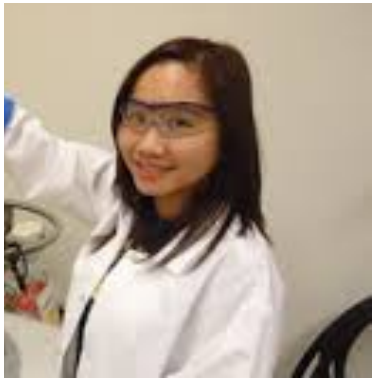
20.109 Spring 2015 Module 2 – Lecture 3
System Engineering and Protein Foundations

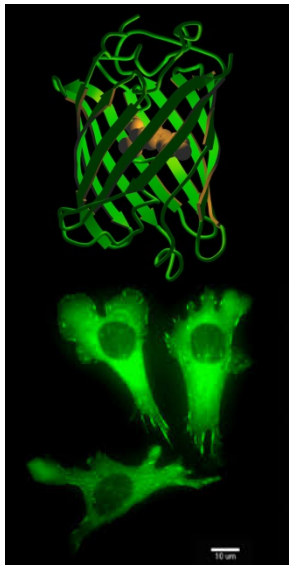


Shannon Hughes
Noreen Lyell
Leslie McLain
Nova Pishesha (TA)
Leona Samson (Lectures)

Zachary Nagel (help with development) Alex Chaim

What experimental question will you ask in Module 2?

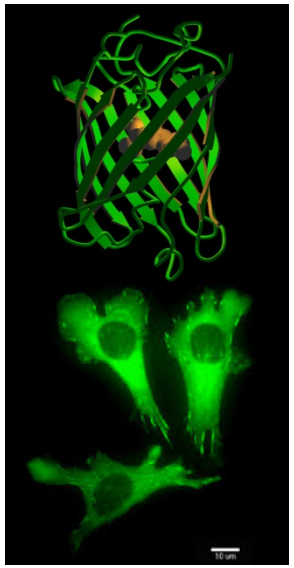


How efficiently does DNA repair by the Non Homologous End Joining (NHEJ) pathway act on DNA damage with different topologies?

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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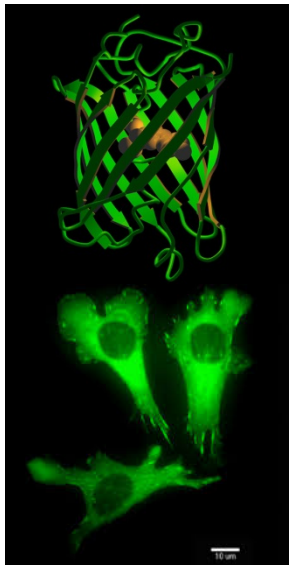

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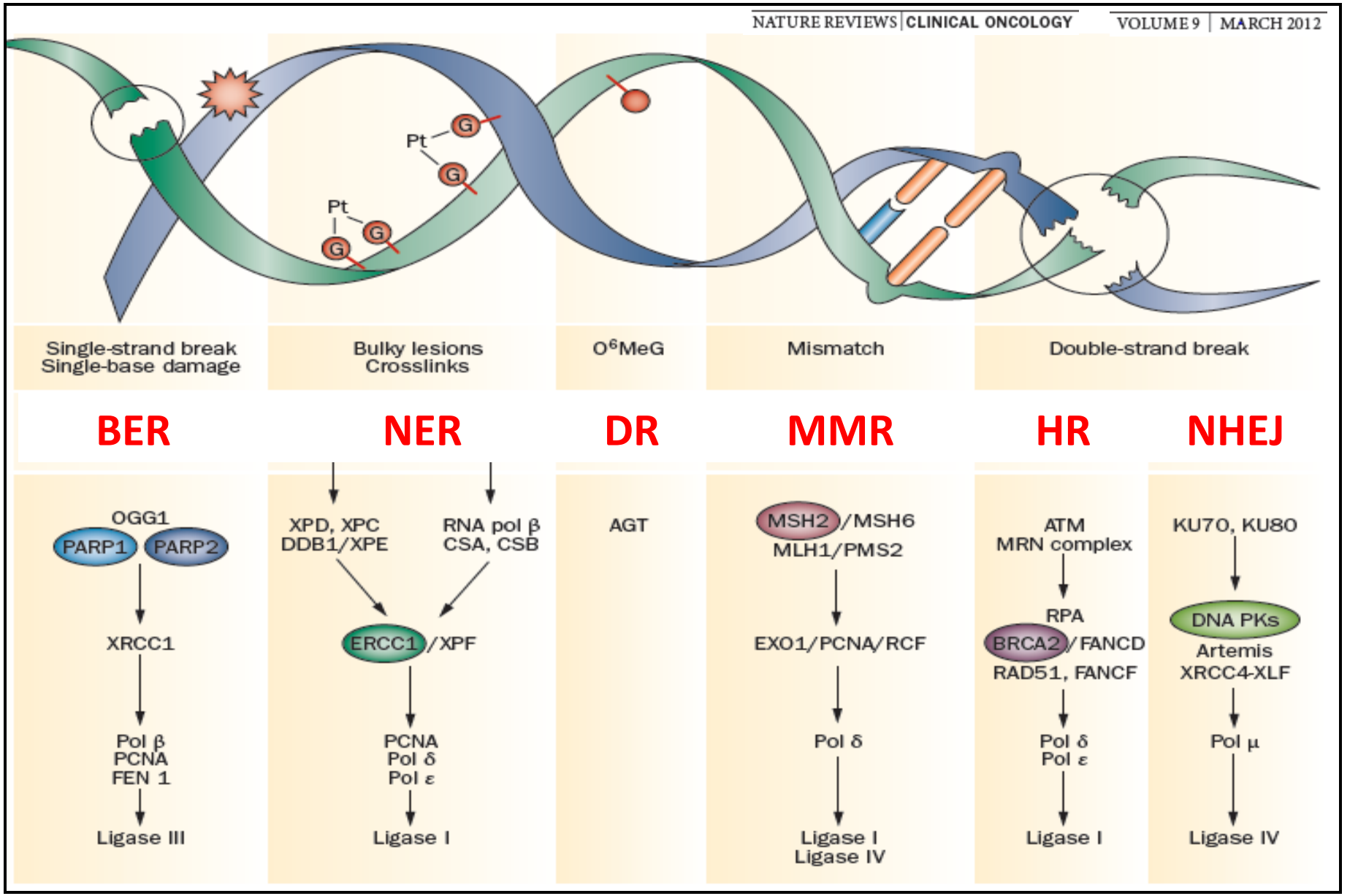
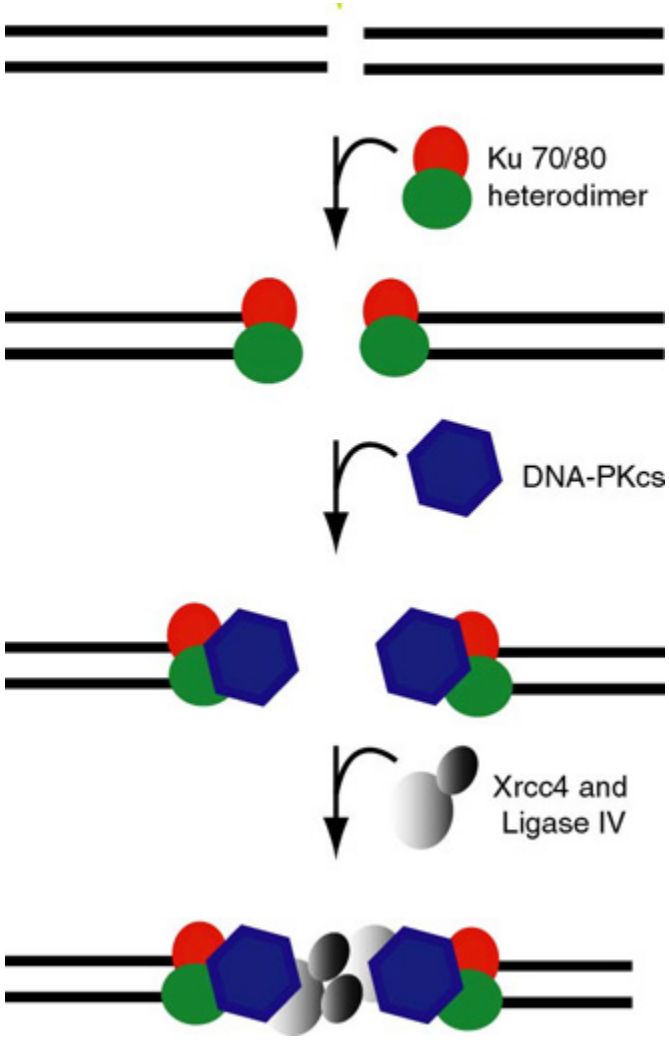
Six Major DNA Repair Pathways



Non-Homologous End Joining (NHEJ)

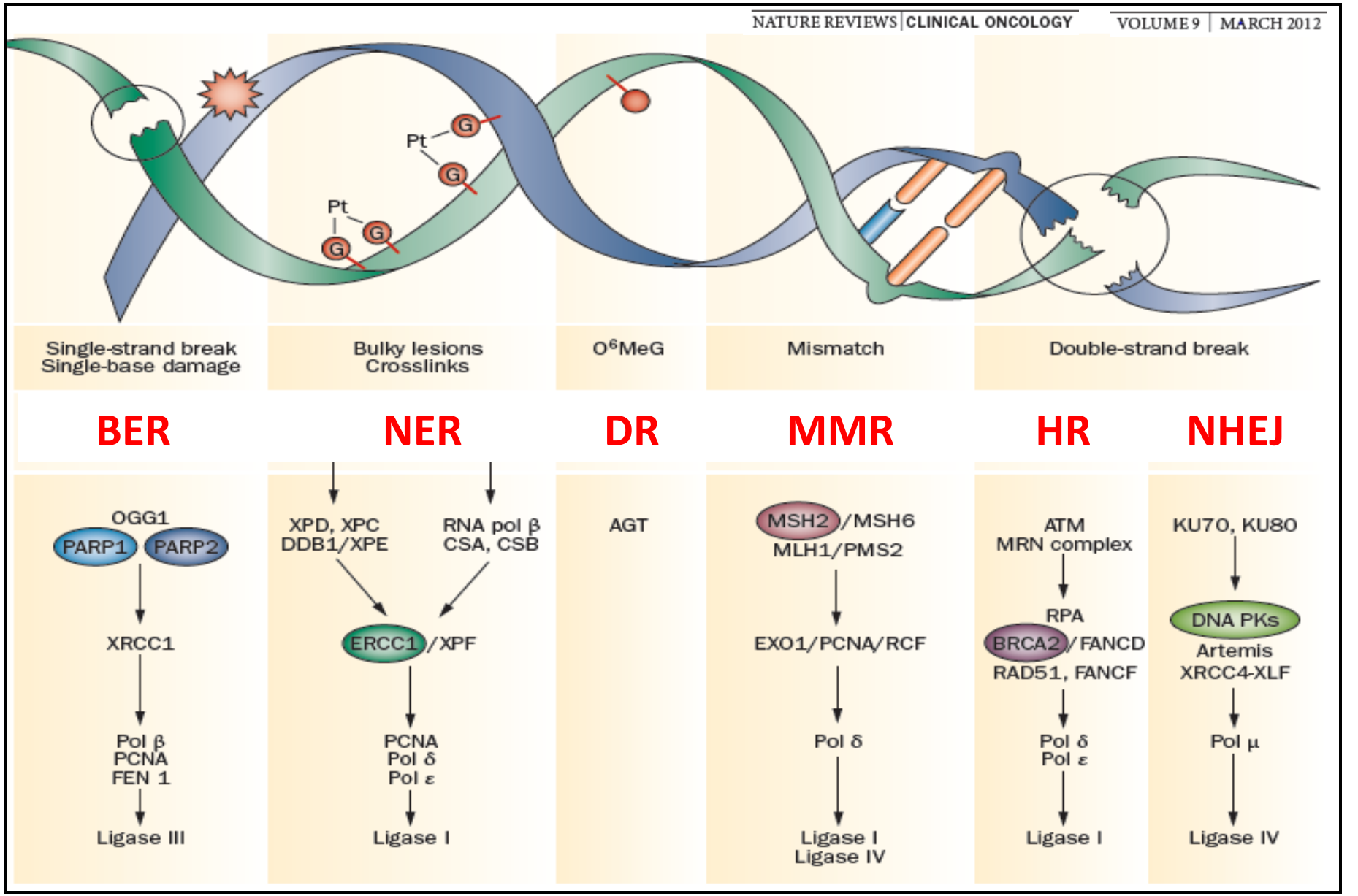


Ku70
Ku80

DNA-PKcs

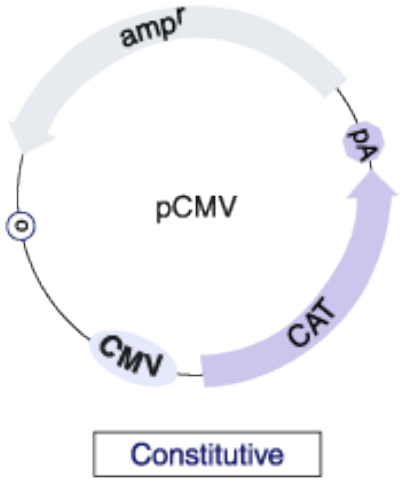
Xrcc4
Ligase IV

Six Major DNA Repair Pathways

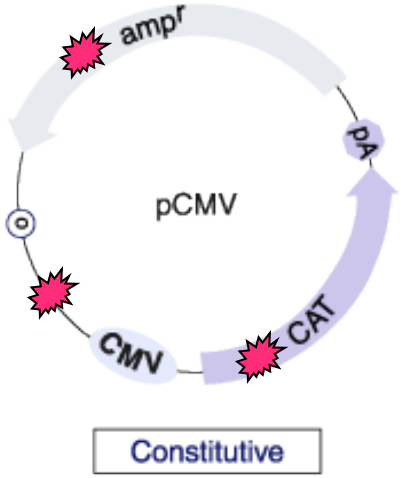


Reactivation of UV damaged DNA by Host cell Reactivation (HCR)

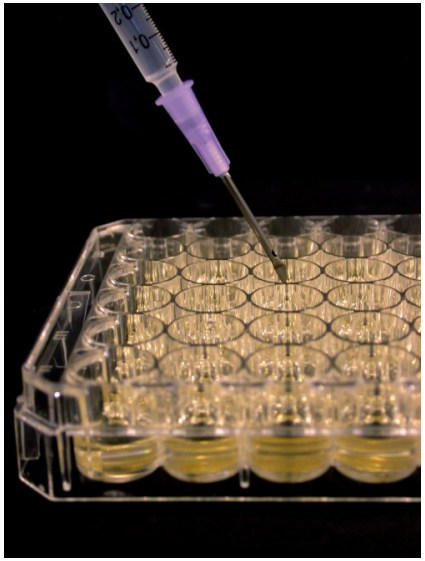
Athas & GROSSMAN
Cancer Res. 1991

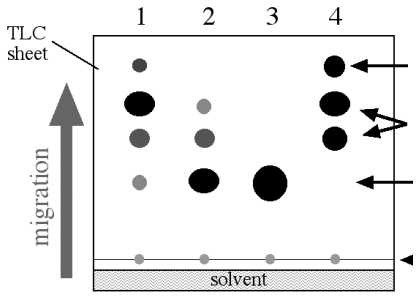


+ UV light



Transient transfection peripheral blood lymphocytes

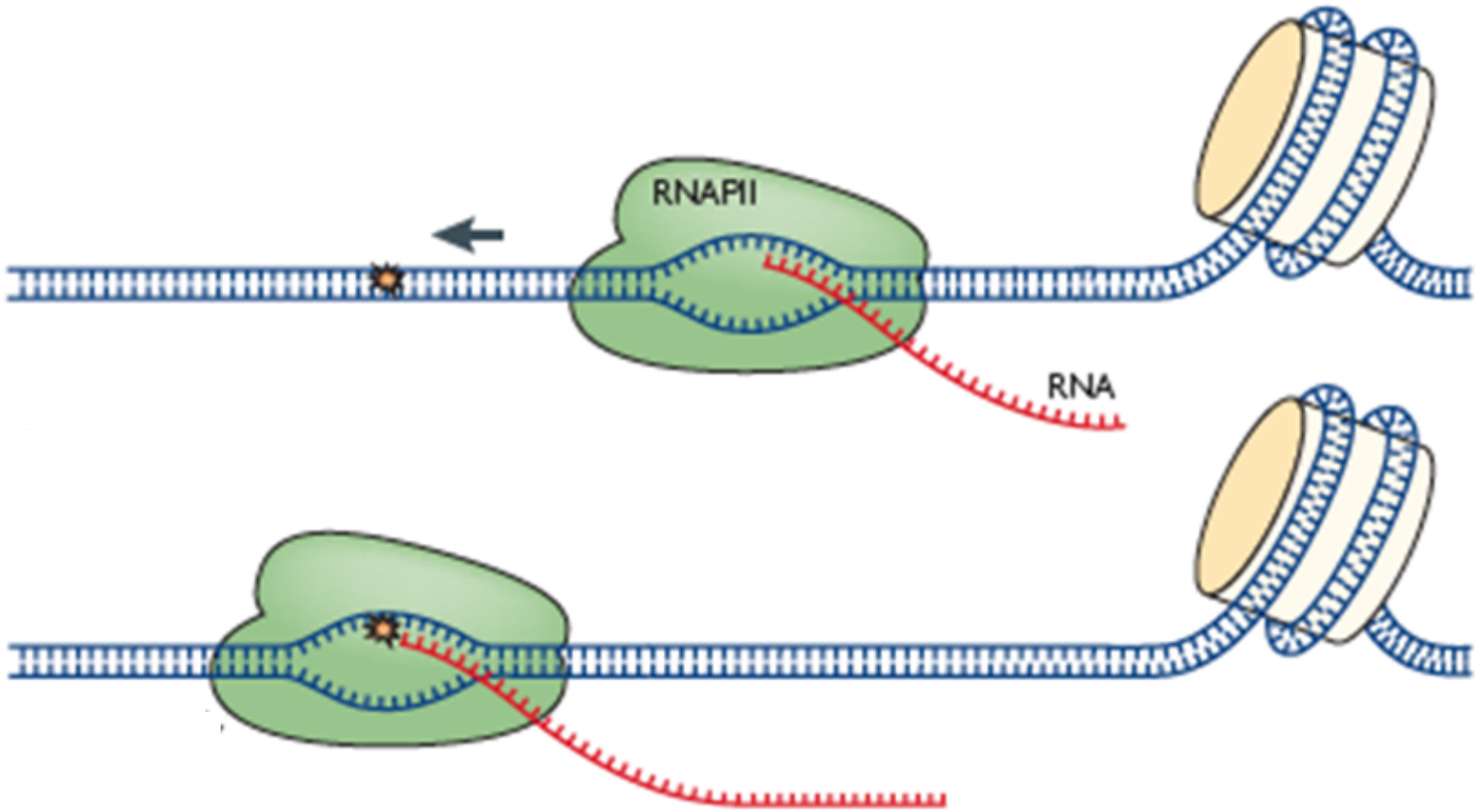




Time to repair

CAT Assay

RNA Polymerase II is exquisitely sensitive to DNA lesions



Nature Reviews Molecular Cell Biology **9**, 958-970

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

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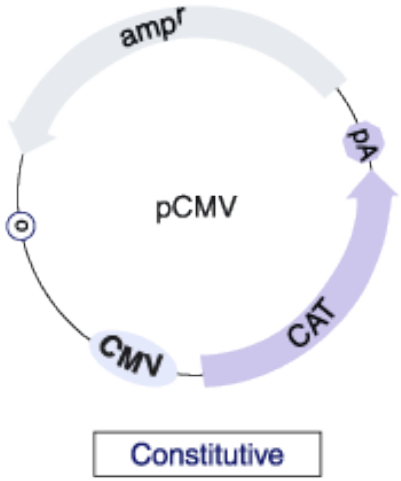
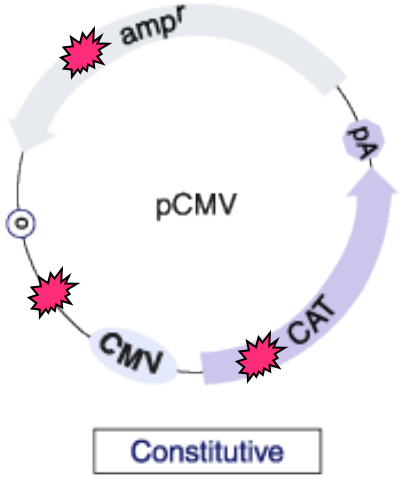
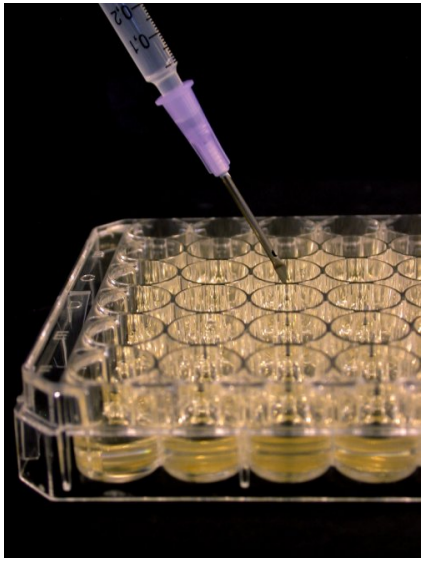
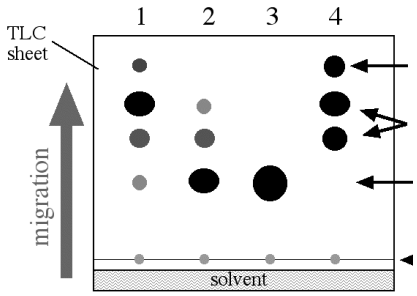


+ UV light



Transient transfection peripheral blood lymphocytes





Time to repair

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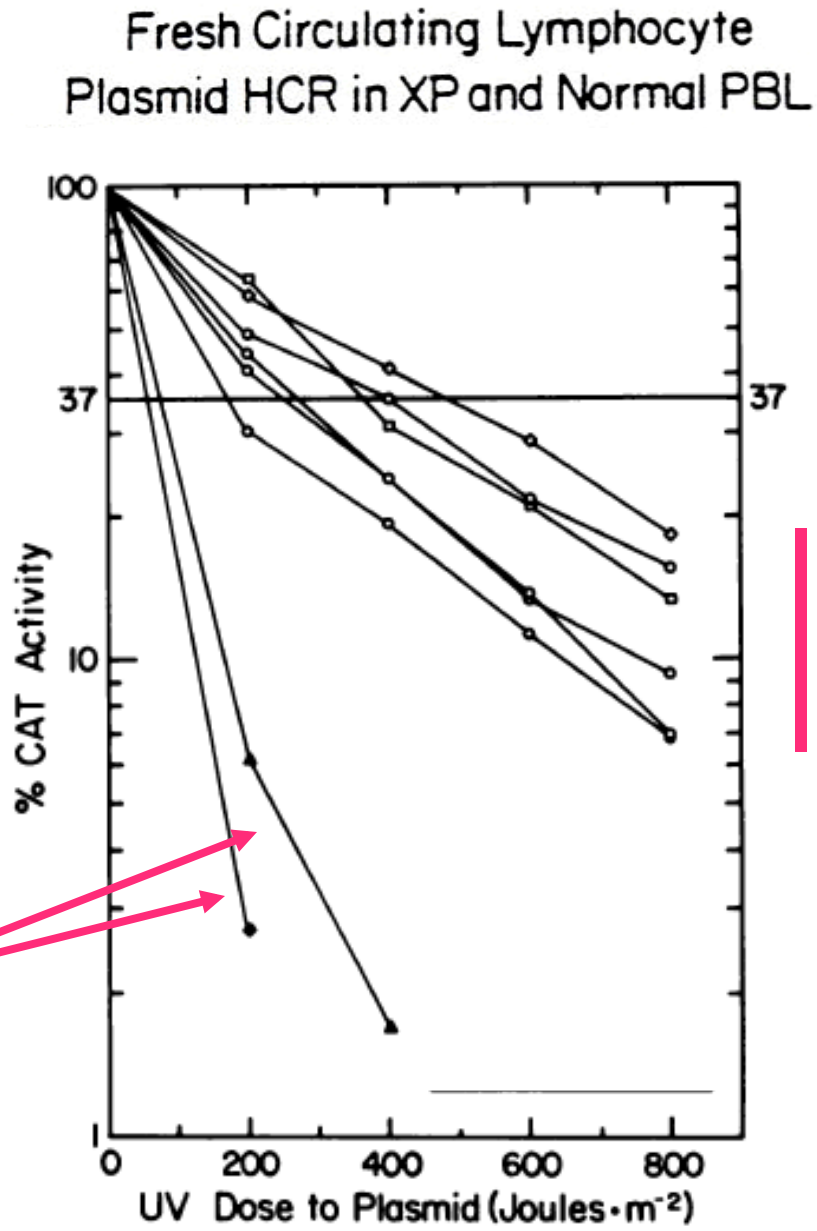


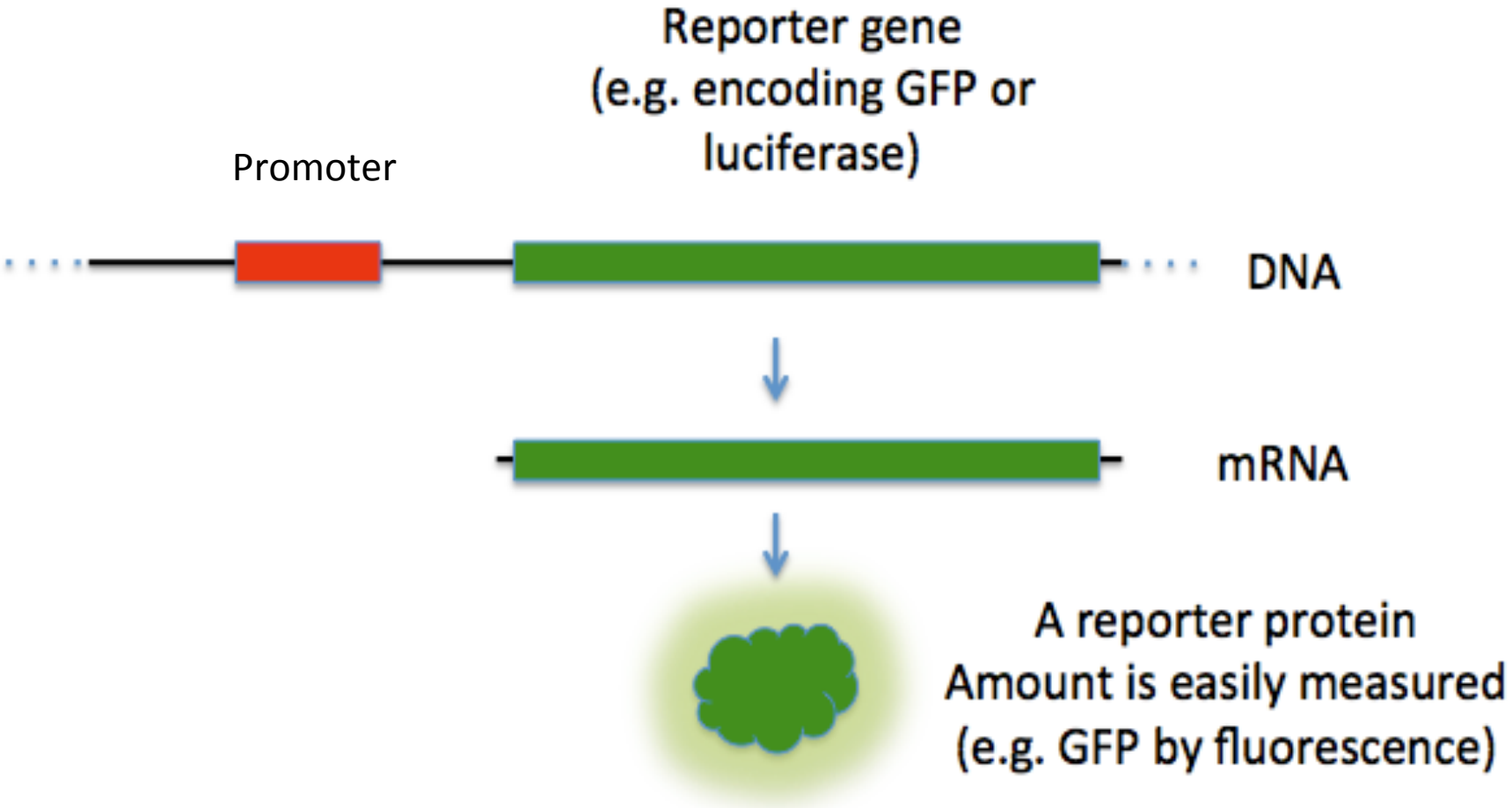
Cells from ‘healthy” people

Cells from XP patients

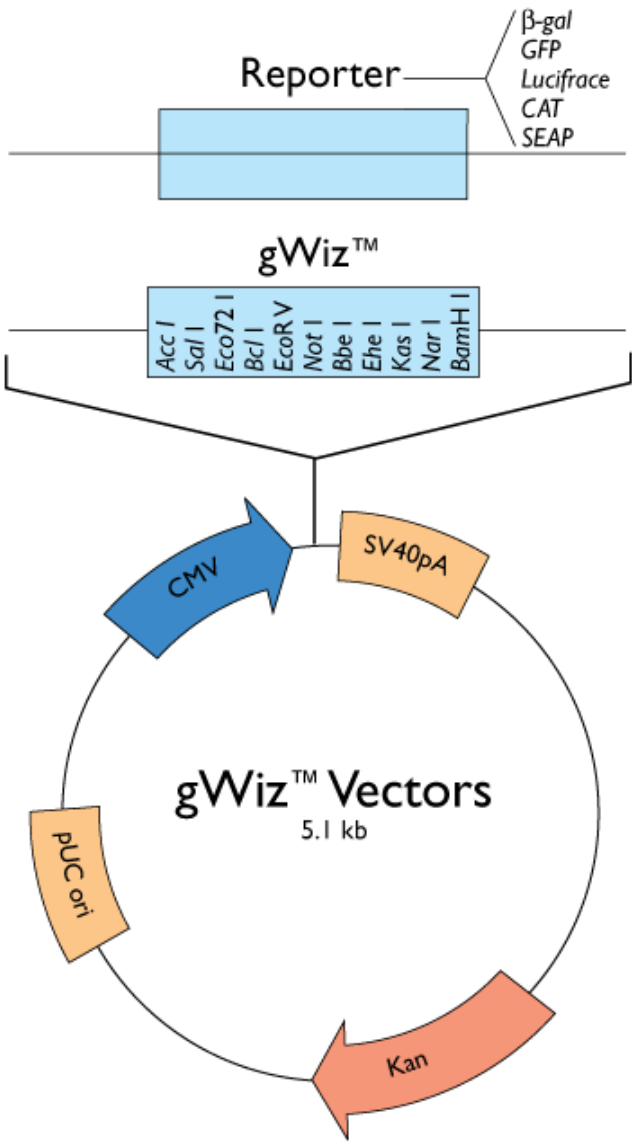
Athas & GROSSMAN

[CANCER RESEARCH 51, 5786–5793, November 1, 1991]

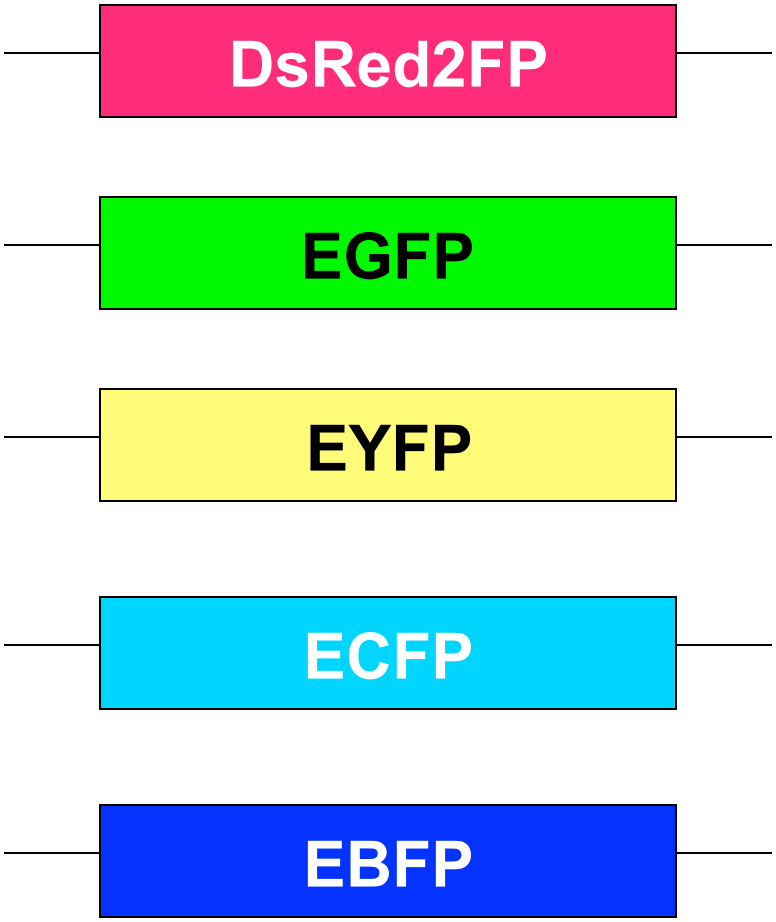
Let's use a different reporter gene that is easy to assay



Reactivation of damaged DNA – multiplexed



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



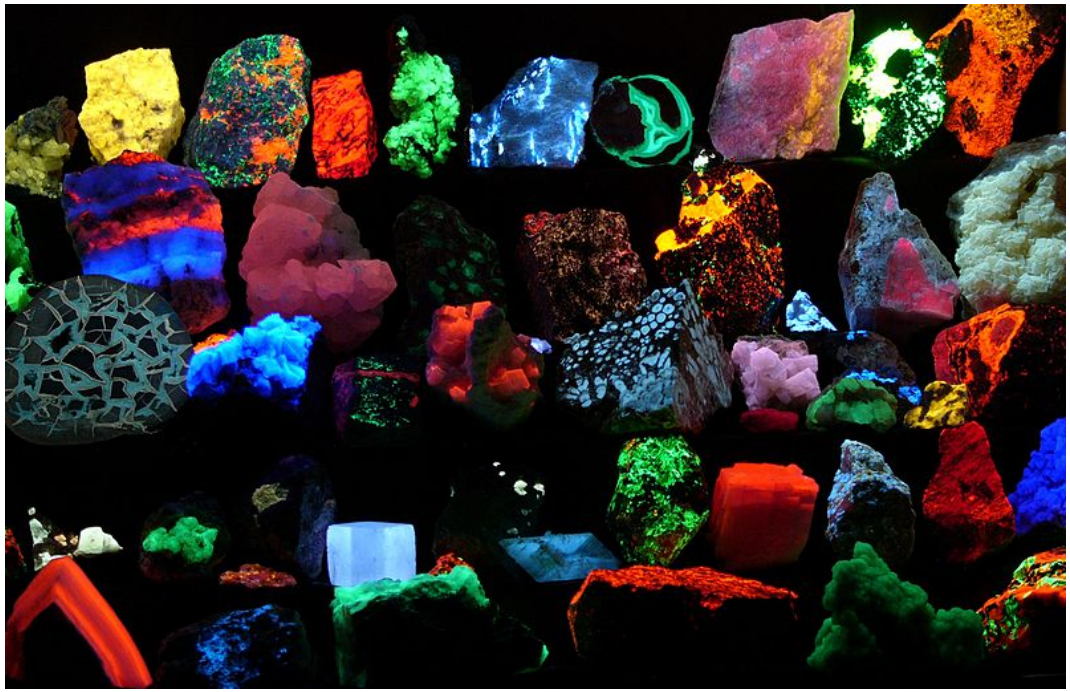
http://www.genlantis.com/corp/images/gWiz_vectmap.gif

fluo·res·cence

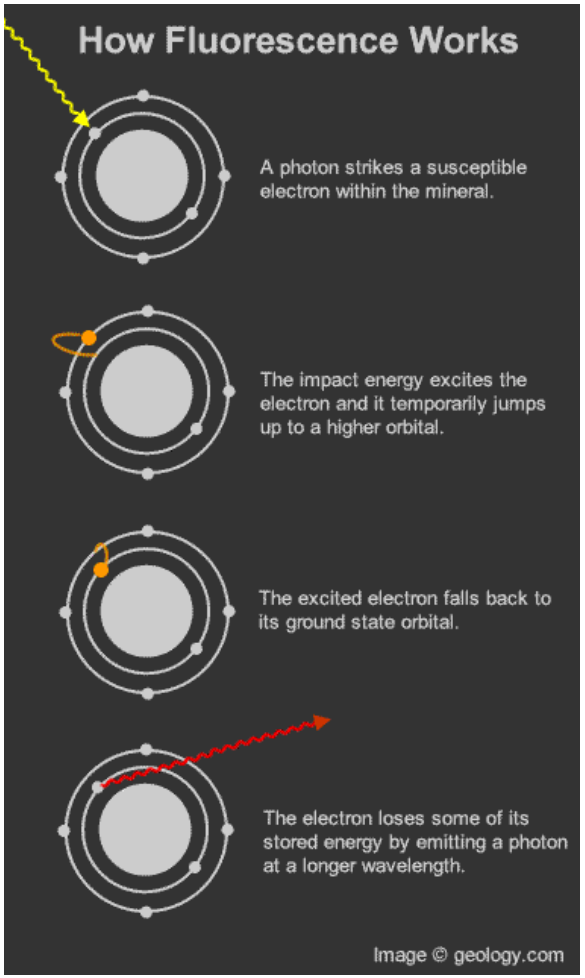
/flo͝o(ə)ˈresəns,flôrˈesəns/

noun

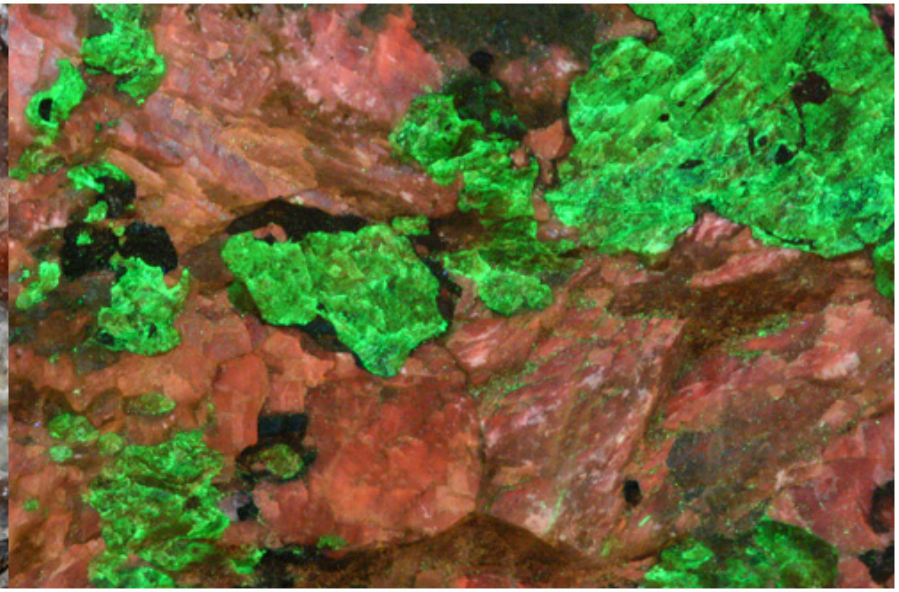
1. the visible or invisible radiation emitted by certain substances as a result of incident radiation of a shorter wavelength such as X-rays or ultraviolet light.



Minerals fluorescing under UV-light

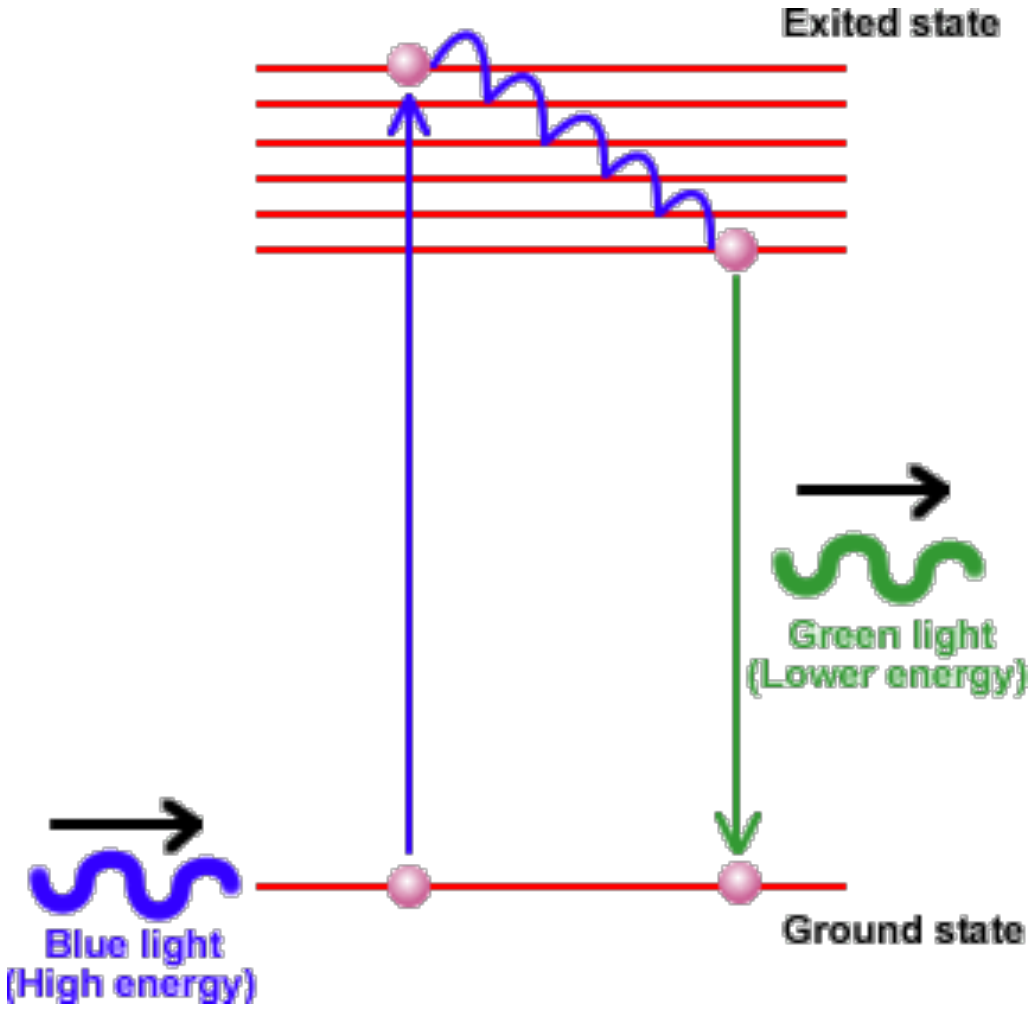


Under White Light



Under UV Light

Theory of Fluorescence

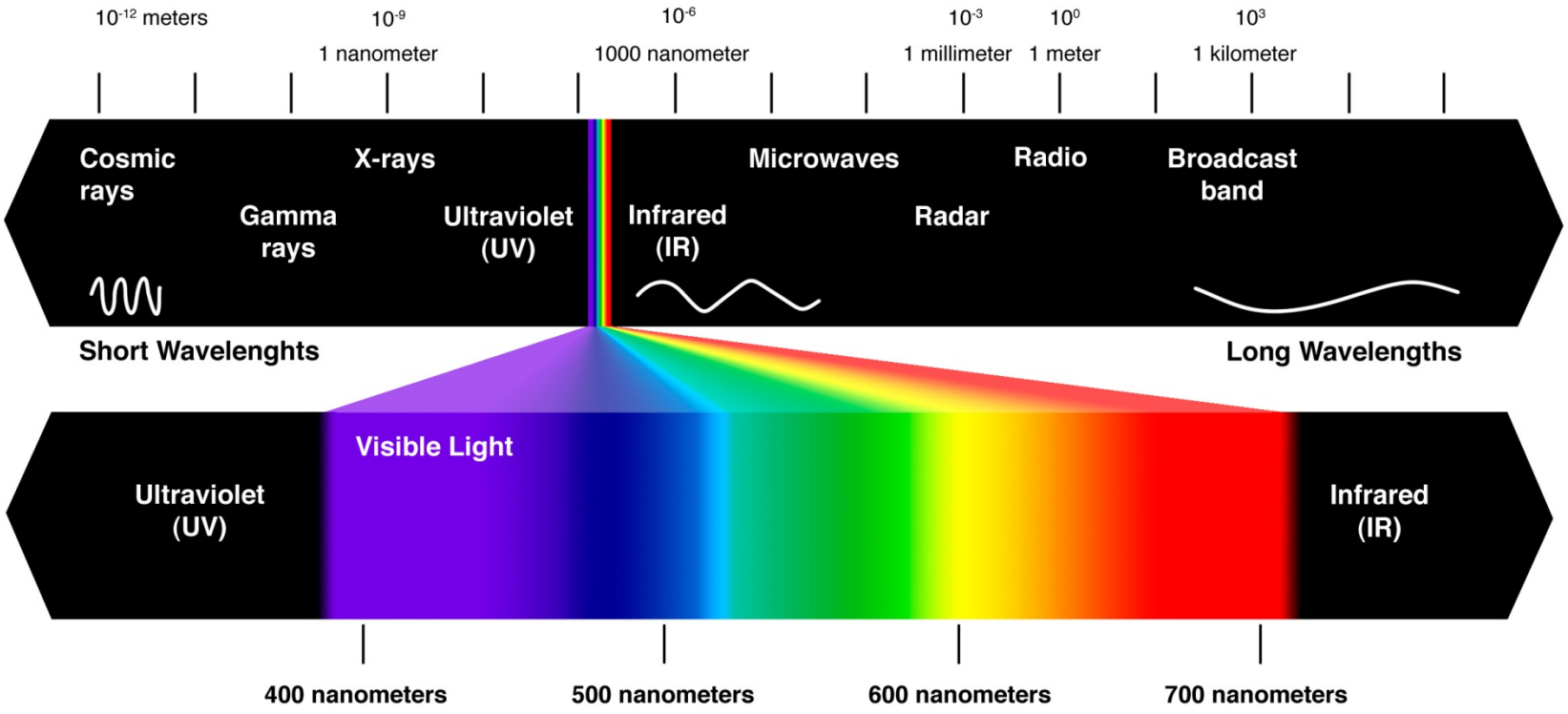


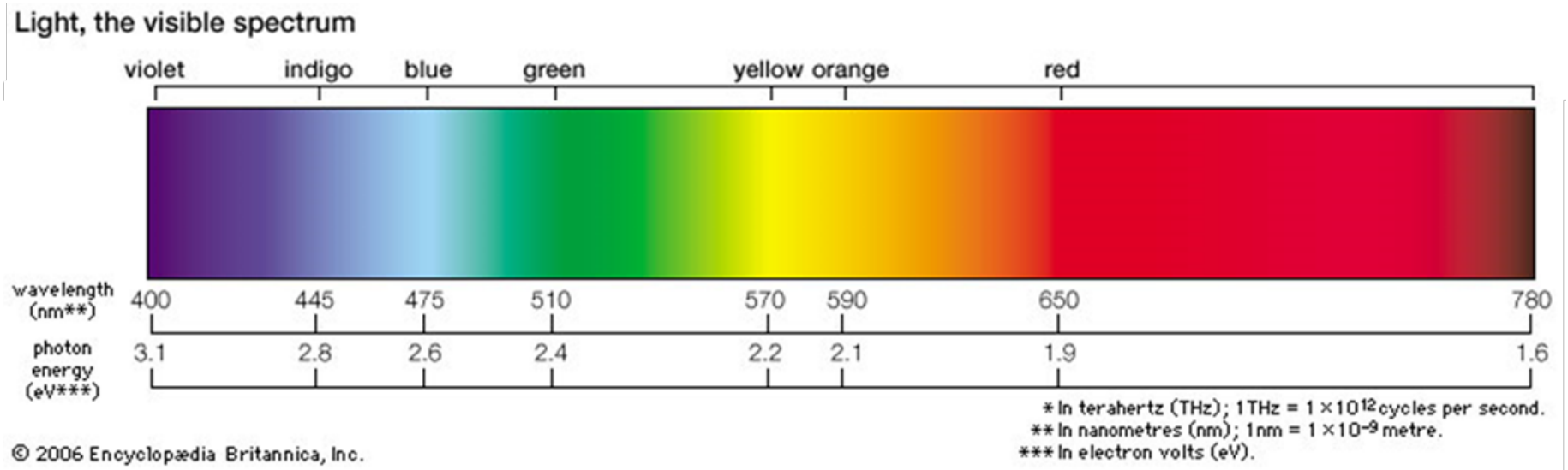
1) Electrons excited by light source
2) Electrons reach a high energy state
3) Energy loss occurs within a few nano seconds
4) Energy loss observed as fluorescent light of a longer wavelength

Excitation

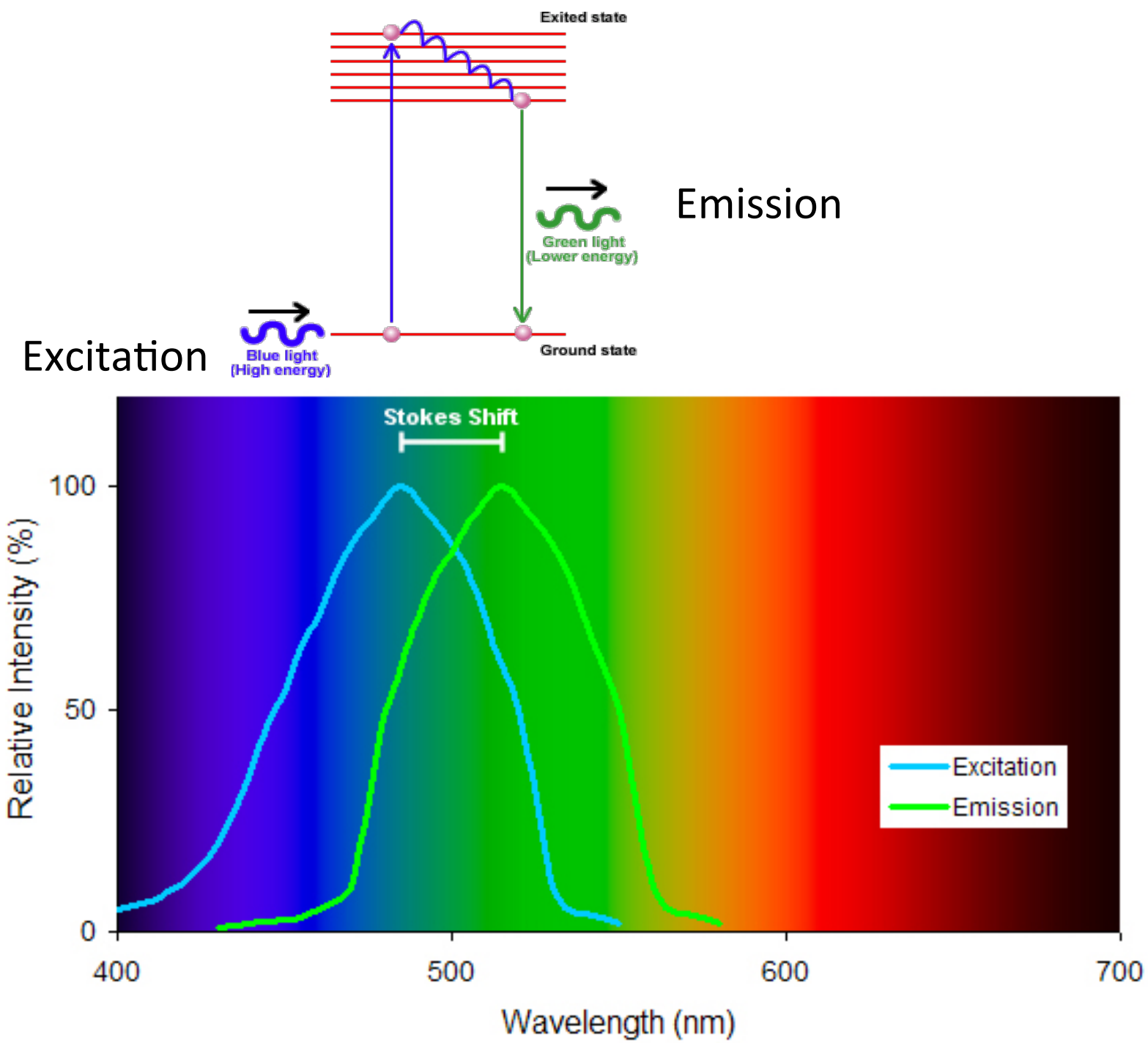
Emission

Electro Magnetic Spectrum and Light

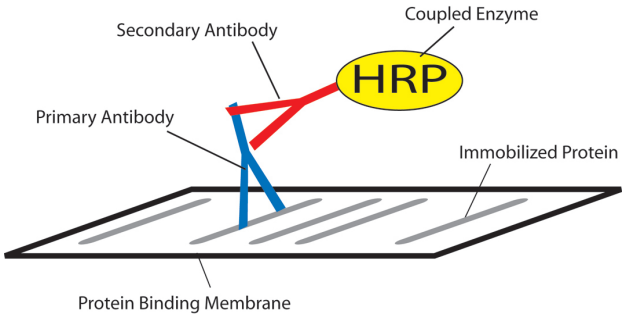


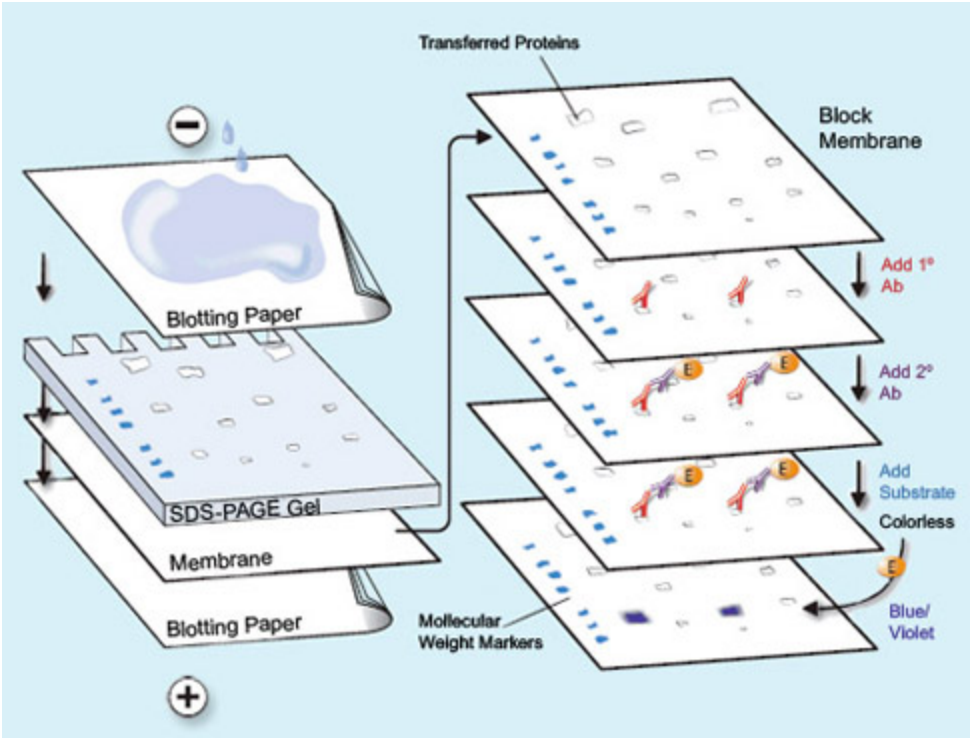


Increasing energy

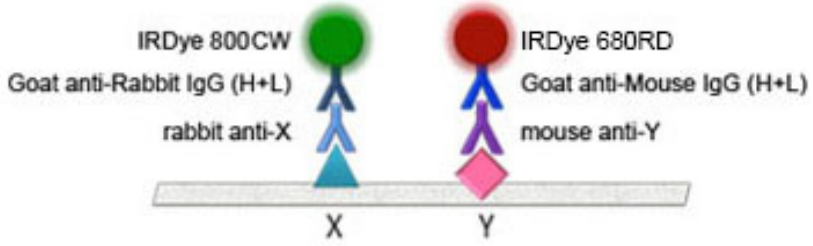

Exited state
Green light
(Lower energy)
Emission
Excitation
Blue light
(High energy)
Ground state
Stokes Shift
100
50
0
Relative Intensity (%)
400
500
600
700
Wavelength (nm)
Excitation
Emission

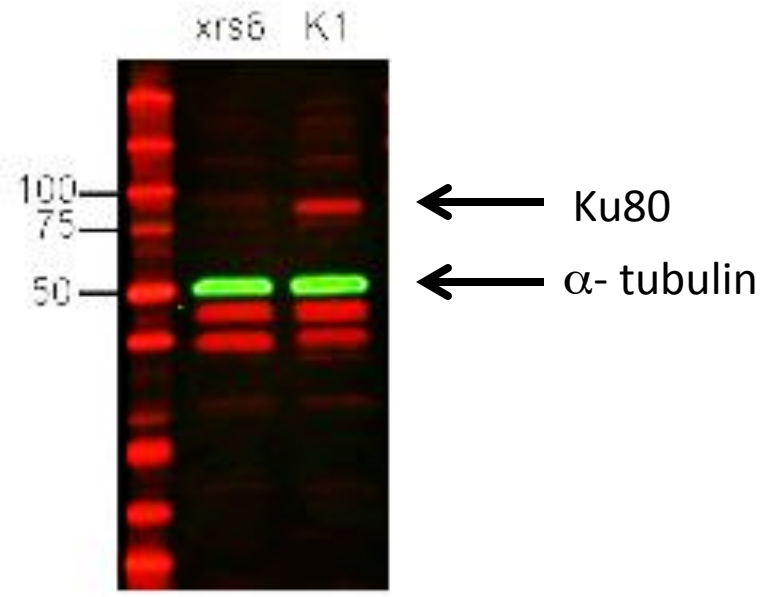
Horseradish Peroxidase

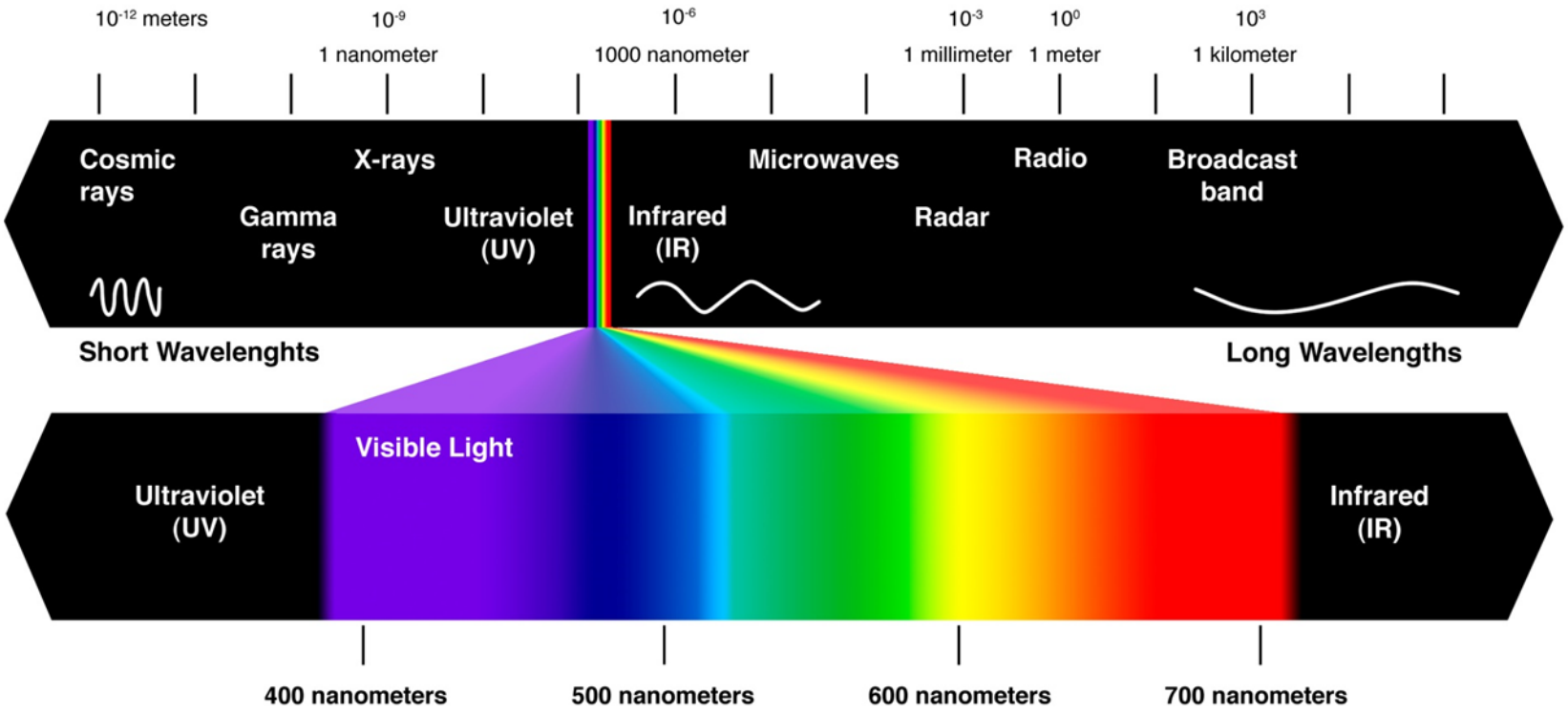


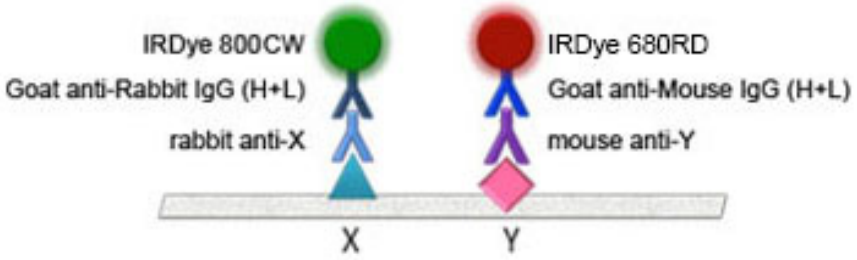


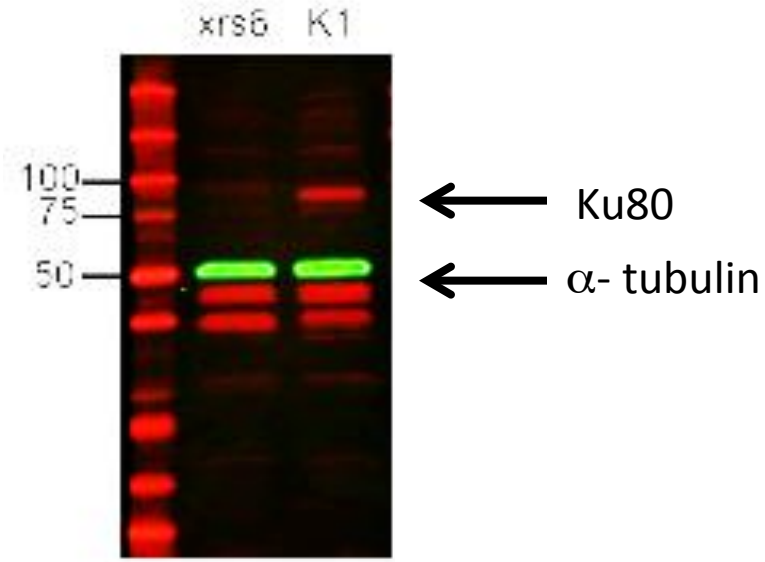
Western Blot Analysis



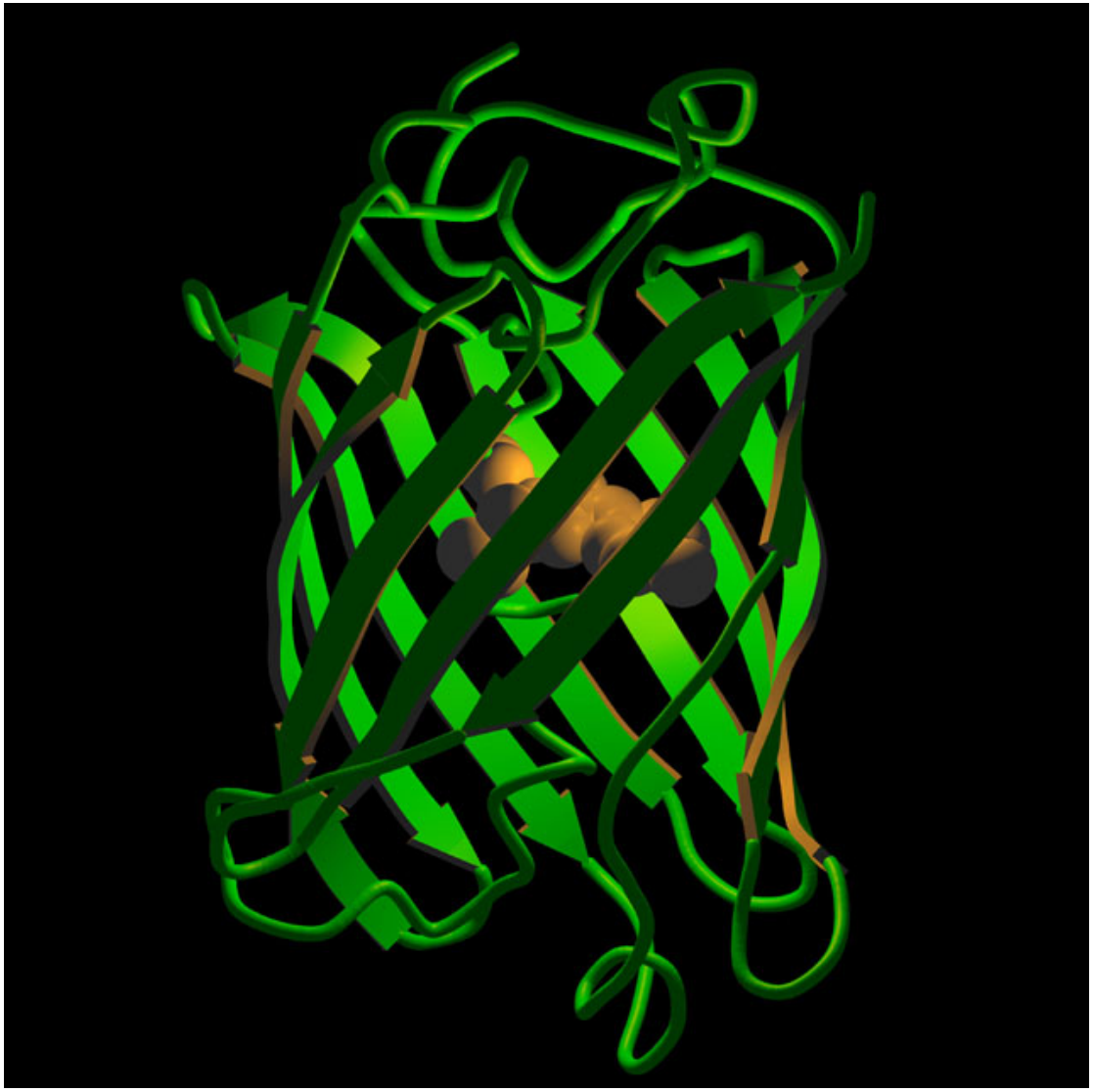



10-12 meters
10-9
1 nanometer
10-6
1000 nanometer
10-3
1 millimeter
100
1 meter
103
1 kilometer
Cosmic rays
X-rays
Gamma rays
Ultraviolet (UV)
Infrared (IR)
Microwaves
Radar
Radio
Broadcast band
Short Wavelenghts
Long Wavelengths
Visible Light
Ultraviolet (UV)
Infrared (IR)
400 nanometers
500 nanometers
600 nanometers
700 nanometers


IRDye 800CW
Goat anti-Rabbit IgG (H+L)
rabbit anti-X
IRDye 680RD
Goat anti-Mouse IgG (H+L)
mouse anti-Y
X
Y


xrs6
K1
100
75
50
Ku80
α- tubulin

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



Osamu Shimomura, 1962

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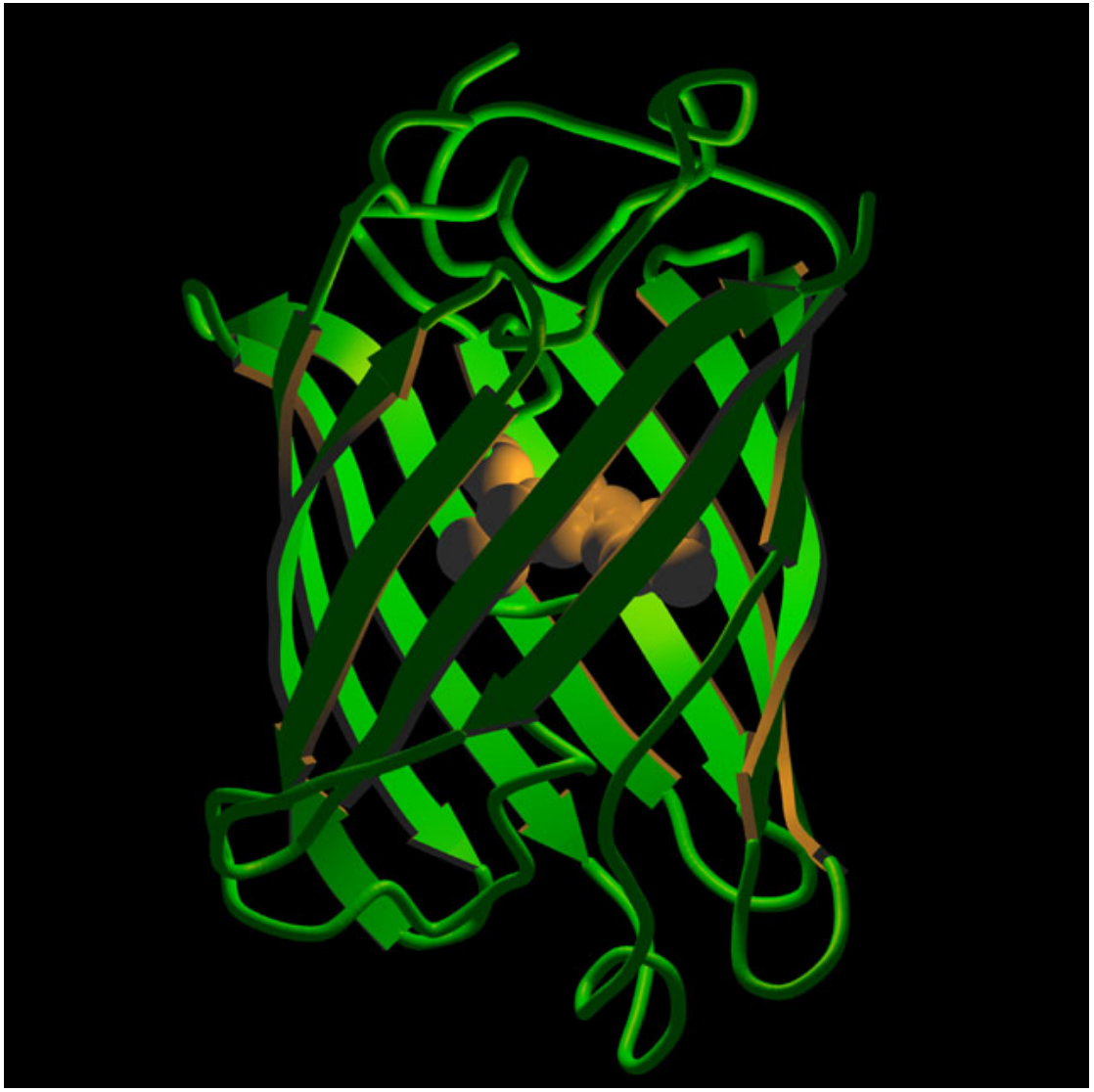

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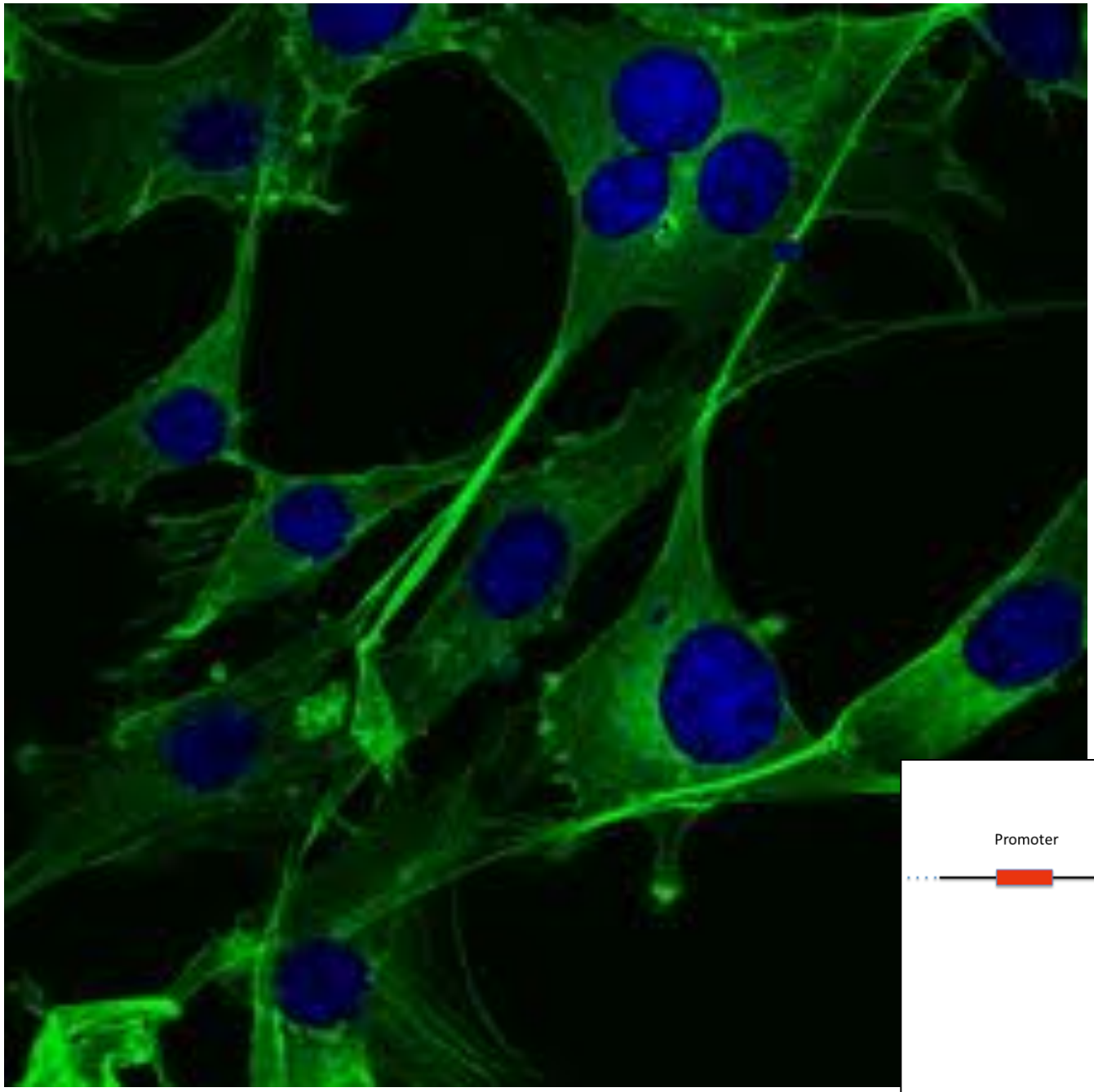
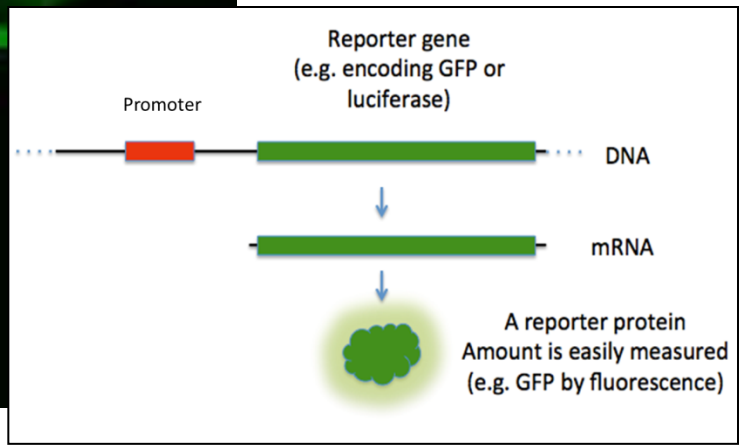


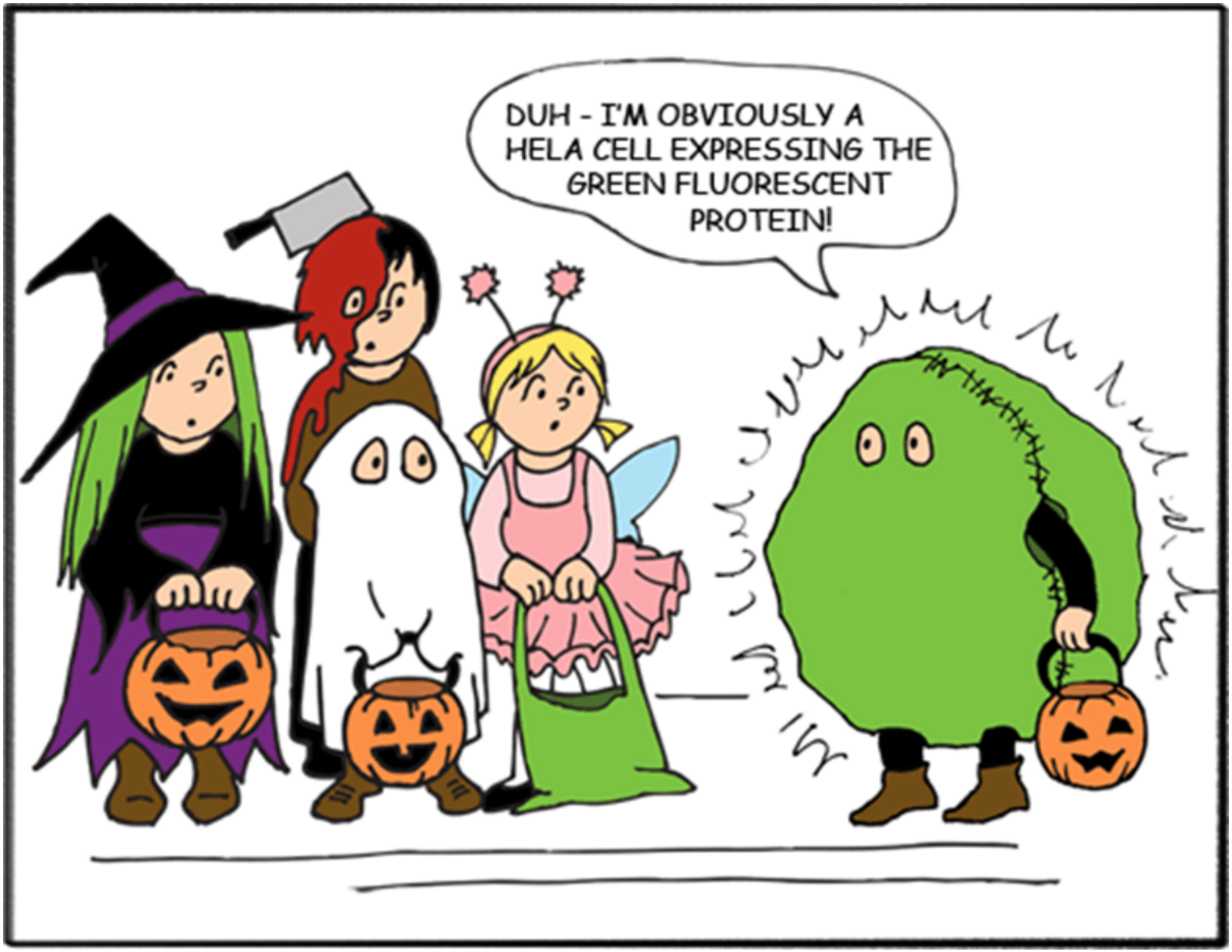
GFP cDNA expressed in human cells

DNA – Blue

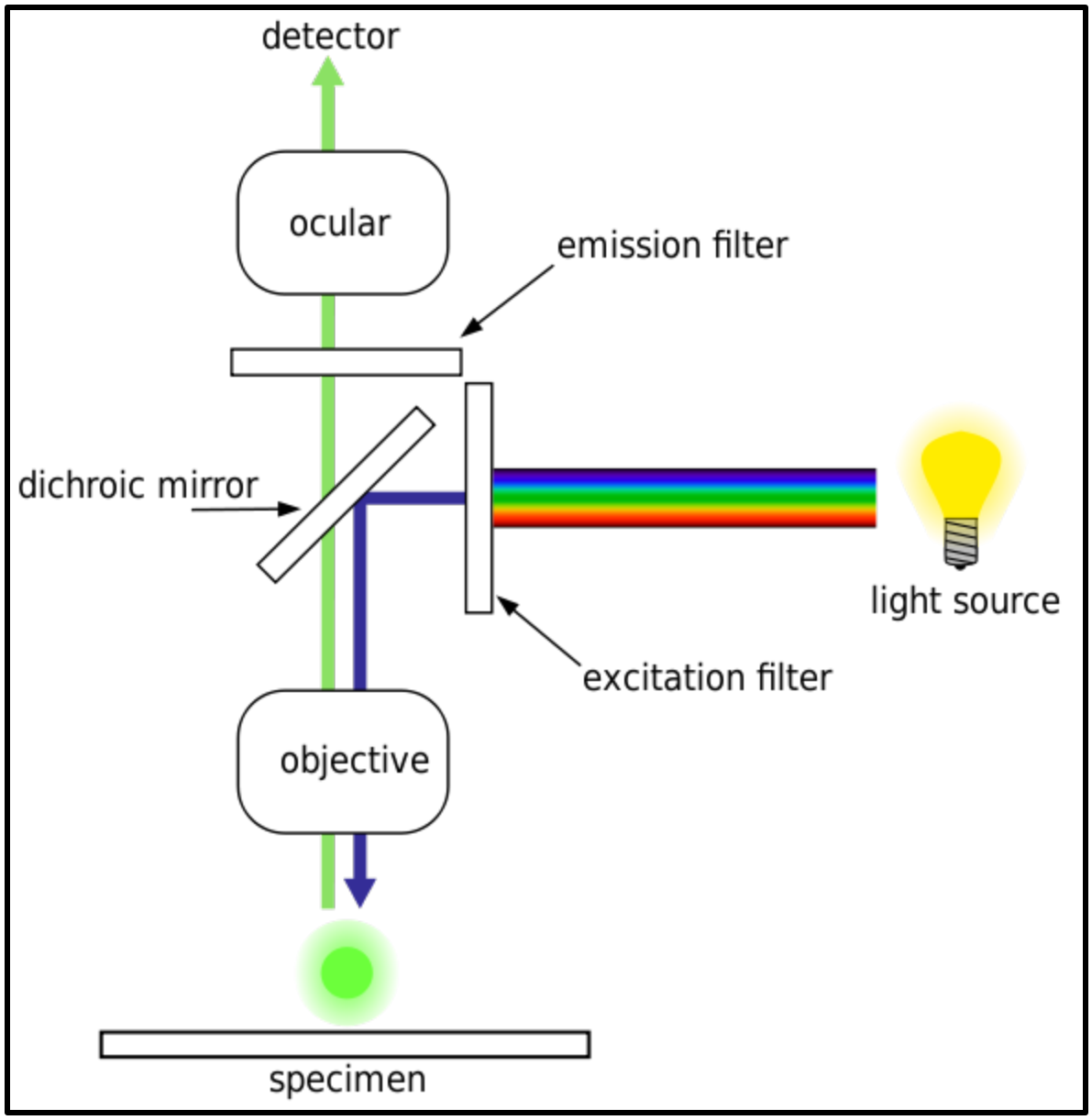
GFP - Green



Next Halloween Costume??

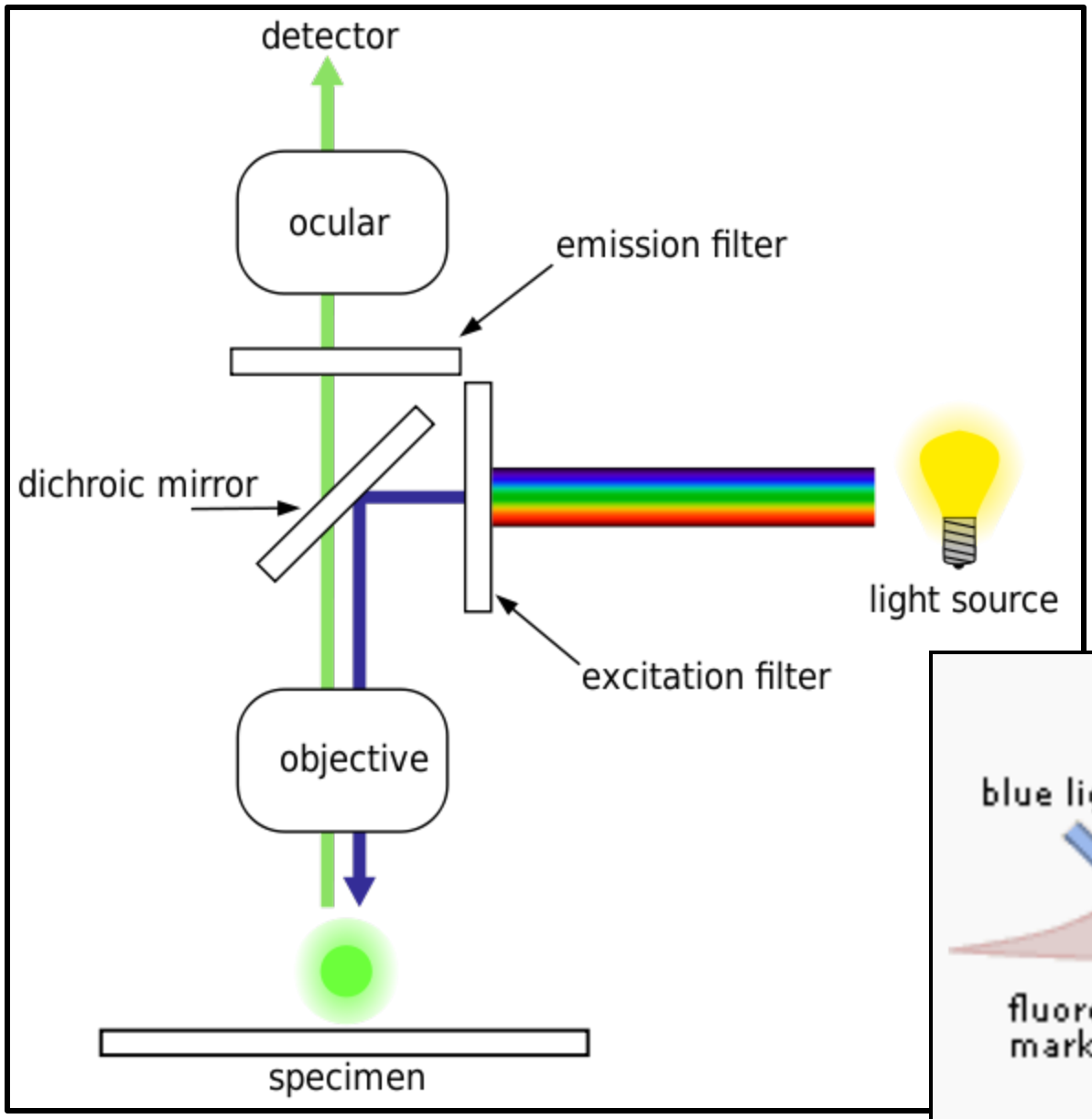


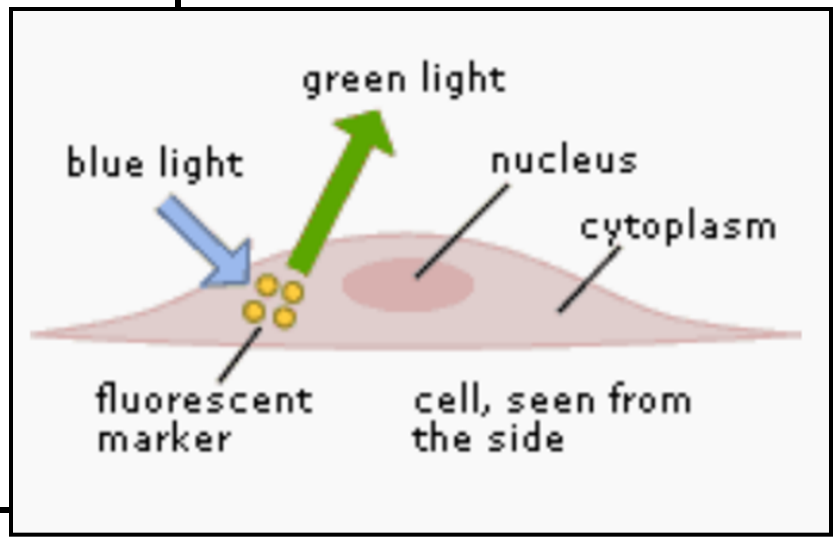
Fluorescence Detection



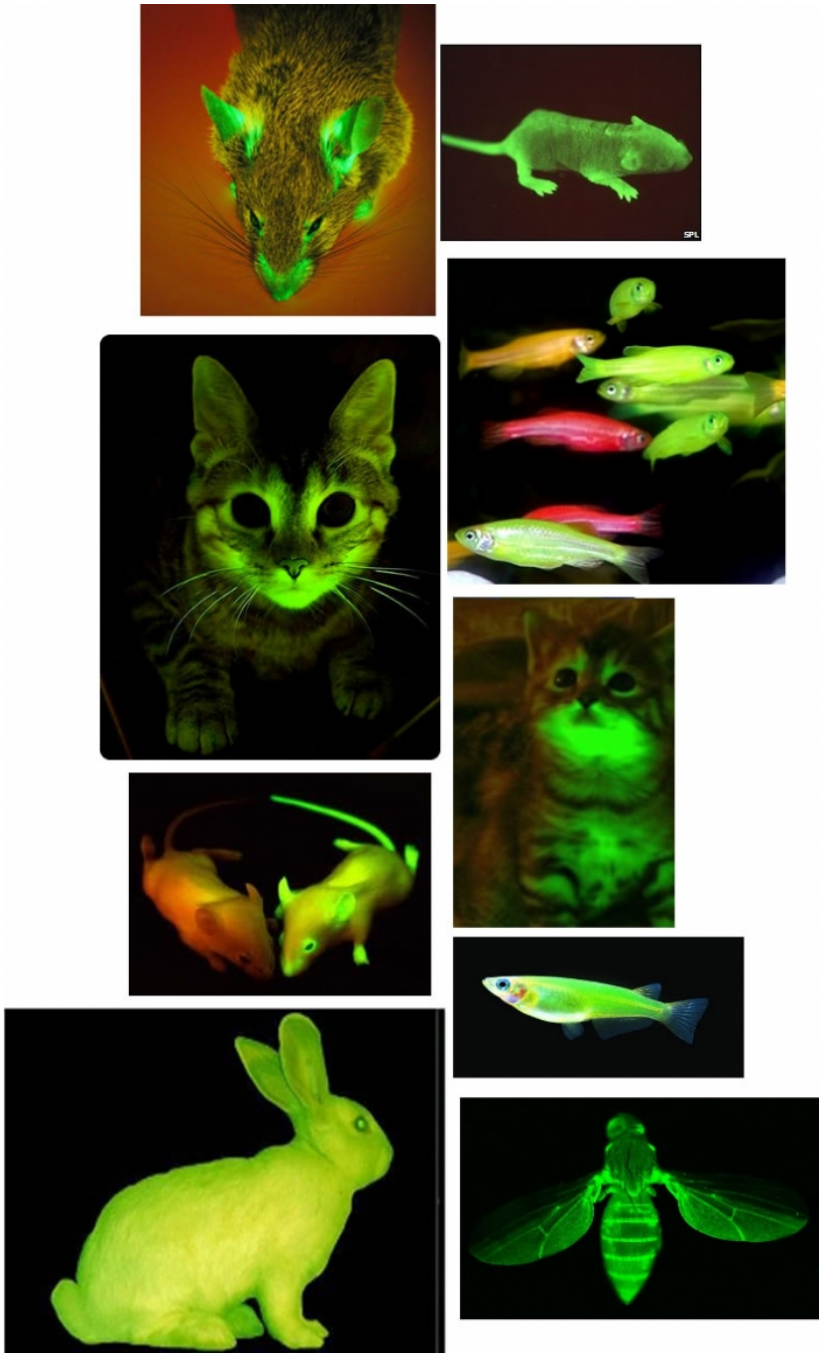
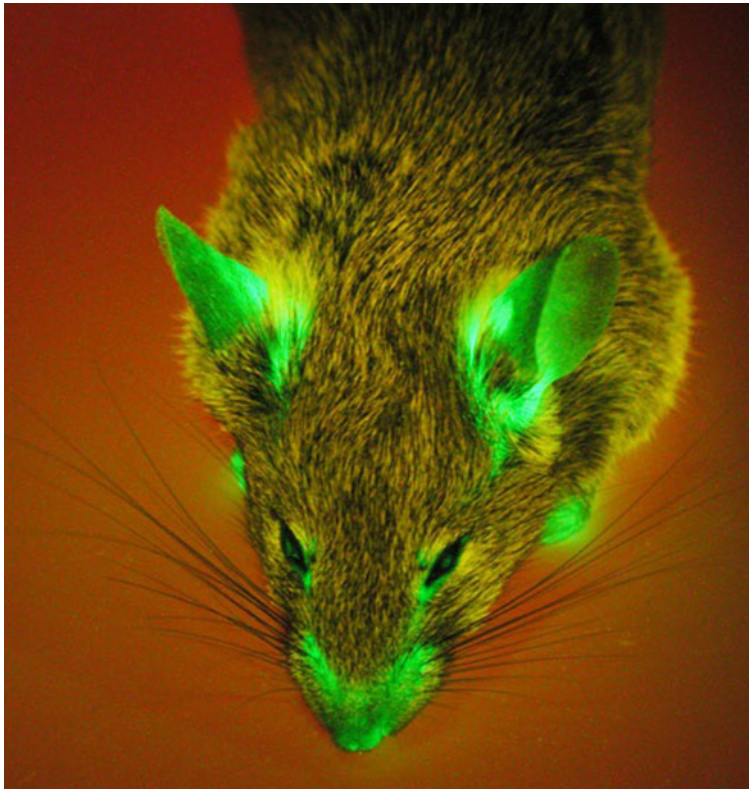
A **dichroic** filter, thin-film filter, or interference filter is a very accurate color filter used to selectively pass light of a small range of colors while reflecting other colors.

Fluorescence Detection





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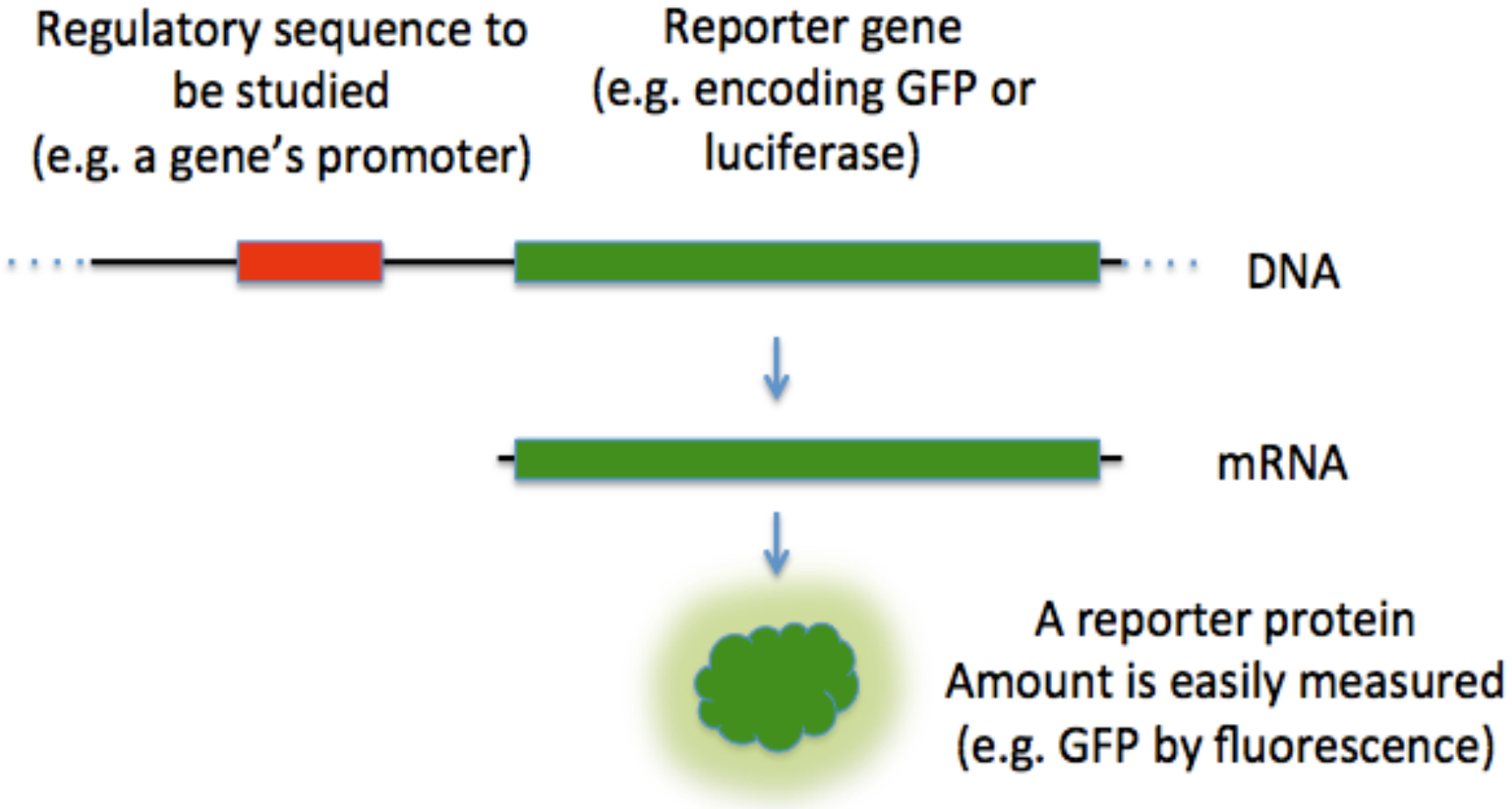
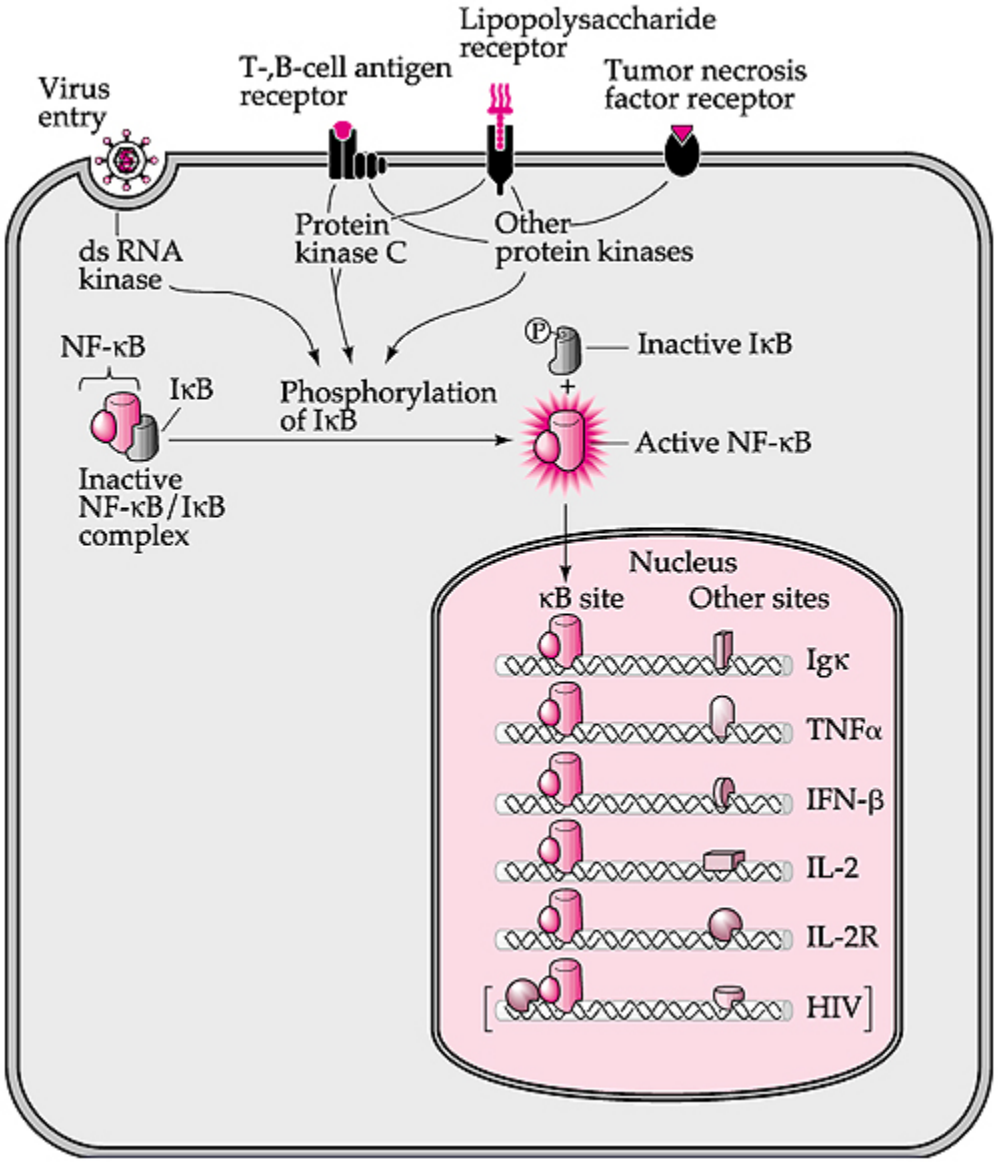
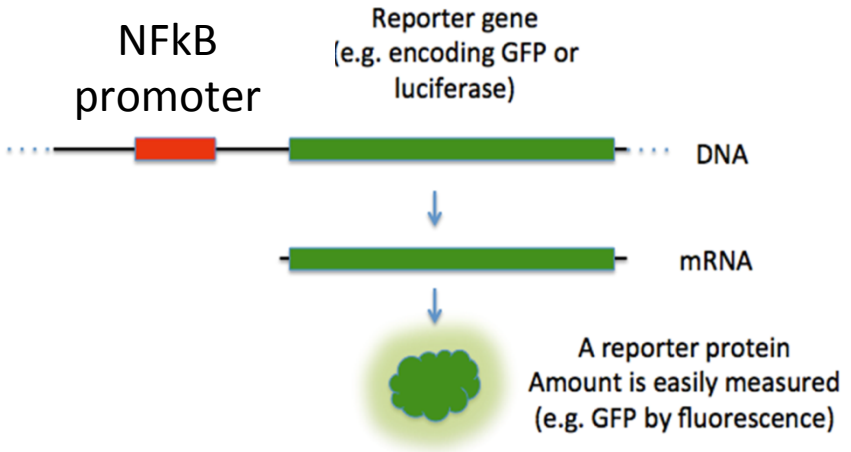


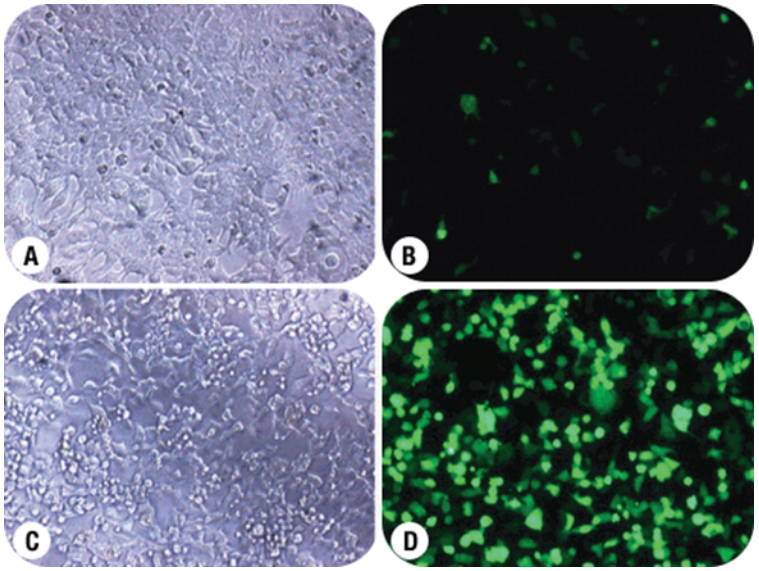
Regulation of gene expression by NFkB

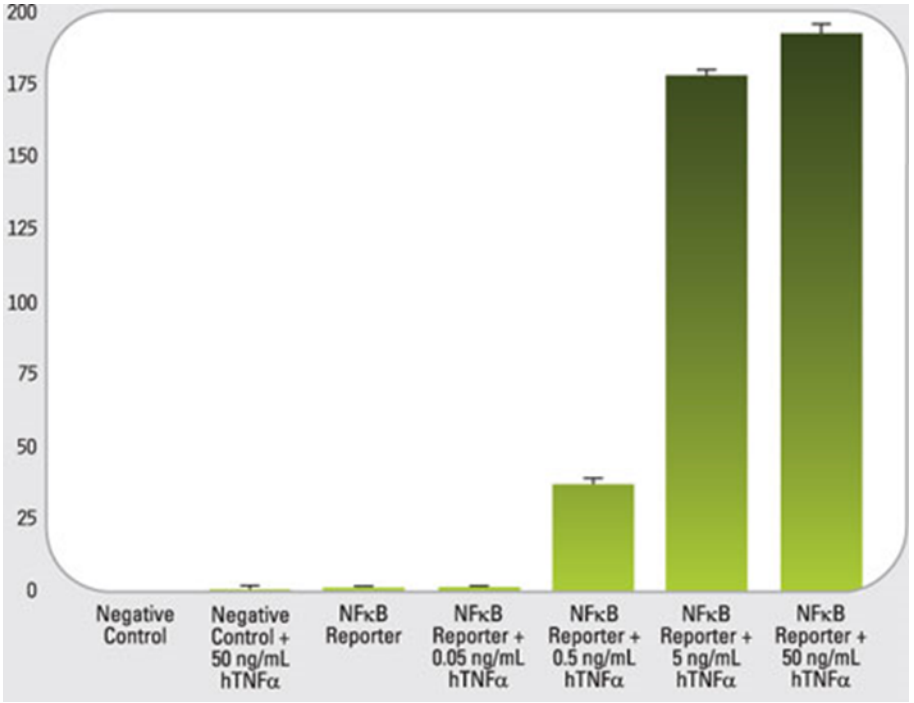


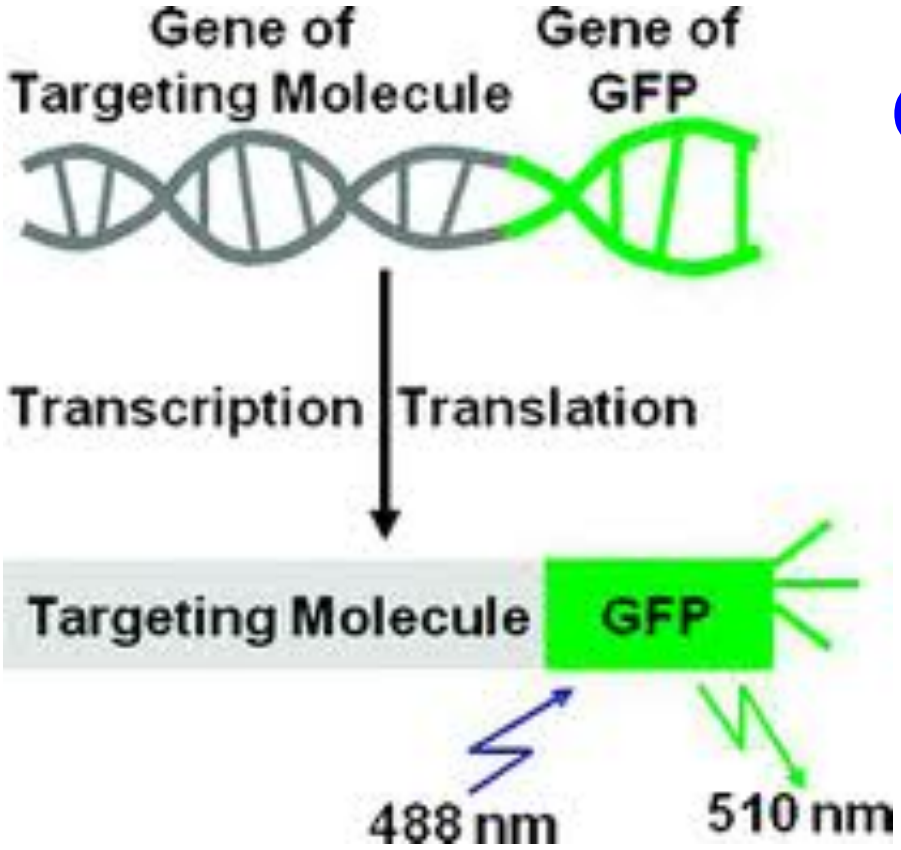
http://10e.devbio.com/article.php?id=31



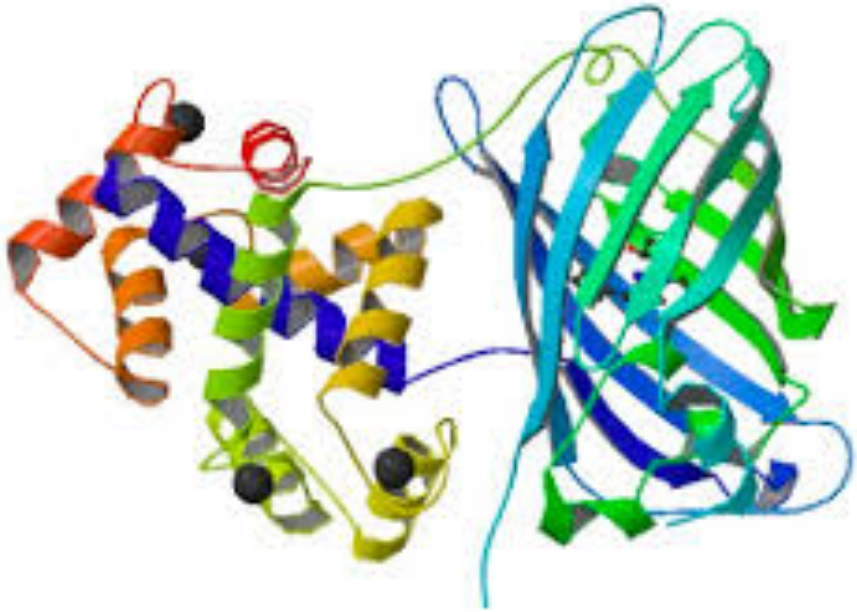
Fluorescent Protein Reporters are often used to report the amount of transcription from a specific promoter







Genetically engineered GFP-Fusion proteins



Fluorescent Protein Gene Fusions for Subcellular Localization Imaging

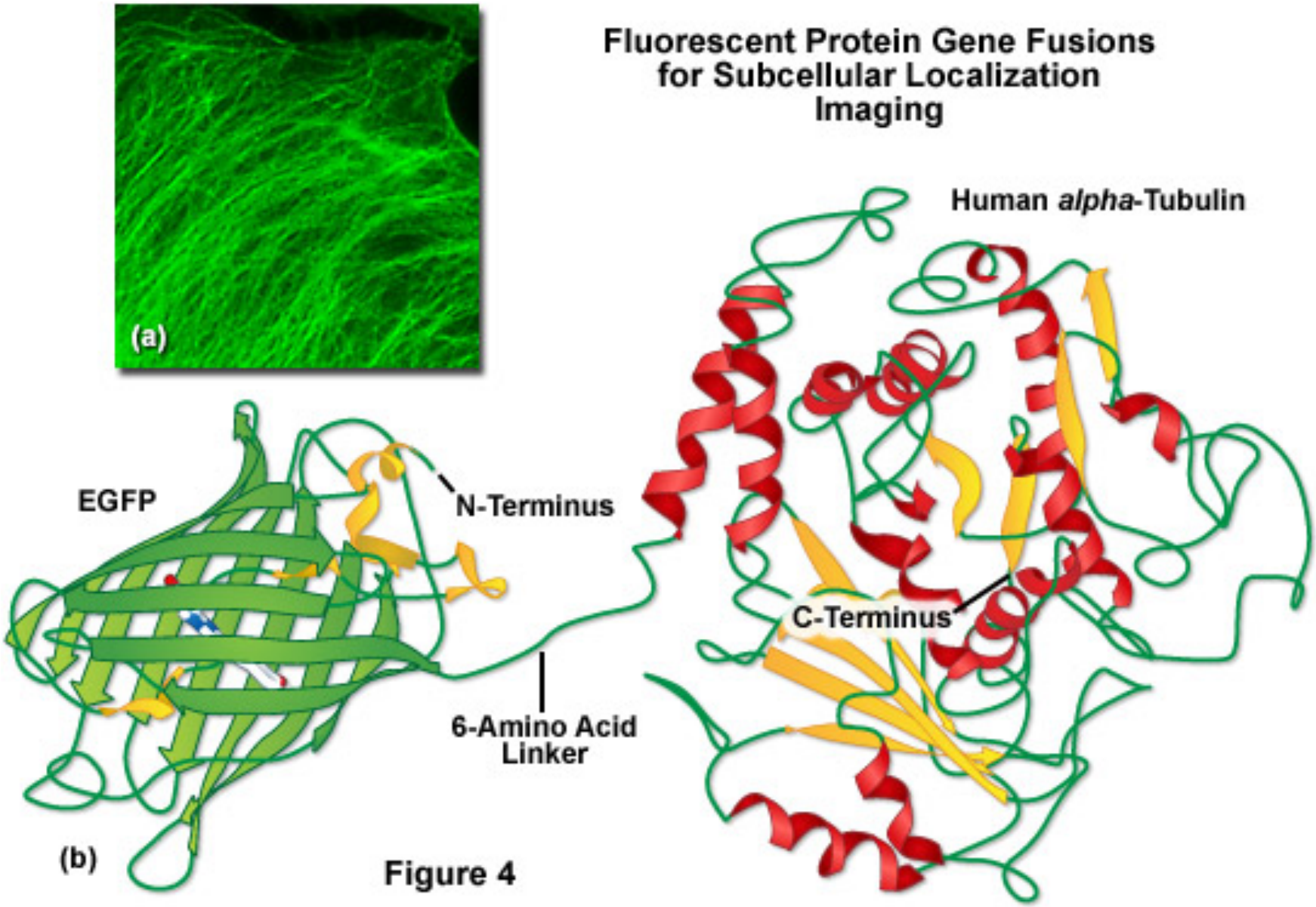
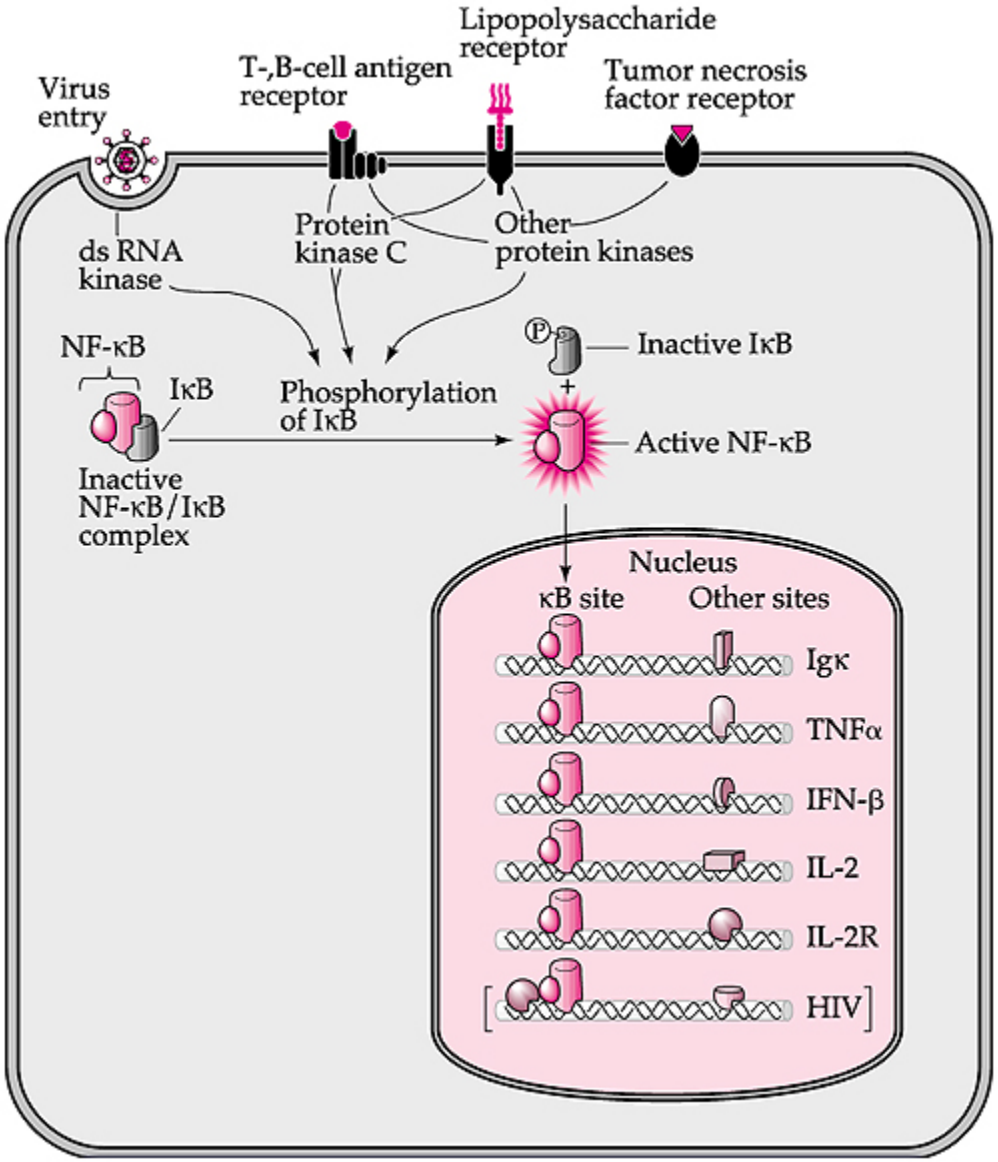


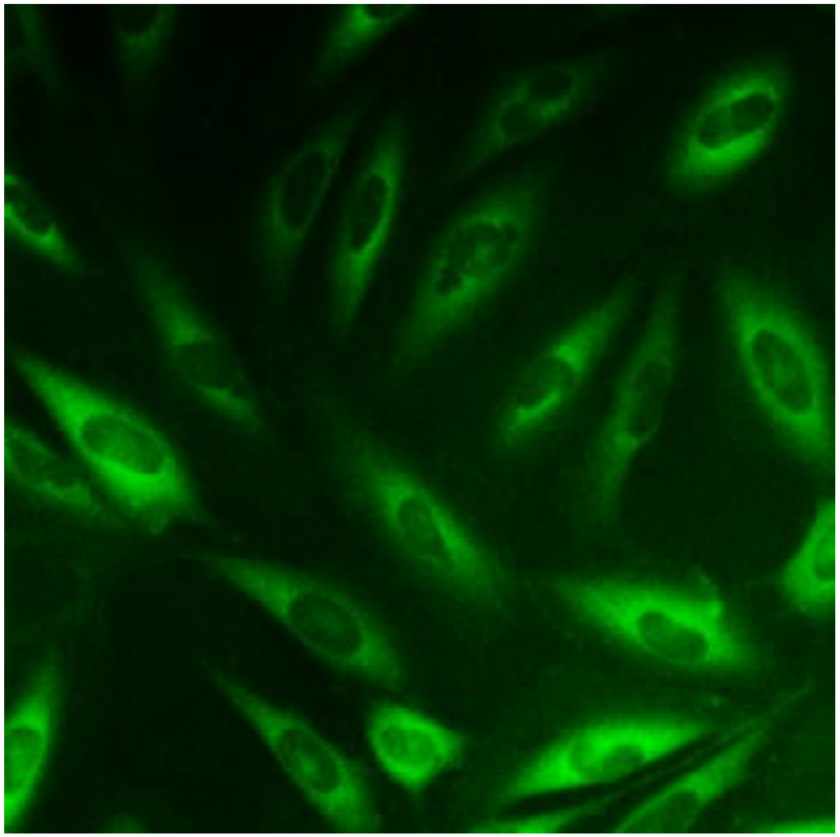
Figure 4

Regulation of gene expression by NFkB

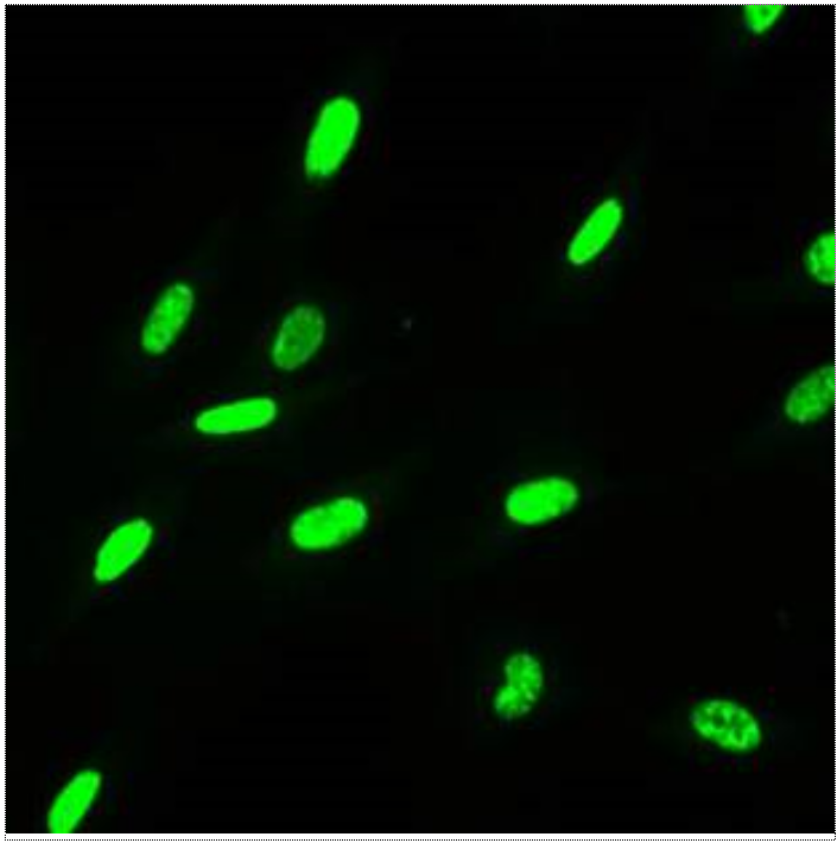


http://10e.devbio.com/article.php?id=31

DNA Damage Induced NFKB Signalling



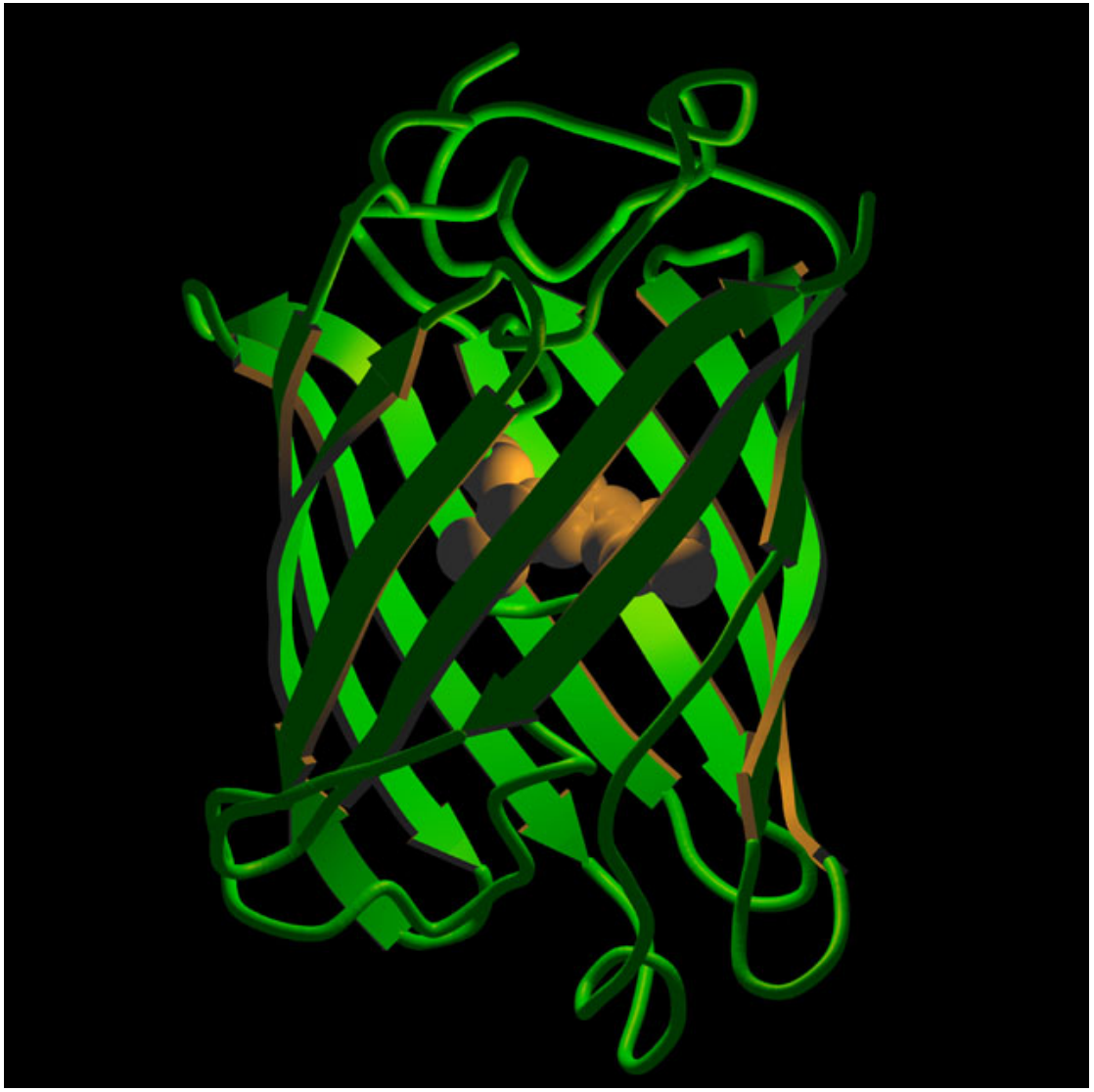
inactive NFKB



cpd induced NFKB activation

A cell line stably expressing a fusion of the NFkB transcription factor and GFP (green fluorescent protein) allows 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

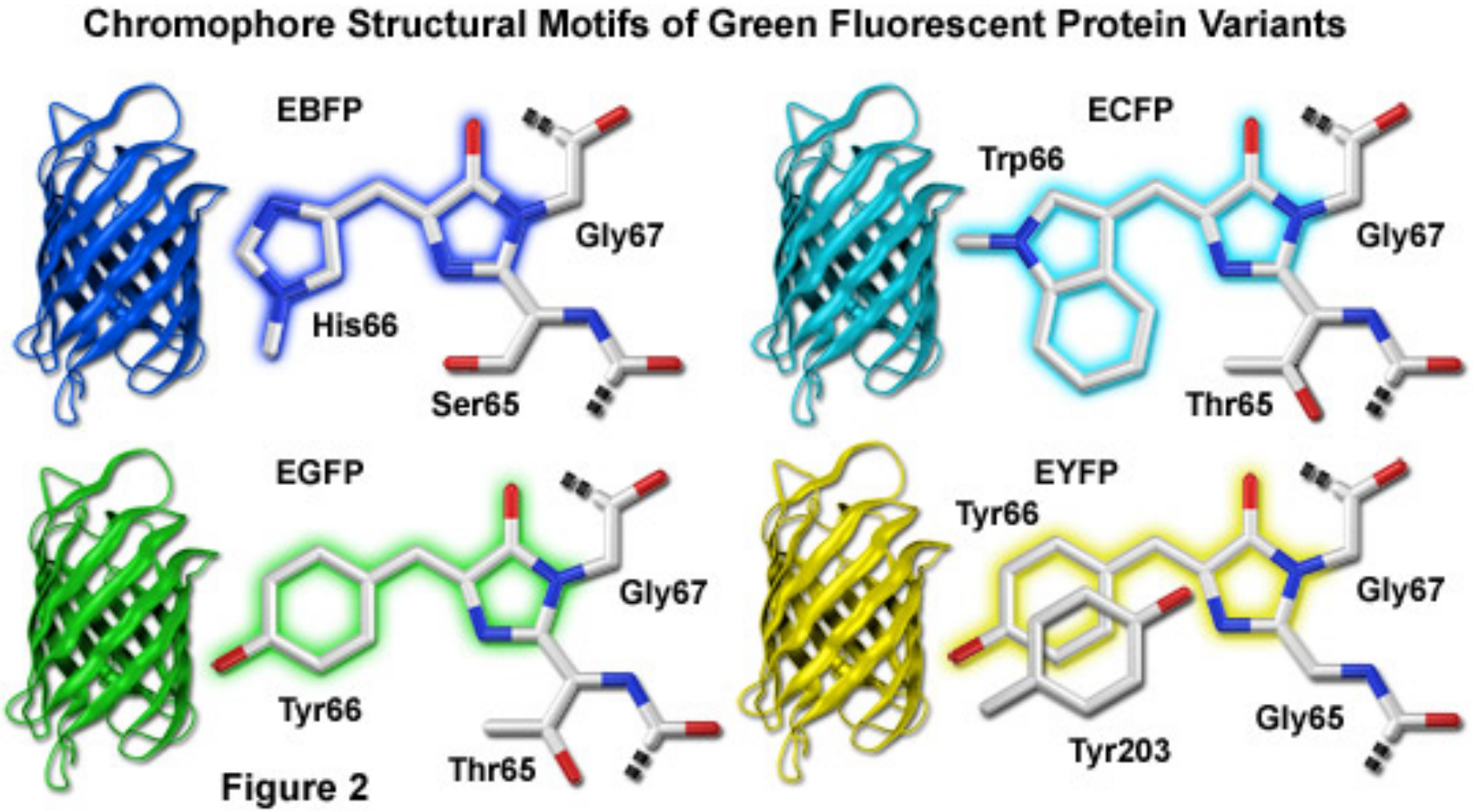
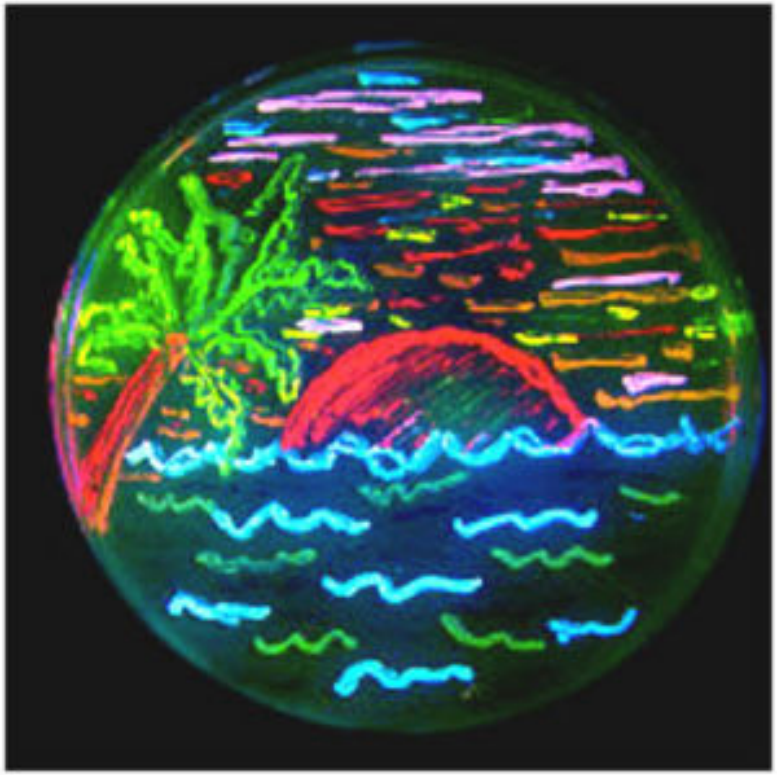


Figure 2

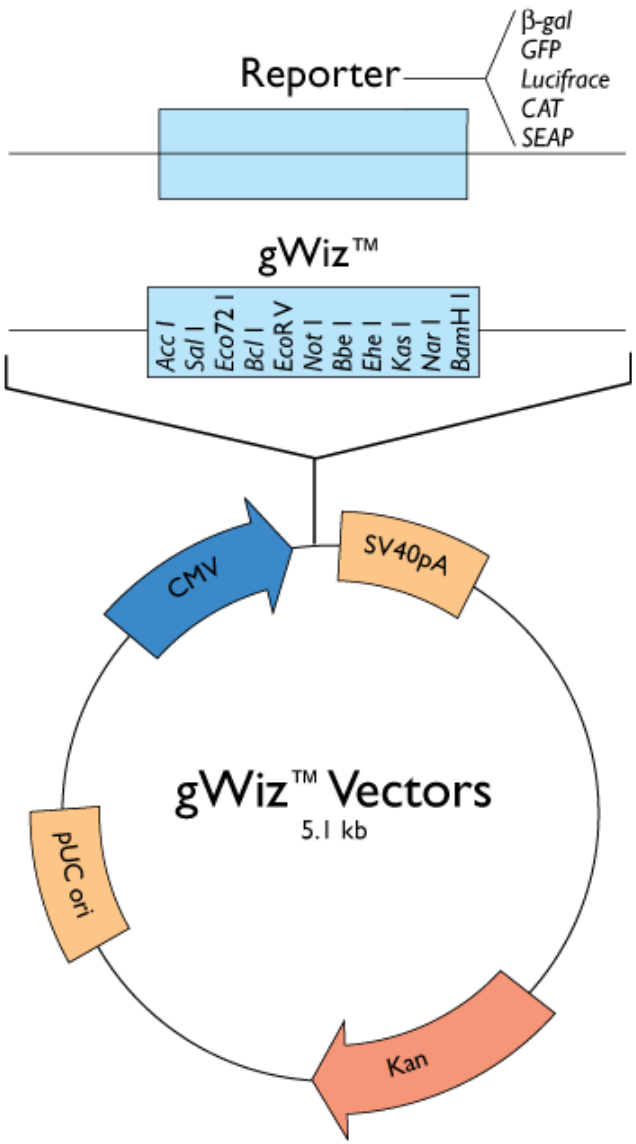
Mushroom Coral

Fluorescent Bulb Anemone (Entacmaea quadricolor)

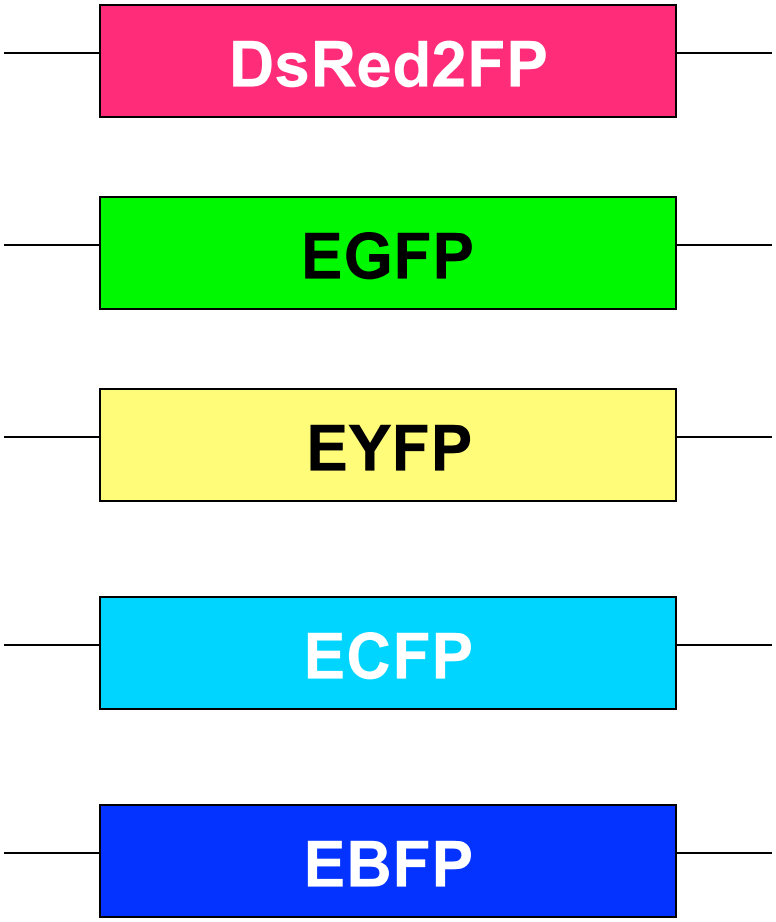


The diversity of fluorescent proteins and genetic mutations is illustrated by this San Diego beach scene drawn with living bacteria expressing 8 different colors of fluorescent proteins.

Reactivation of damaged DNA – multiplexed

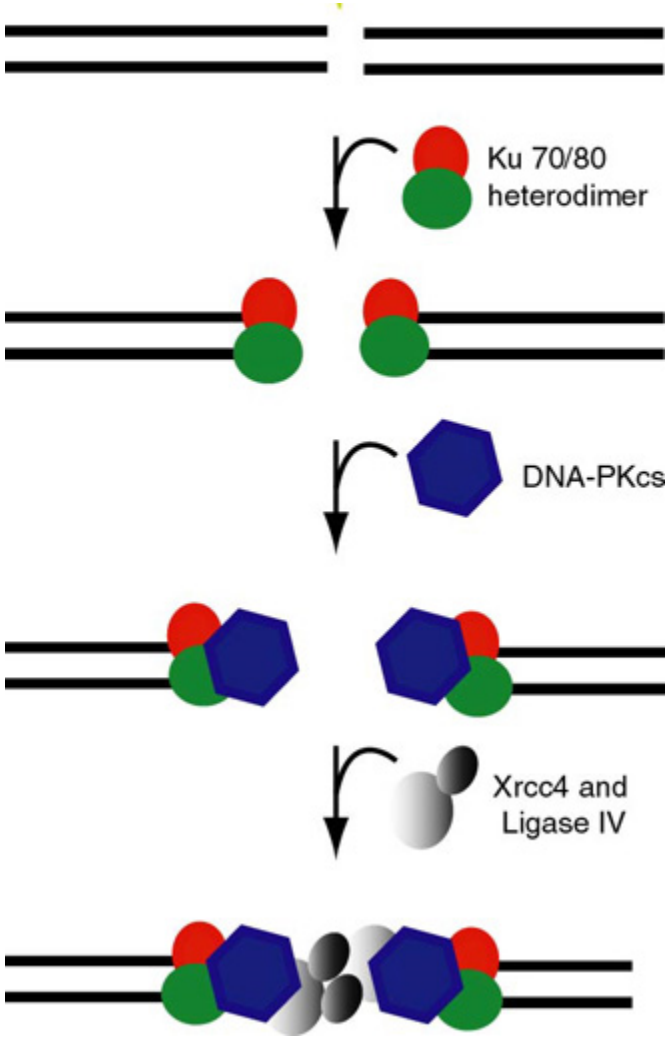


Each Fluorescent Protein gene will harbor a different type of DNA damage



http://www.genlantis.com/corp/images/gWiz_vectmap.gif

Non-Homologous End Joining (NHEJ)

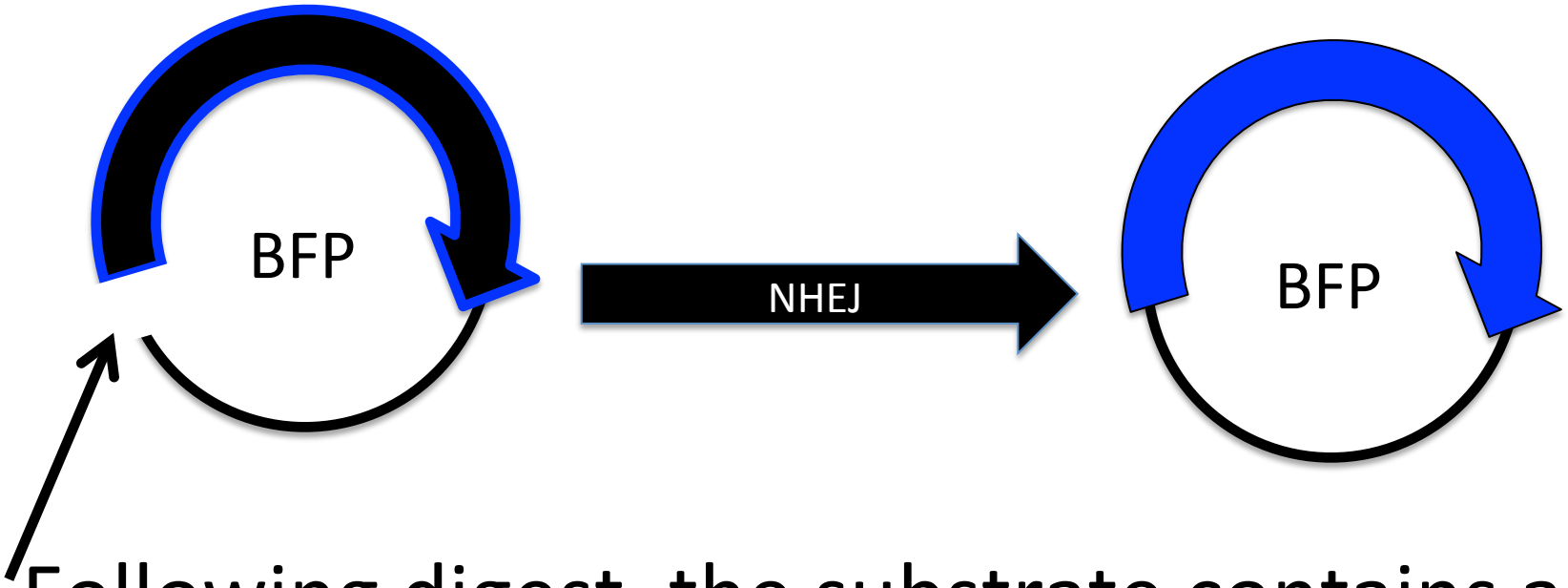


Ku70
Ku80

DNA-PKcs

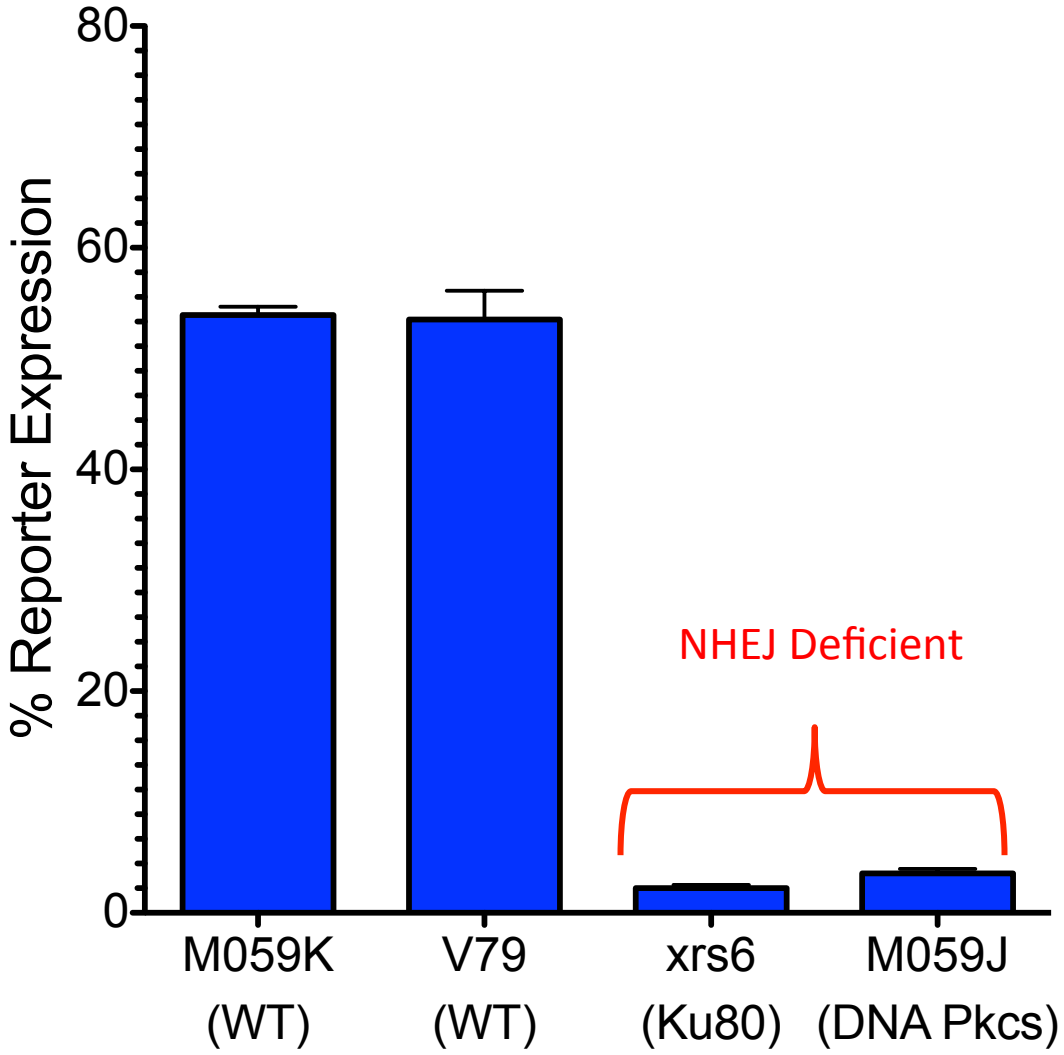
Xrcc4
Ligase IV

Basis for the fluorescent reporter assay:

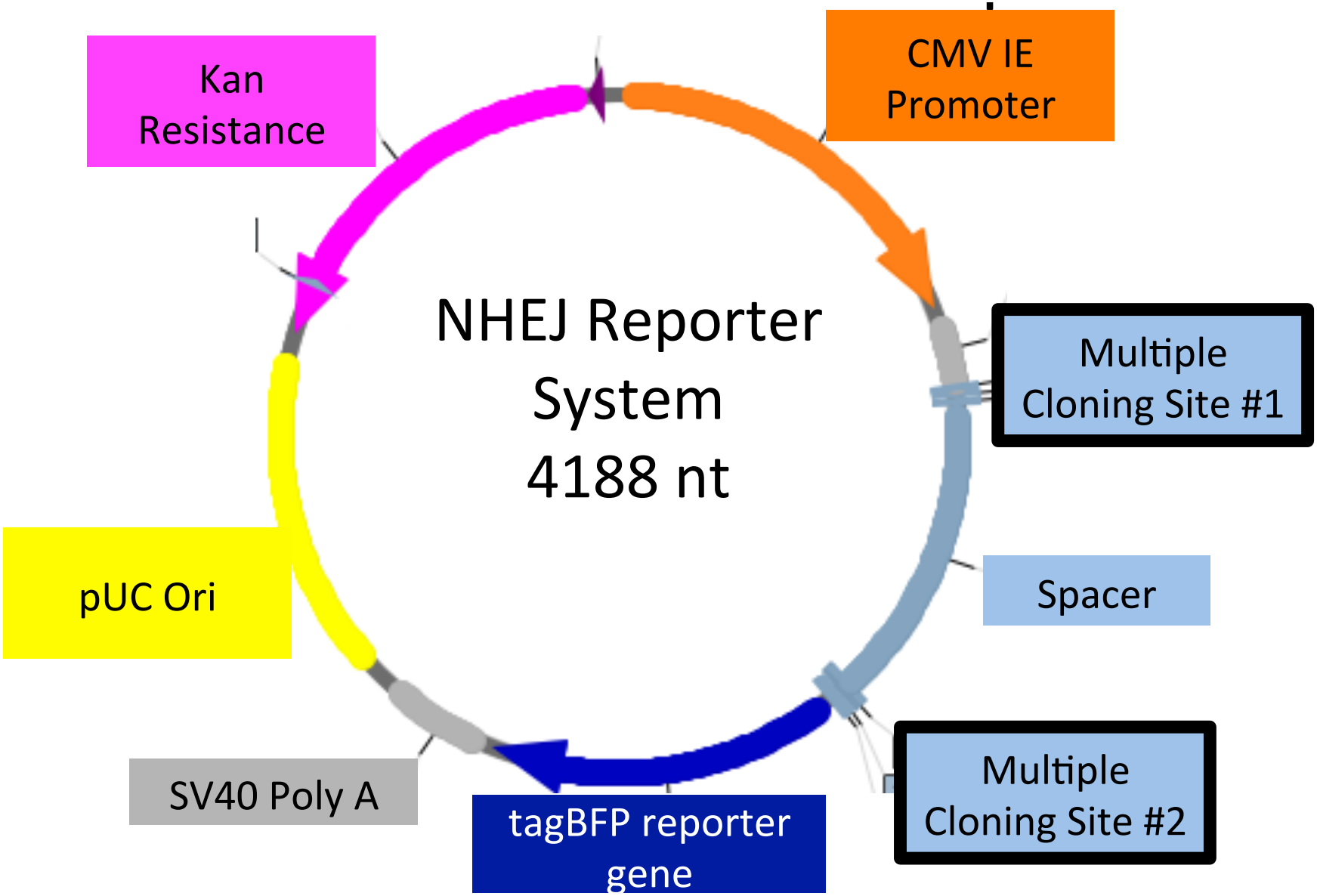


Following digest, the substrate contains a DSB in the 5’ UTR that prevents fluorescent reporter expression

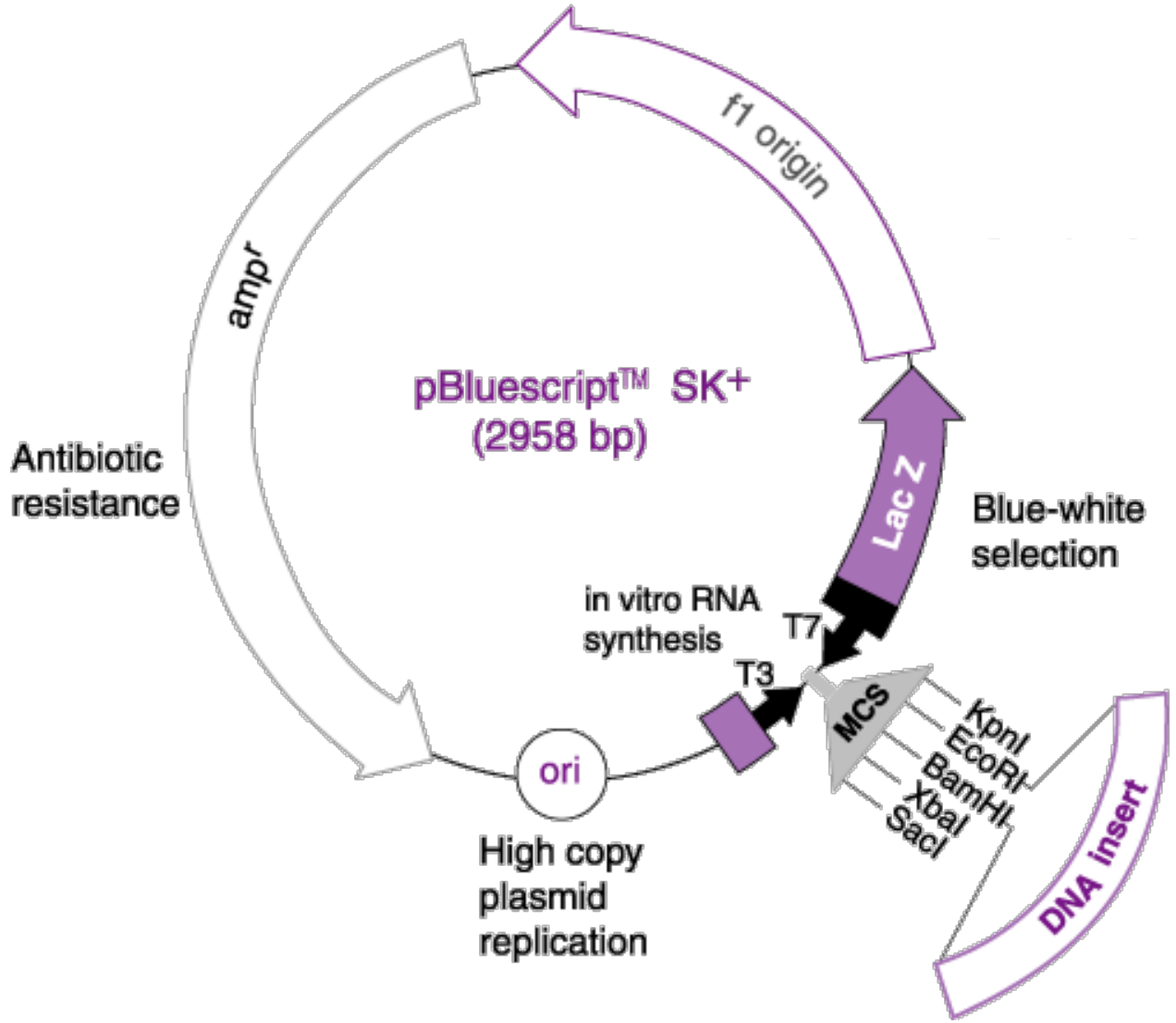
NHEJ HCR in WT and NHEJ defective cells at 18 hours post-transfection:



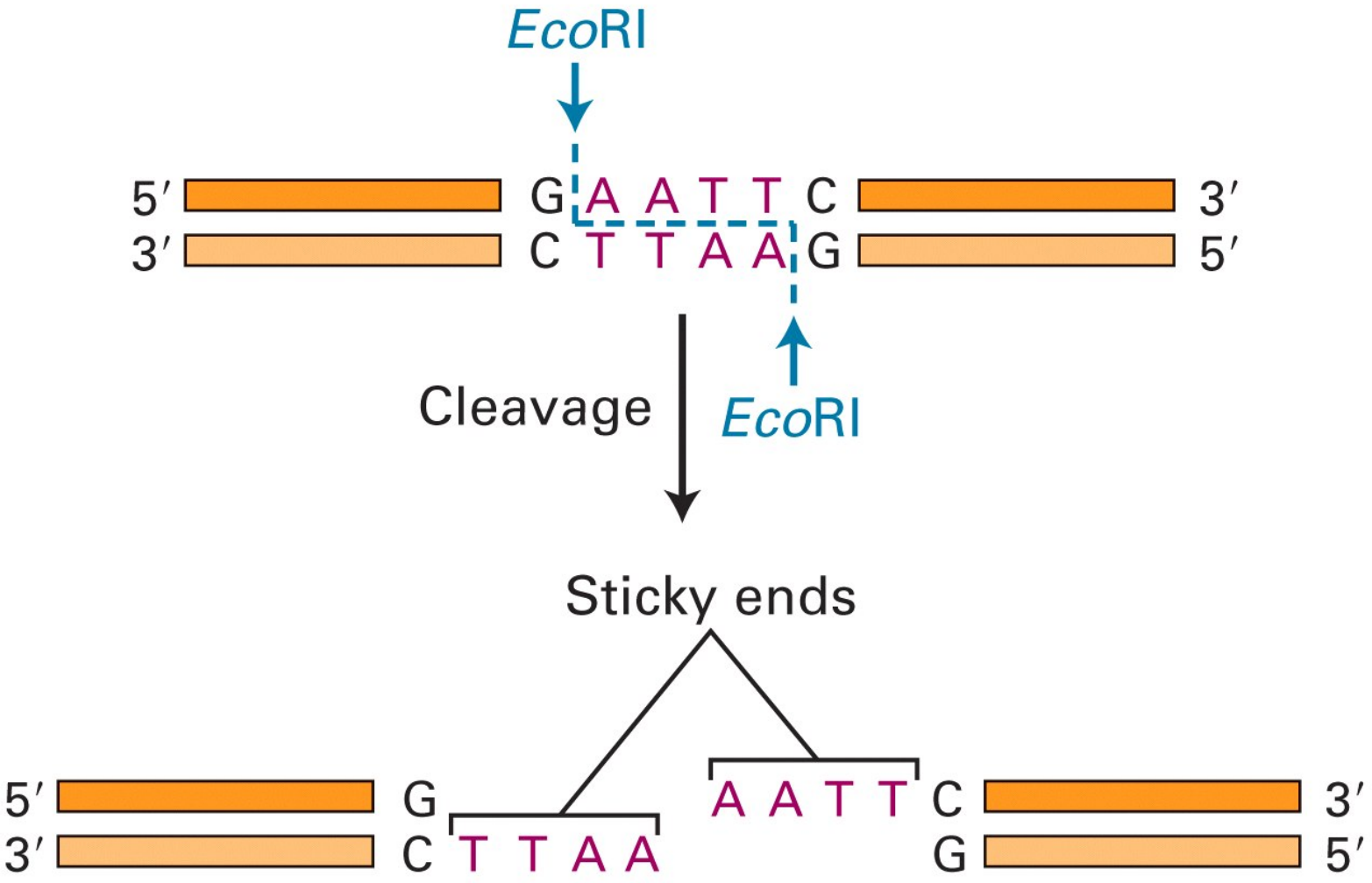
Overall Structure of the Reporter:



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.

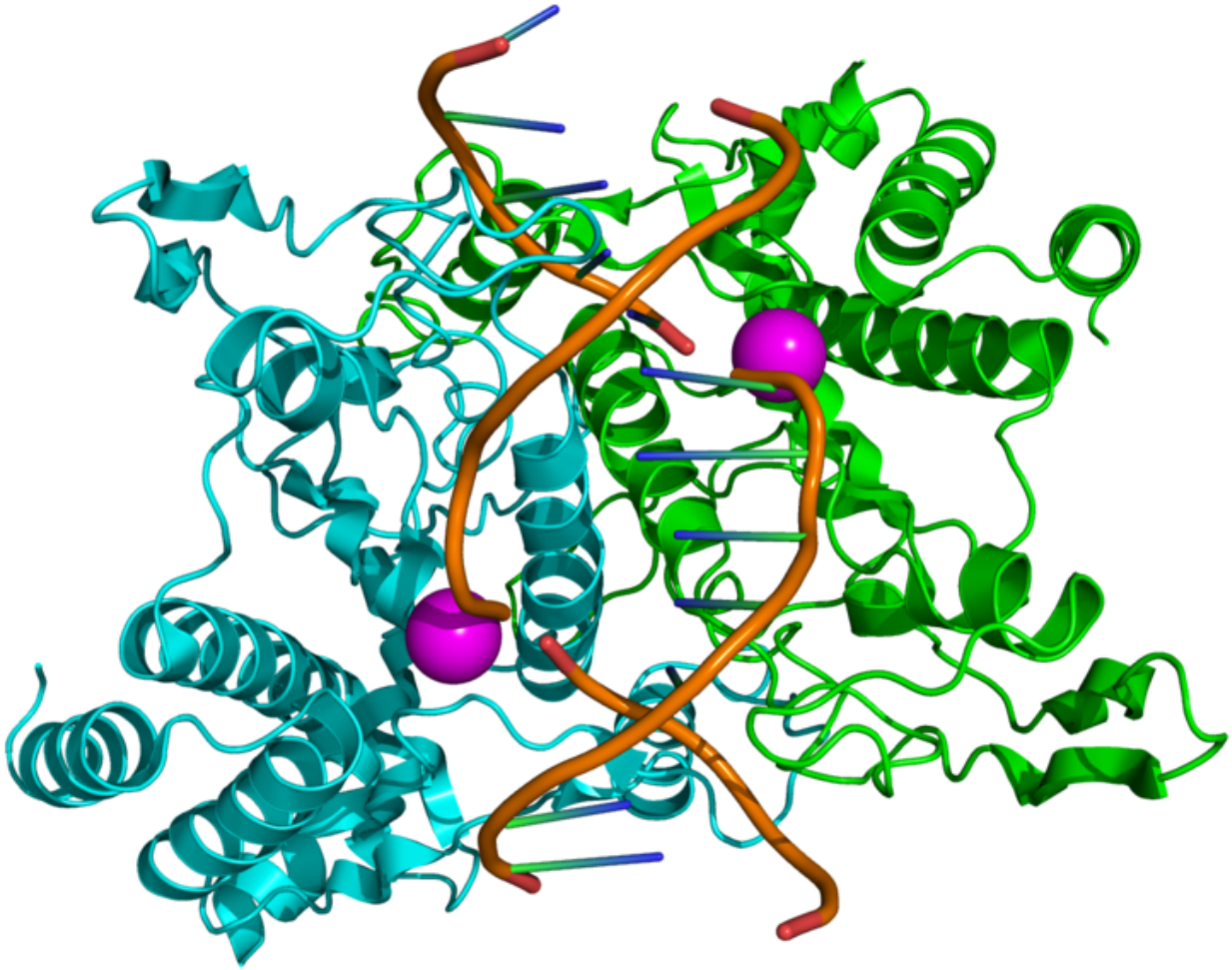
Flee to me, remote elf.

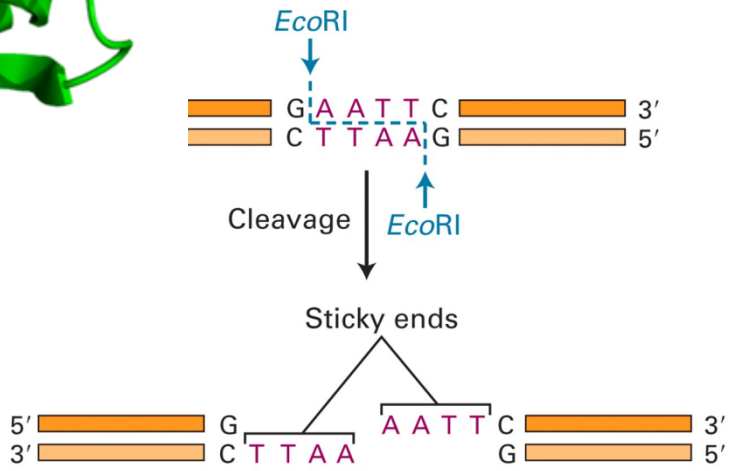
Gnu dung.

Lager, Sir, is regal.

Tuna nut.

What is a Restriction Enzyme?

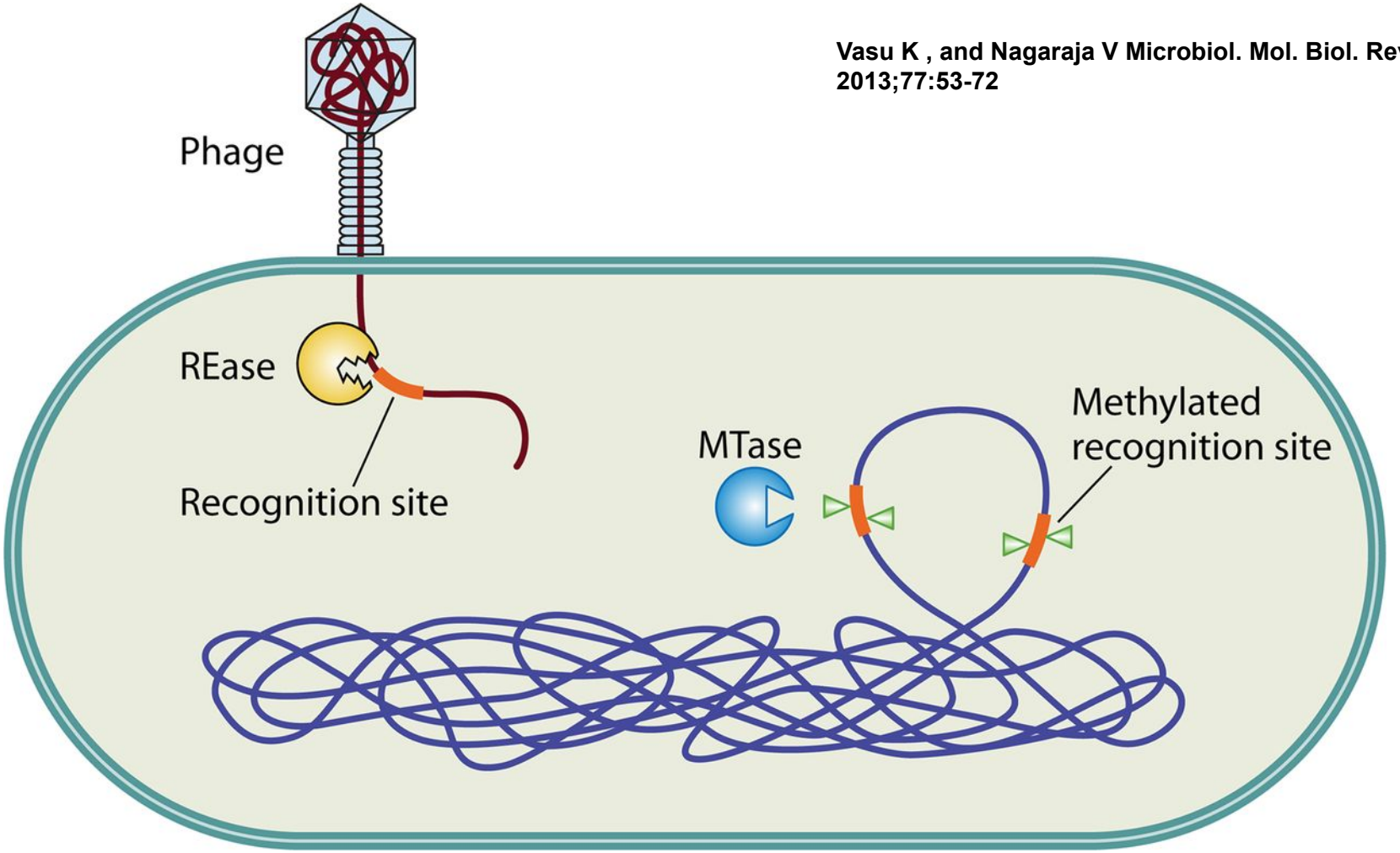




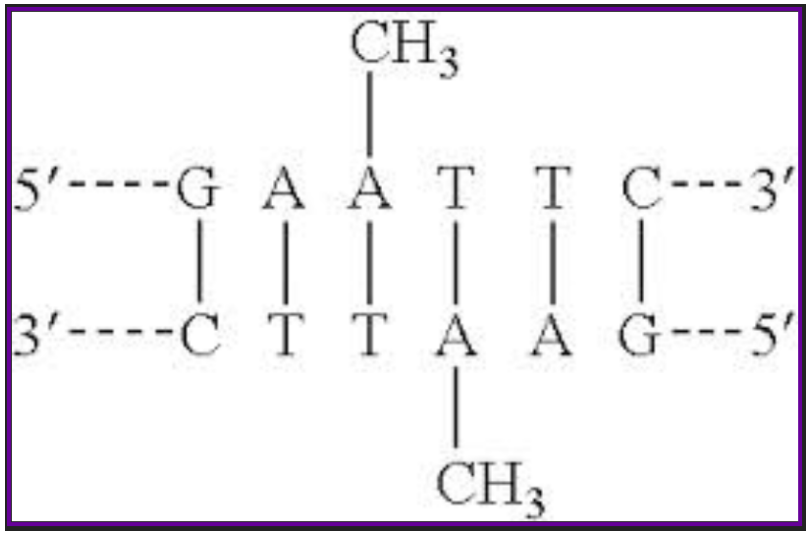
Why do Restriction Enzymes exist?

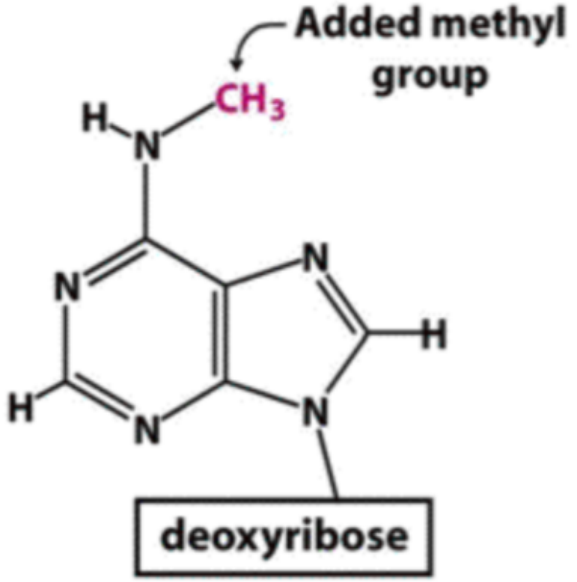
Restriction-modification (R-M) systems as defense mechanisms.

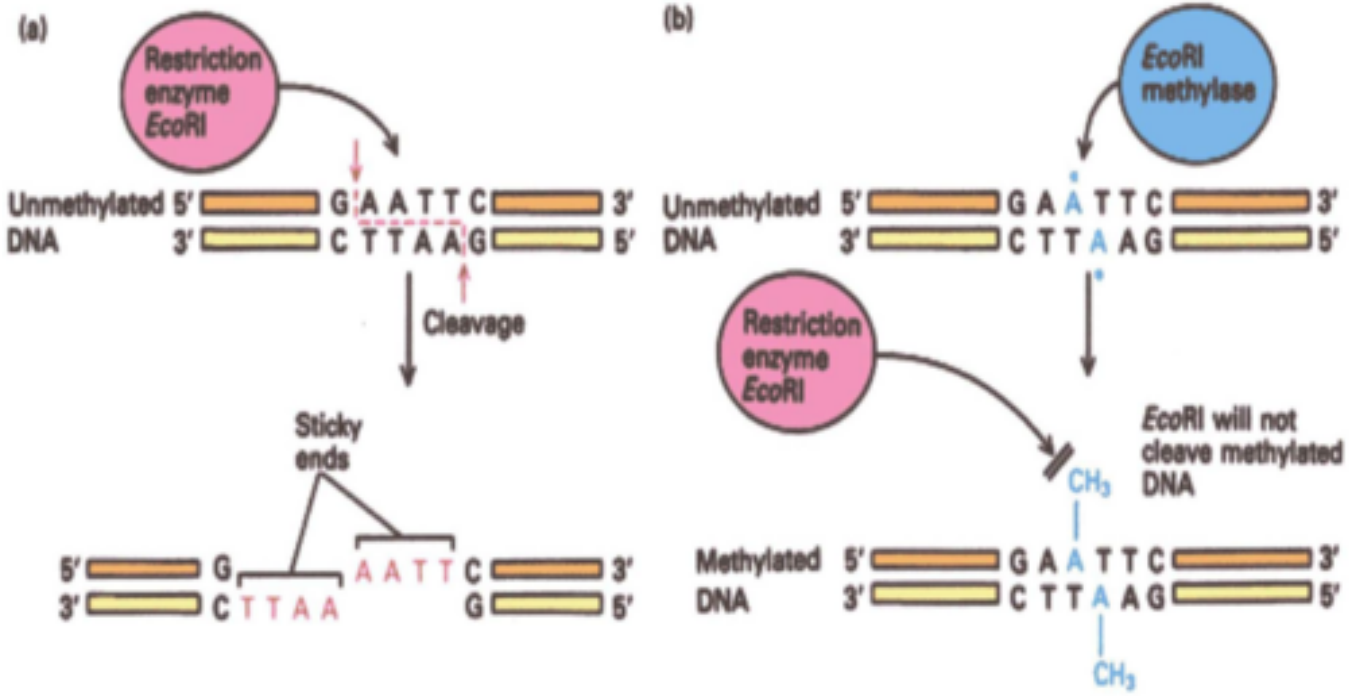
Vasu K , and Nagaraja V Microbiol. Mol. Biol. Rev. 2013;77:53-72



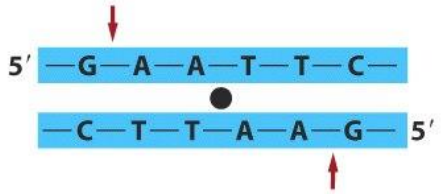
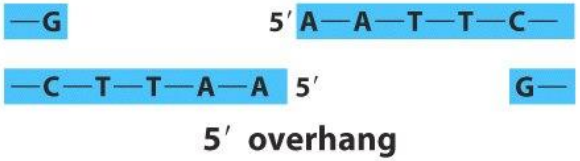
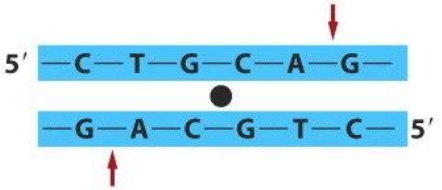
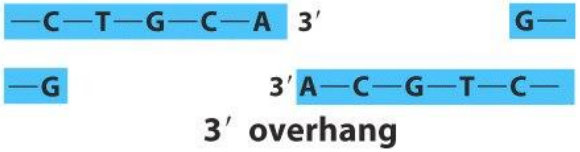
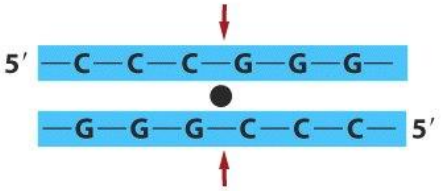
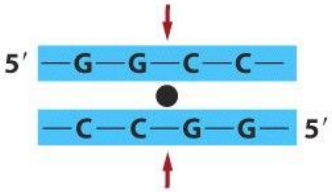
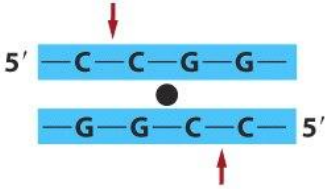
R-M systems recognize methylation status of incoming foreign DNA, e.g., phage genomes. Methylated sequences are recognized as self, while recognition sequences on the incoming DNA lacking methylation are recognized as nonself and are cleaved by the restriction endonuclease (REase). The methylation status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


CH3
5′----G A A T T C---3′
3′----C T T A A G---5′
CH3

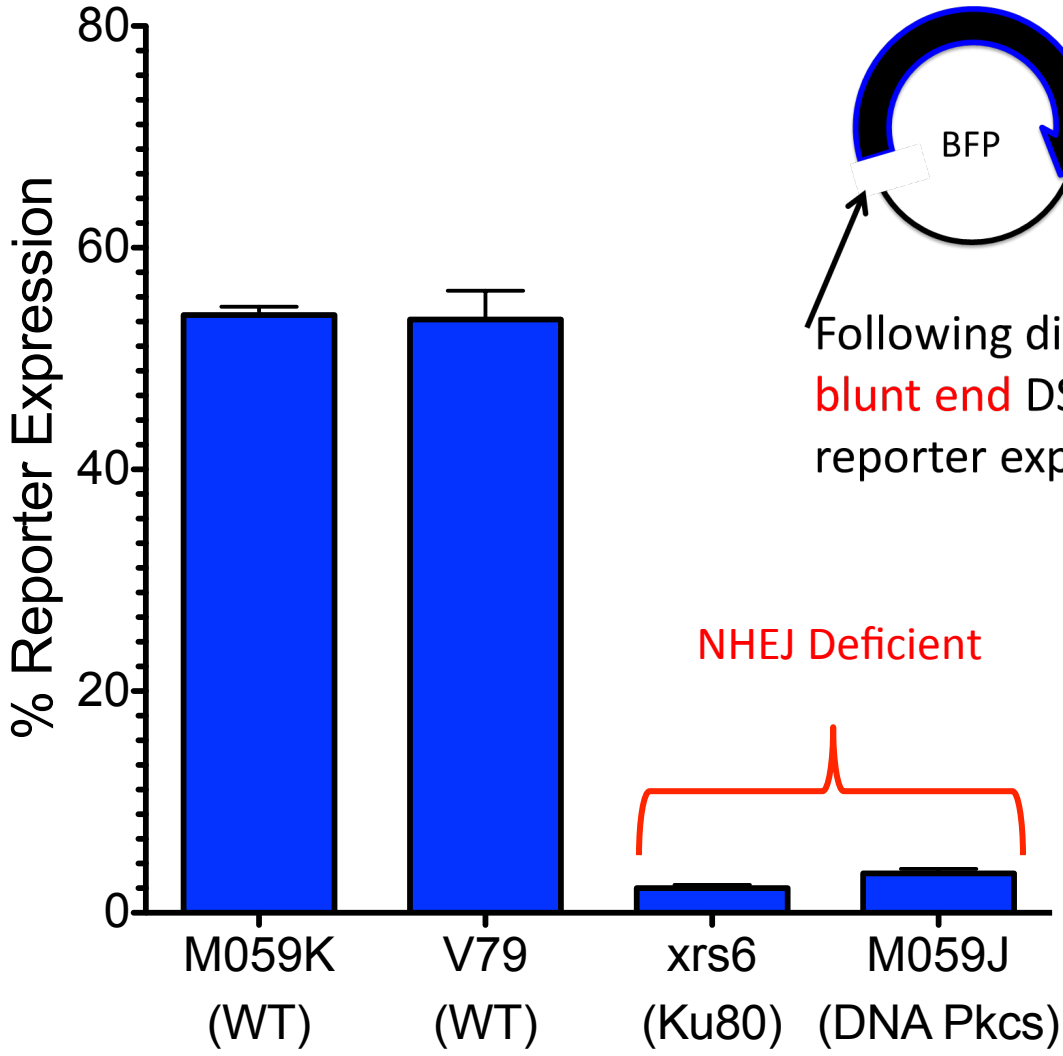

Added methyl group
CH3
H
N
N
N
H
H
N
N
deoxyribose

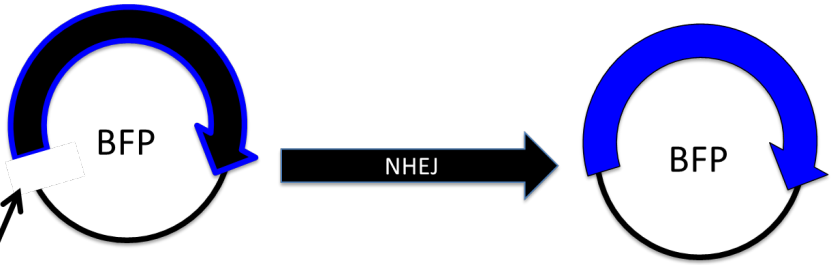

(a)
Restriction enzyme EcoRI
Unmethylated DNA
5′ G A A T T C 3′
3′ C T T A A G 5′
Cleavage
Sticky ends
5′ G A A T T C 3′
3′ C T T A A G 5′
(b)
EcoRI methylase
Unmethylated DNA
5′ G A A T T C 3′
3′ C T T A A G 5′
Restriction enzyme EcoRI
EcoRI will not cleave methylated DNA
CH3
Methylated DNA
5′ G A A T T C 3′
3′ C T T A A G 5′
CH3

Some restriction enzymes

Enzyme	Source organism	Restriction recognition site in double-stranded DNA	Structure of the cleaved products
(a)			
*Eco*RI	*Escherichia coli*		
*Pst*I	*Providencia stuartii*		
*Sma*I	*Serratia marcescens*		
(b)			
*Hae*III	*Haemophilus aegyptius*		
*Hpa*II	*Haemophilus parainfluenzae*		

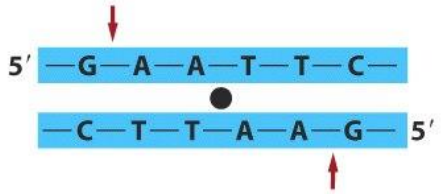
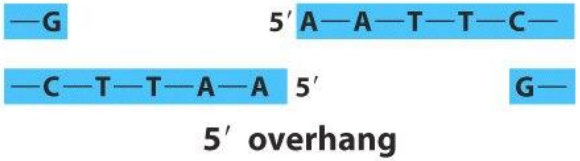
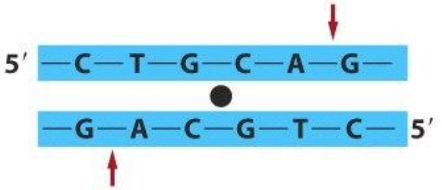
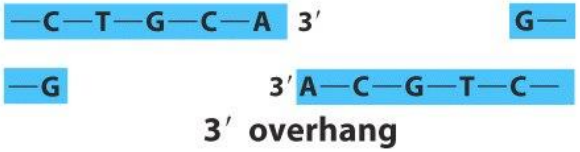
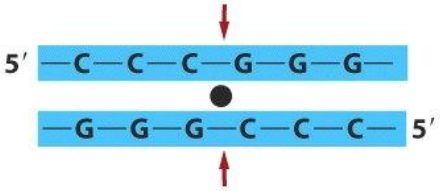
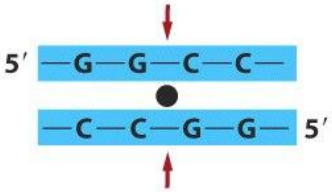
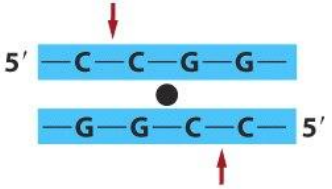
NHEJ HCR in WT and NHEJ defective cells at 18 hours post-transfection:



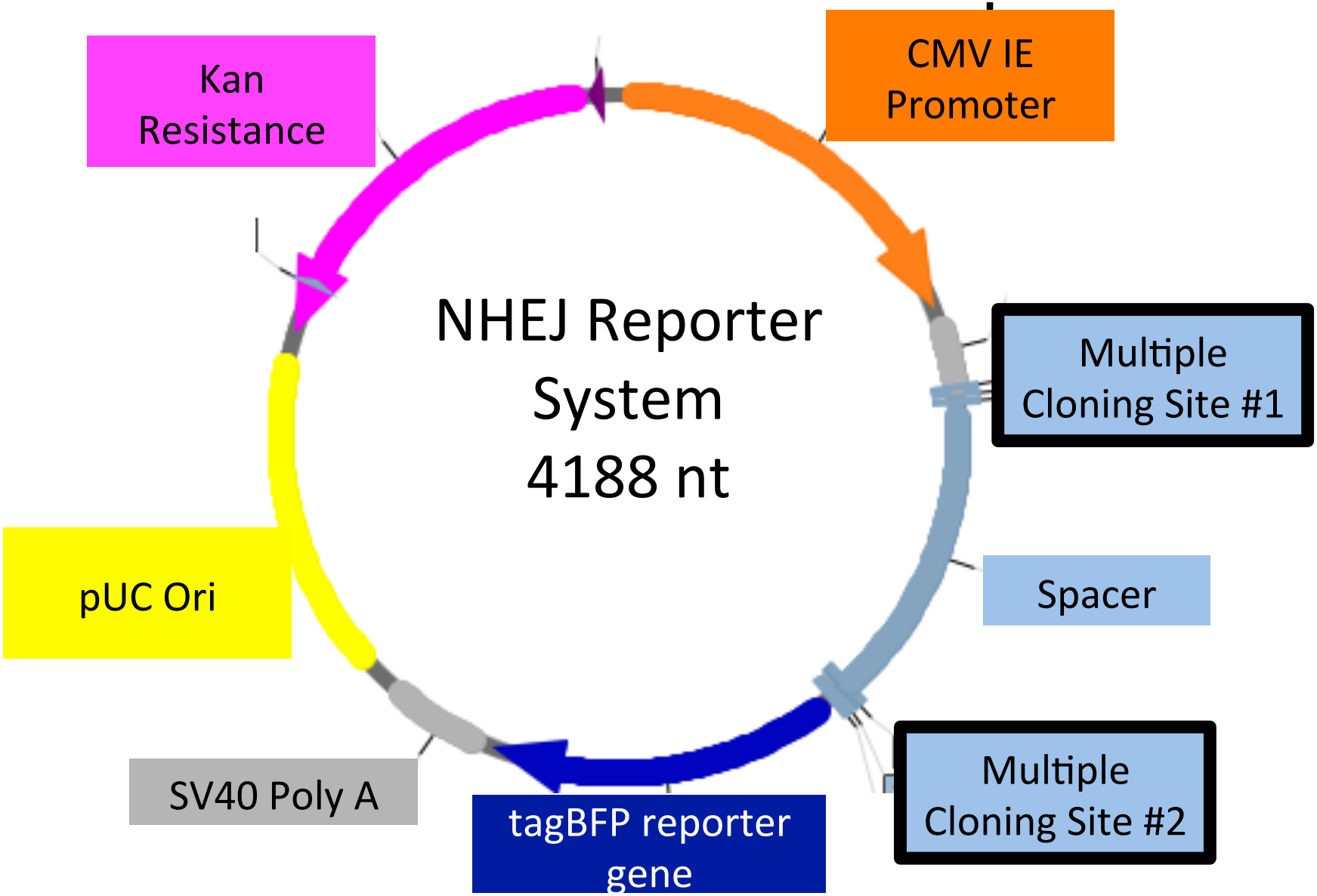


Following digest, the substrate contains a blunt end DSB that prevents fluorescent reporter expression

Some restriction enzymes

Enzyme	Source organism	Restriction recognition site in double-stranded DNA	Structure of the cleaved products
(a)			
*Eco*RI	*Escherichia coli*		
*Pst*I	*Providencia stuartii*		
*Sma*I	*Serratia marcescens*		
(b)			
*Hae*III	*Haemophilus aegyptius*		
*Hpa*II	*Haemophilus parainfluenzae*		

Overall Structure of the Reporter:



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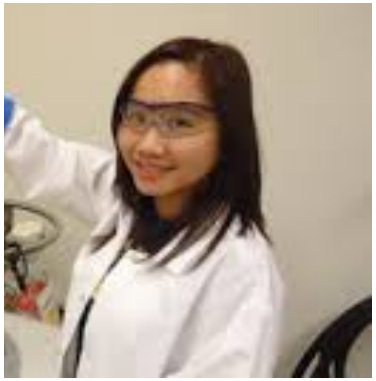

Shannon Hughes
Noreen Lyell
Leslie McLain
Nova Pishesha (TA)
Leona Samson (Lectures)

Zachary Nagel (help with development) Alex Chaim