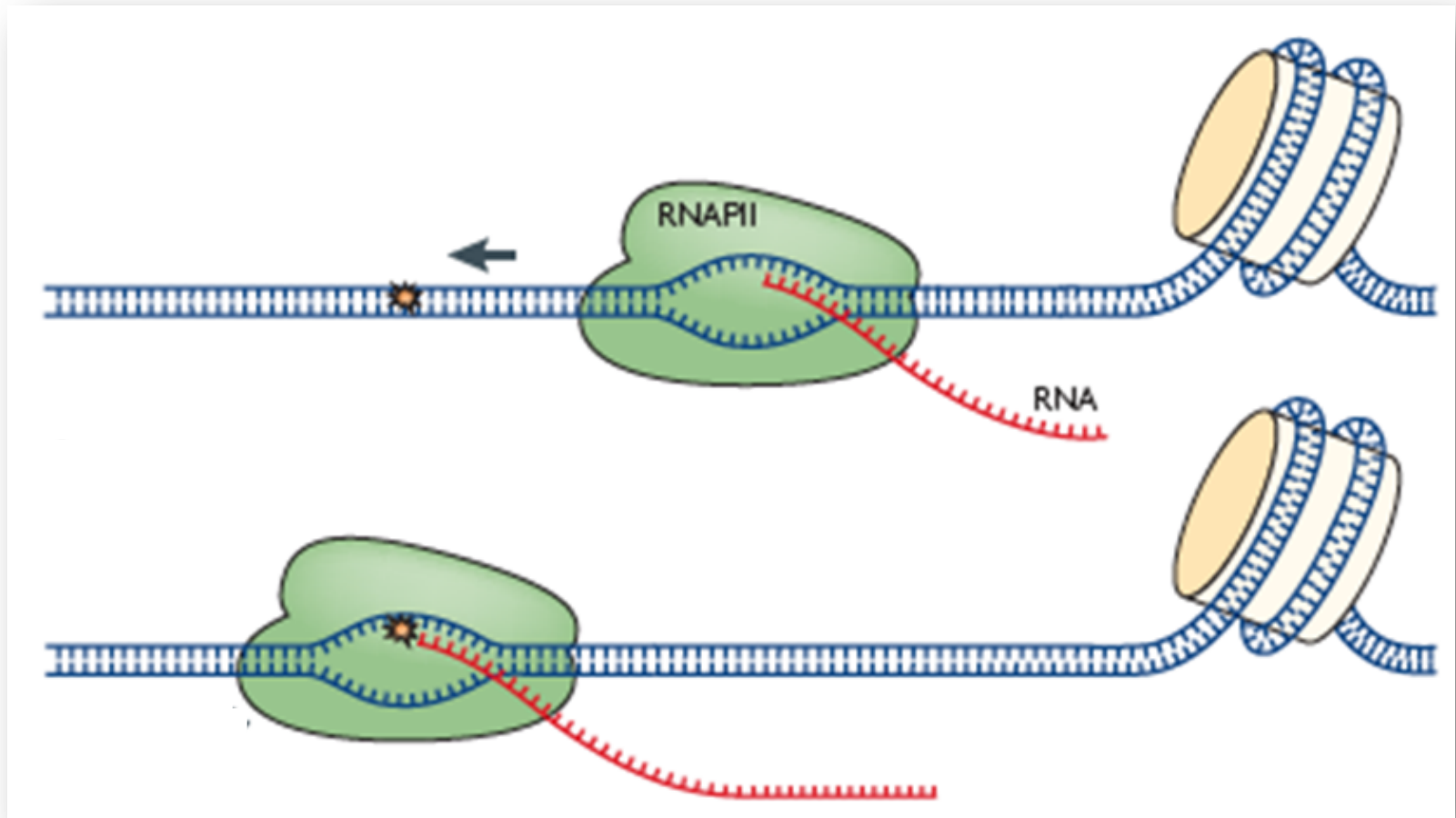
CAT Assay



Time to repair

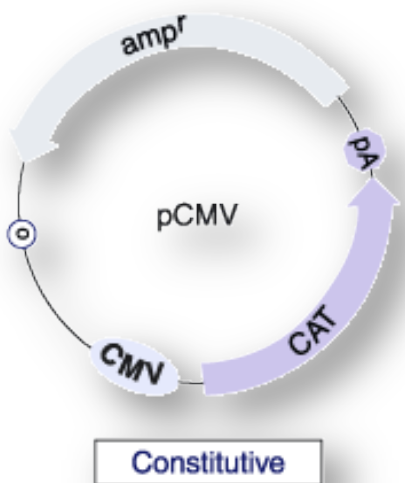


RNA Polymerase II is exquisitely sensitive to DNA lesions

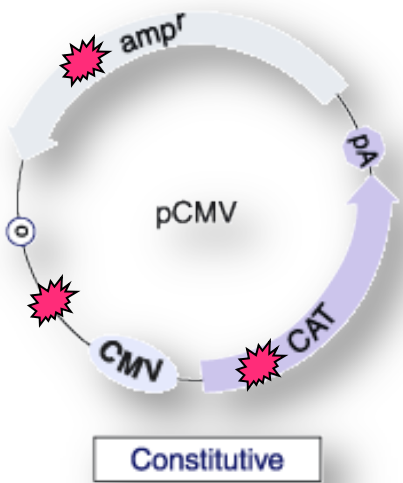


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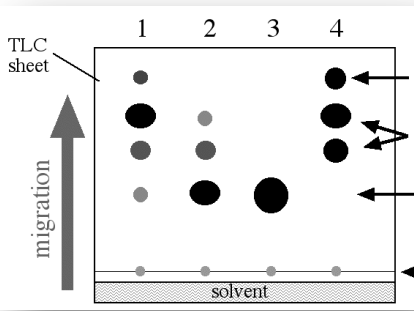
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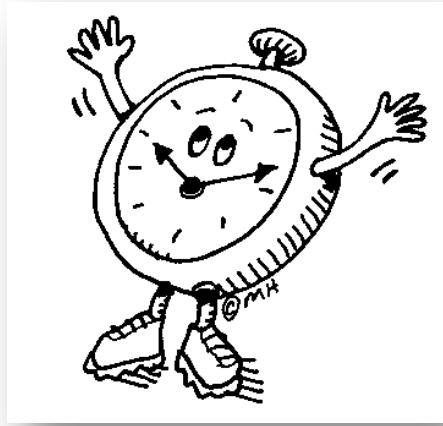
+ UV
light



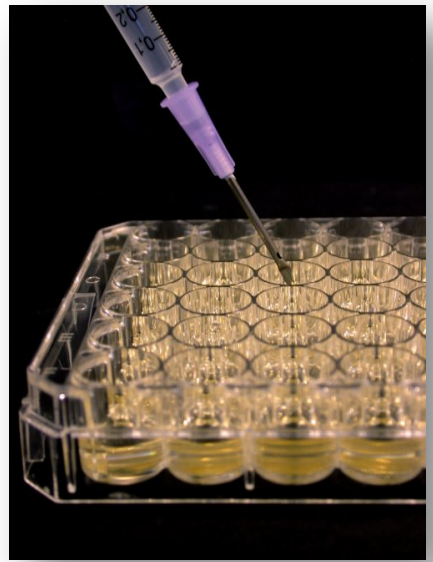
Transient
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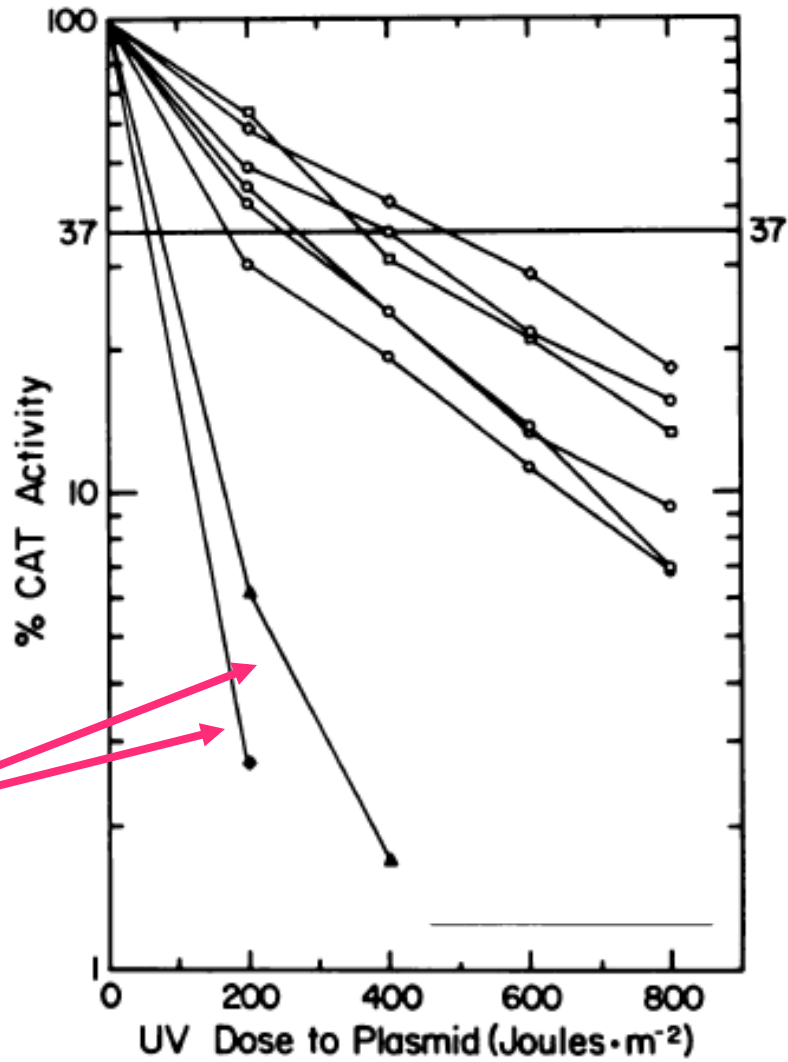
CAT Assay



Time to repair

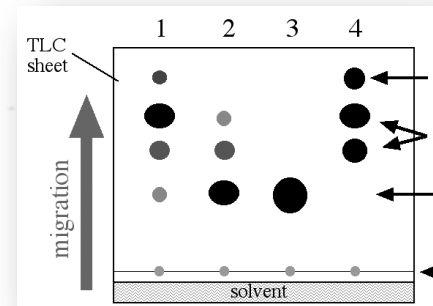


Fresh Circulating Lymphocyte Plasmid HCR in XP and Normal PBL

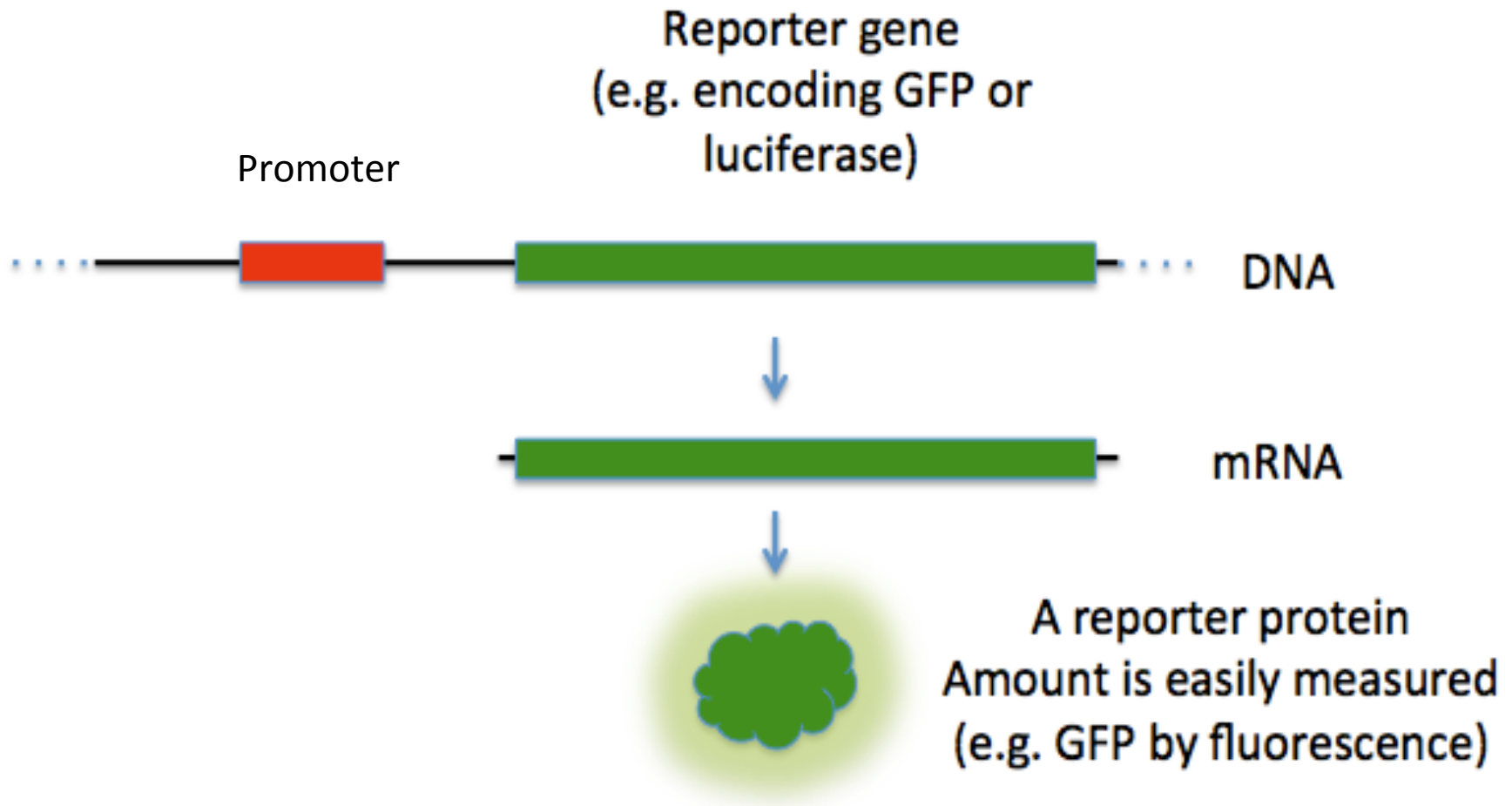


**Cells
from XP
patients**

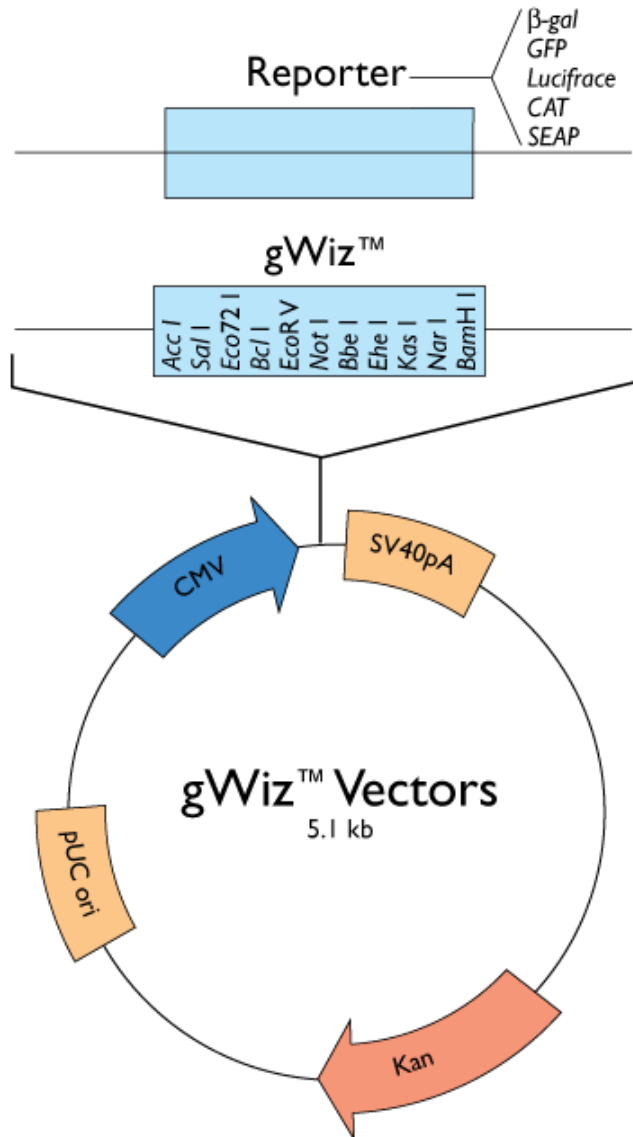
**Cells from
‘healthy’ people**



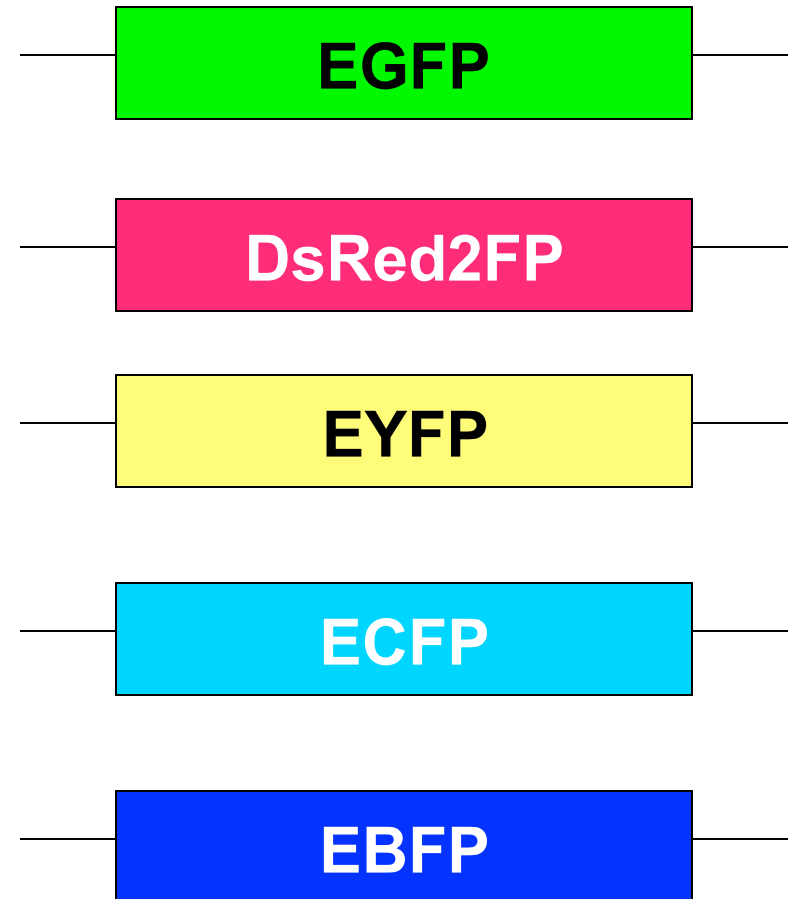
Let's use a different reporter gene that is easy to assay and works *in vivo*




Reactivation of damaged DNA - multiplexed



Each Fluorescent Protein gene will harbor a different type of DNA damage

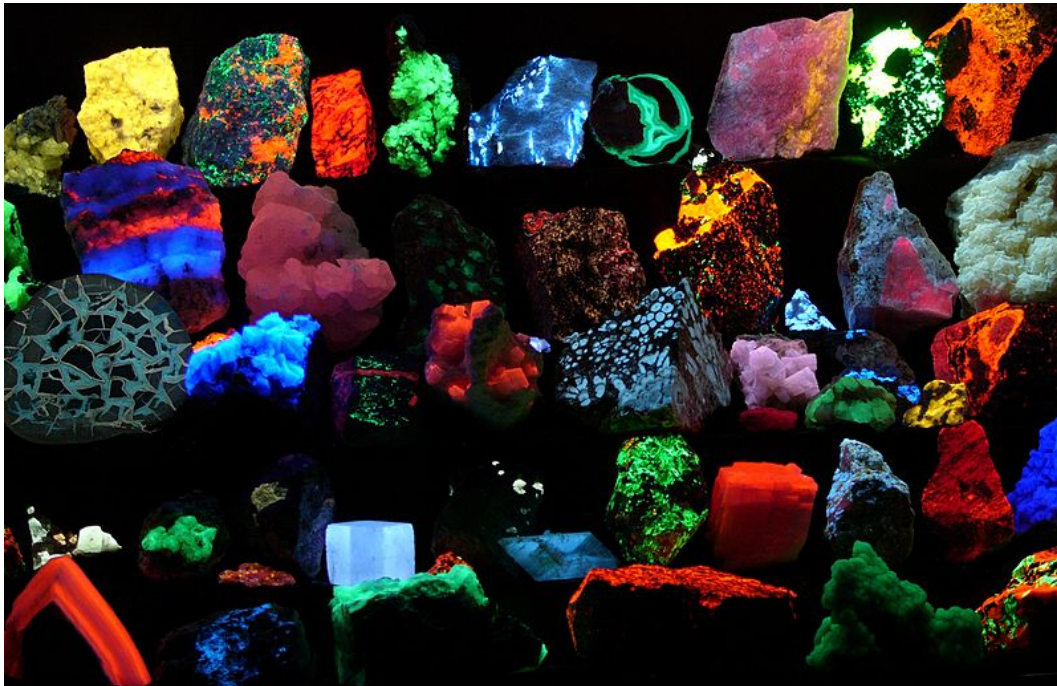


fluo·res·cence

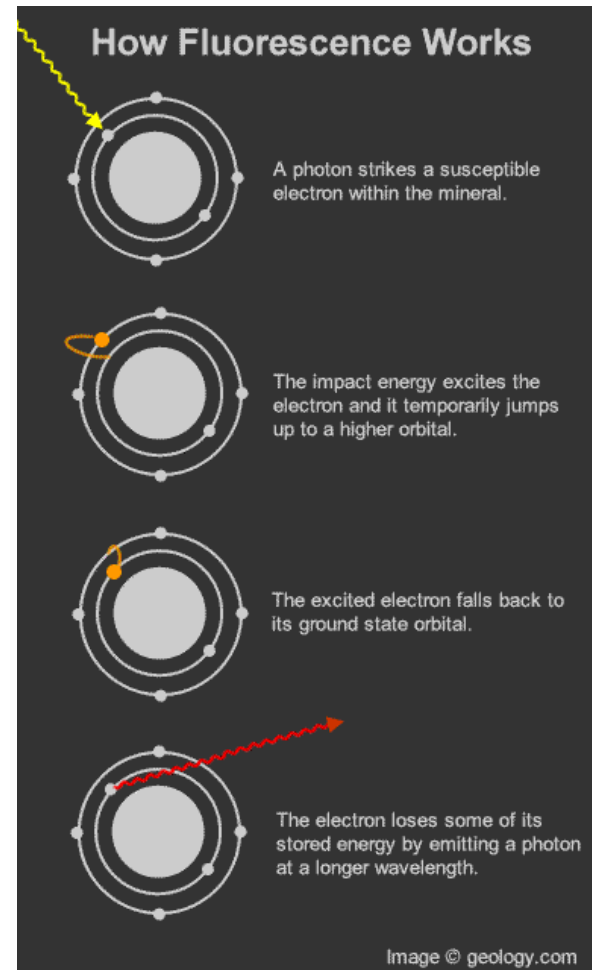
/flō(ə)'resəns,flôr'esəns/ 

noun

1. the visible or invisible radiation emitted by certain substances as a result of incident radiation of a shorter wavelength such as X-rays or ultraviolet light.

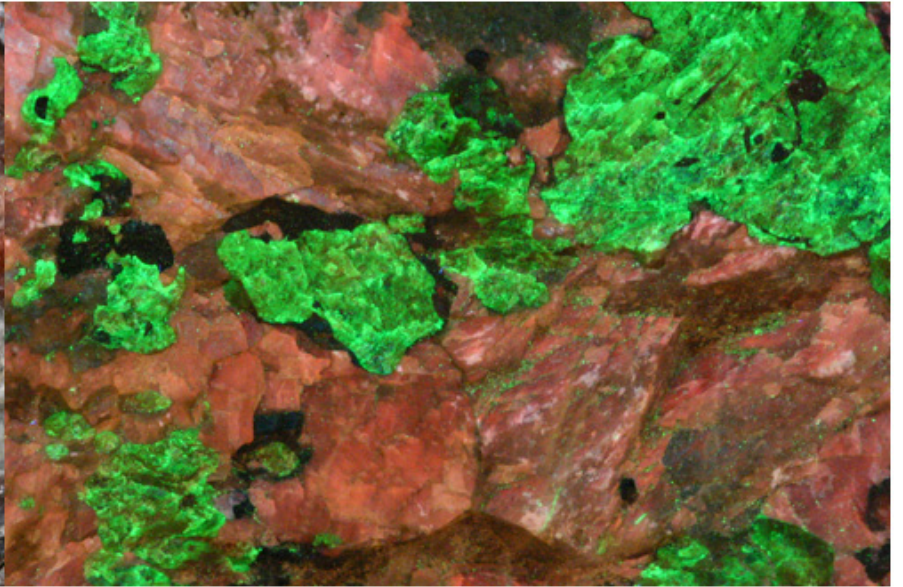


Minerals fluorescing under
UV-light



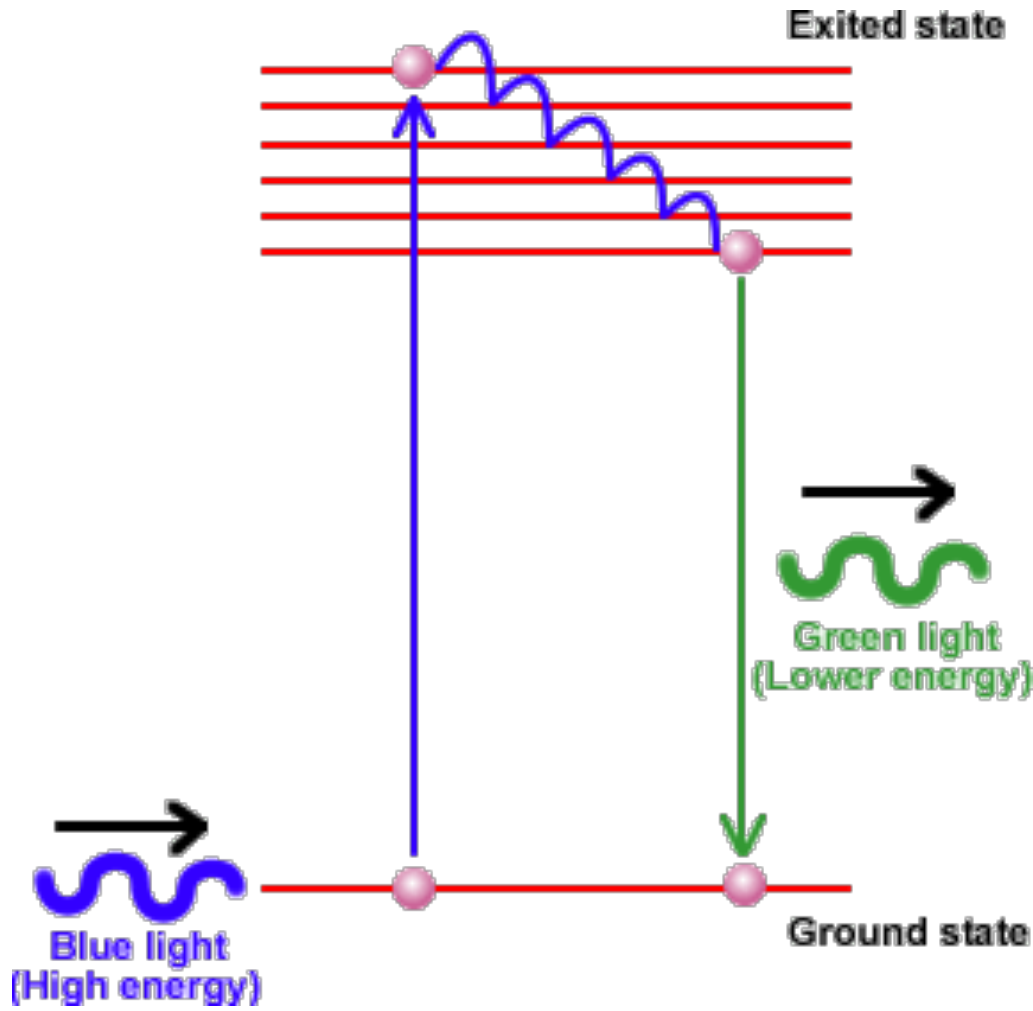


Under White Light



Under UV Light

Theory of Fluorescence

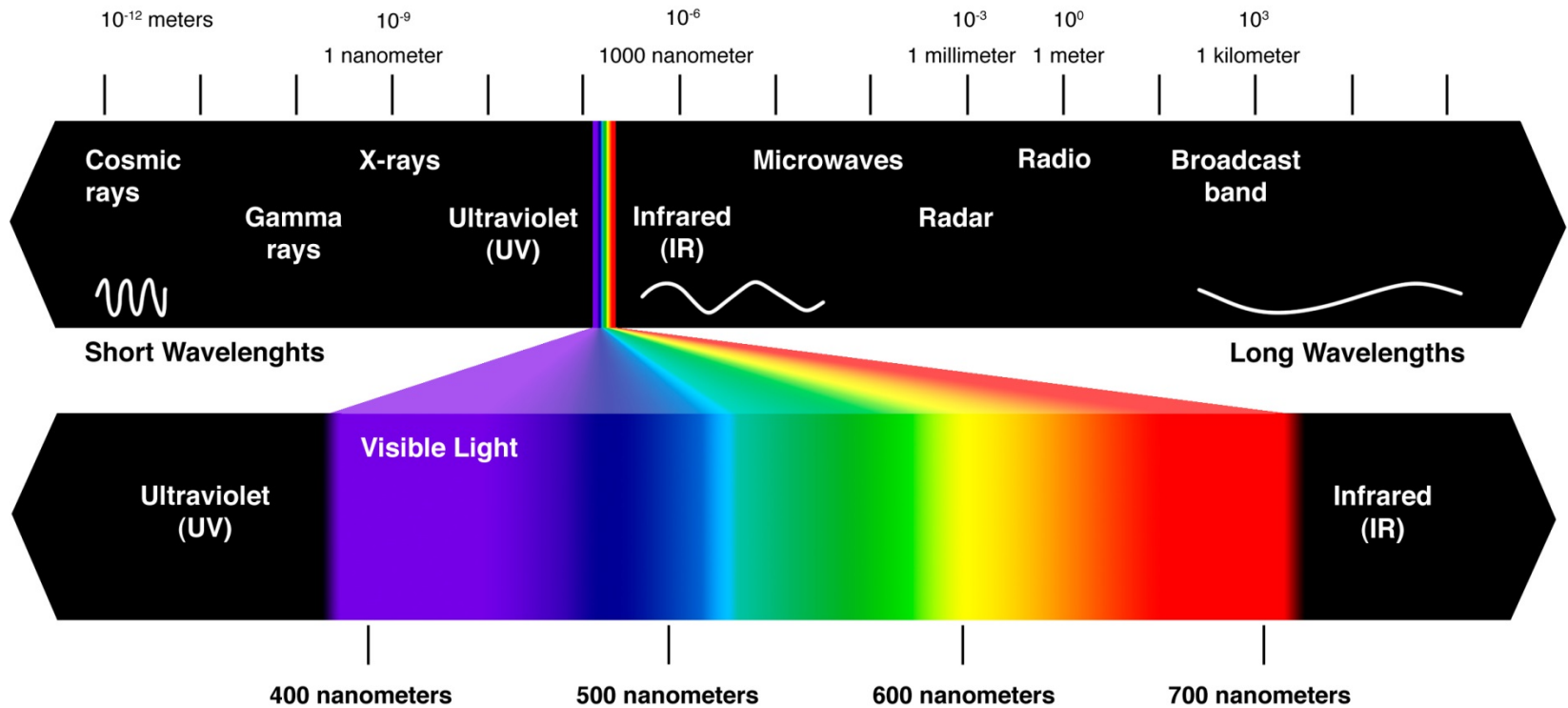


- 1) Electrons excited by light source
- 2) Electrons reach a high energy state
- 3) Energy loss occurs within a few nano seconds
- 4) Energy loss observed as fluorescent light of a longer wavelength

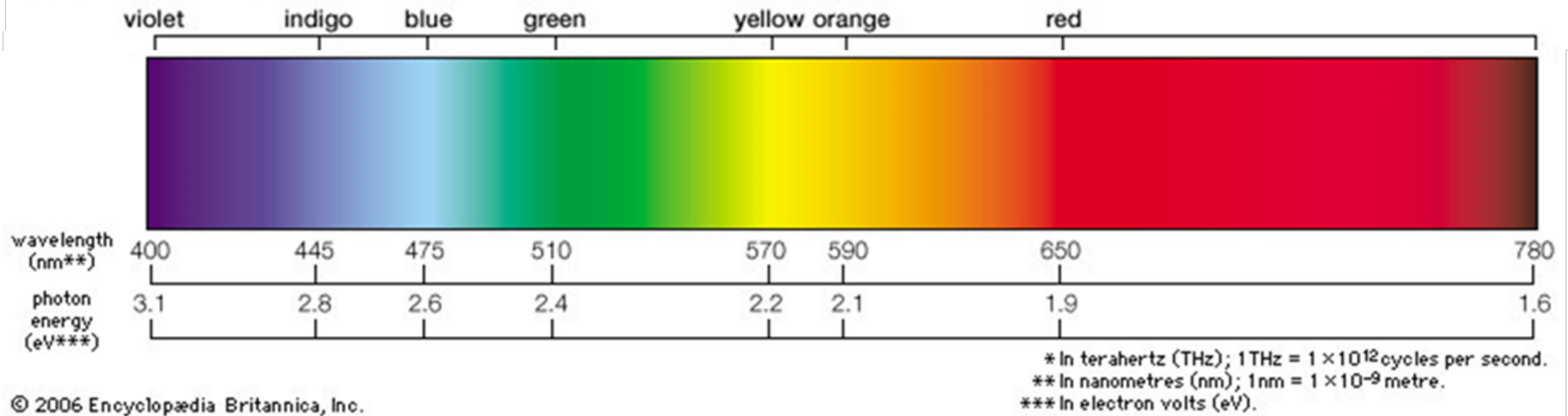
Excitation

Emission

Electro Magnetic Spectrum and Light



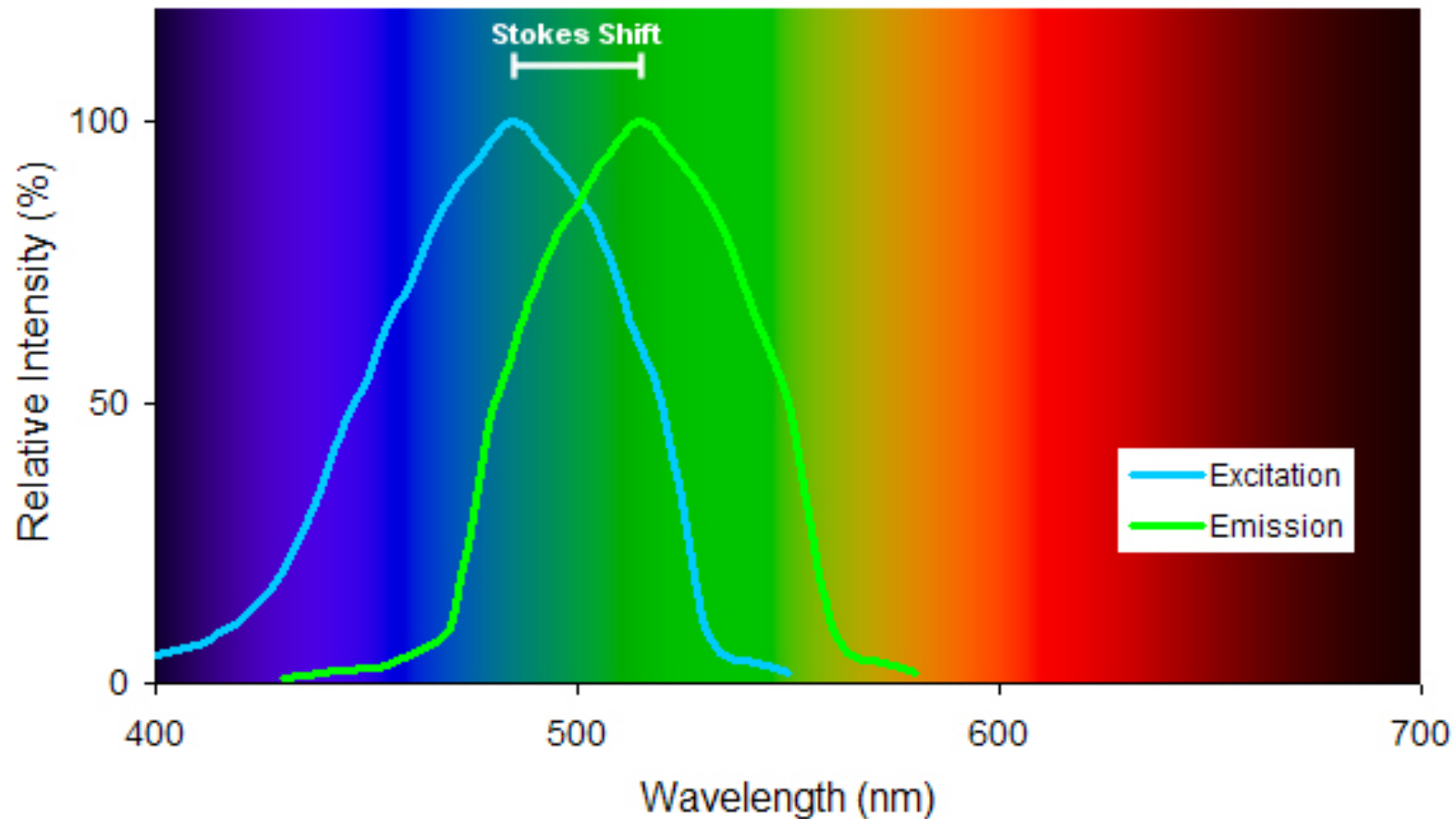
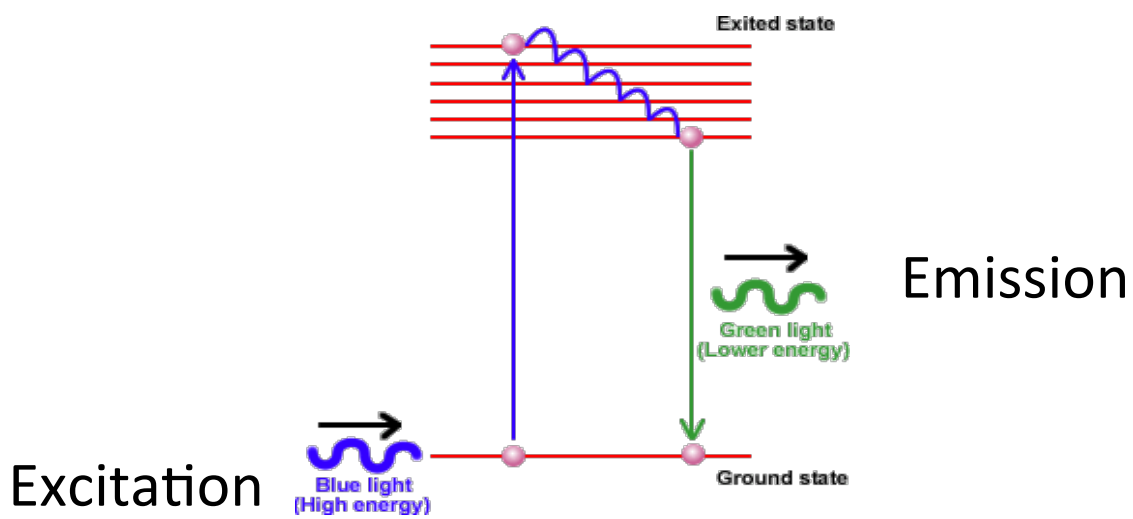
Light, the visible spectrum



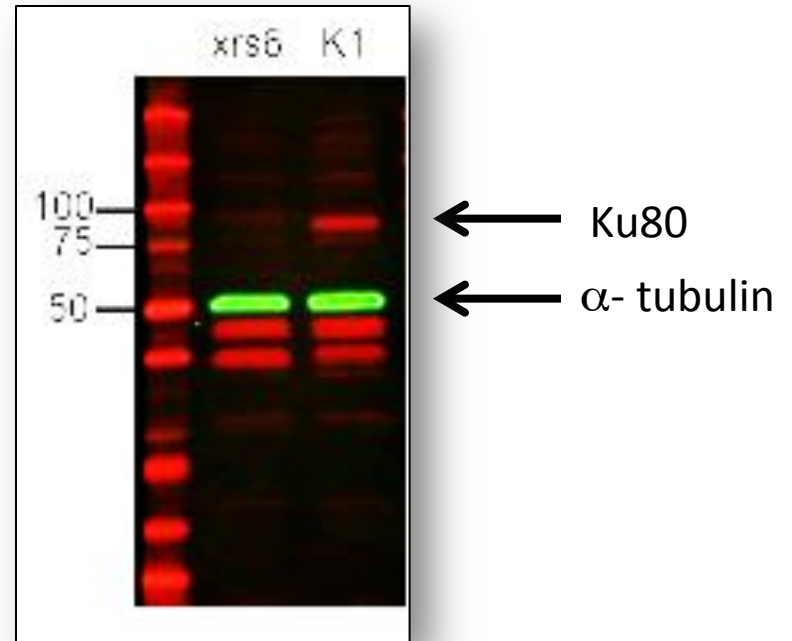
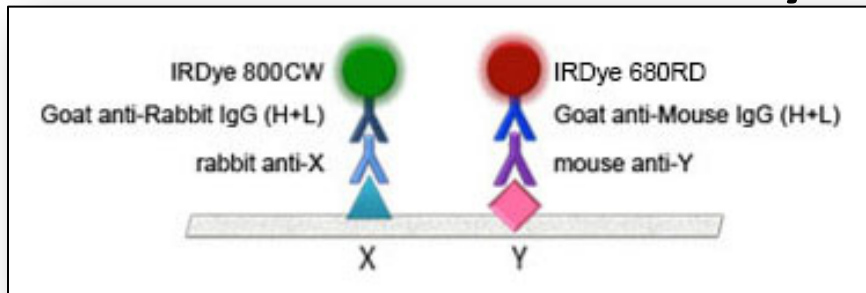
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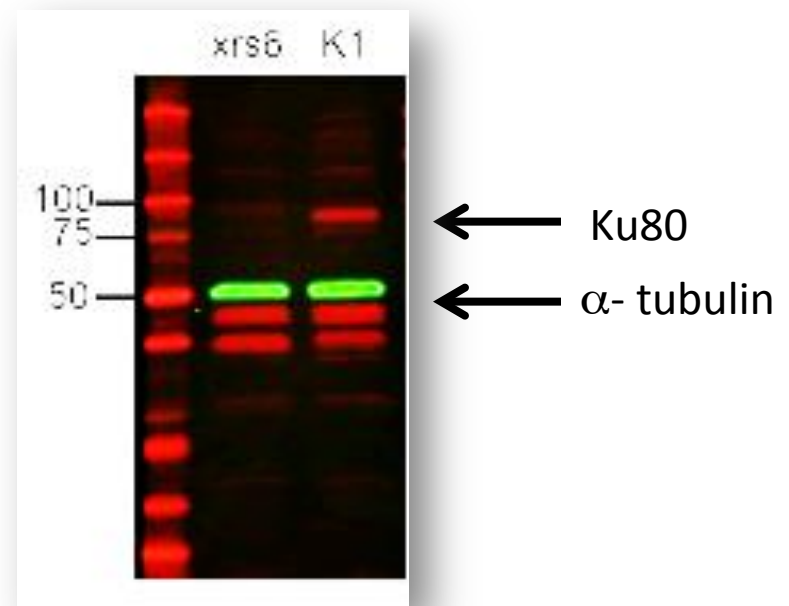
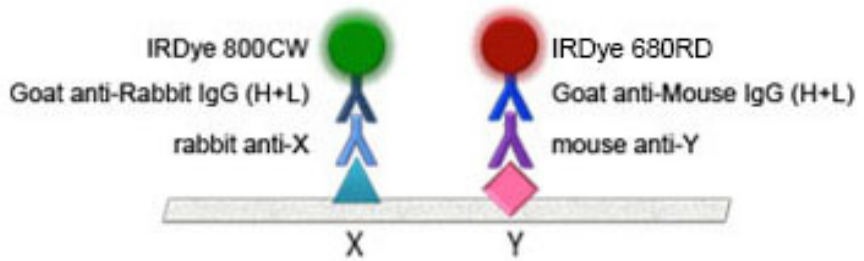
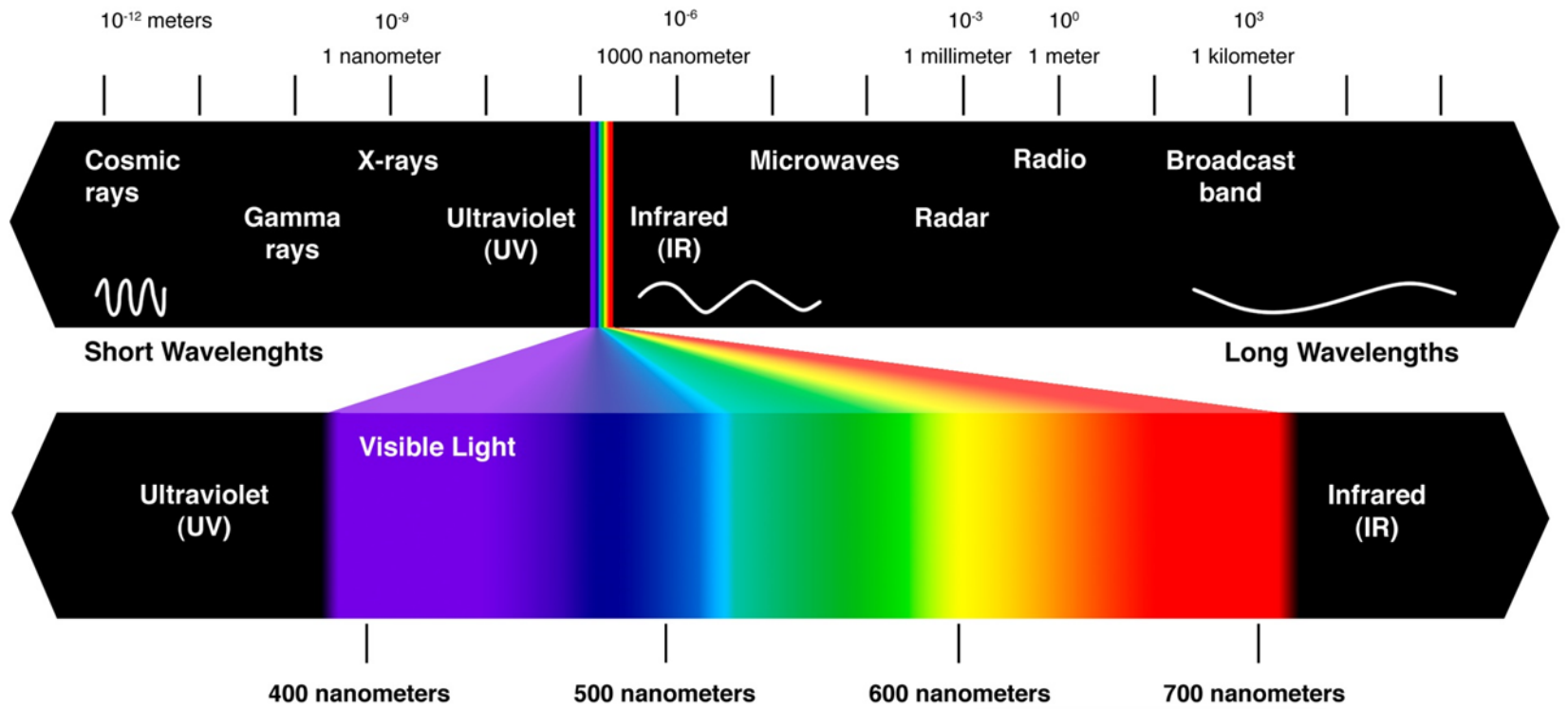
Increasing energy



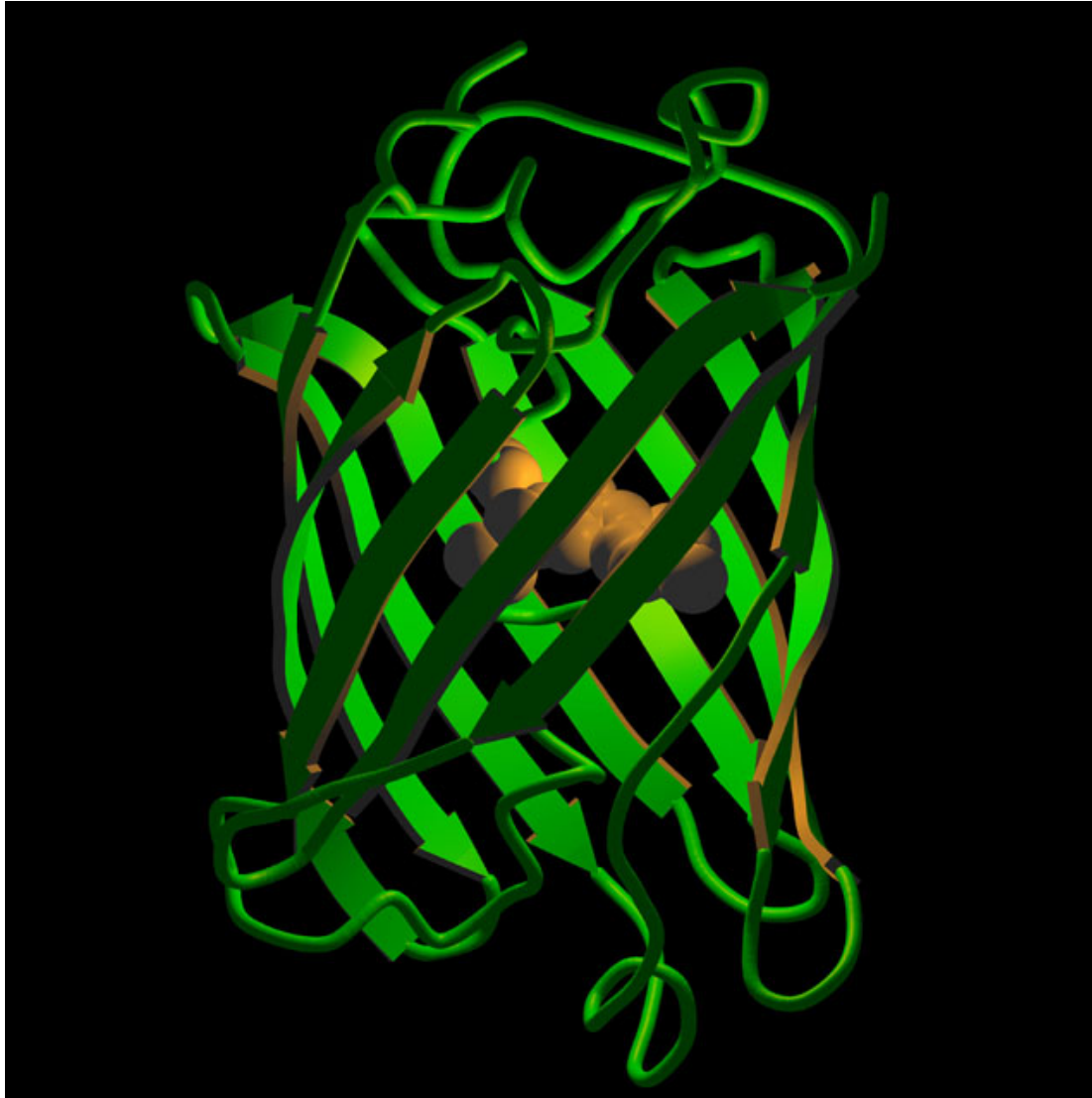


Western Blot Analysis

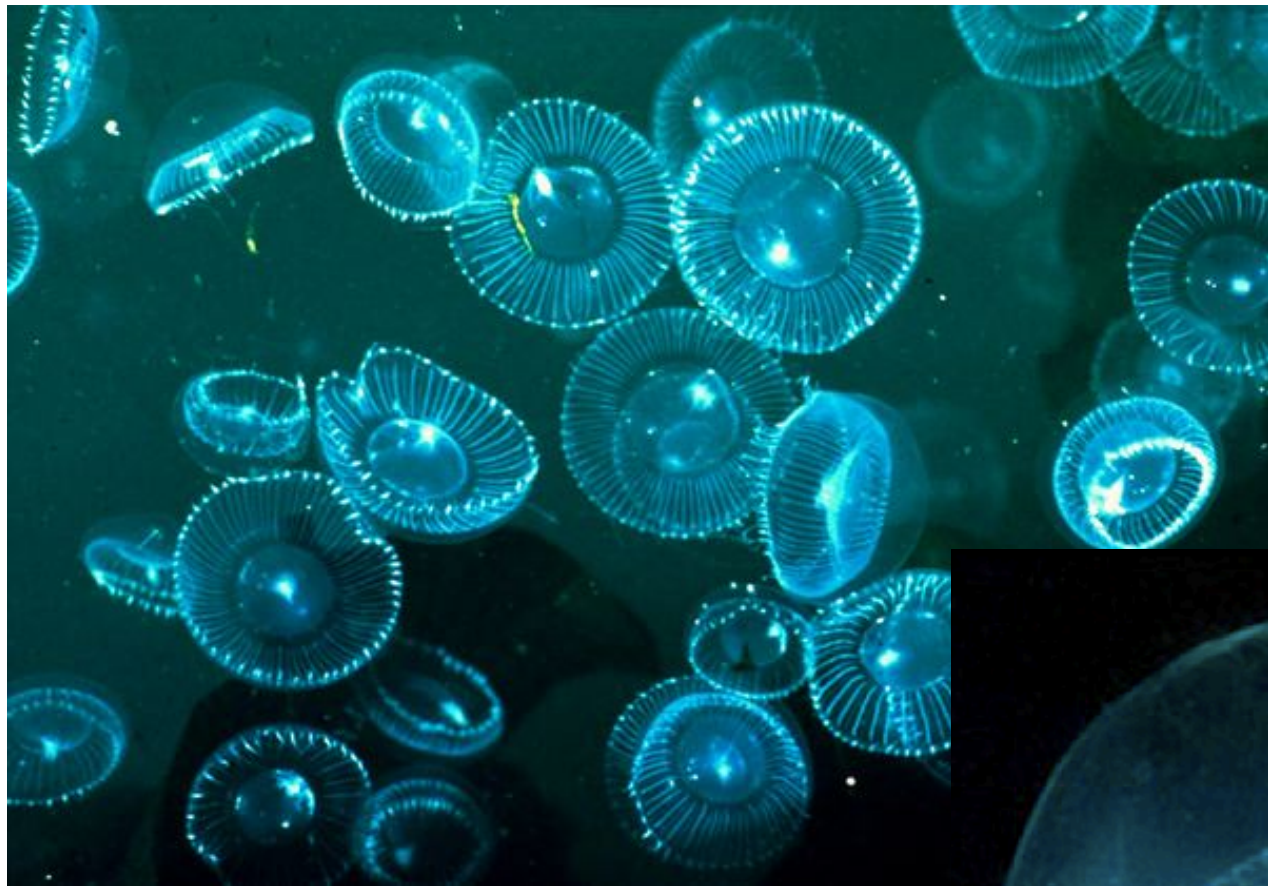




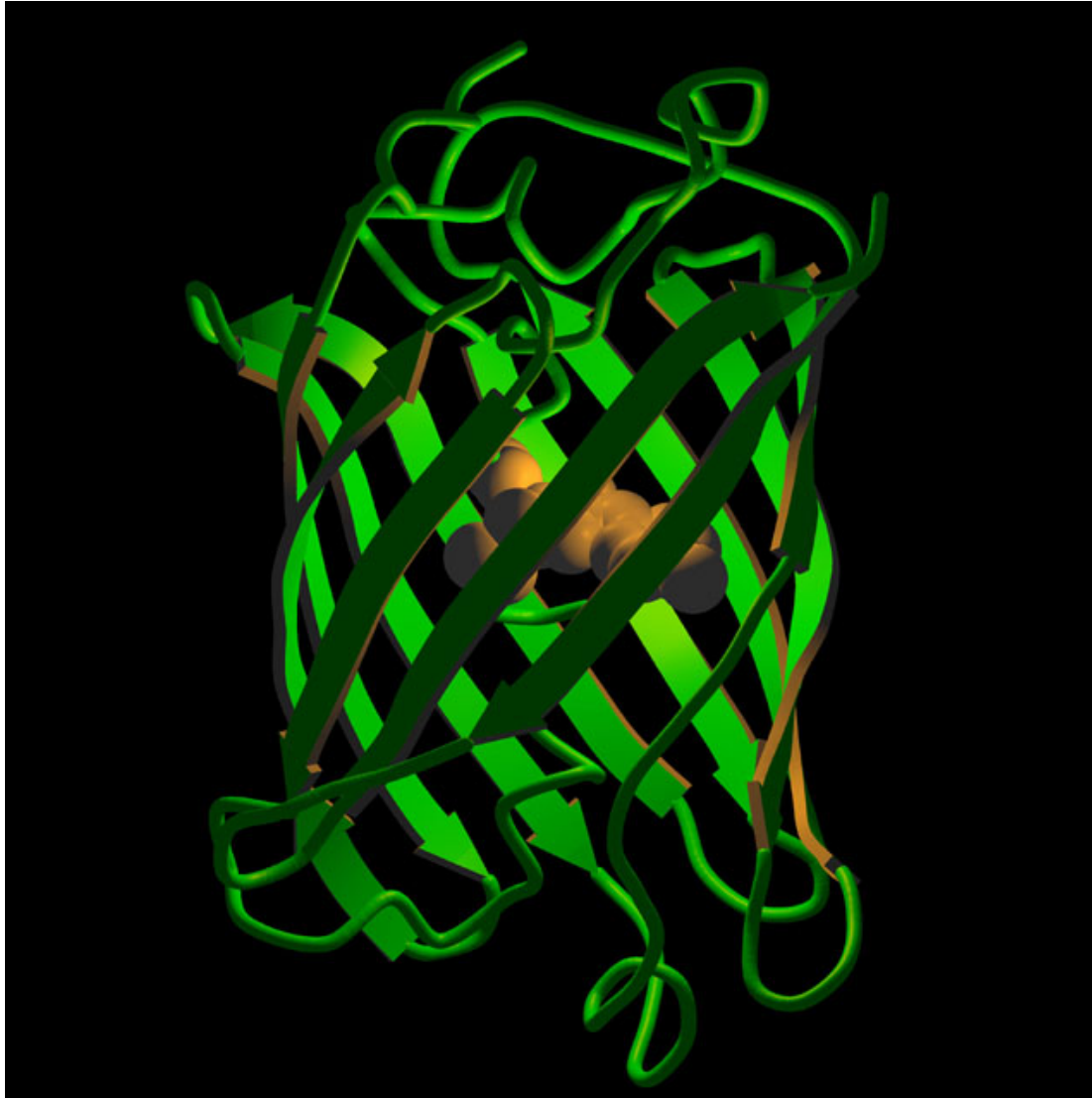
Green Fluorescent Protein (GFP) first isolated from
crystal jellyfish (*Aequorea victoria*).



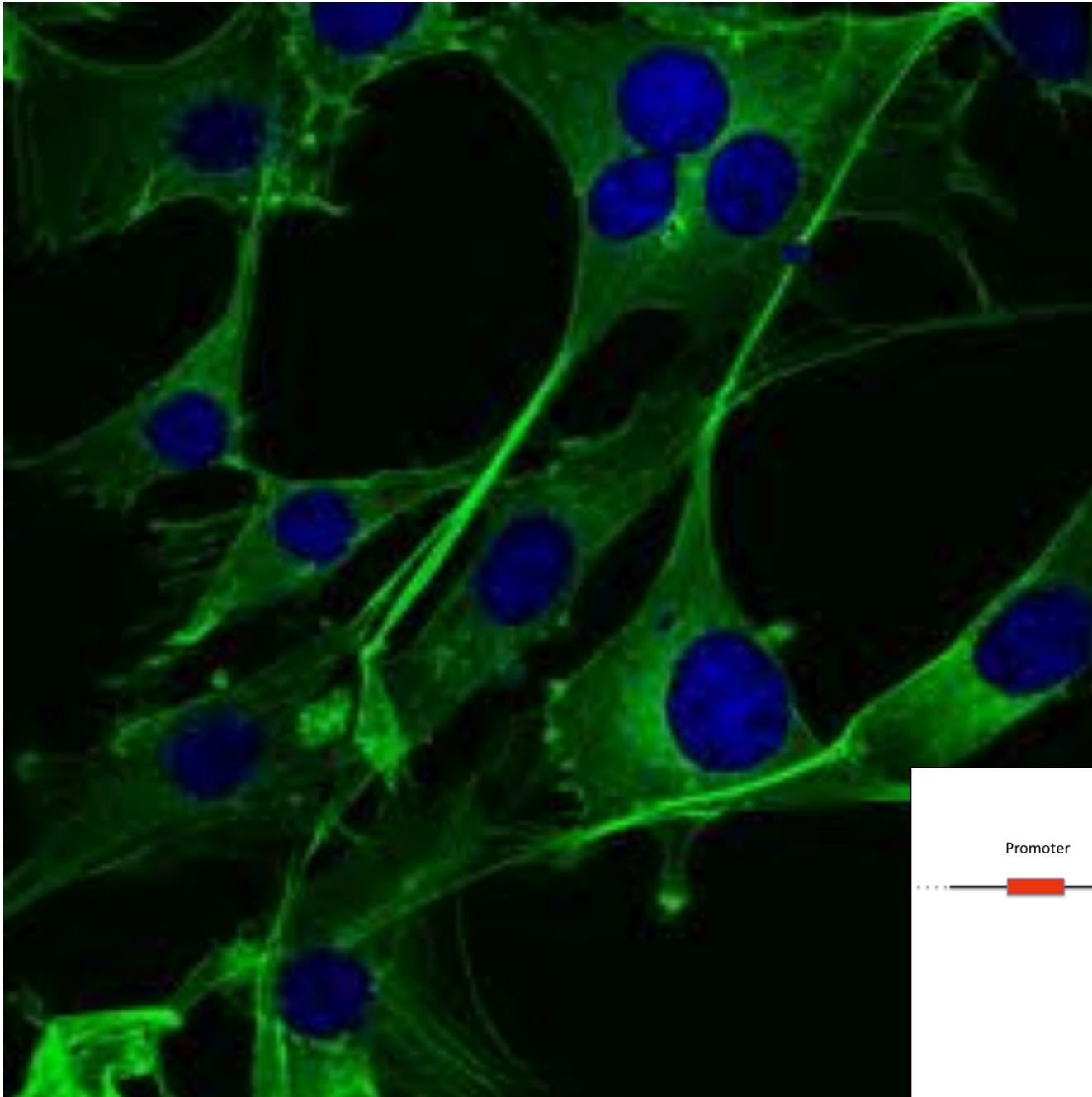
Osamu
Shimomura, 1962



Green Fluorescent Protein (GFP) first isolated from
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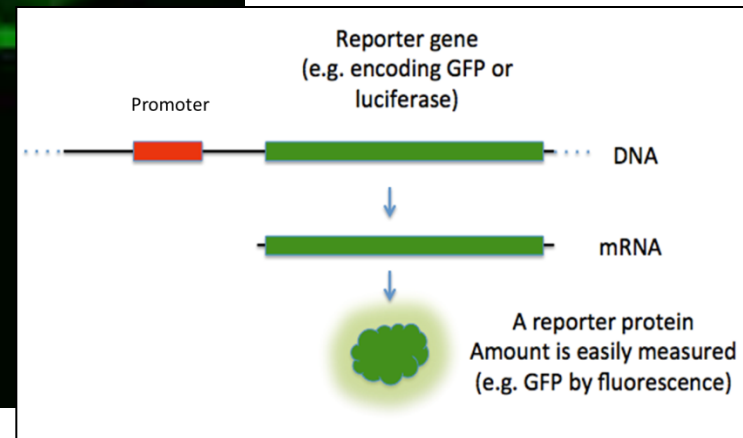
Osamu
Shimomura, 1962



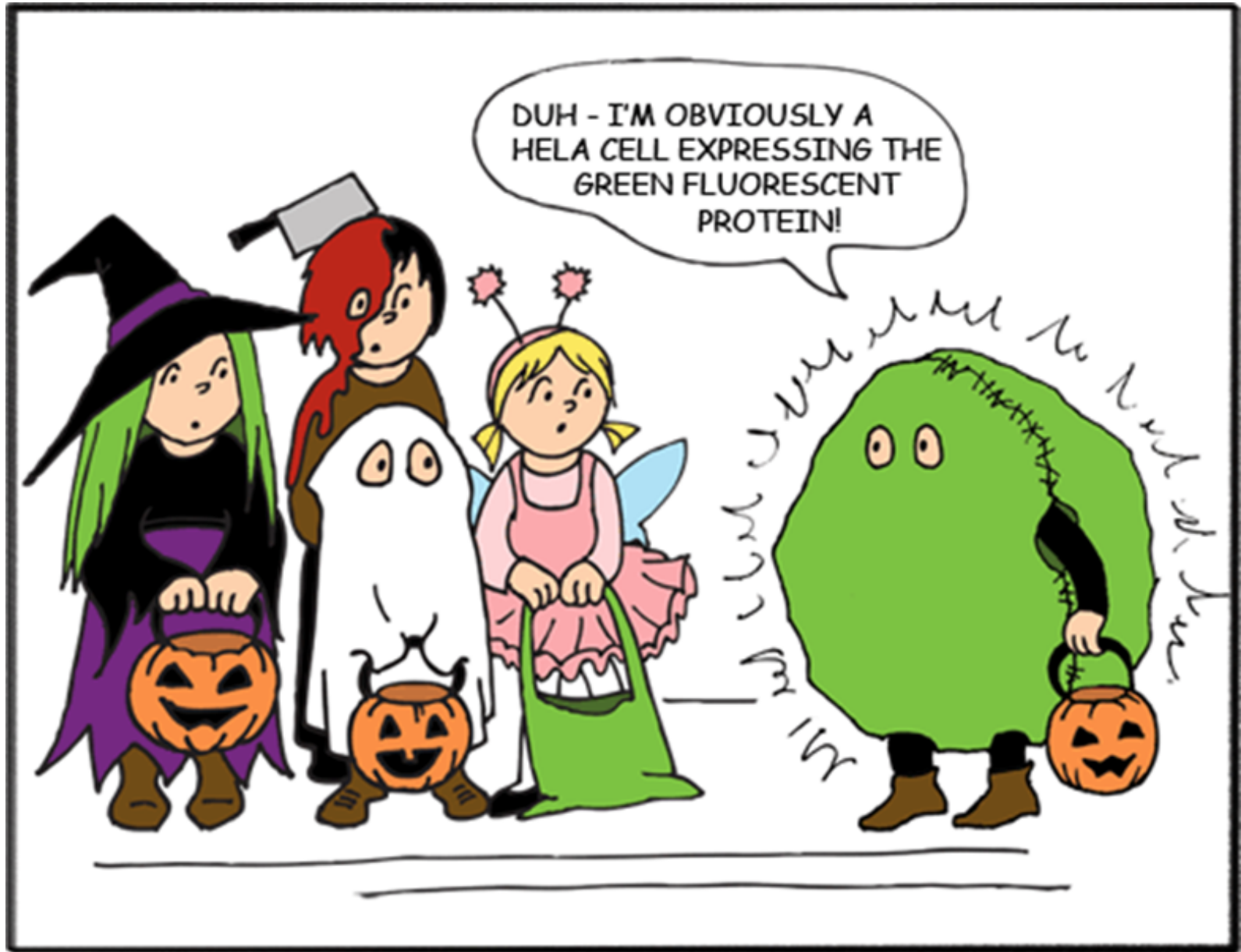
GFP cDNA
expressed in
human cells

DNA – Blue

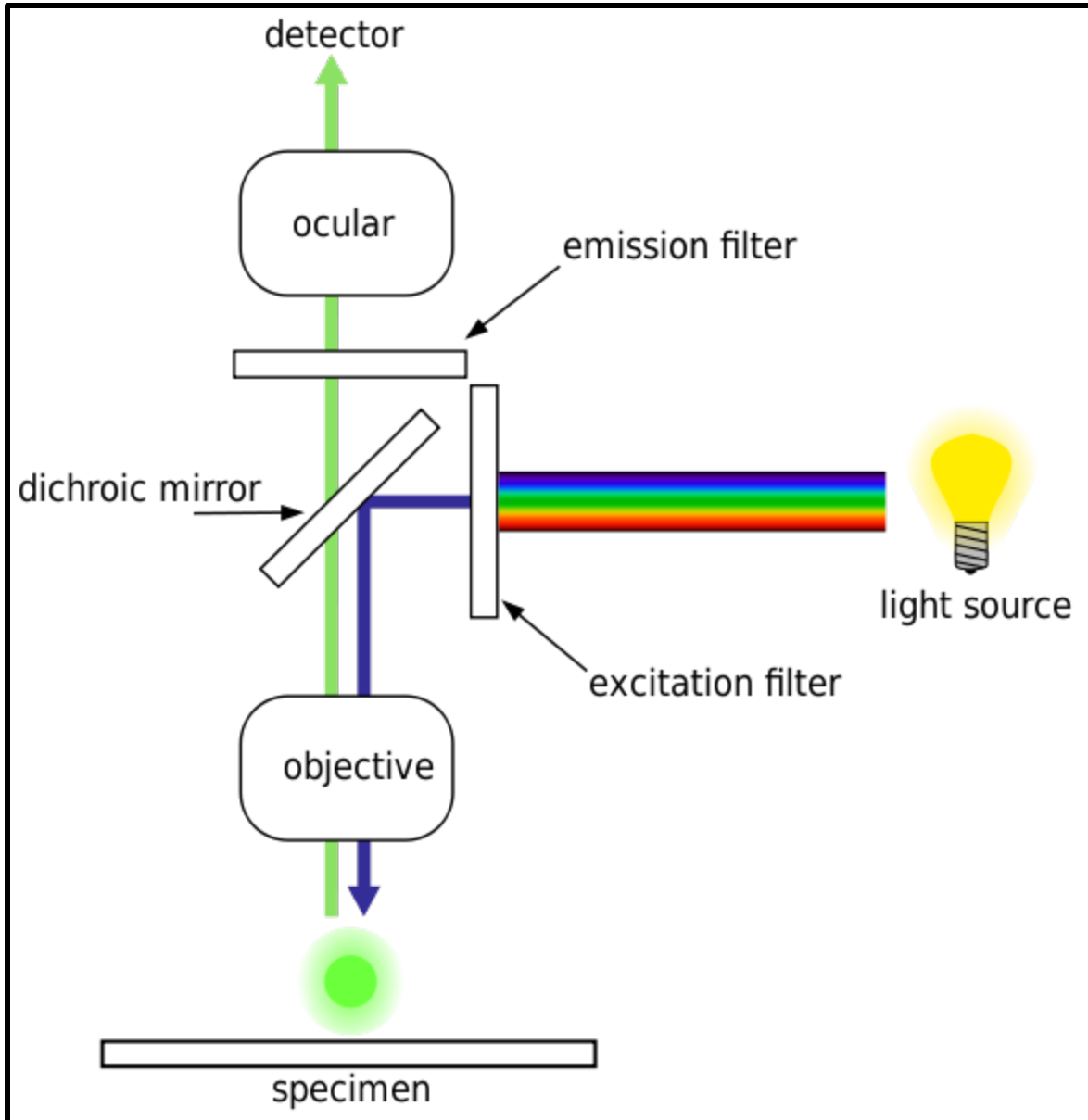
GFP - Green



Next Halloween Costume??



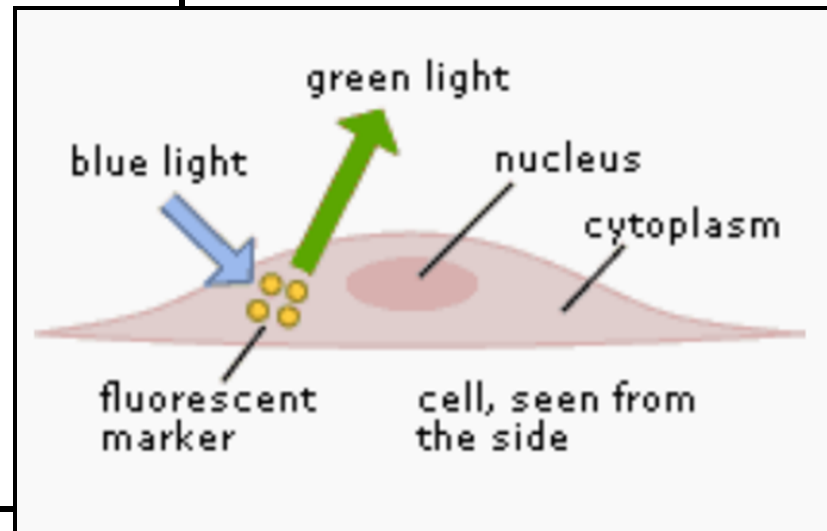
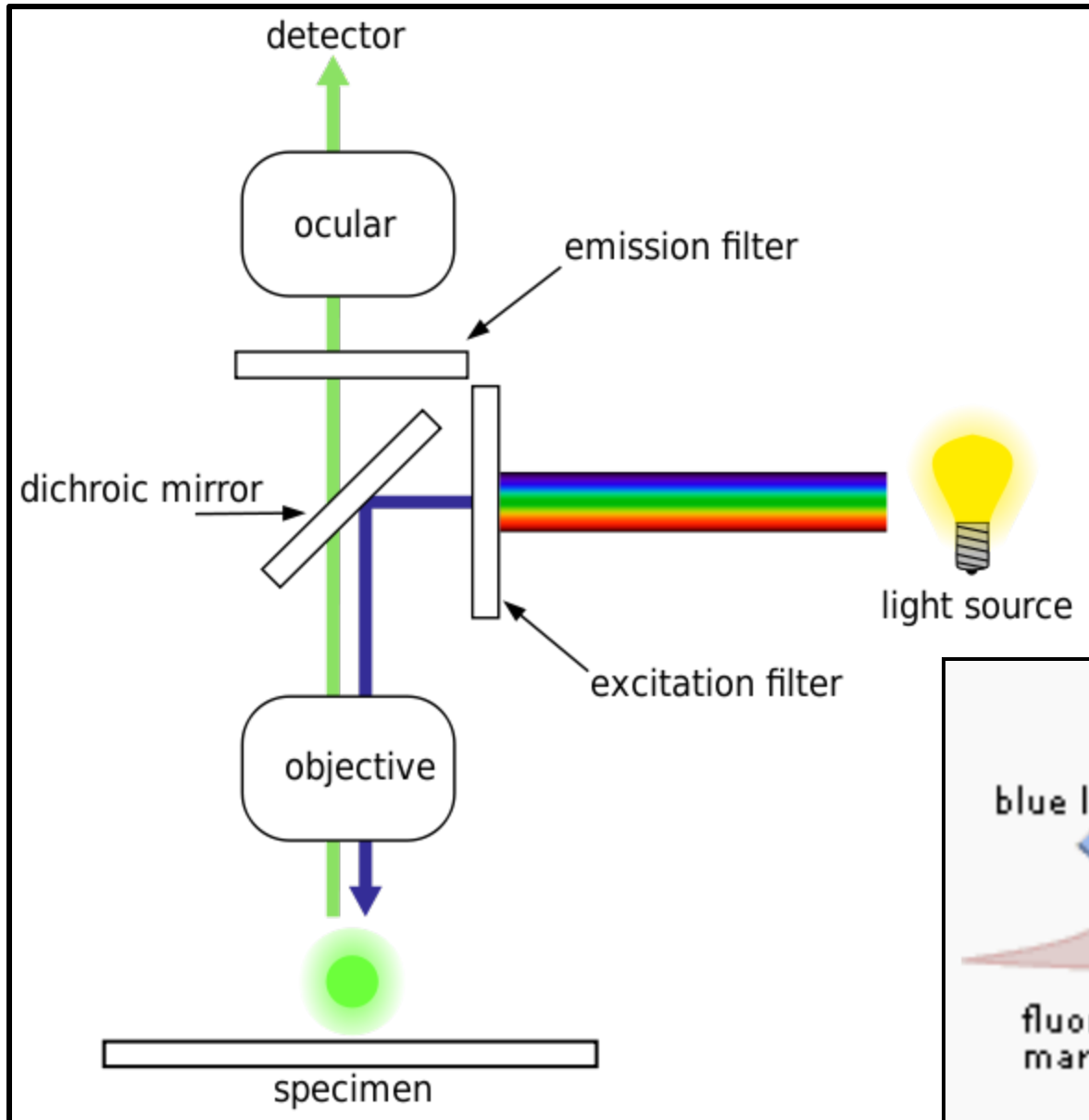
Fluorescence Detection



A **dichroic** filter, thin-film filter, or interference filter is a very accurate color filter used to selectively pass light of a small range of colors while reflecting other colors.

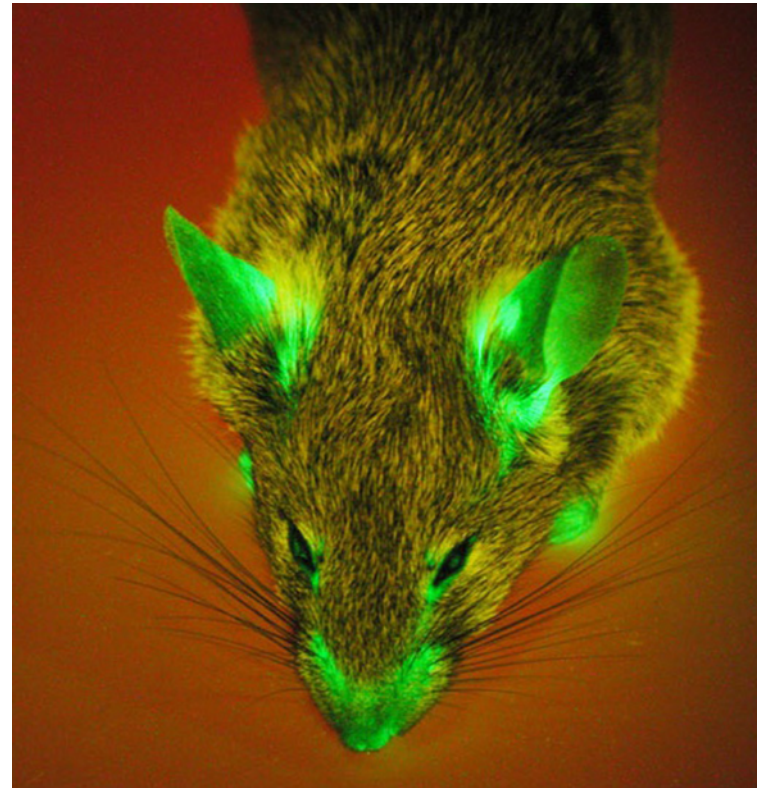
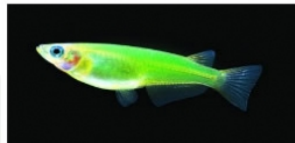
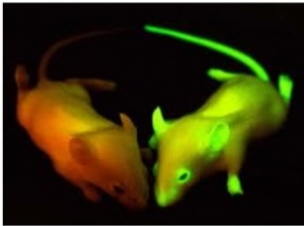
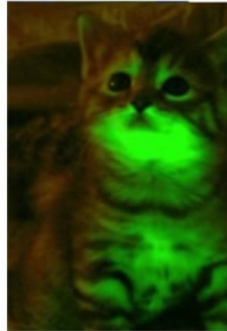


Fluorescence Detection

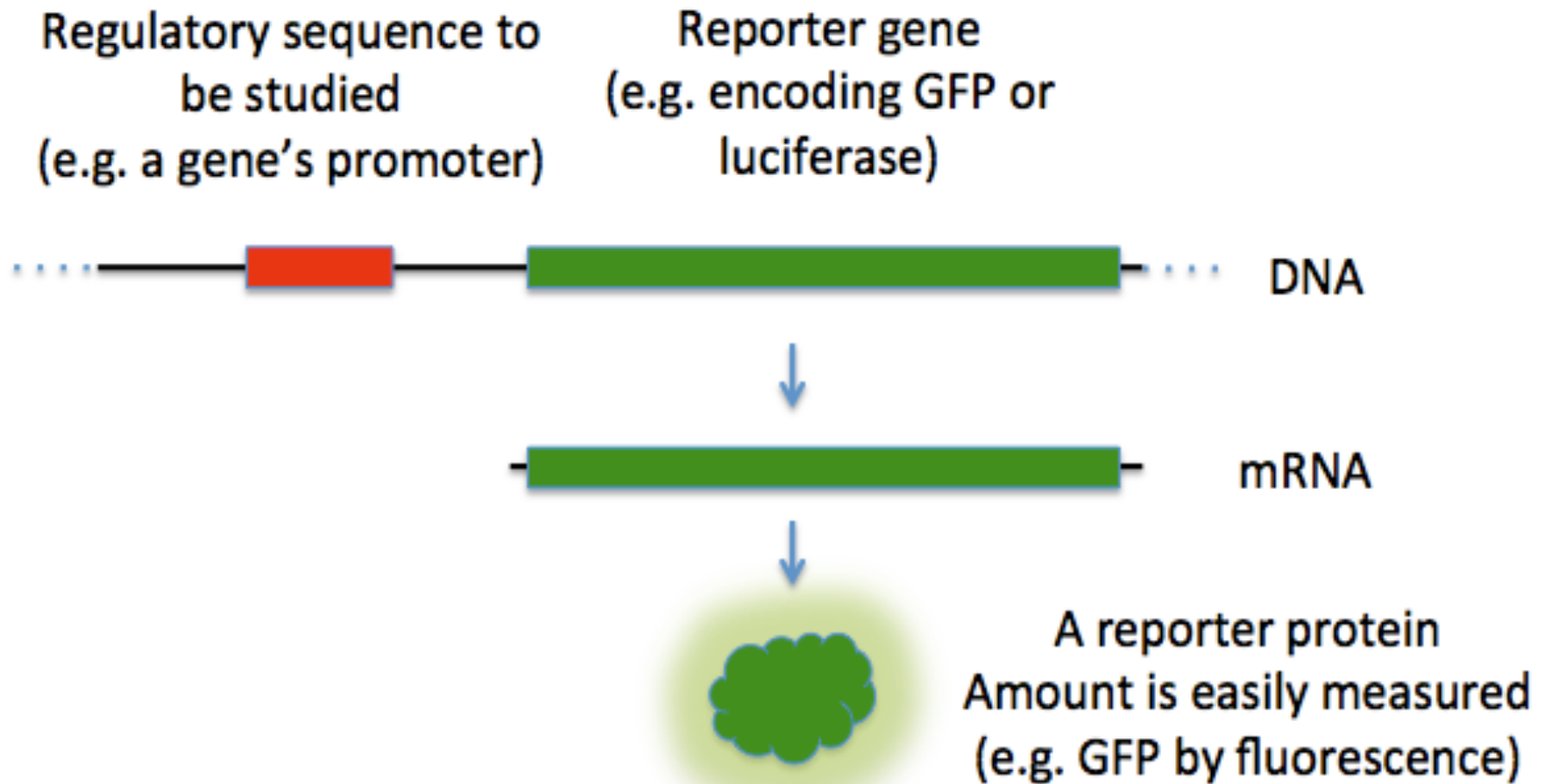




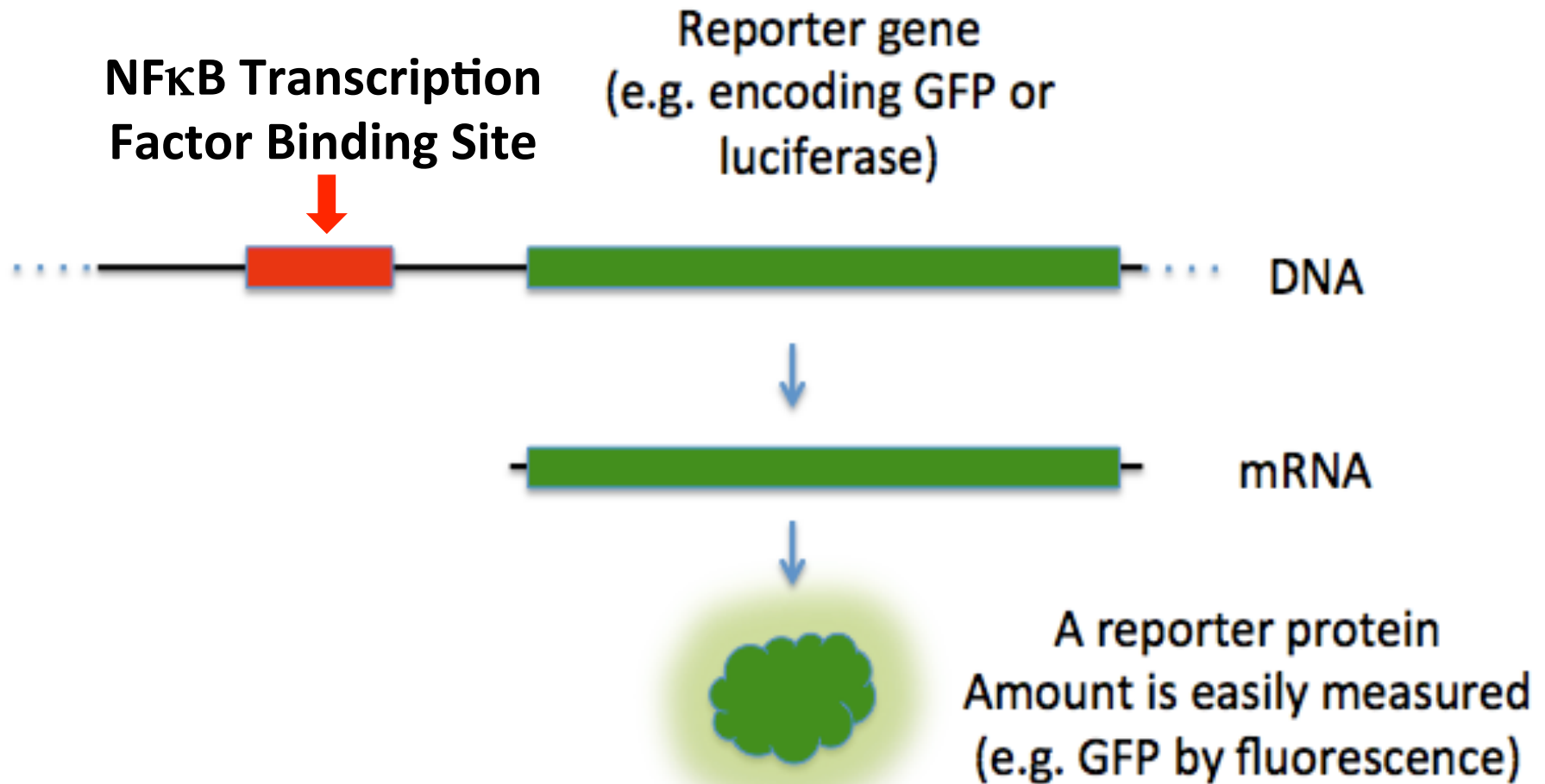
<http://www.glofish.com/meet-glofish/glofish-gallery/>



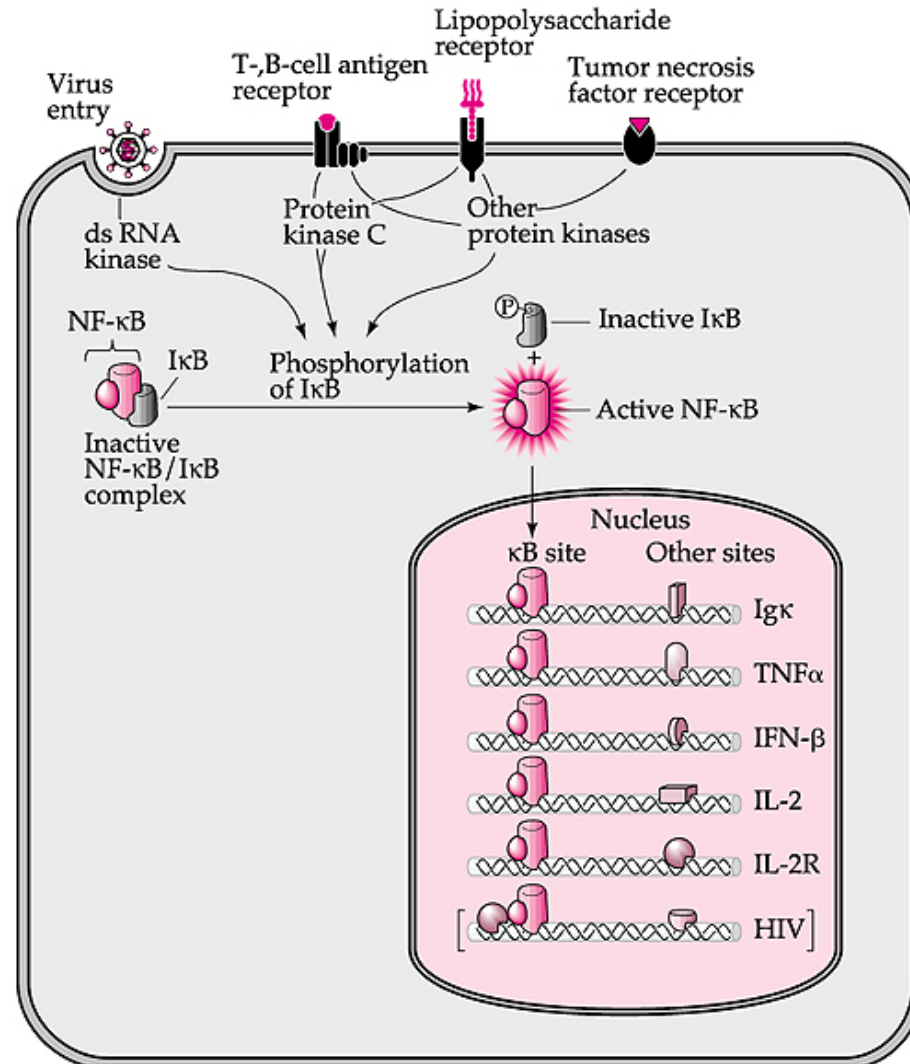
Let's use a different reporter gene that is easy to assay and works *in vivo*



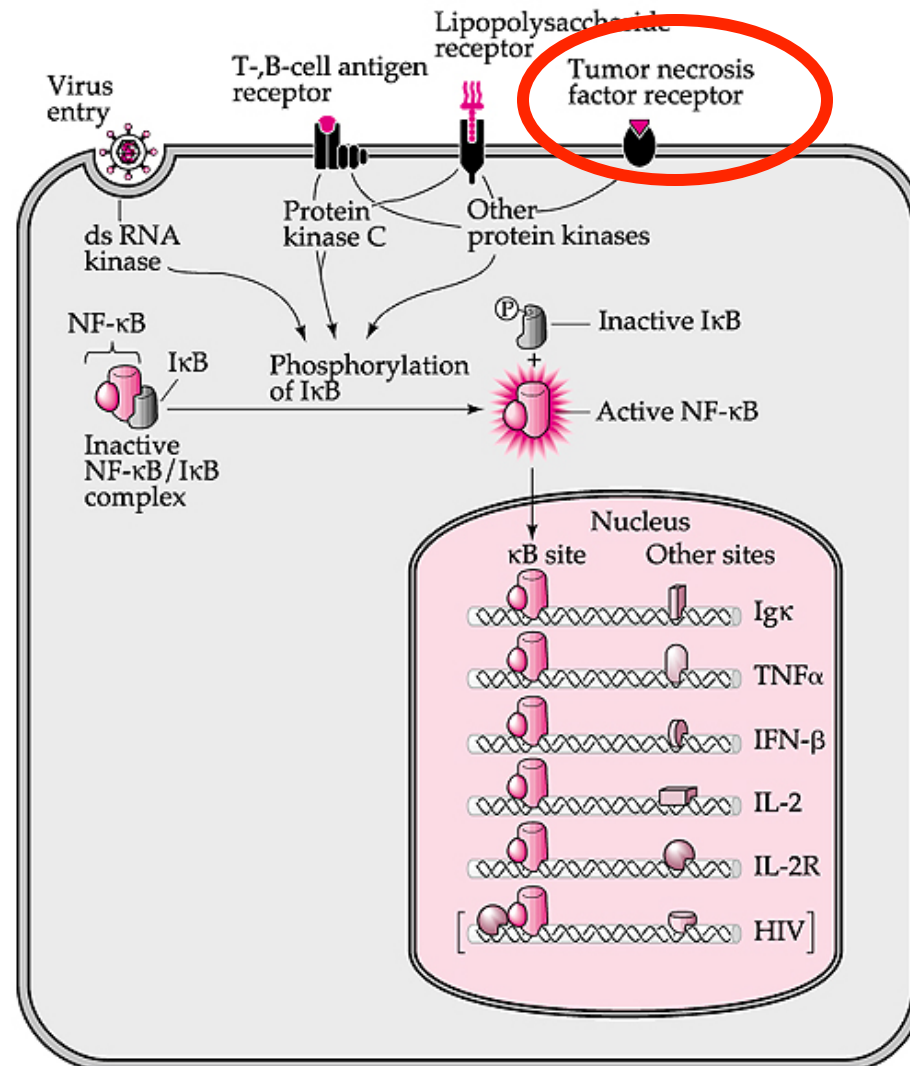
Let's use a different reporter gene that is easy to assay and works *in vivo*

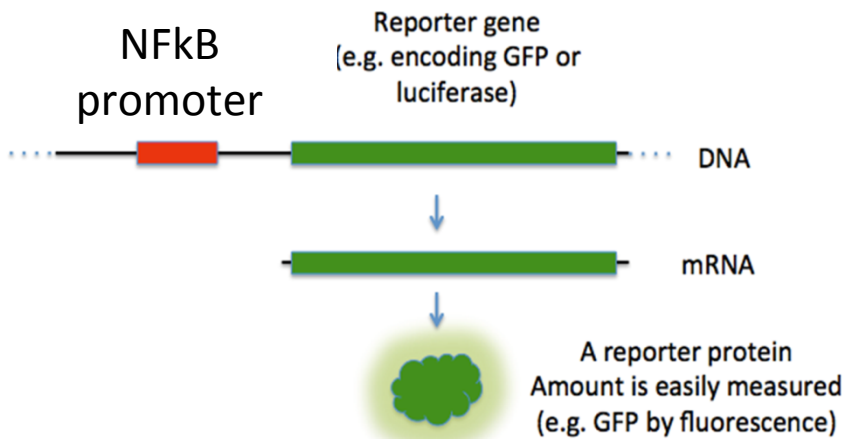


Regulation of gene expression by the NFκB Transcription Factor

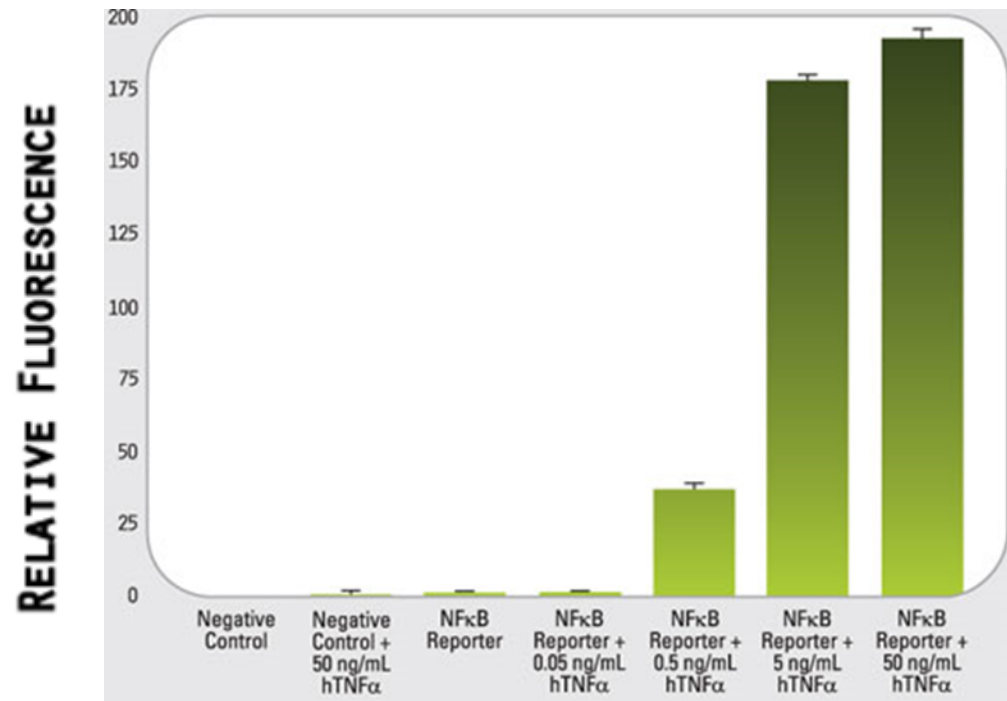
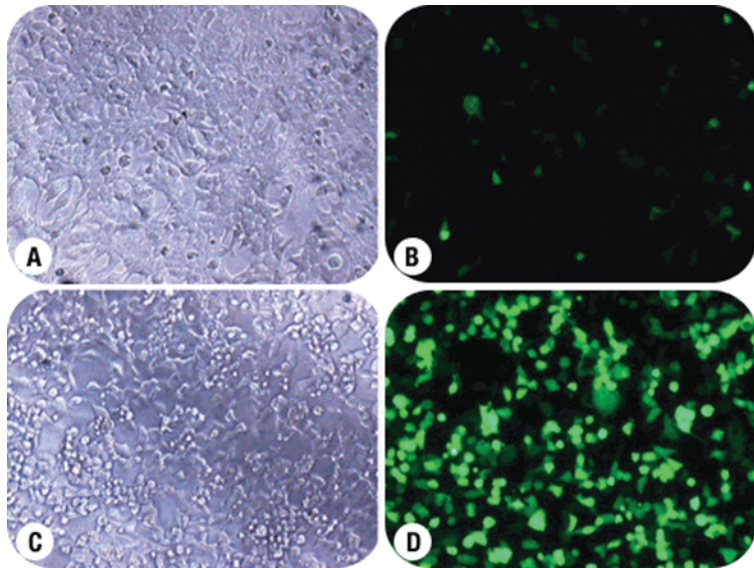


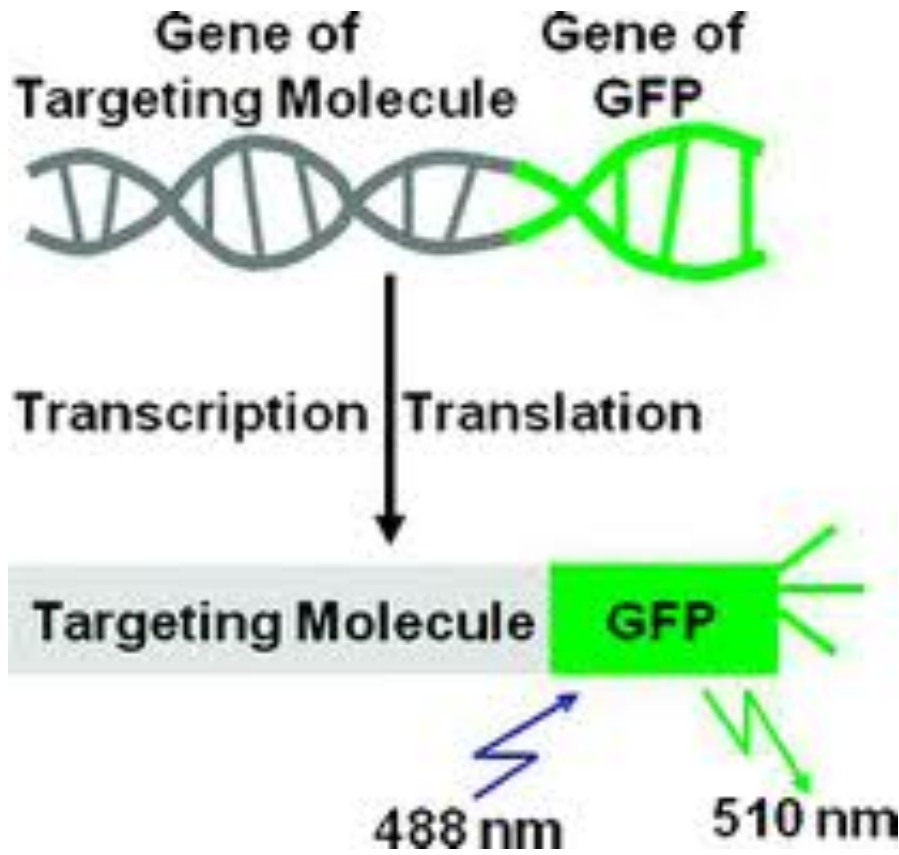
Regulation of gene expression by the NFκB Transcription Factor



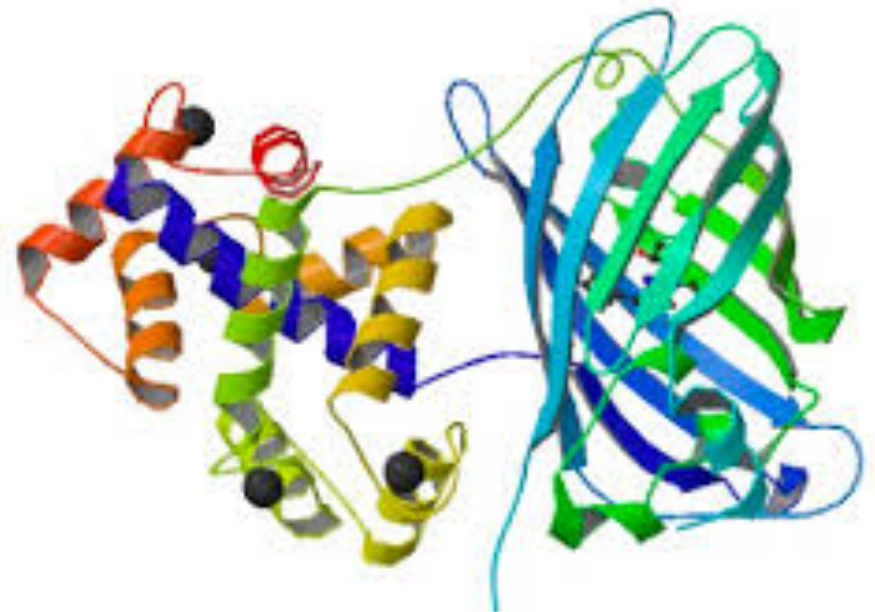


Fluorescent Protein Reporters are often used to report the amount of transcription from a specific promoter





Genetically engineered GFP-Fusion proteins



Fluorescent Protein Gene Fusions for Subcellular Localization Imaging

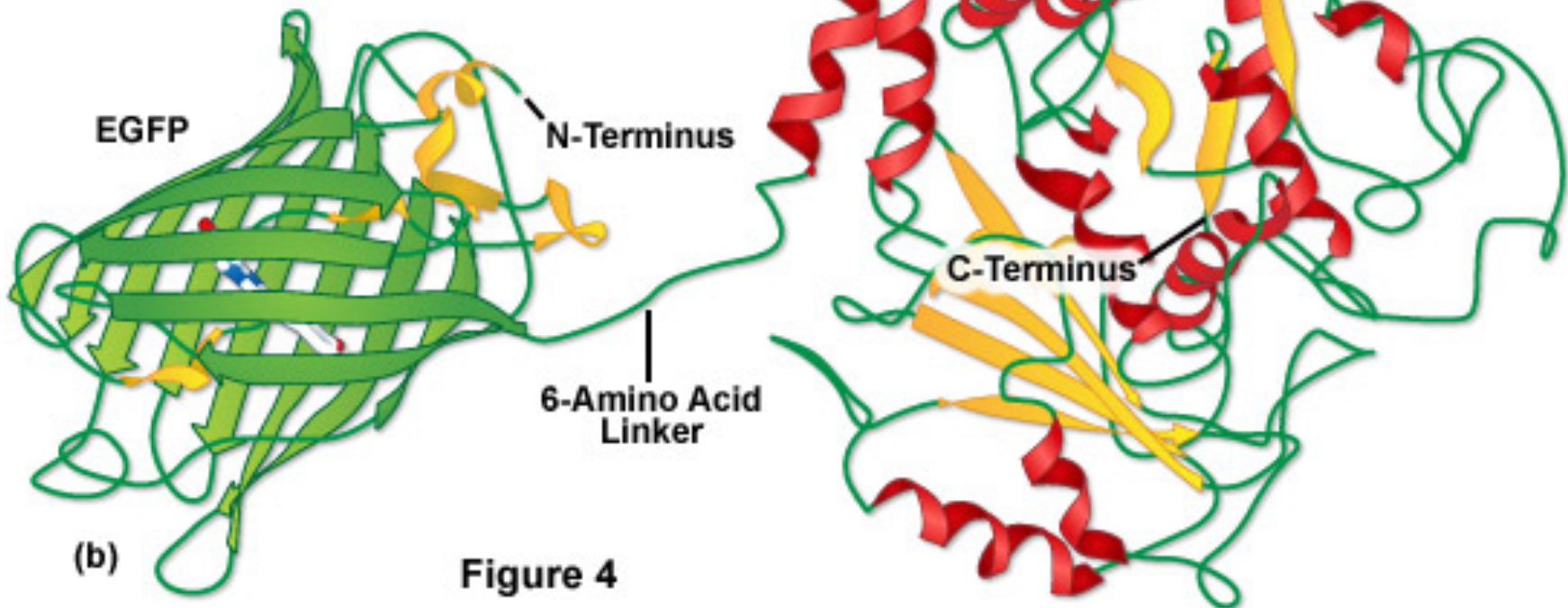
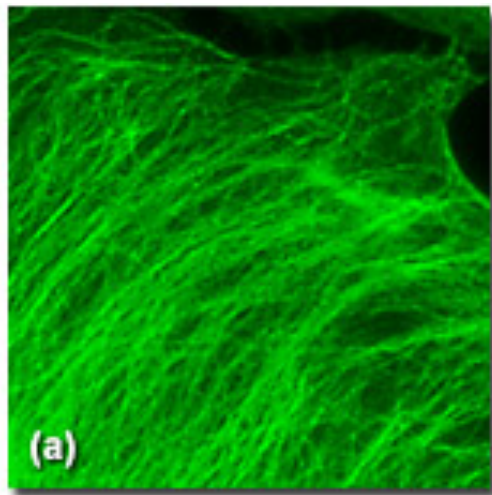
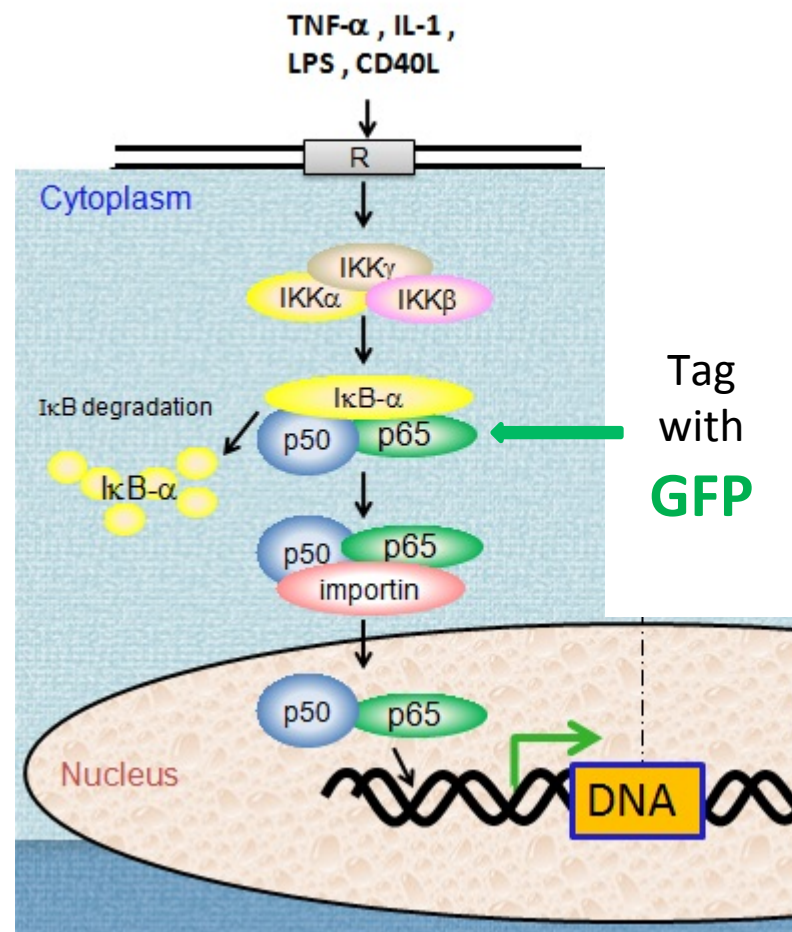
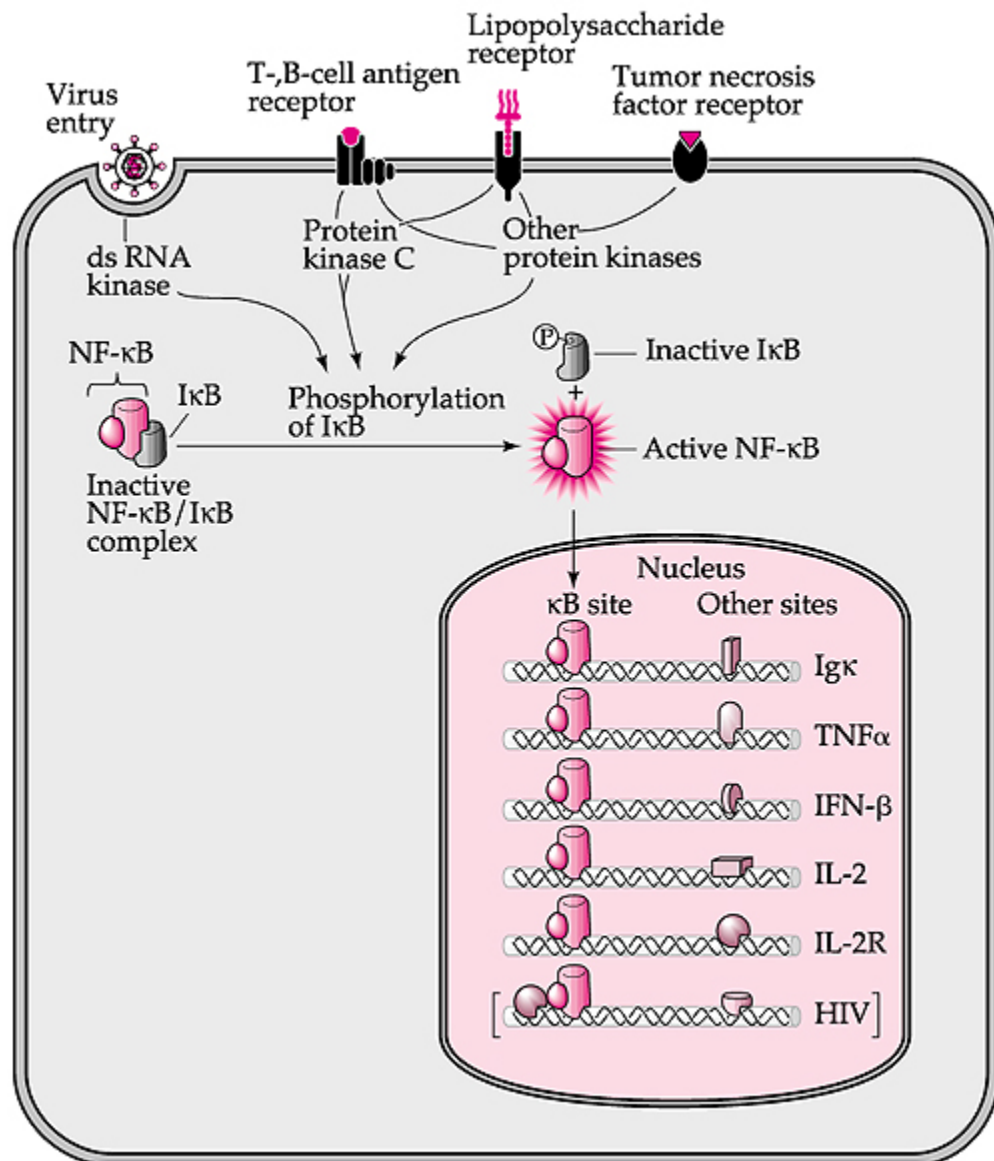
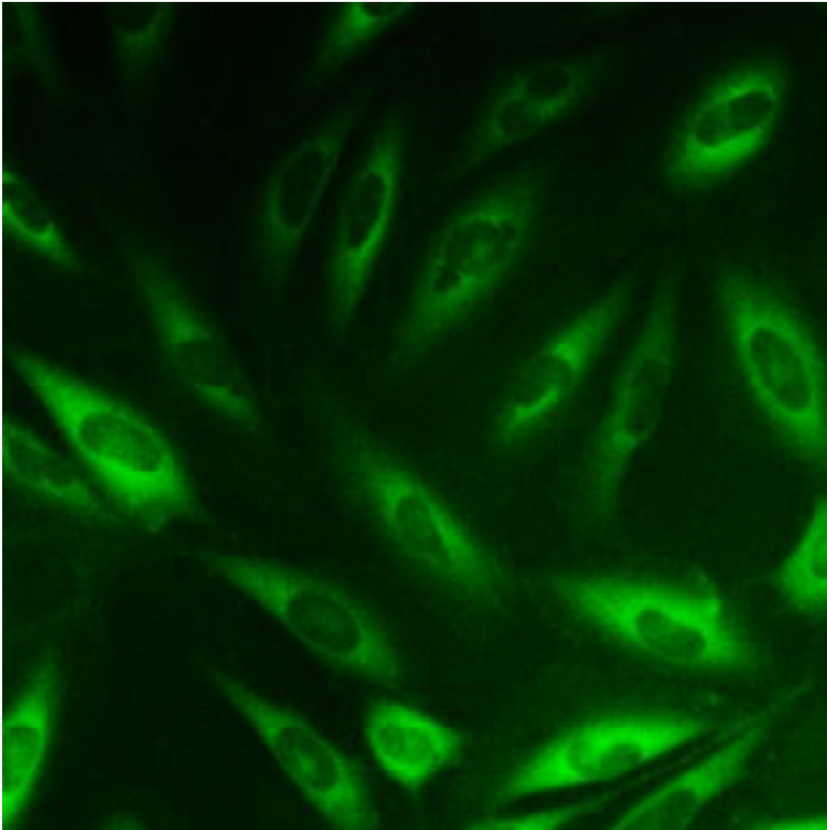


Figure 4

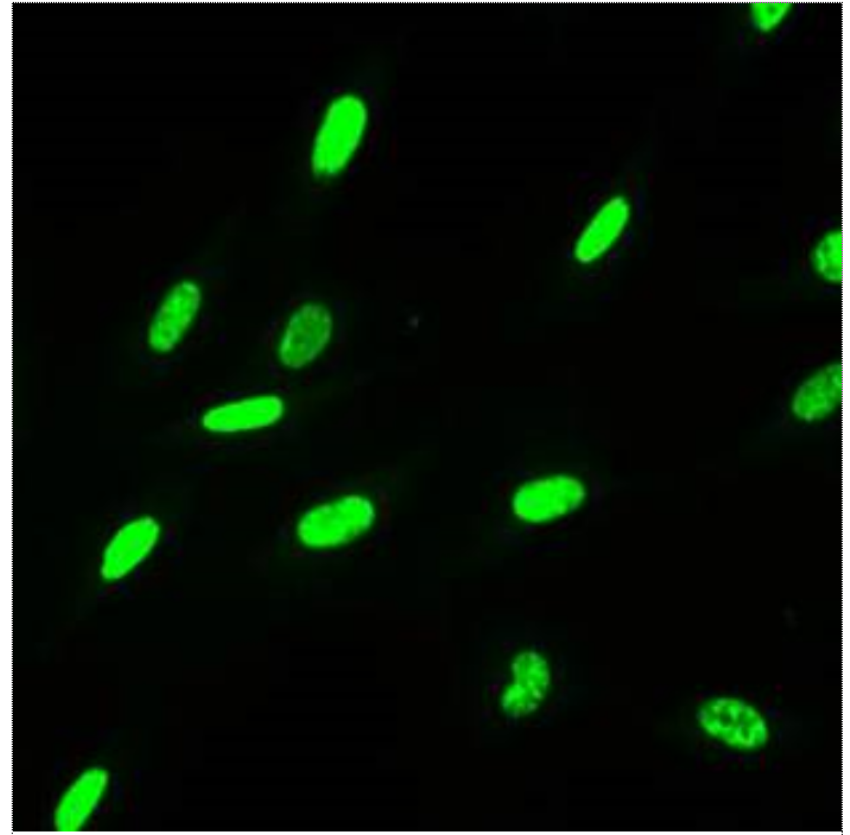
Regulation of subcellular location of NFκB



DNA Damage Induced NFkB Signalling



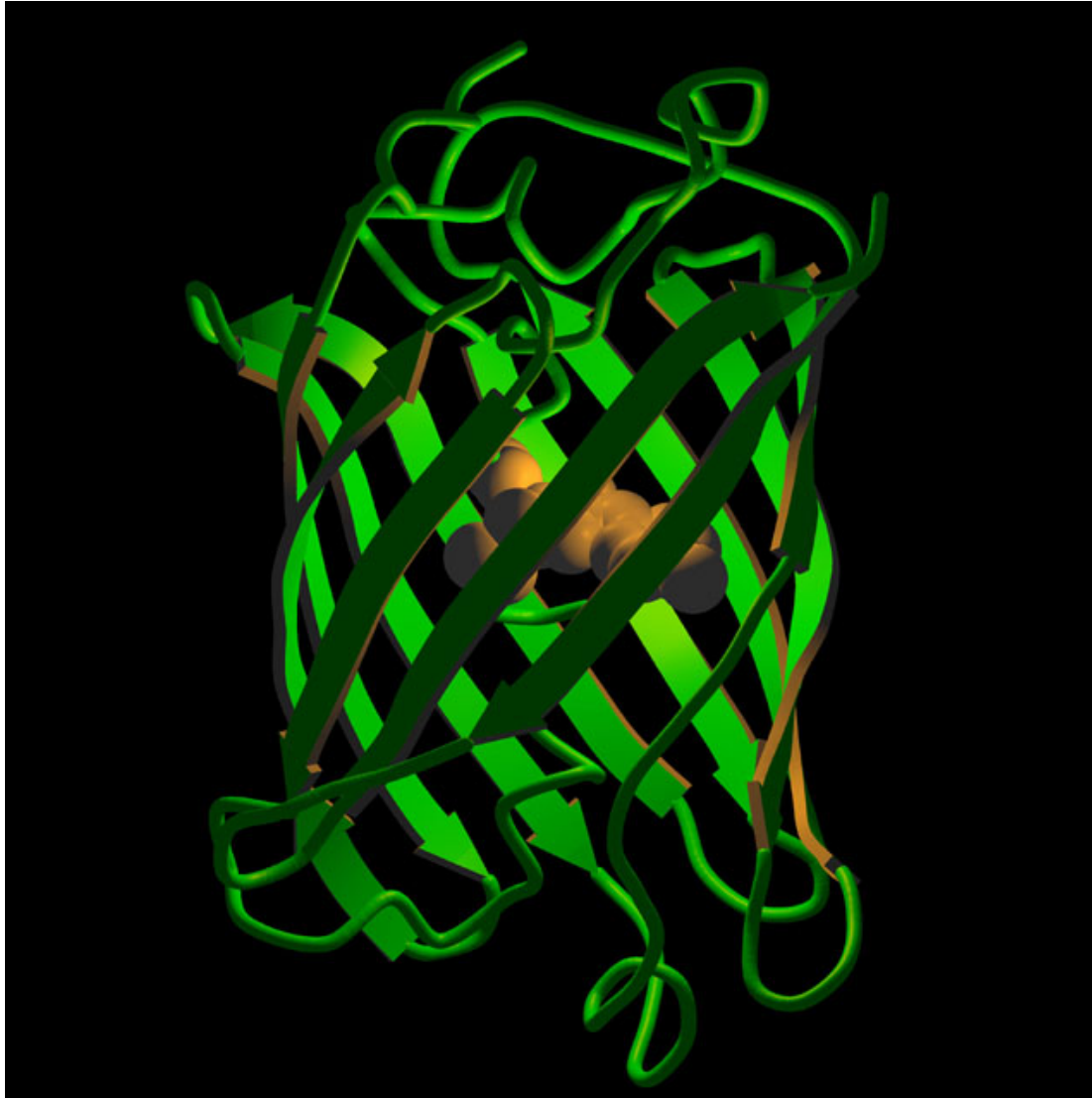
inactive NFkB



cpd induced NFkB activation

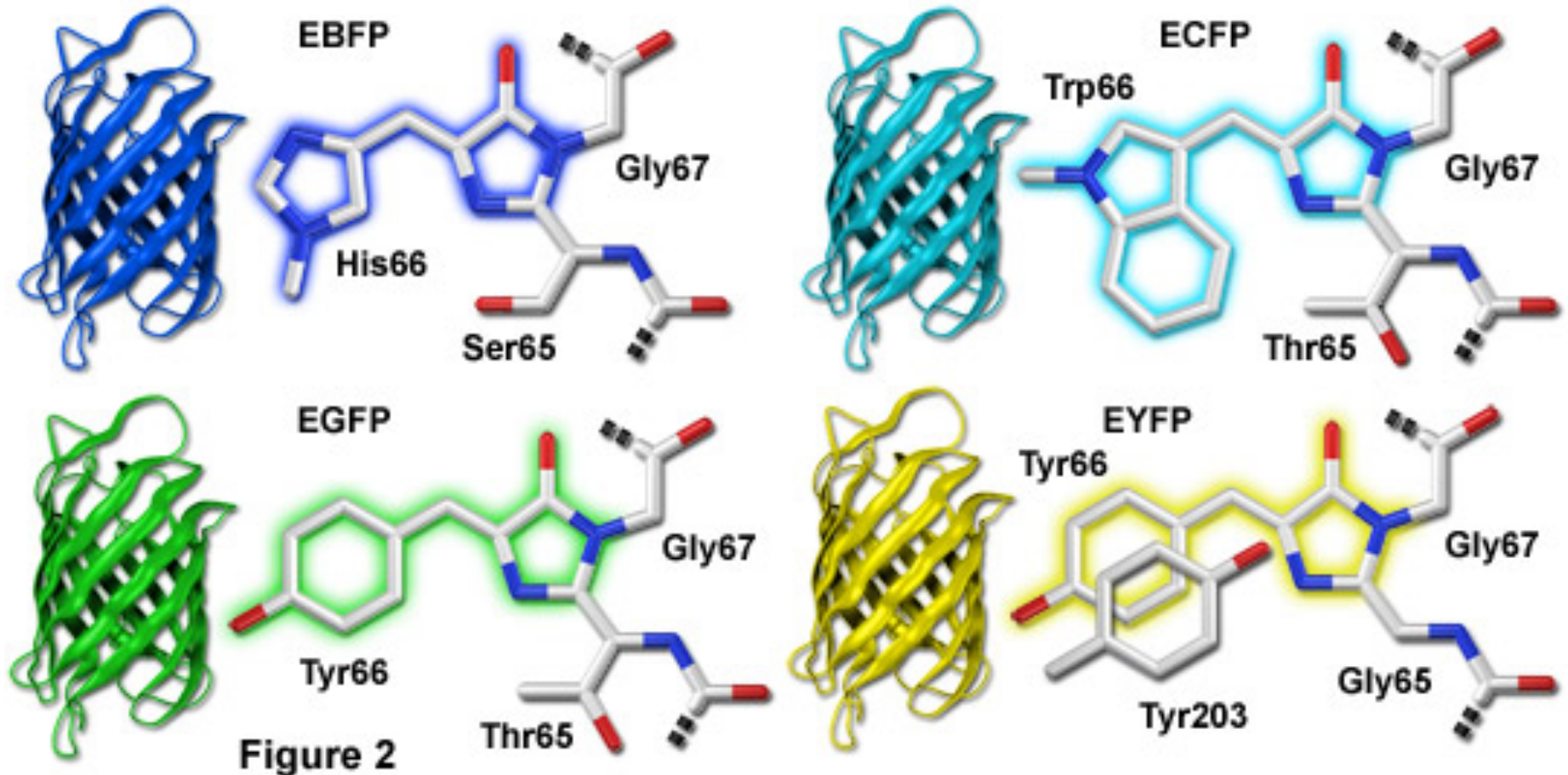
A cell line stably expressing a fusion of the NFkB transcription factor subunit with GFP allows one to monitor NFkB activation (translocation to nucleus) by compound induced DNA damage.

Green Fluorescent Protein (GFP) first isolated from
crystal jellyfish (*Aequorea victoria*).



GFP modified to Enhanced GFP (EGFP) and EGFP modified to fluoresce at different wavelengths

Chromophore Structural Motifs of Green Fluorescent Protein Variants

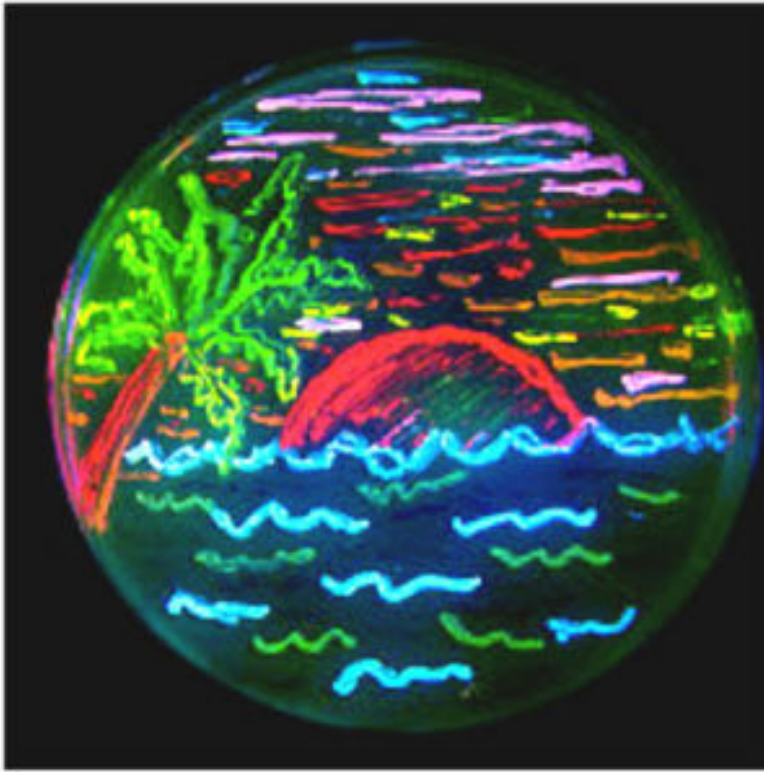




Mushroom Coral

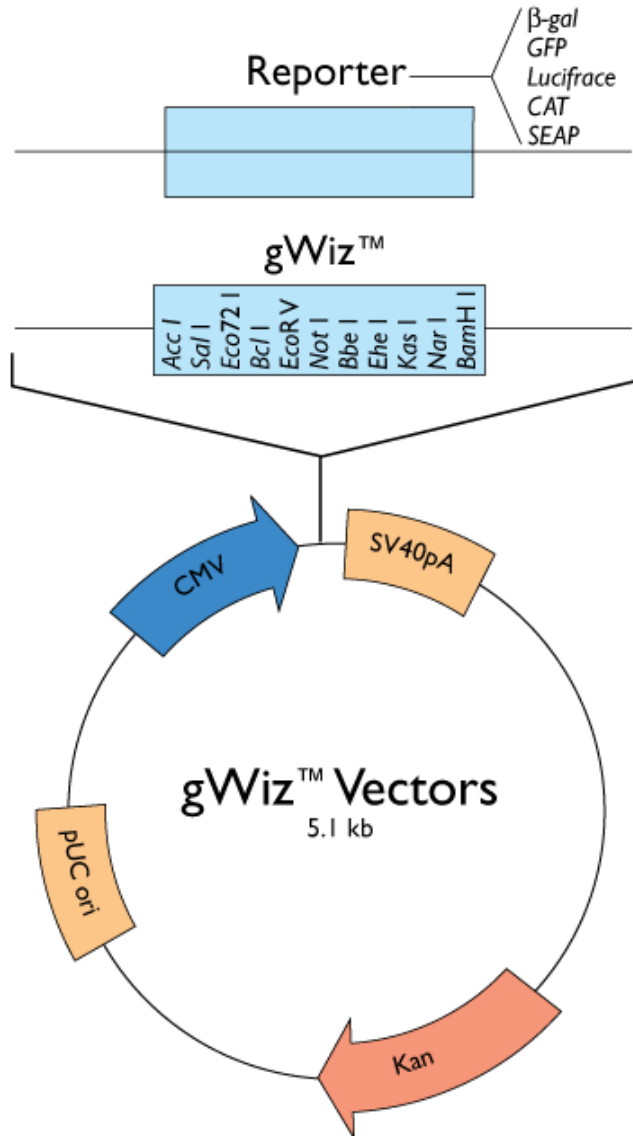
Fluorescent Bulb
Anemone (*Entacmaea
quadricolor*)



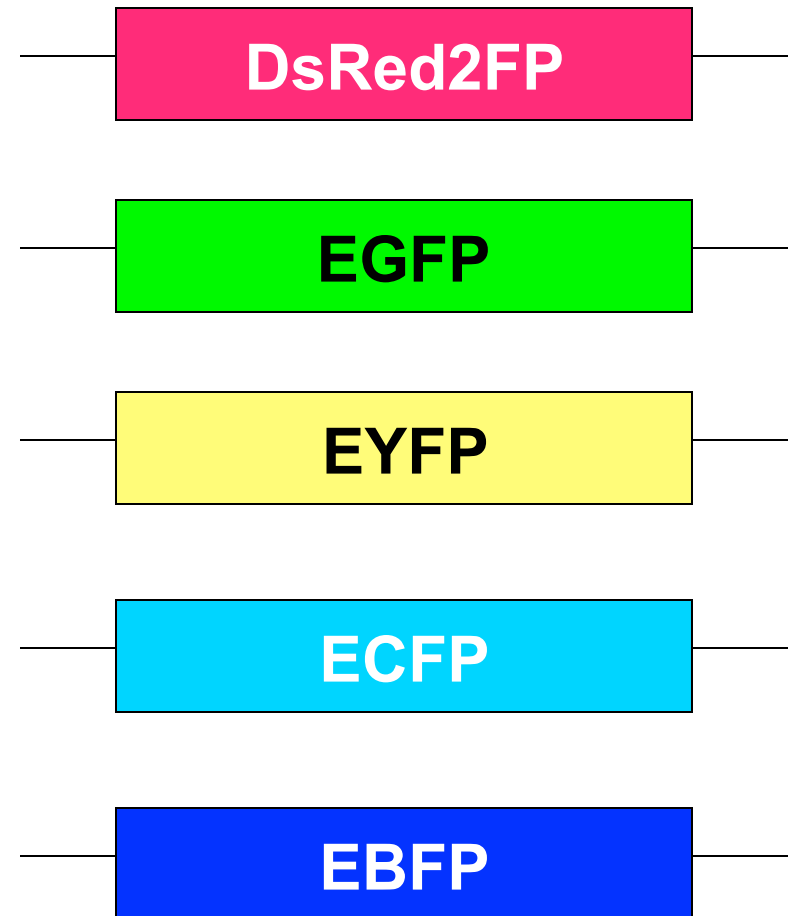


The diversity of fluorescent proteins and genetic mutations is illustrated by this San Diego beach scene drawn with living bacteria expressing 8 different colors of fluorescent proteins.

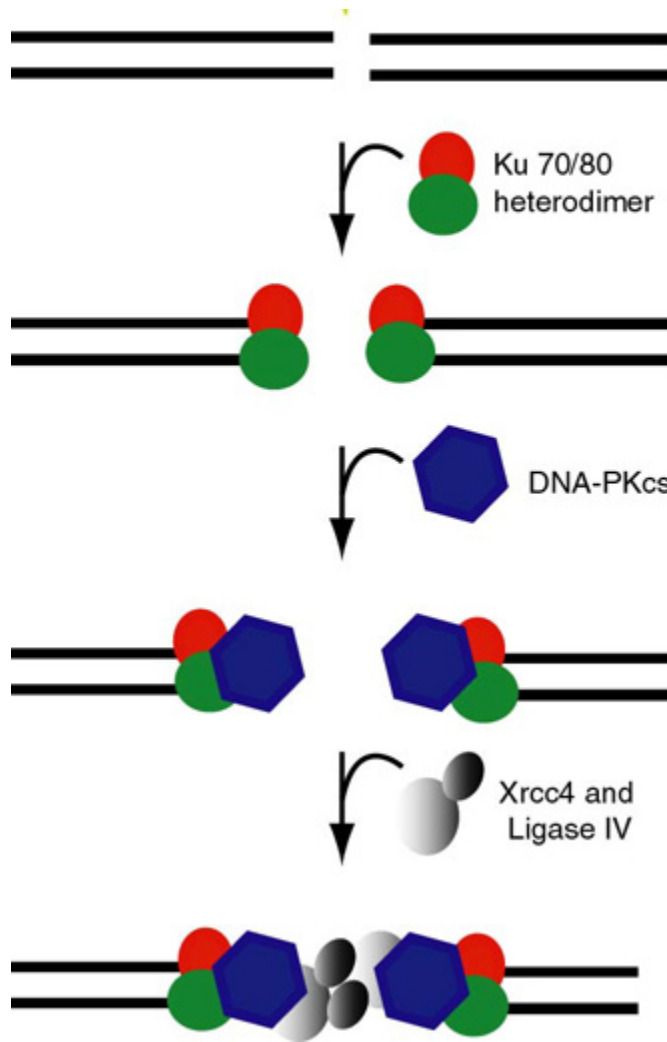
Reactivation of damaged DNA - multiplexed



Each Fluorescent Protein gene will harbor a different type of DNA damage



Non-Homologous End Joining (NHEJ)



Ku70

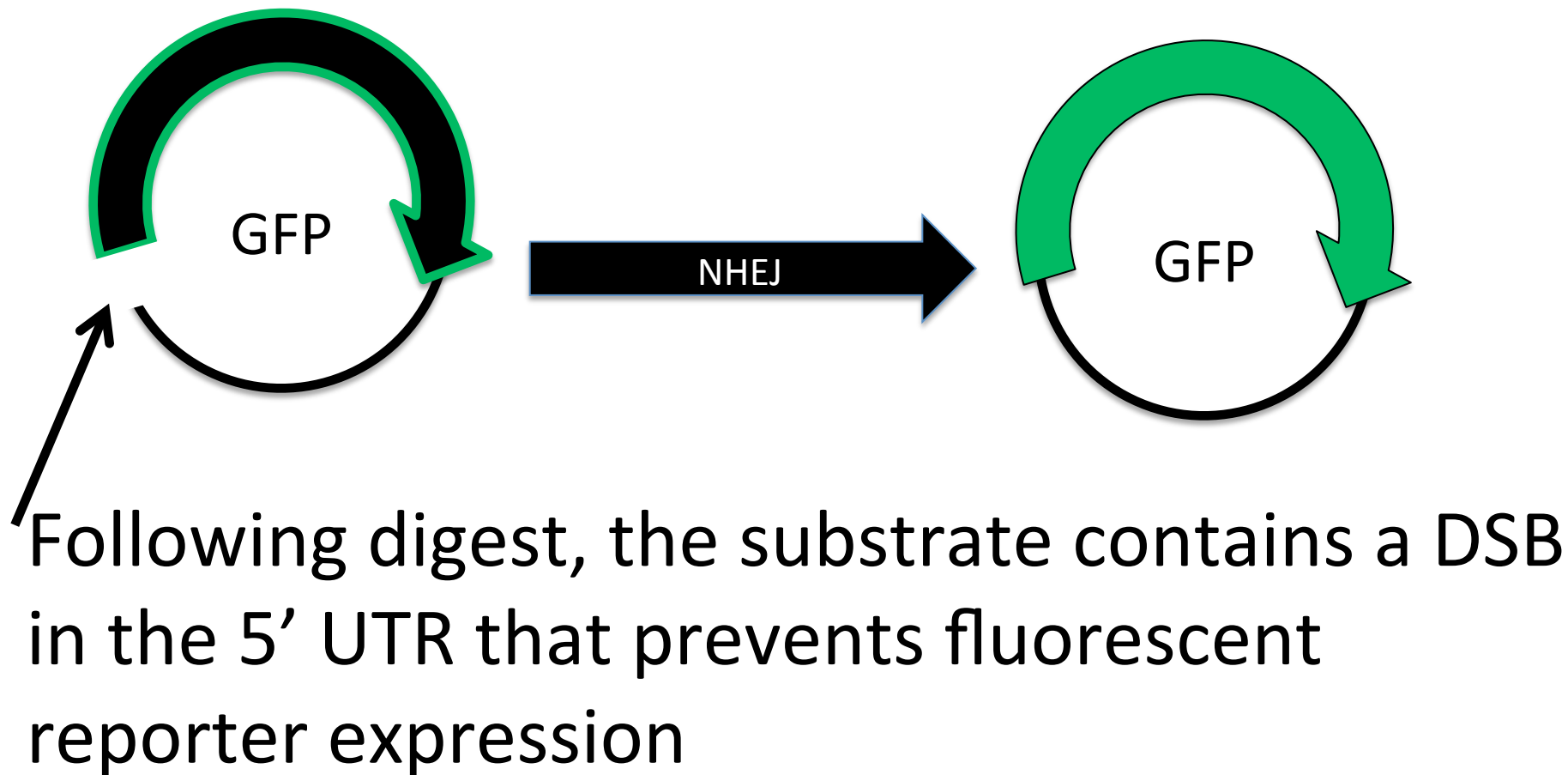
Ku80

DNA-PKcs

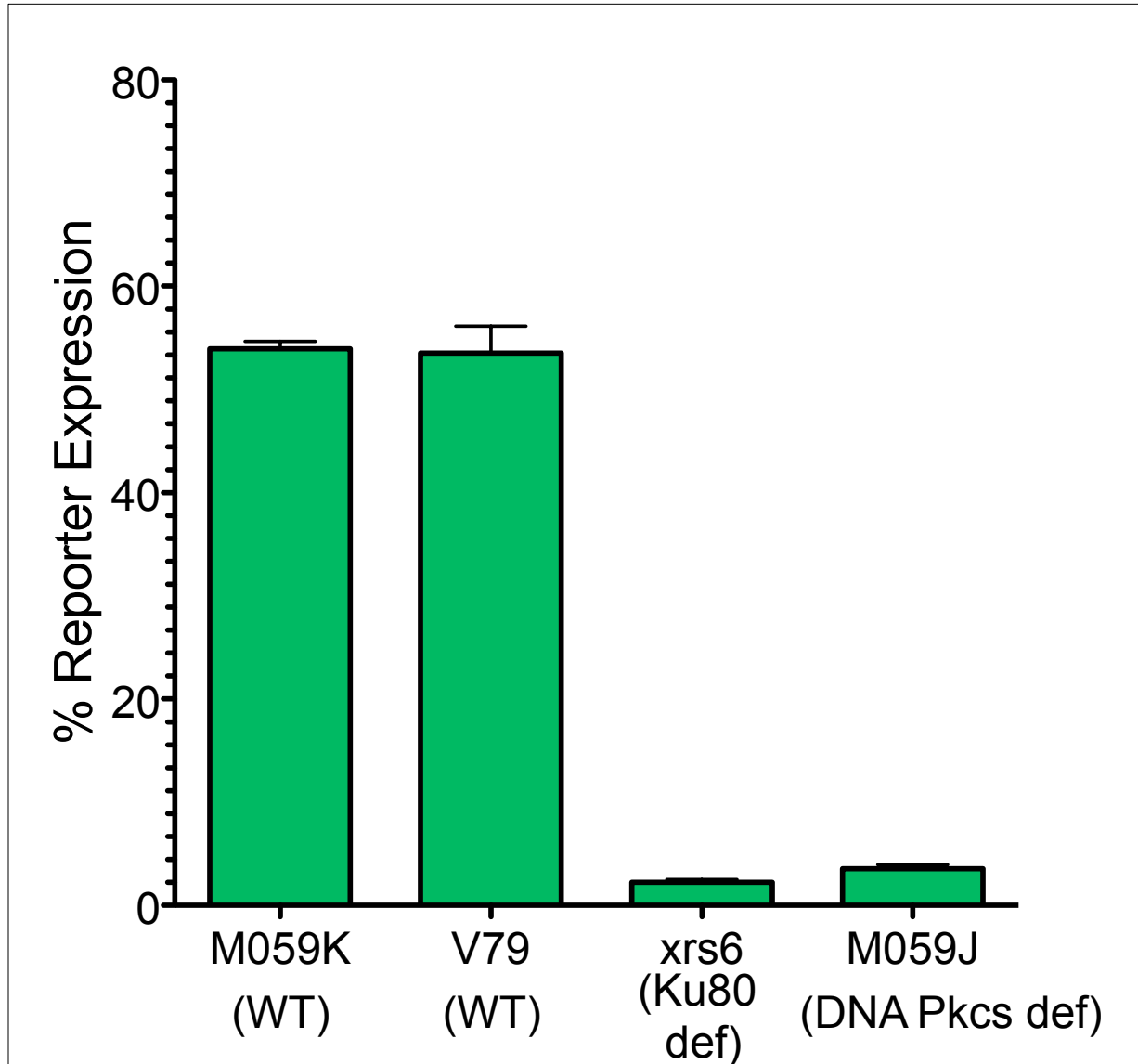
Xrcc4

Ligase IV

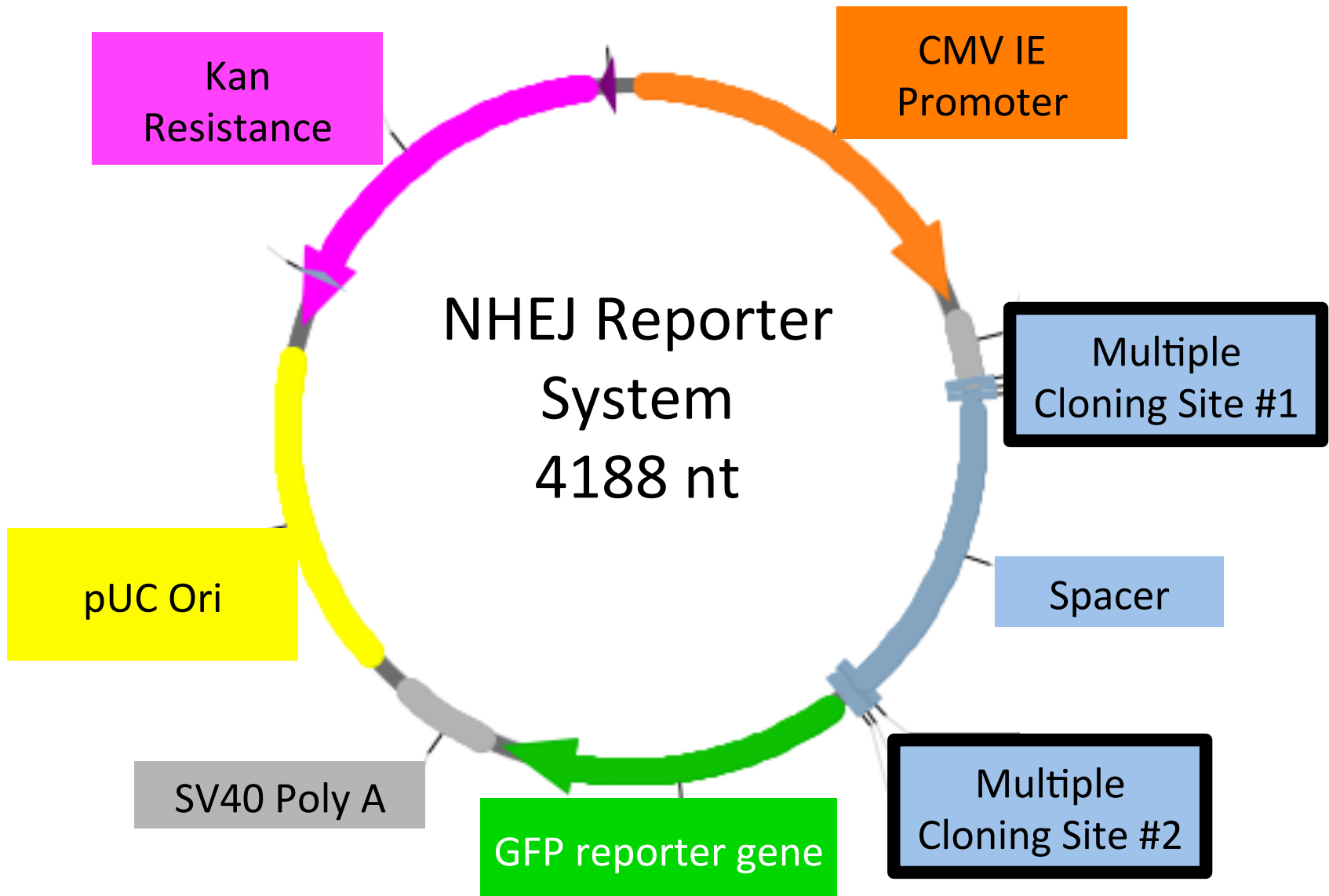
Basis for the fluorescent reporter assay:



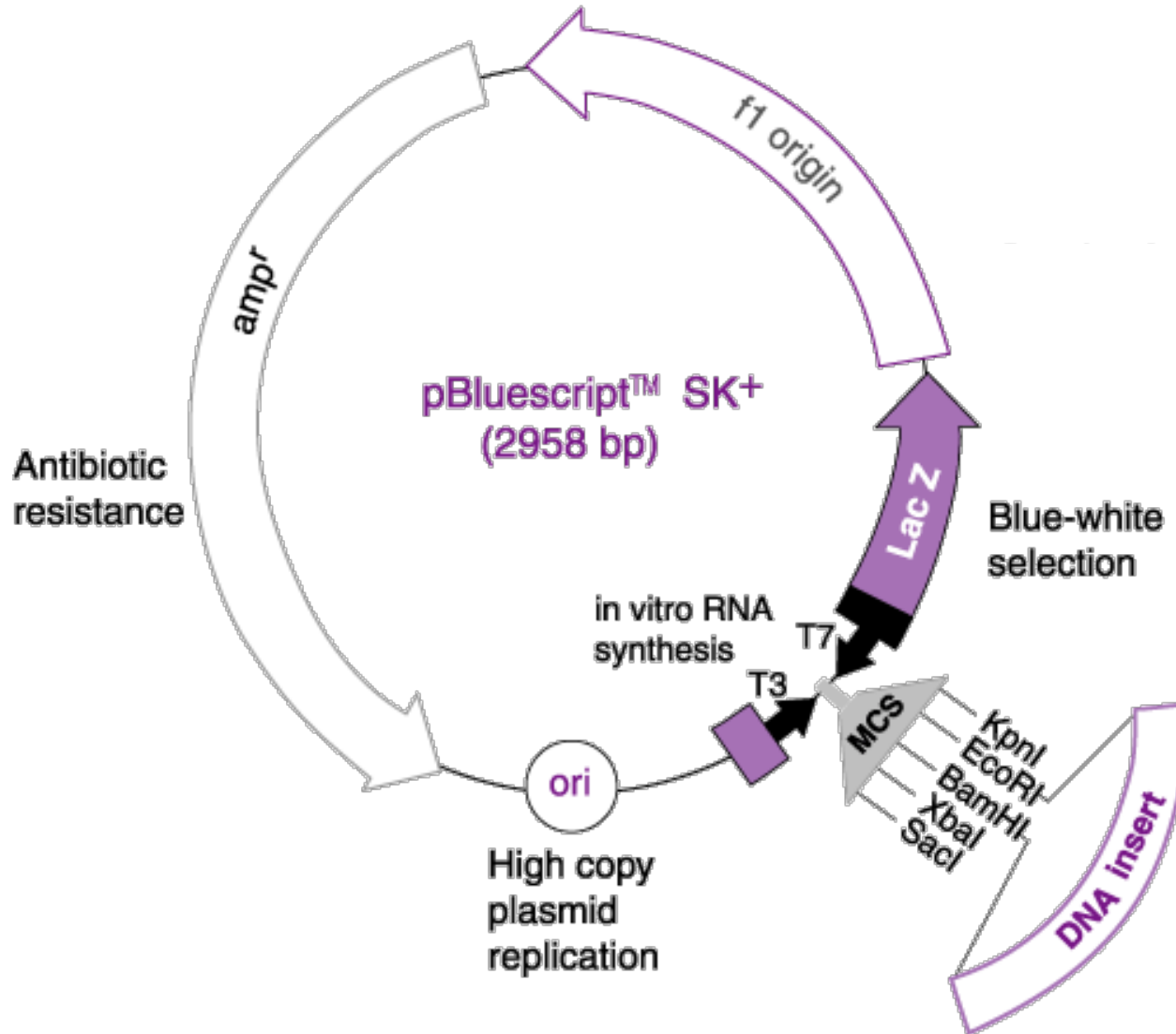
NHEJ HCR in WT and NHEJ defective cells at 18 hours post-transfection:



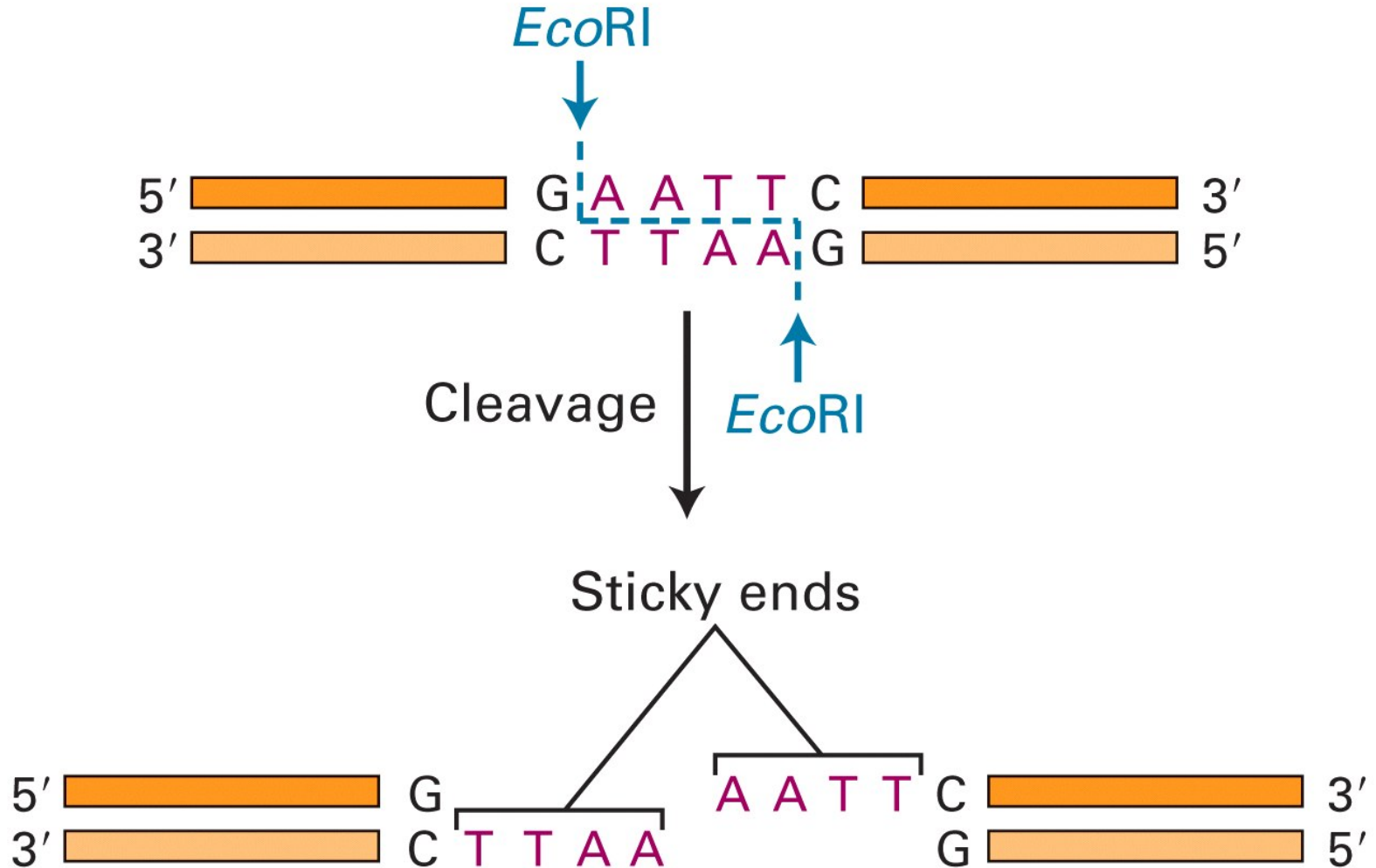
Overall Structure of the Reporter:



What is a Multiple Cloning Site (MCS)? (sometimes called a polylinker)



What is a Restriction Enzyme?



Palindromes

Madam I'm Adam.

Sit on a potato pan, Otis!

Cigar? Toss it in a can, it is so tragic.

U.F.O. tofu.

Golf? No sir, prefer prison flog.

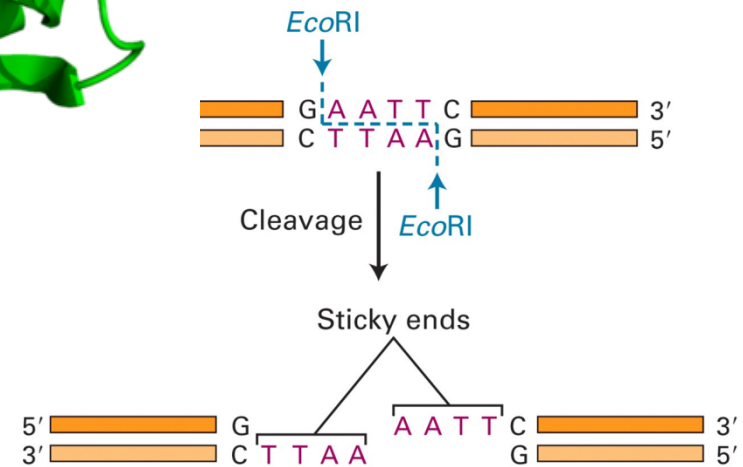
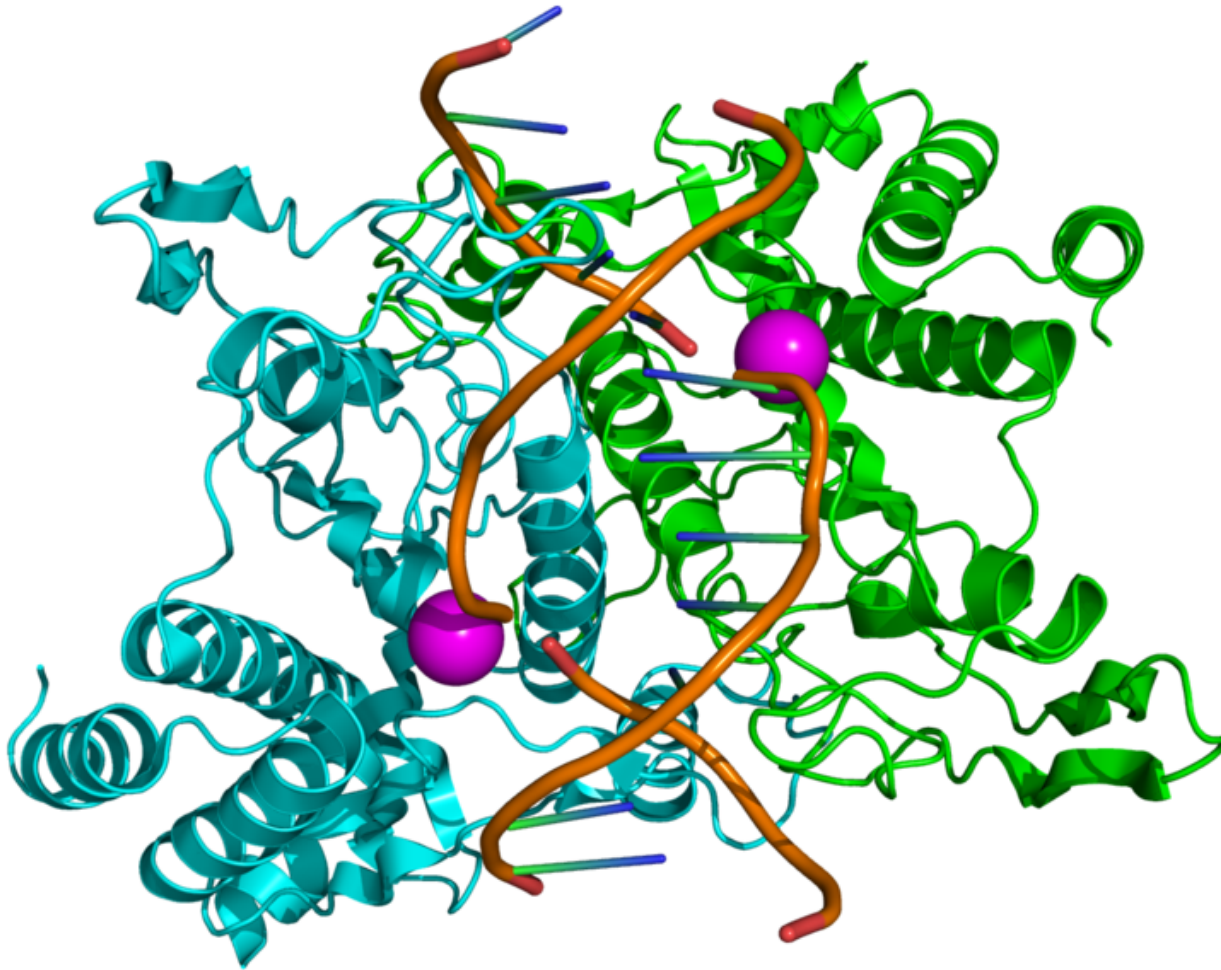
Flee to me remote elf.

Gnu dung.

Lager, Sir, is regal.

Tuna nut.

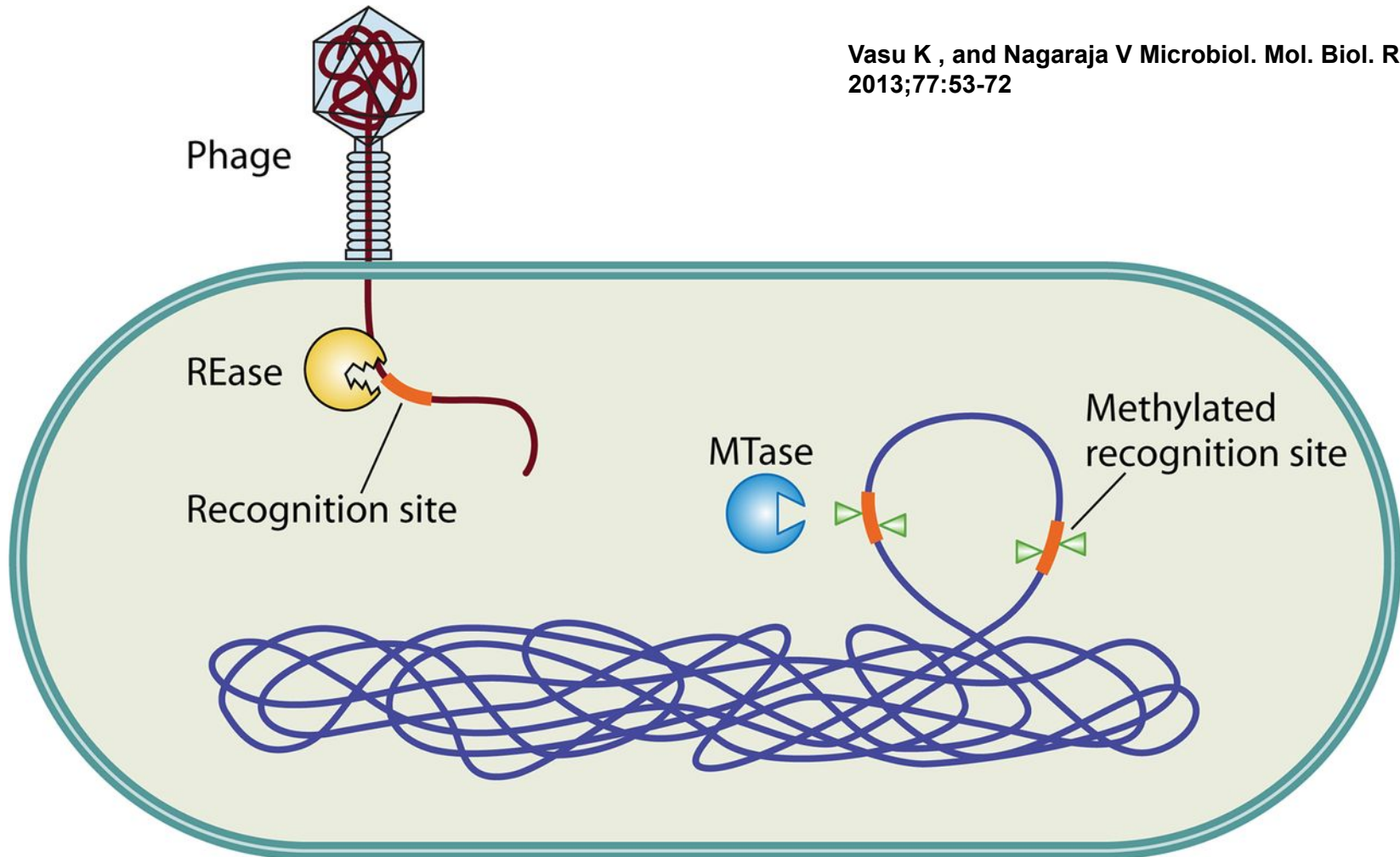
What is a Restriction Enzyme?



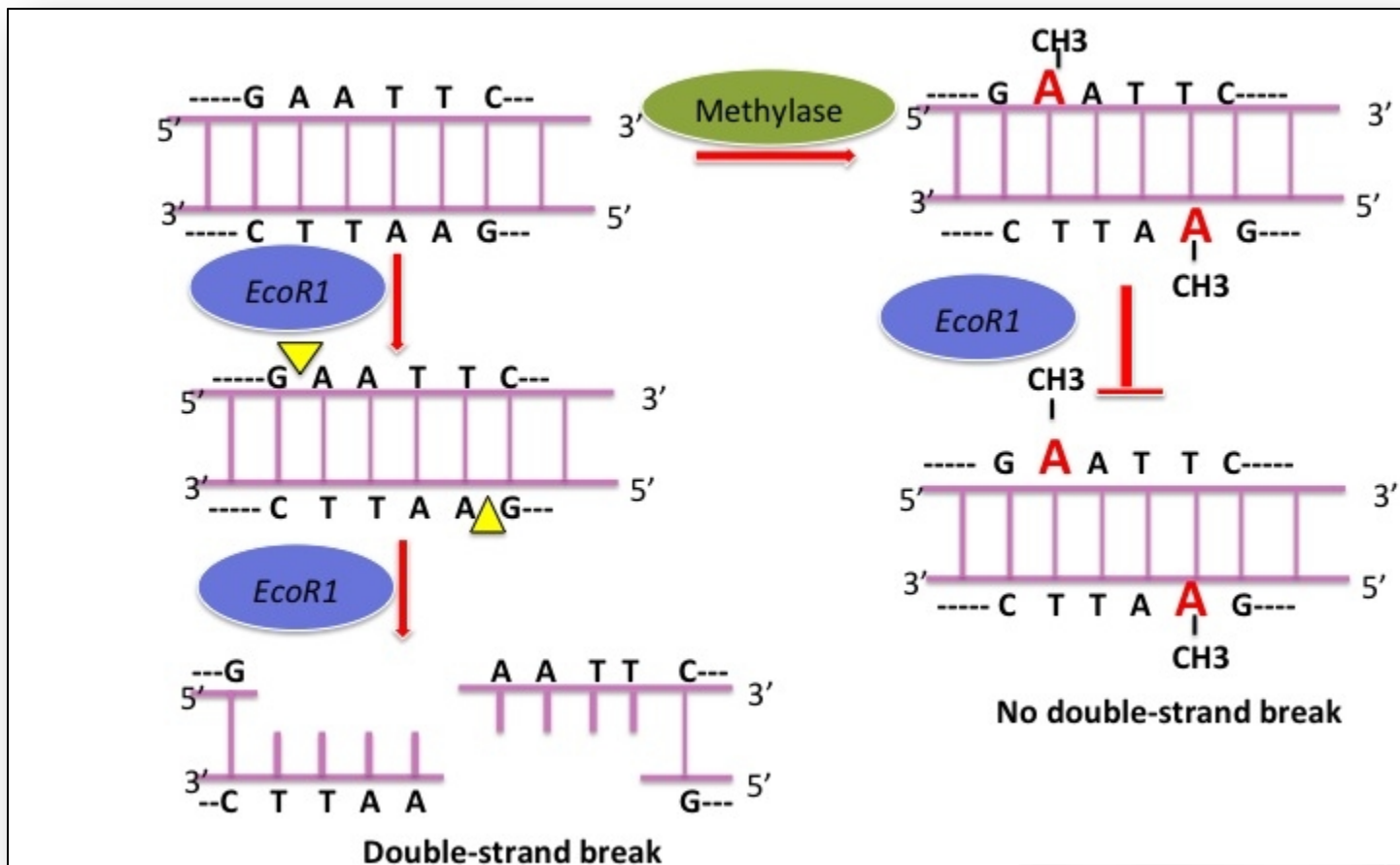
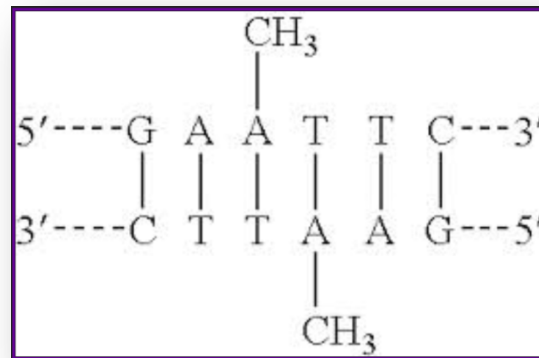
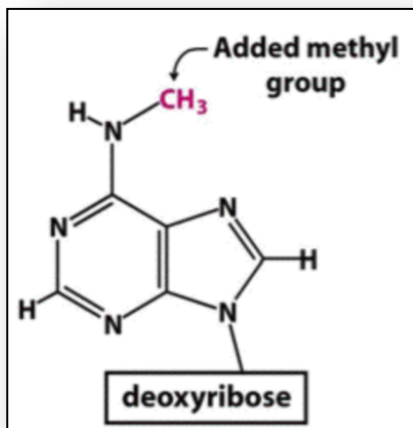
Why do Restriction Enzymes exist?

Restriction-modification (R-M) systems as defense mechanisms.

Vasu K , and Nagaraja V *Microbiol. Mol. Biol. Rev.* 2013;77:53-72



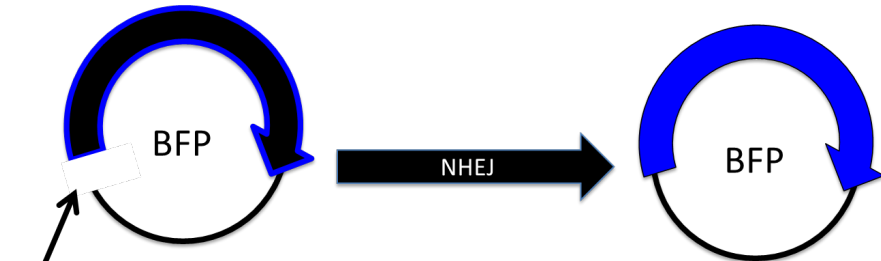
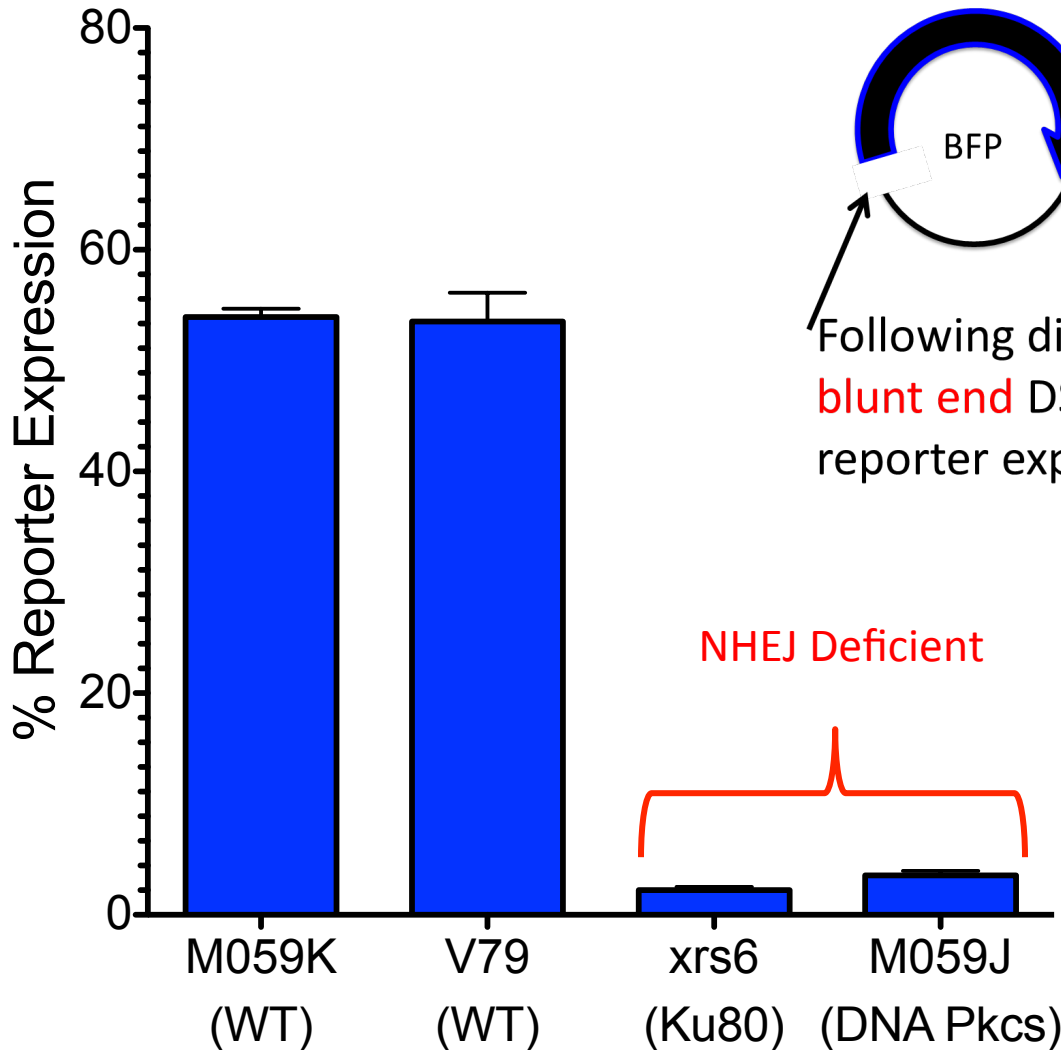
R-M systems recognize methylation status of incoming foreign DNA, e.g., phage genomes. Methylated sequences are recognized as self, while recognition sequences on the incoming DNA lacking methylation are recognized as nonself and are cleaved by the restriction endonuclease (REase). The methylation status at the genomic recognition sites is maintained by the cognate methyltransferase (MTase) of the R-M system



Some restriction enzymes

Enzyme	Source organism	Restriction recognition site in double-stranded DNA	Structure of the cleaved products
(a) <i>EcoRI</i>	<i>Escherichia coli</i>		<p style="text-align: right;">5' overhang</p>
<i>PstI</i>	<i>Providencia stuartii</i>		<p style="text-align: right;">3' overhang</p>
<i>SmaI</i>	<i>Serratia marcescens</i>		<p style="text-align: right;">Blunt ends</p>
(b) <i>HaeIII</i>	<i>Haemophilus aegyptius</i>		<p style="text-align: right;">Blunt ends</p>
<i>HpaII</i>	<i>Haemophilus parainfluenzae</i>		<p style="text-align: right;">5' overhang</p>

NHEJ HCR in WT and NHEJ defective cells at 18 hours post-transfection:



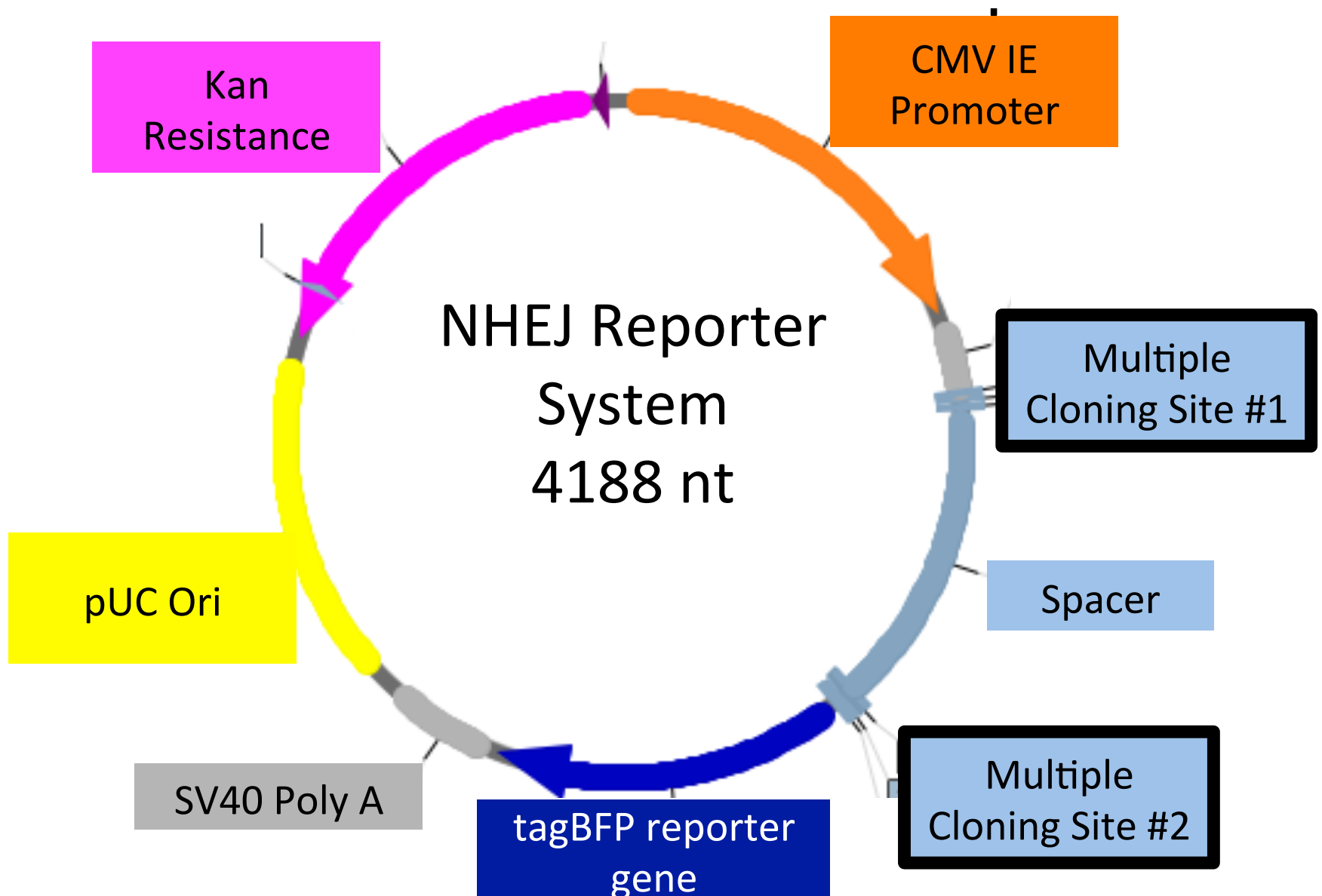
Following digest, the substrate contains a **blunt end** DSB that prevents fluorescent reporter expression

NHEJ Deficient

Some restriction enzymes

Enzyme	Source organism	Restriction recognition site in double-stranded DNA	Structure of the cleaved products
(a) <i>EcoRI</i>	<i>Escherichia coli</i>	<p>5' —G—A—A—T—T—C— —C—T—T—A—A—G— 5'</p>	<p>—G 5' A—A—T—T—C— —C—T—T—A—A 5' G— 5' overhang</p>
<i>PstI</i>	<i>Providencia stuartii</i>	<p>5' —C—T—G—C—A—G— —G—A—C—G—T—C— 5'</p>	<p>—C—T—G—C—A 3' G— —G 3' A—C—G—T—C— 3' overhang</p>
<i>SmaI</i>	<i>Serratia marcescens</i>	<p>5' —C—C—C—G—G—G— —G—G—G—C—C—C— 5'</p>	<p>—C—C—C G—G—G— —G—G—G C—C—C— Blunt ends</p>
(b) <i>HaellI</i>	<i>Haemophilus aegyptius</i>	<p>5' —G—G—C—C— —C—C—G—G— 5'</p>	<p>—G—G 5' C—C— —C—C 5' G—G— Blunt ends</p>
<i>HpaII</i>	<i>Haemophilus parainfluenzae</i>	<p>5' —C—C—G—G— —G—G—C—C— 5'</p>	<p>—C C—G—G— —G—G—C 5' C— 5' overhang</p>

Overall Structure of the Reporter:



20.109 Spring 2016 Module 2 – Lecture 3

System Engineering (March 15th, 2016)



Noreen Lyell
Leslie McLain
Maxine Jonas
Jing Zhang(TA)

Leona Samson (Lectures)

Zachary Nagel (help with development) Alex Chaim