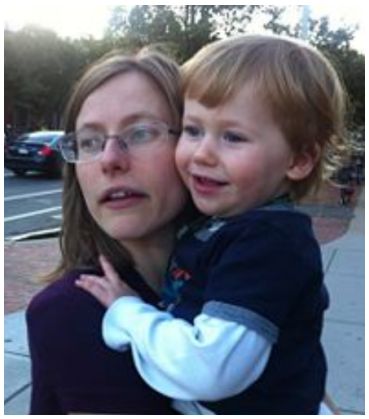
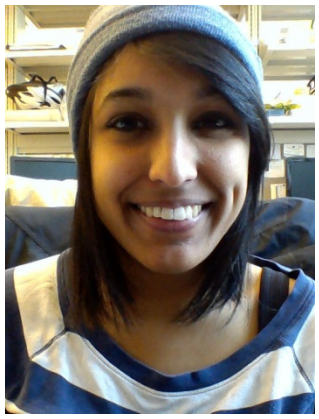
20.109 Spring 2014 Module 2
System Engineering and Protein Foundations



Agi Stachowiak

Shannon Hughes

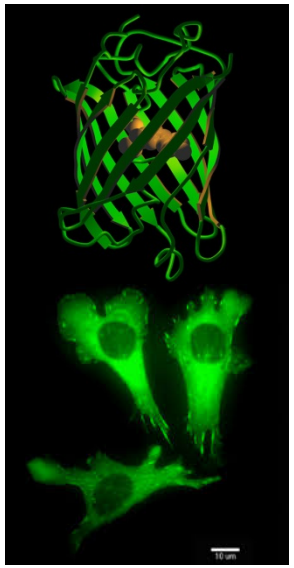
Aneesh Ramaswamy

Suhani Vora (TA)

Leona Samson (Lectures)

Zachary Nagel (help with development)

What experimental question will you ask in Module 2?

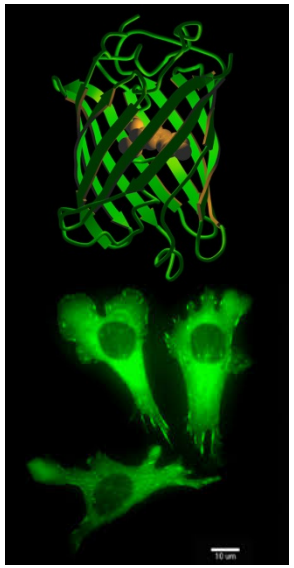


How efficiently does DNA repair by the Non Homologous End Joining (NHEJ) pathway act on DNA damage with different topologies?

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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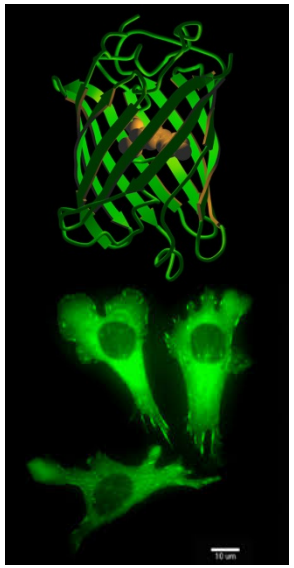

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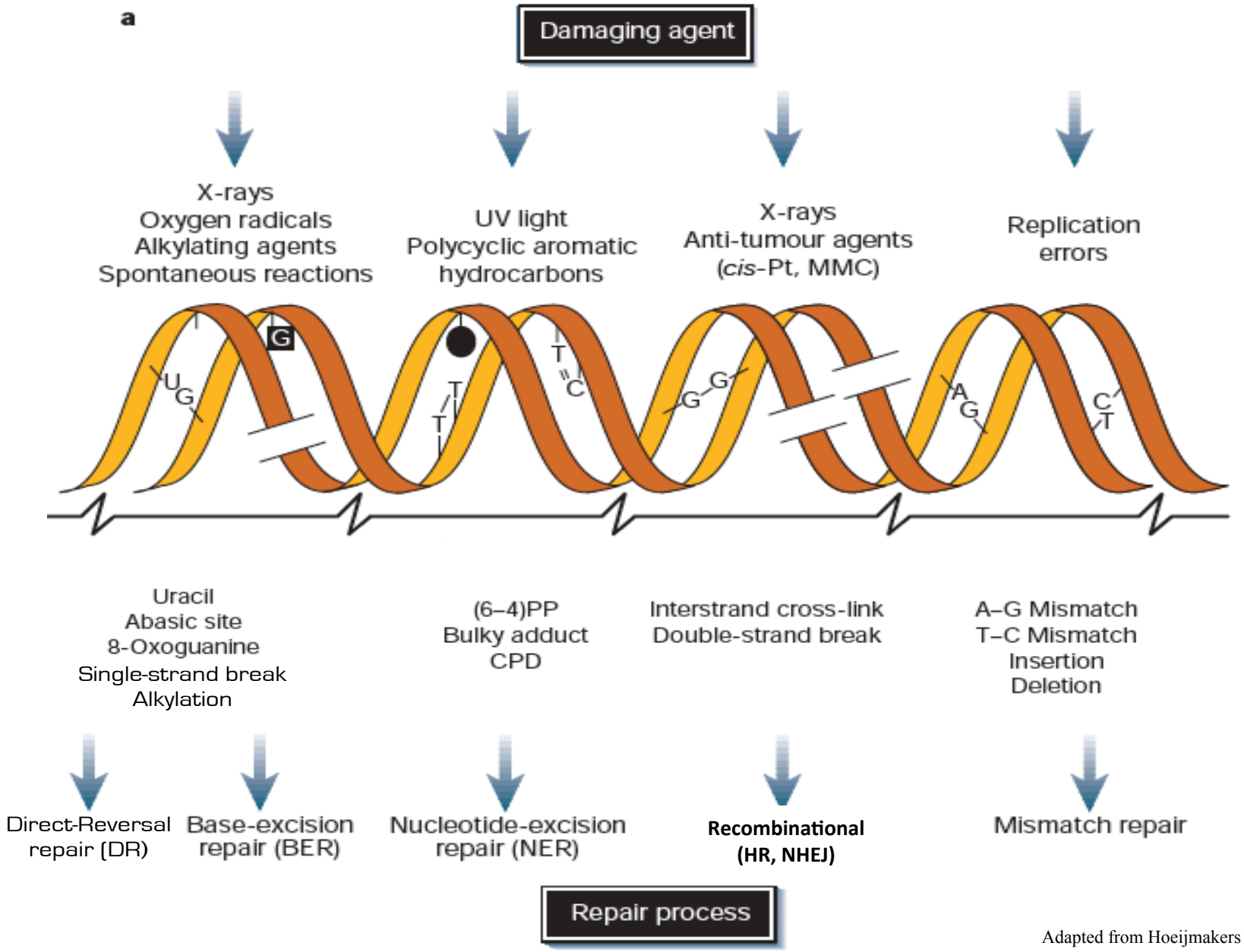
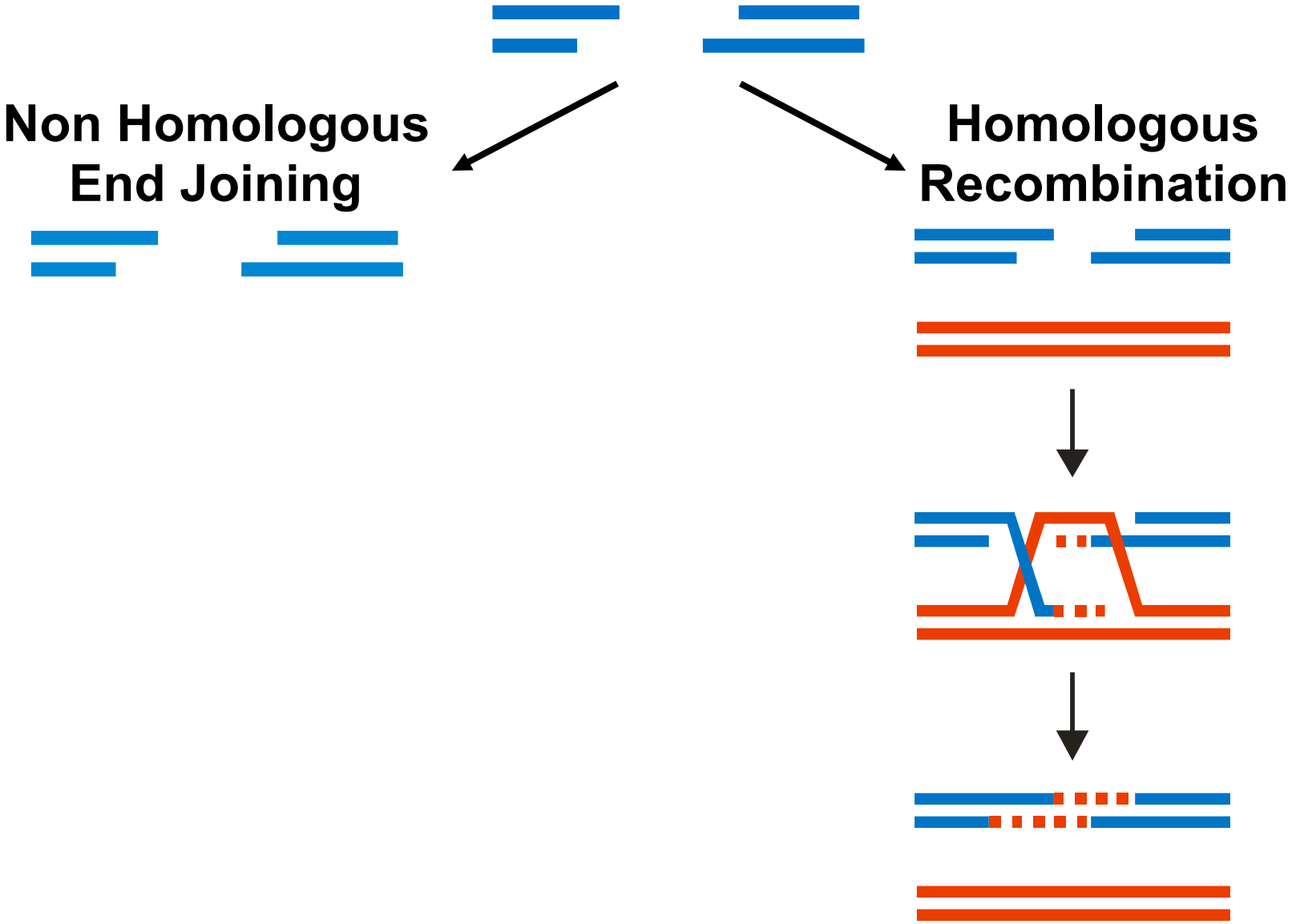
DNA Damage and Repair

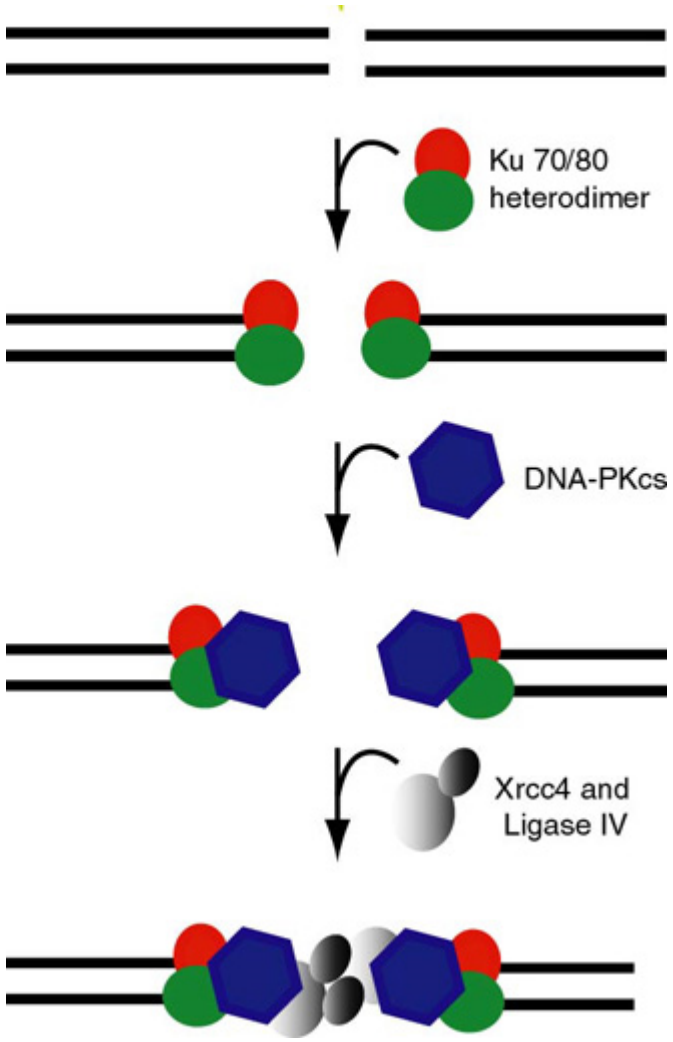


Adapted from Hoeijmakers **2001** *Nature*

DNA double-strand break repair



Non-Homologous End Joining (NHEJ)

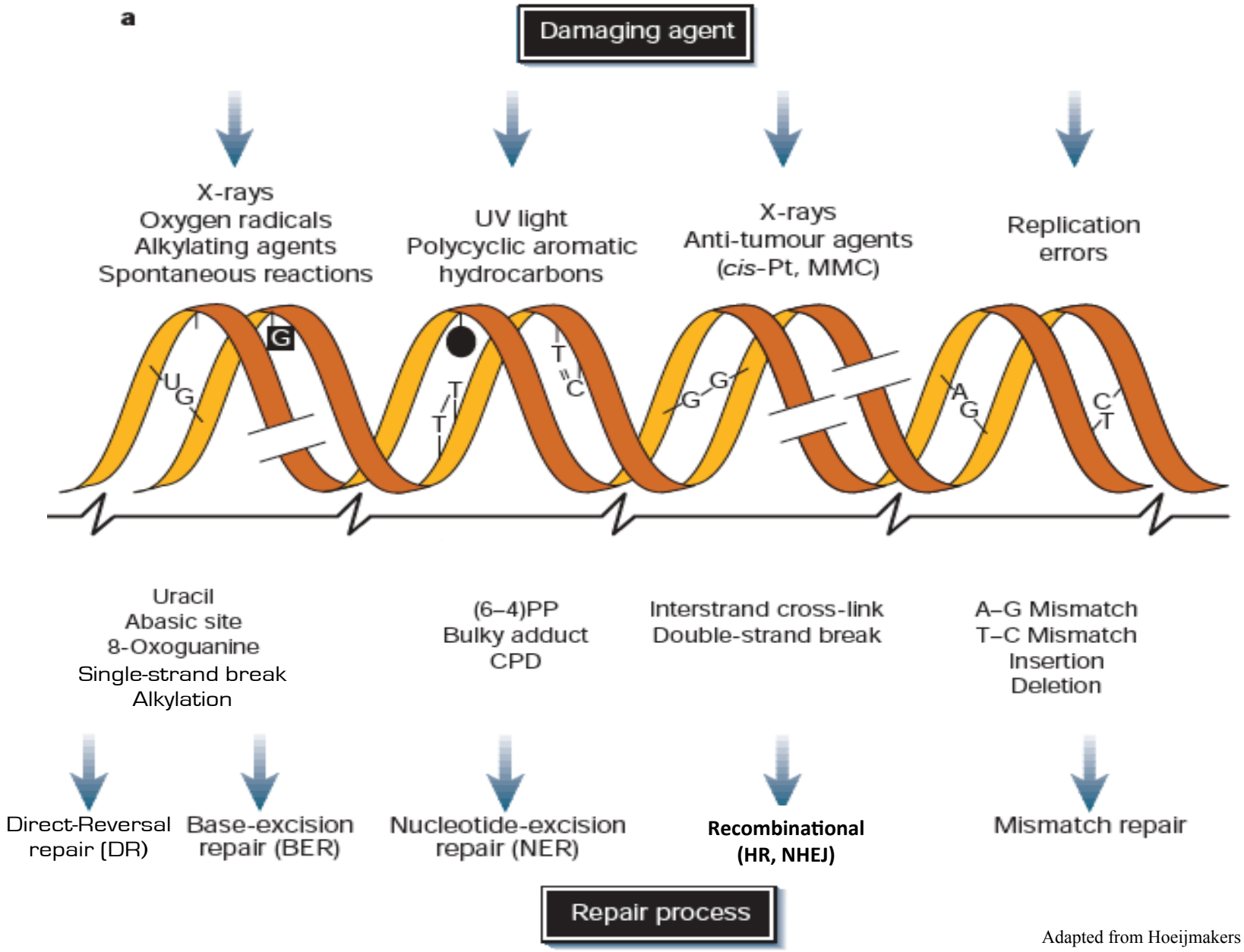


Ku70
Ku80

DNA-PKcs

Xrcc4
Ligase IV

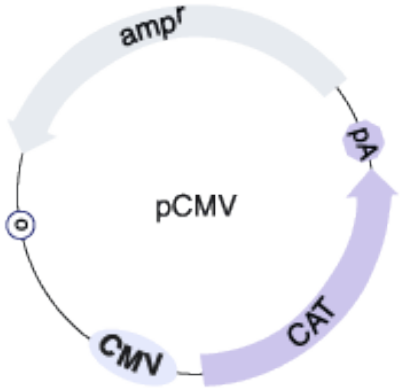
DNA Damage and Repair



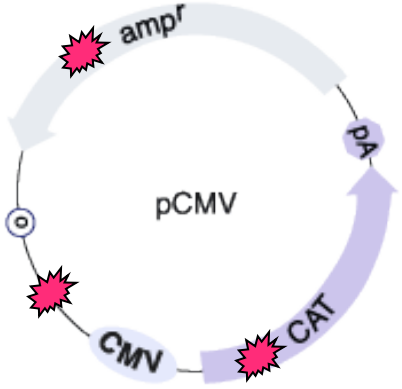
Adapted from Hoeijmakers **2001** *Nature*

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

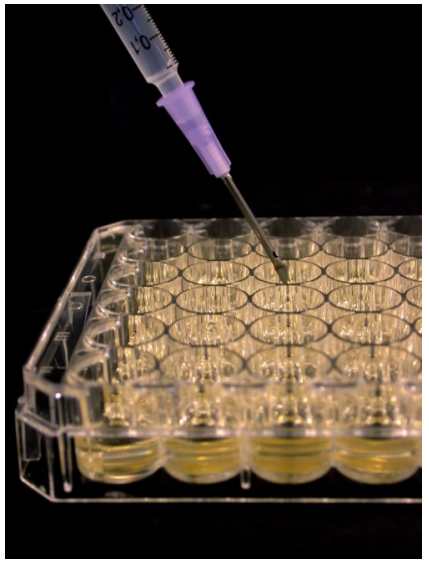
Athas & GROSSMAN
Cancer Res. 1991

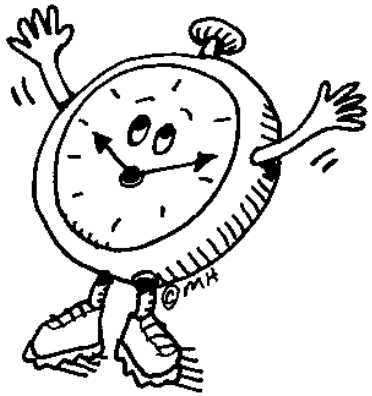


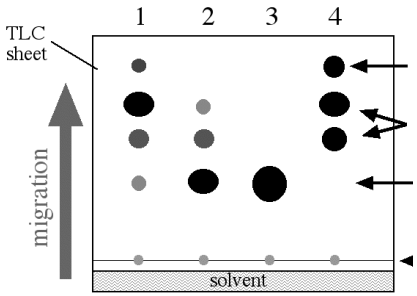
+ UV light



Transient transfection peripheral blood lymphocytes



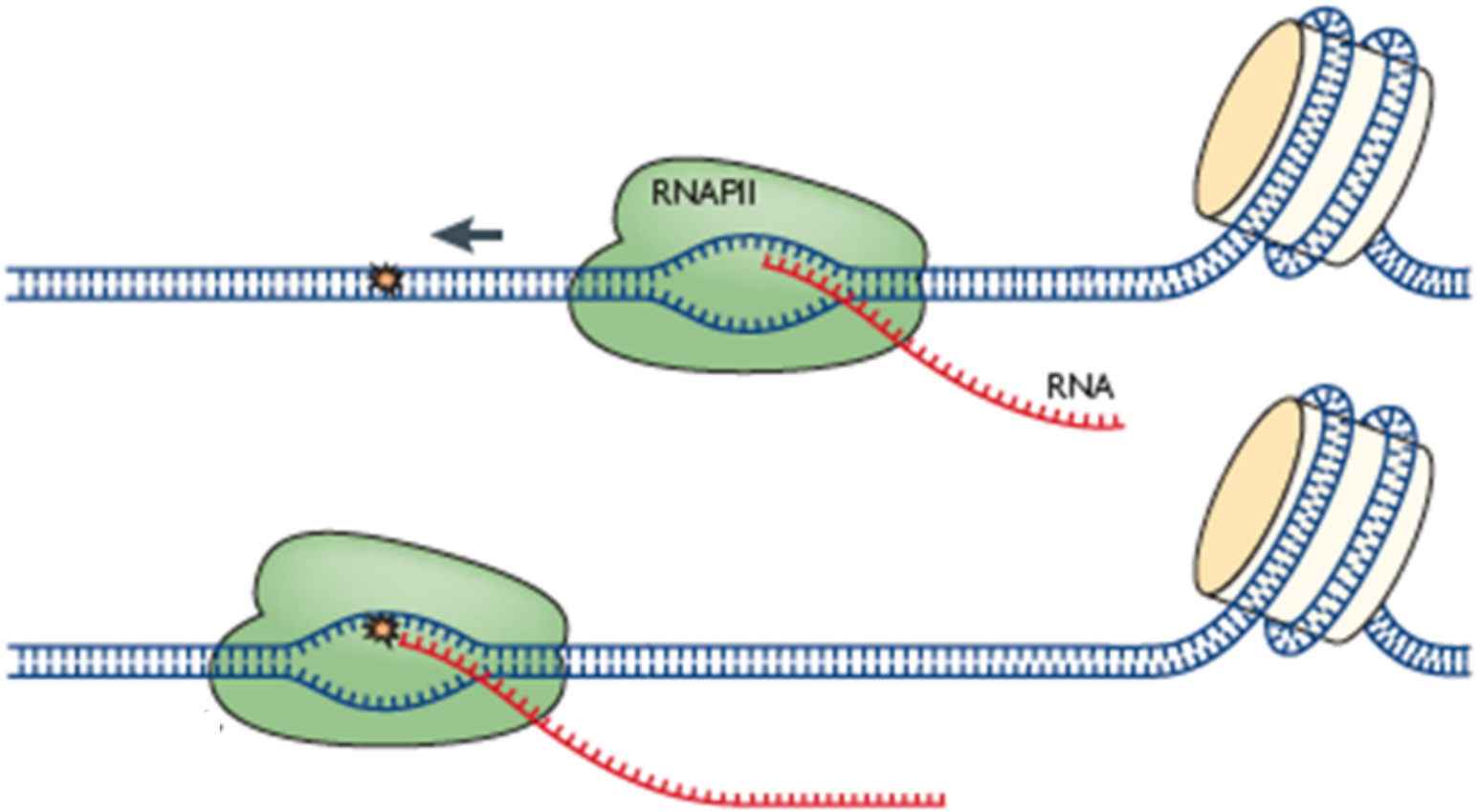




CAT Assay

Time to repair

RNA Polymerase II is exquisitely sensitive to DNA lesions



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

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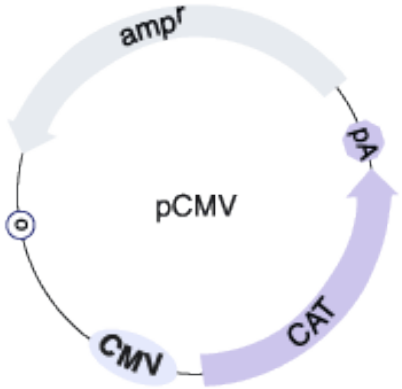
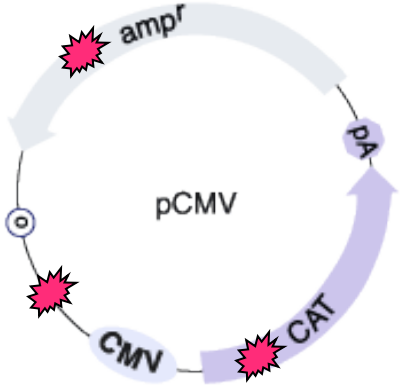
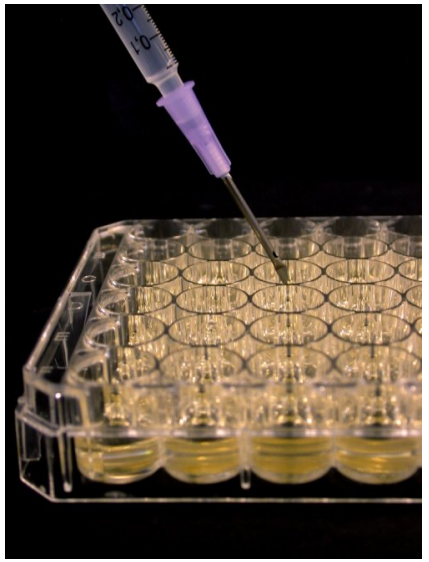
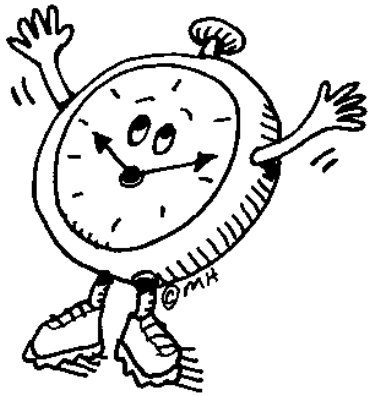


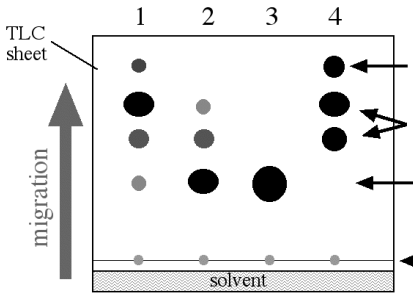
+ UV light



Transient transfection peripheral blood lymphocytes

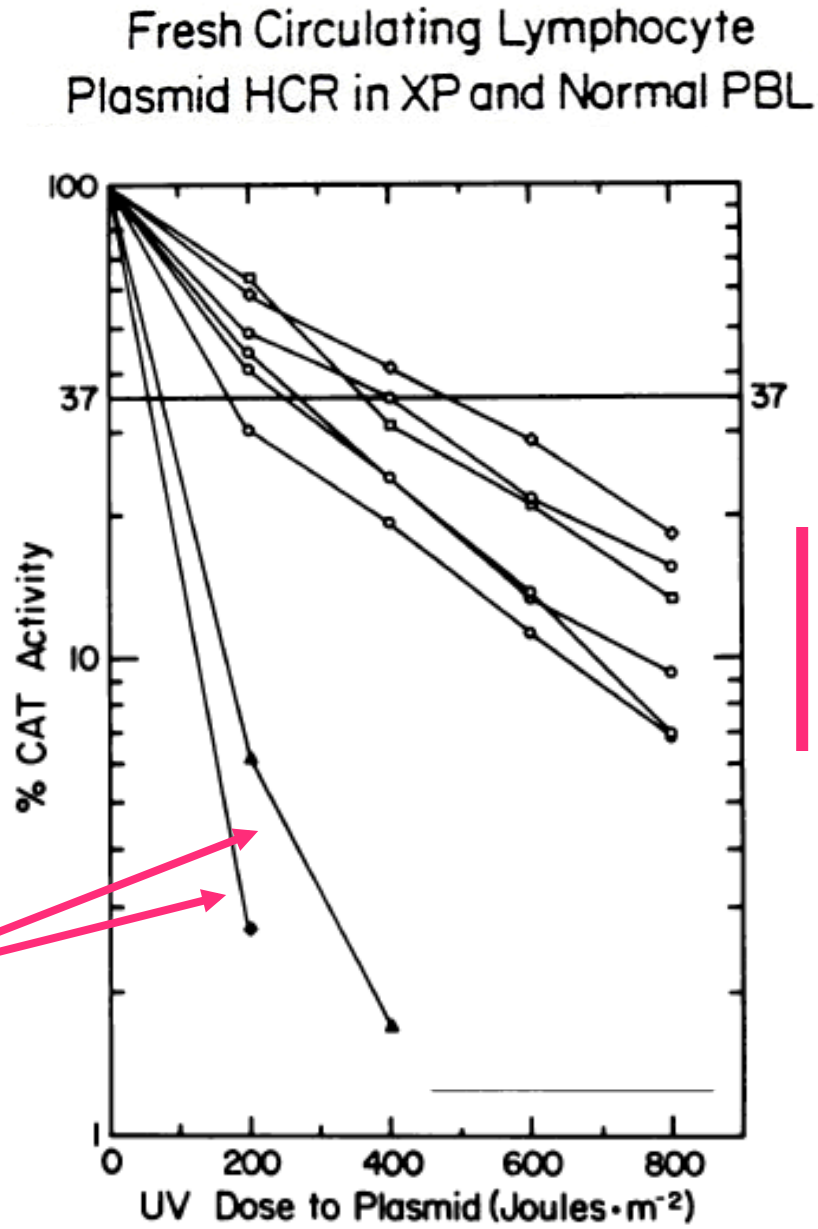






CAT Assay

Time to repair



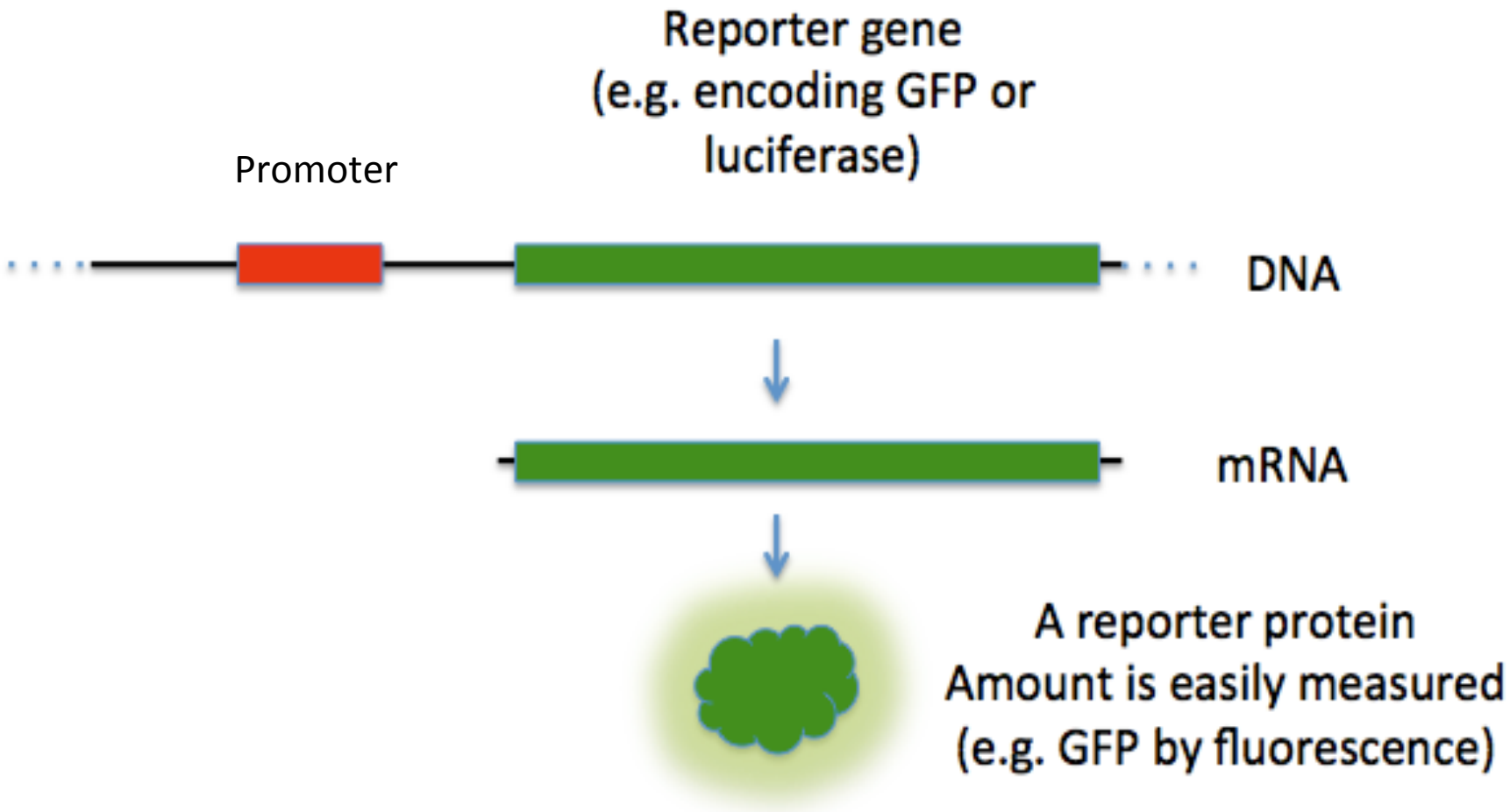
Cells from ‘healthy” people

Cells from XP patients

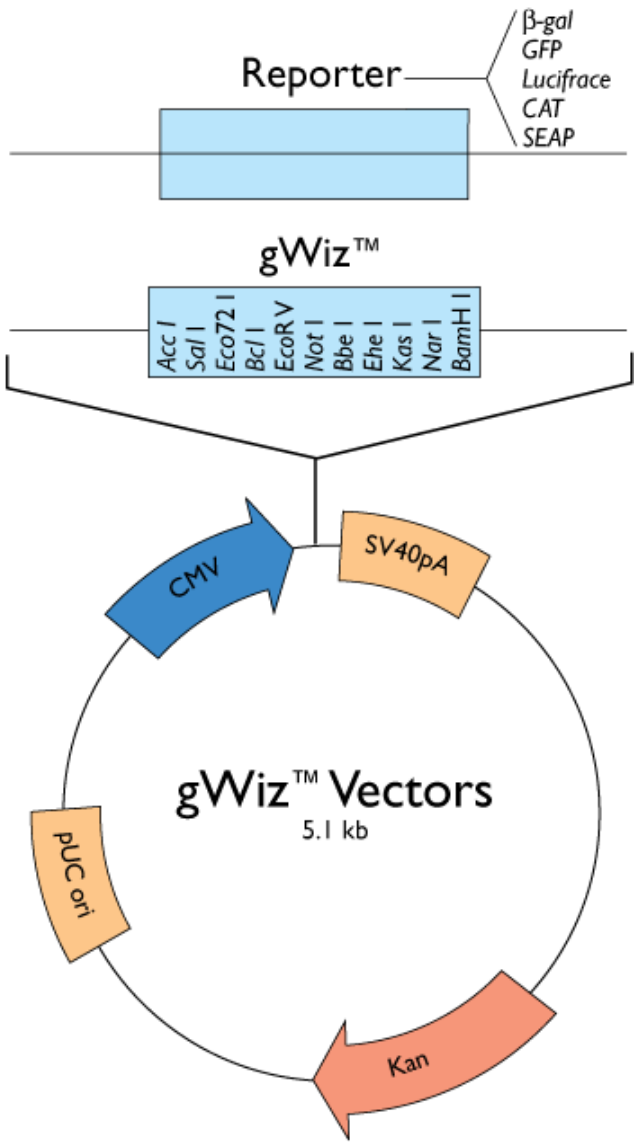
Athas & GROSSMAN

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

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

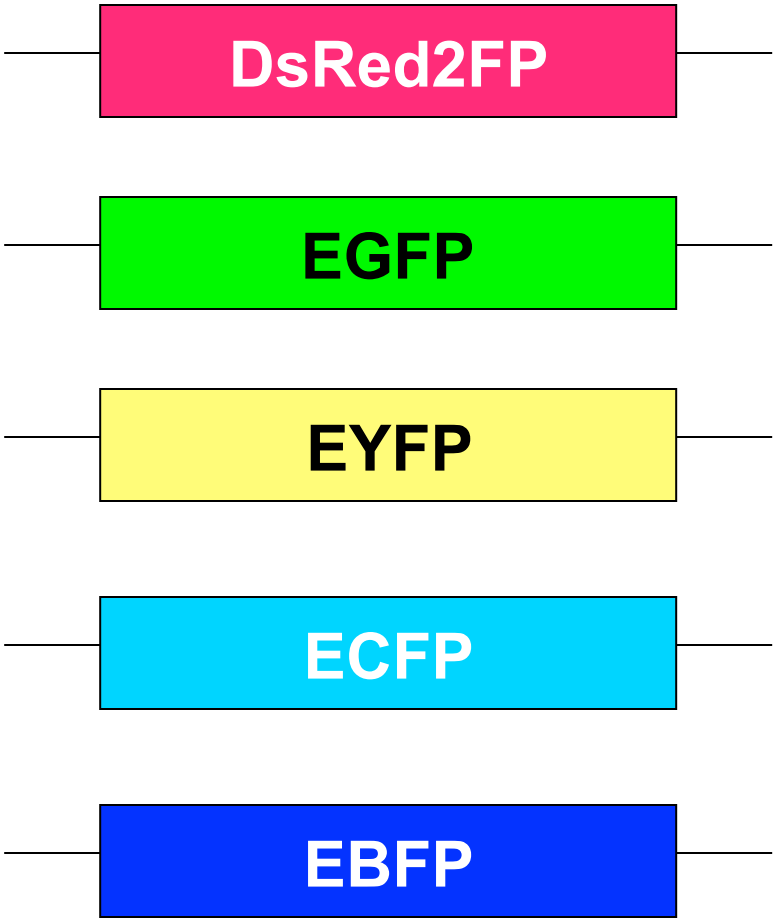


Reactivation of damaged DNA – multiplexed



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

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

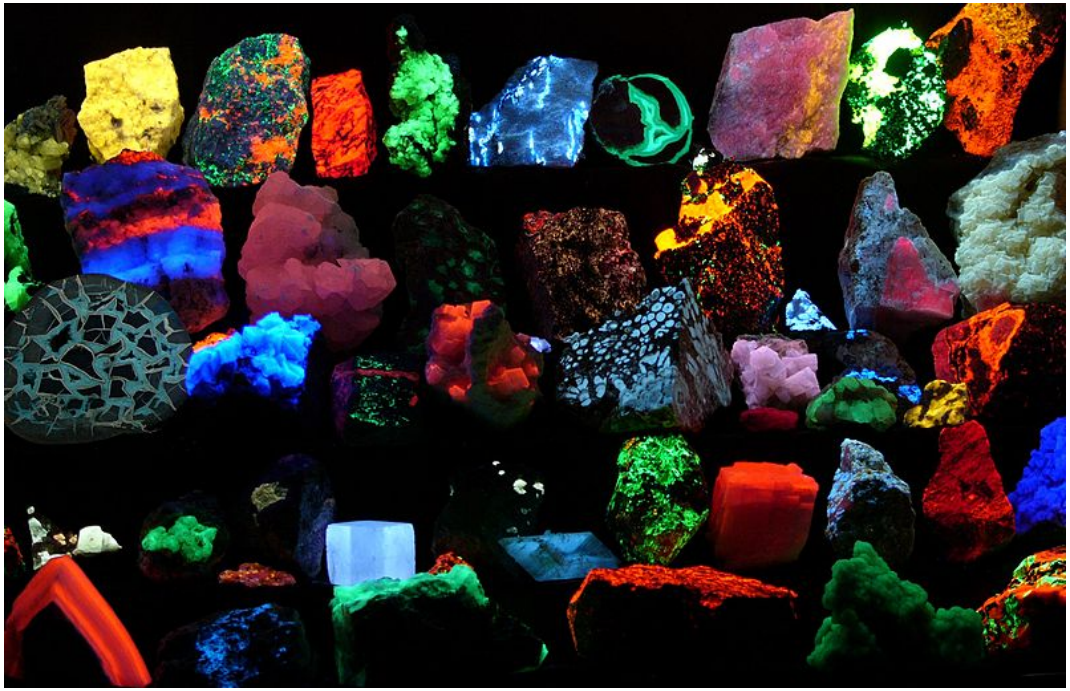


fluo·res·cence

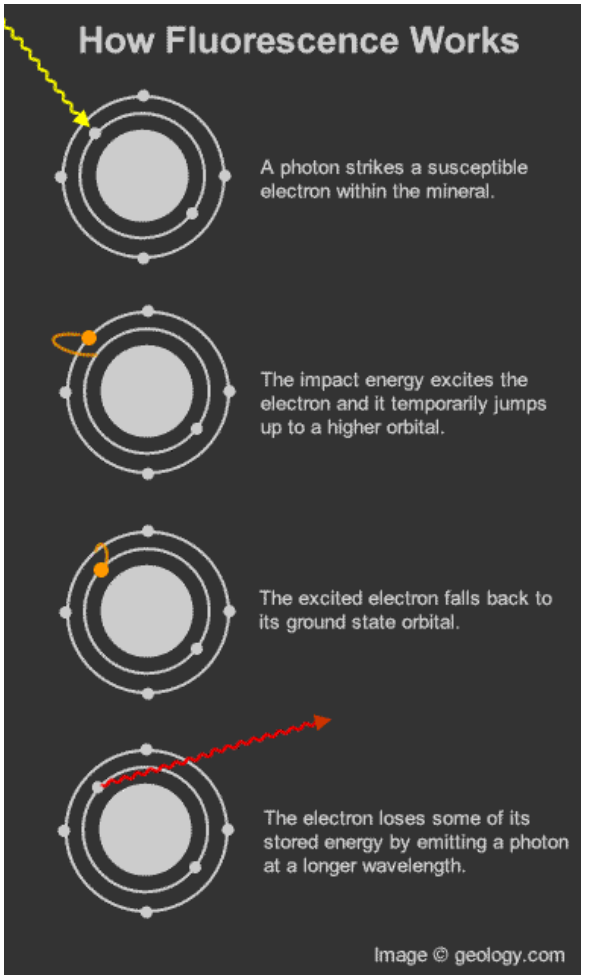
/flo͝o(ə)ˈresəns,flôrˈesəns/

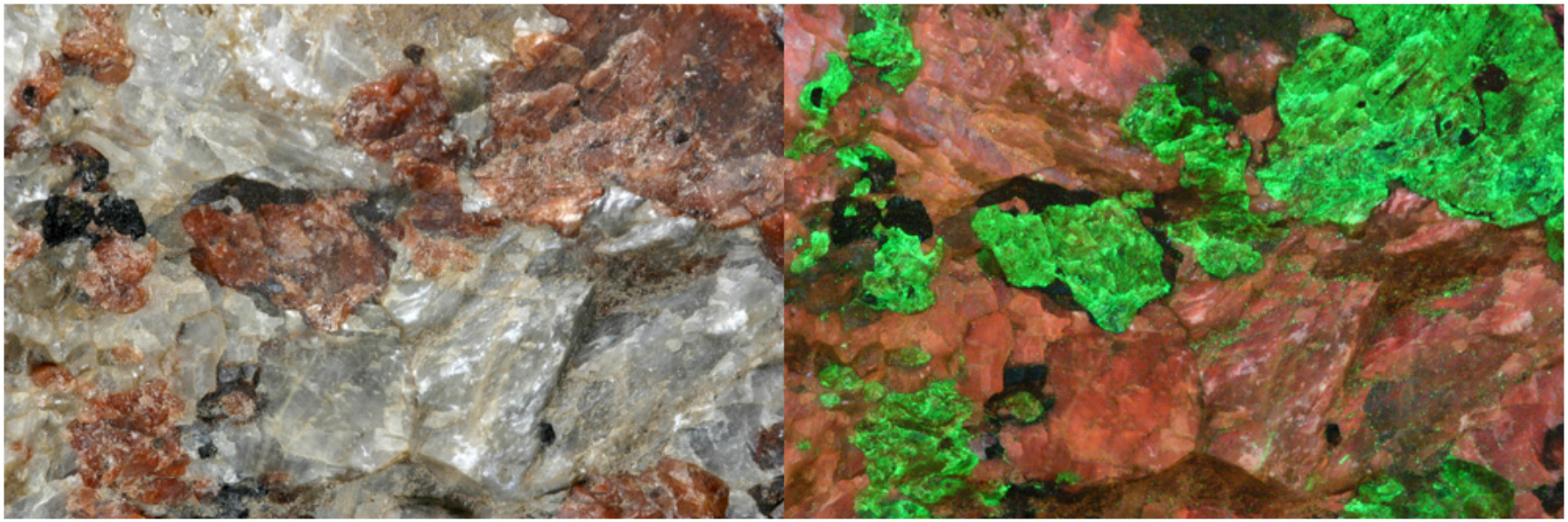
noun

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

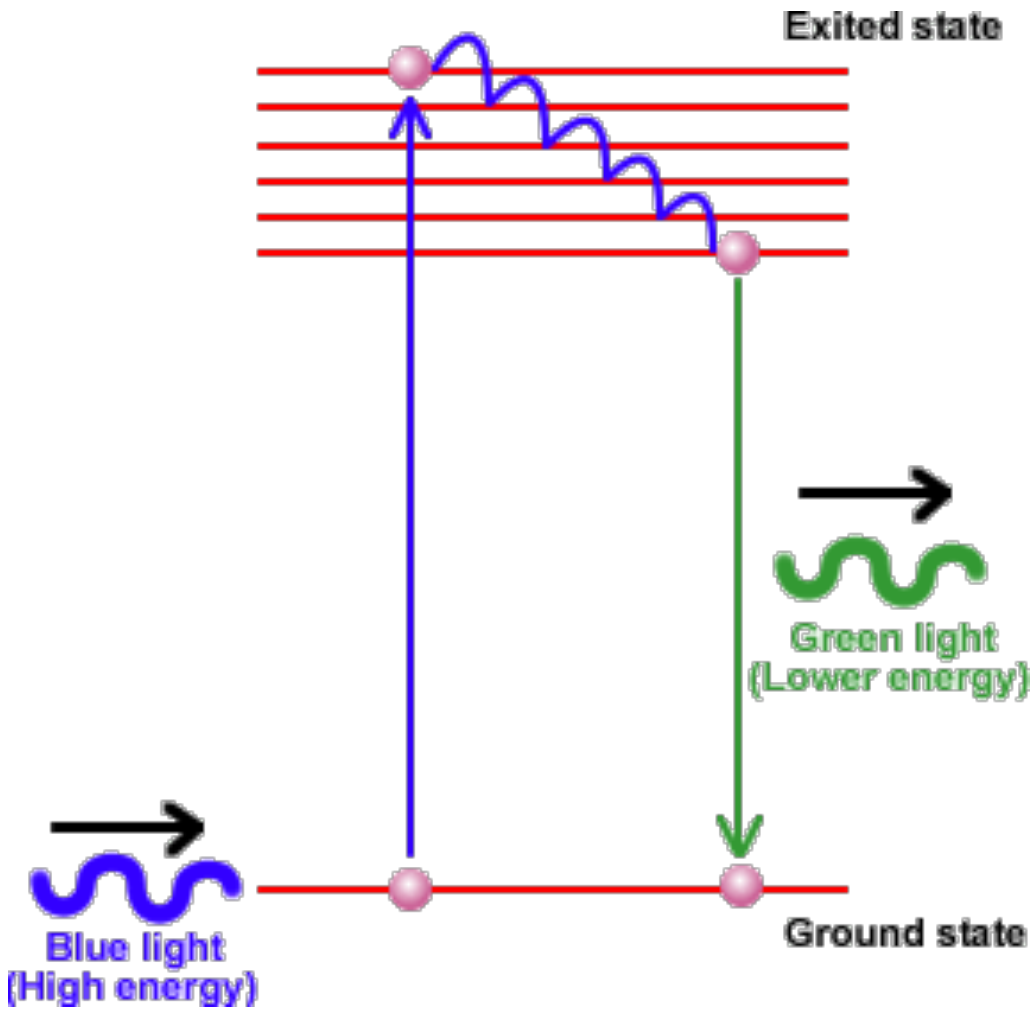




Under White Light

Under UV Light

Theory of Fluorescence

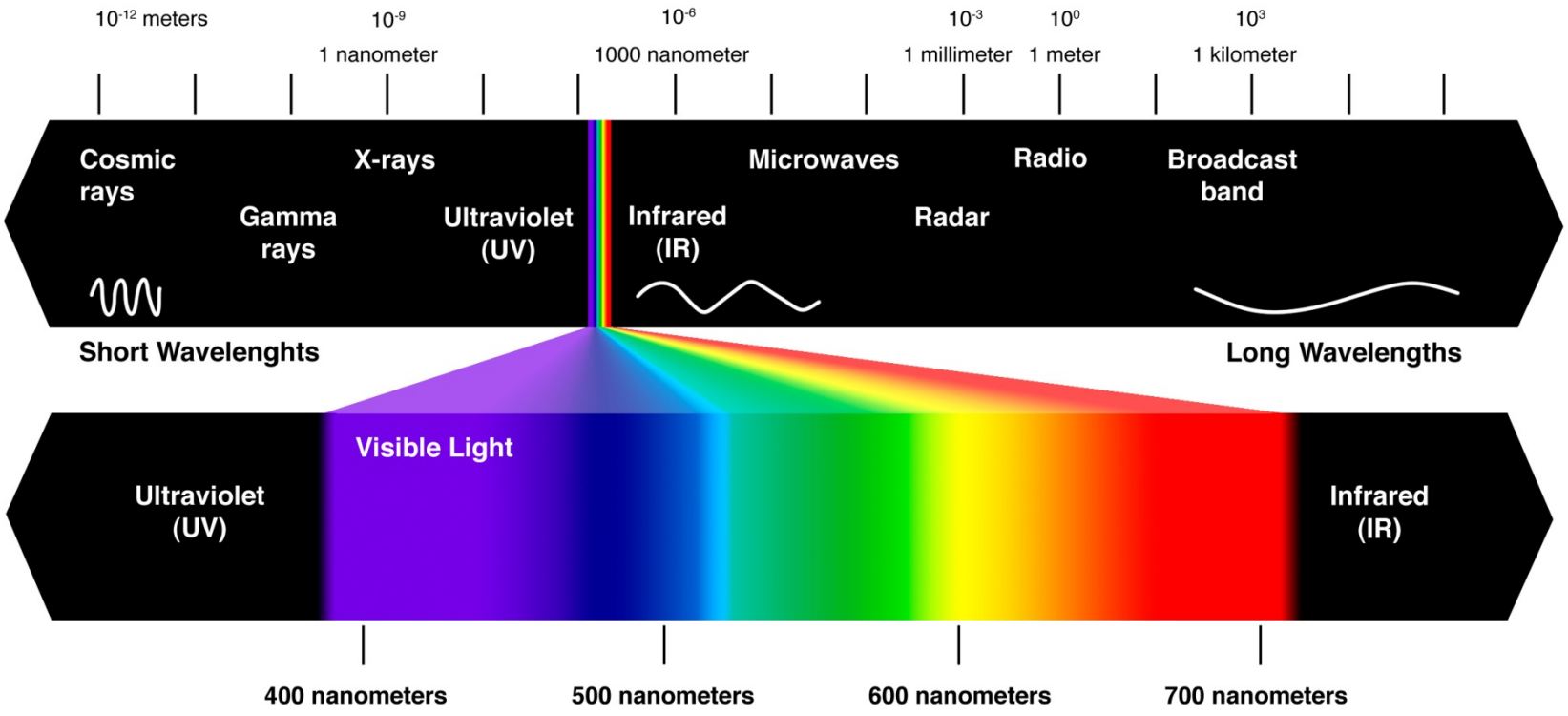


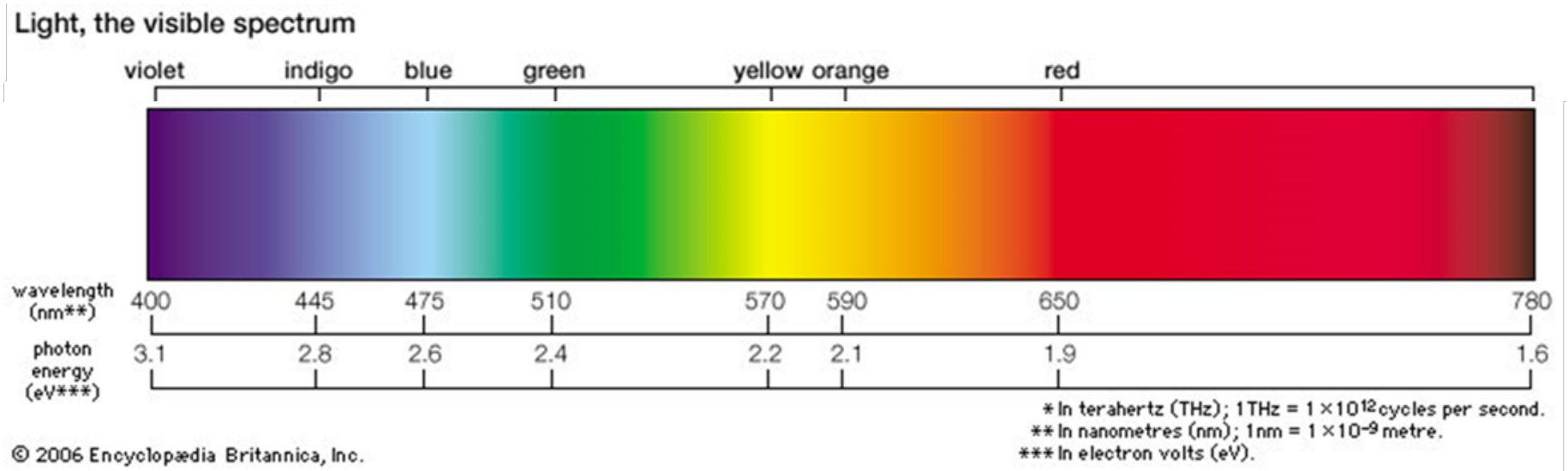
1) Electrons excited by light source
2) Electrons reach a high energy state
3) Energy loss occurs within a few nano seconds
4) Energy loss observed as fluorescent light of a longer wavelength

Excitation

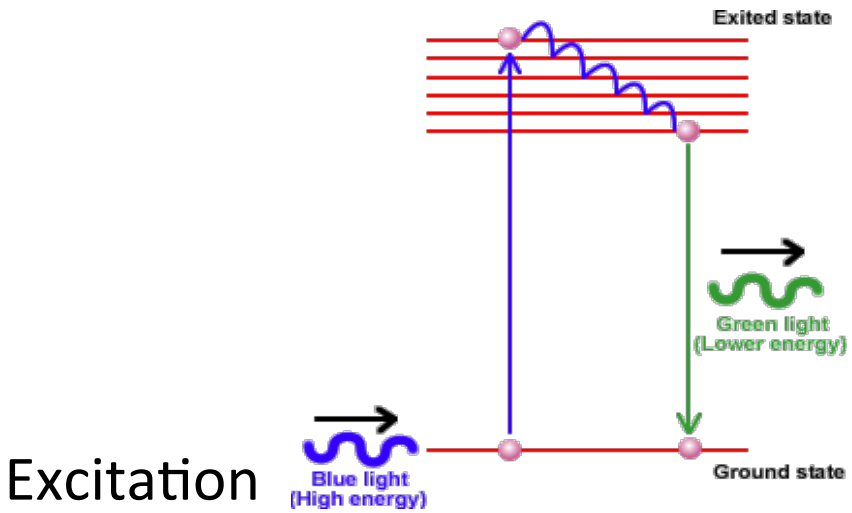
Emission

Electro Magnetic Spectrum and Light



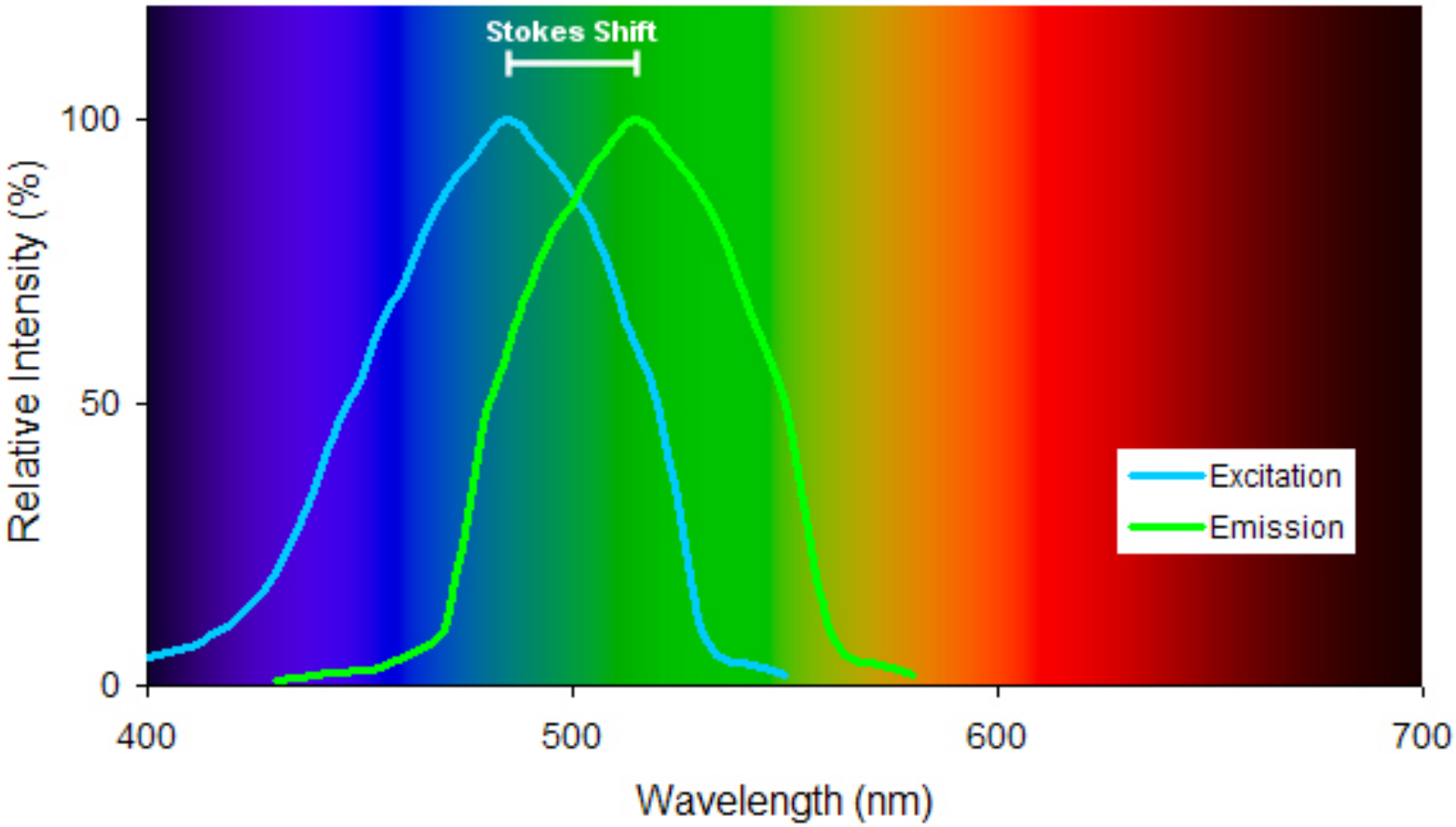


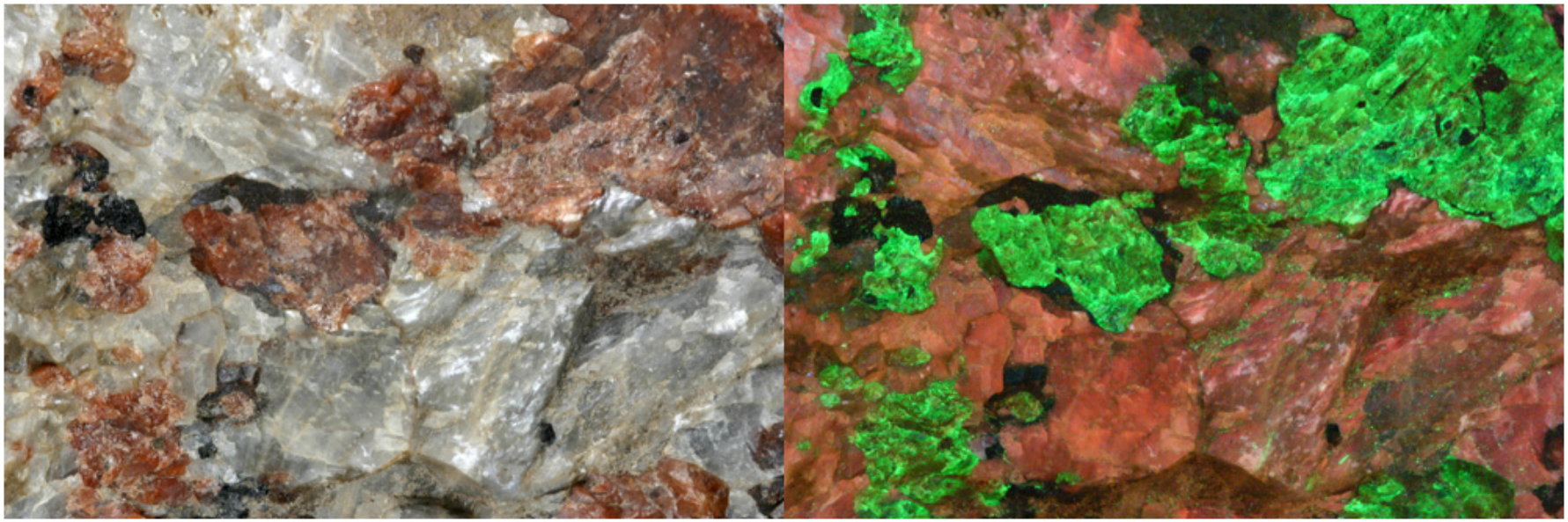
Increasing energy



Emission

Excitation

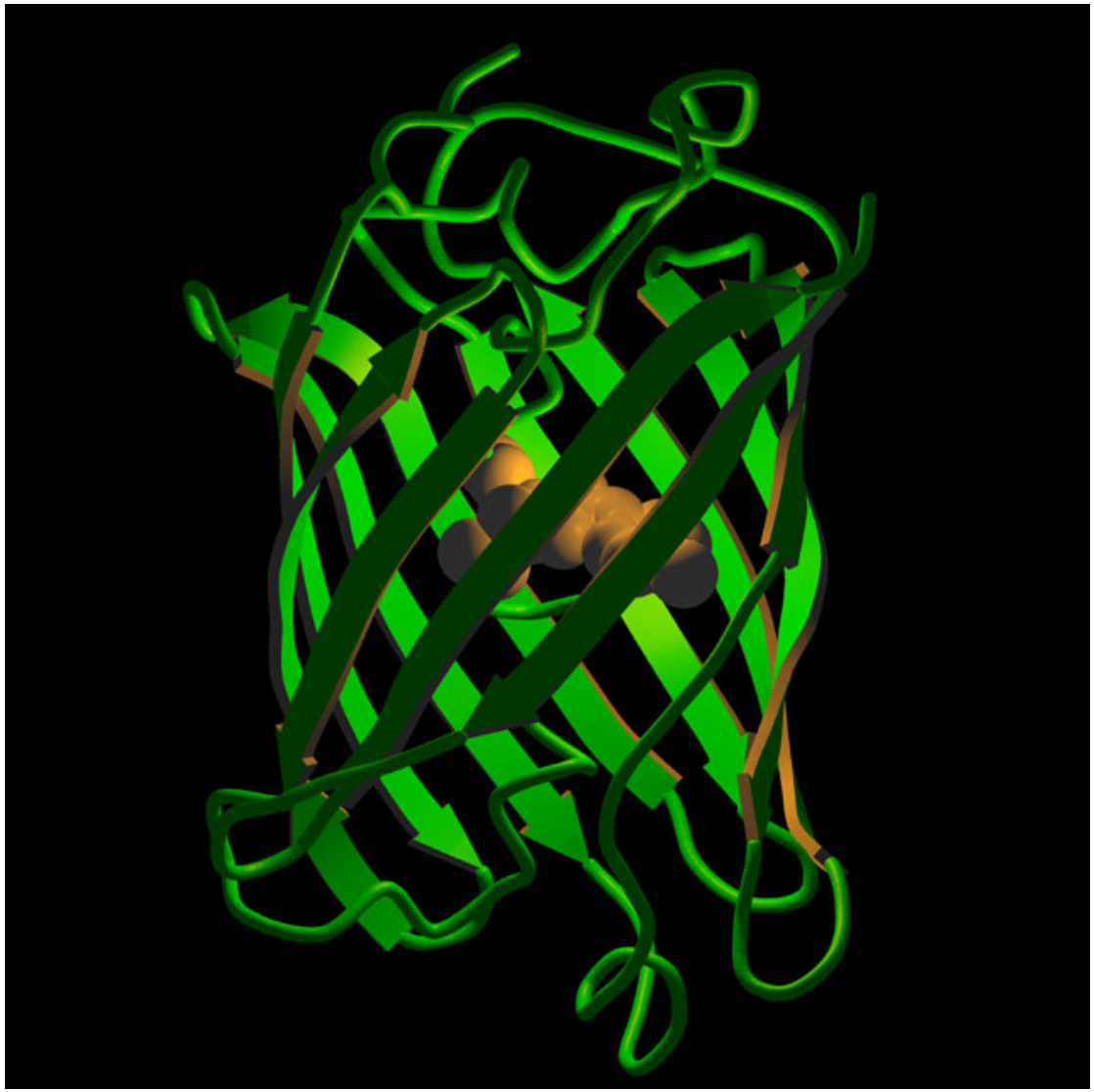




Under White Light

Under UV Light

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



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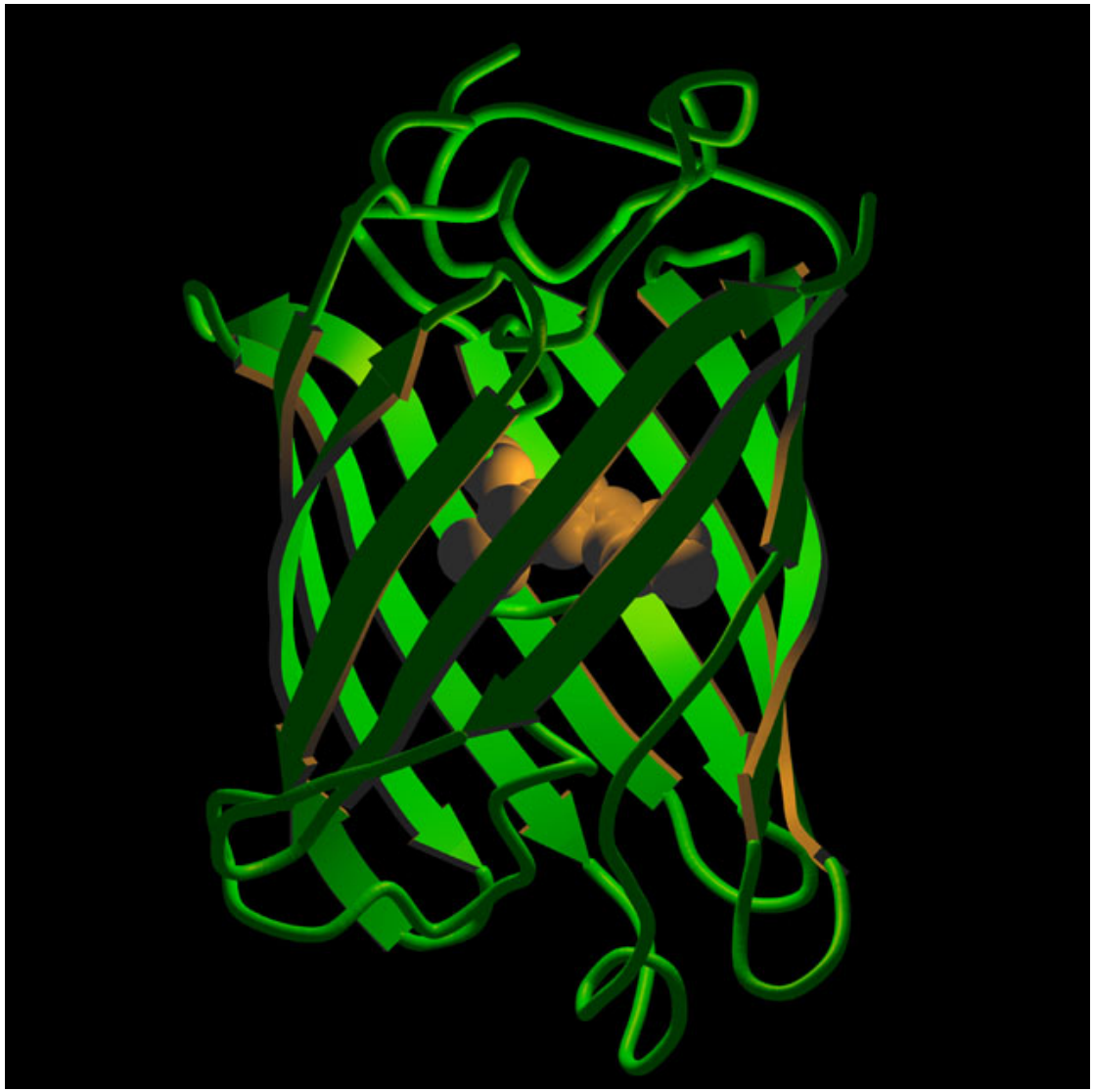
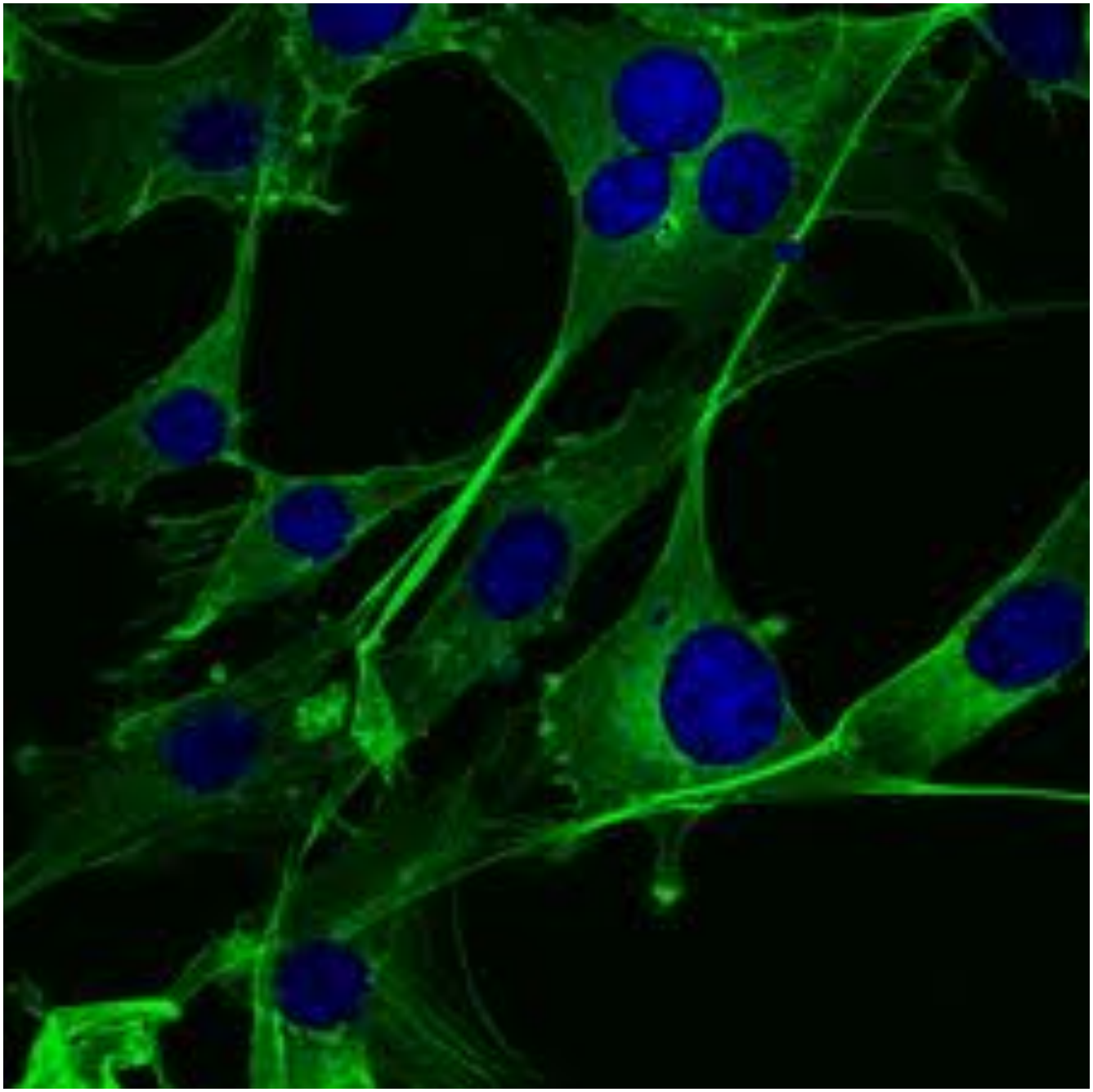
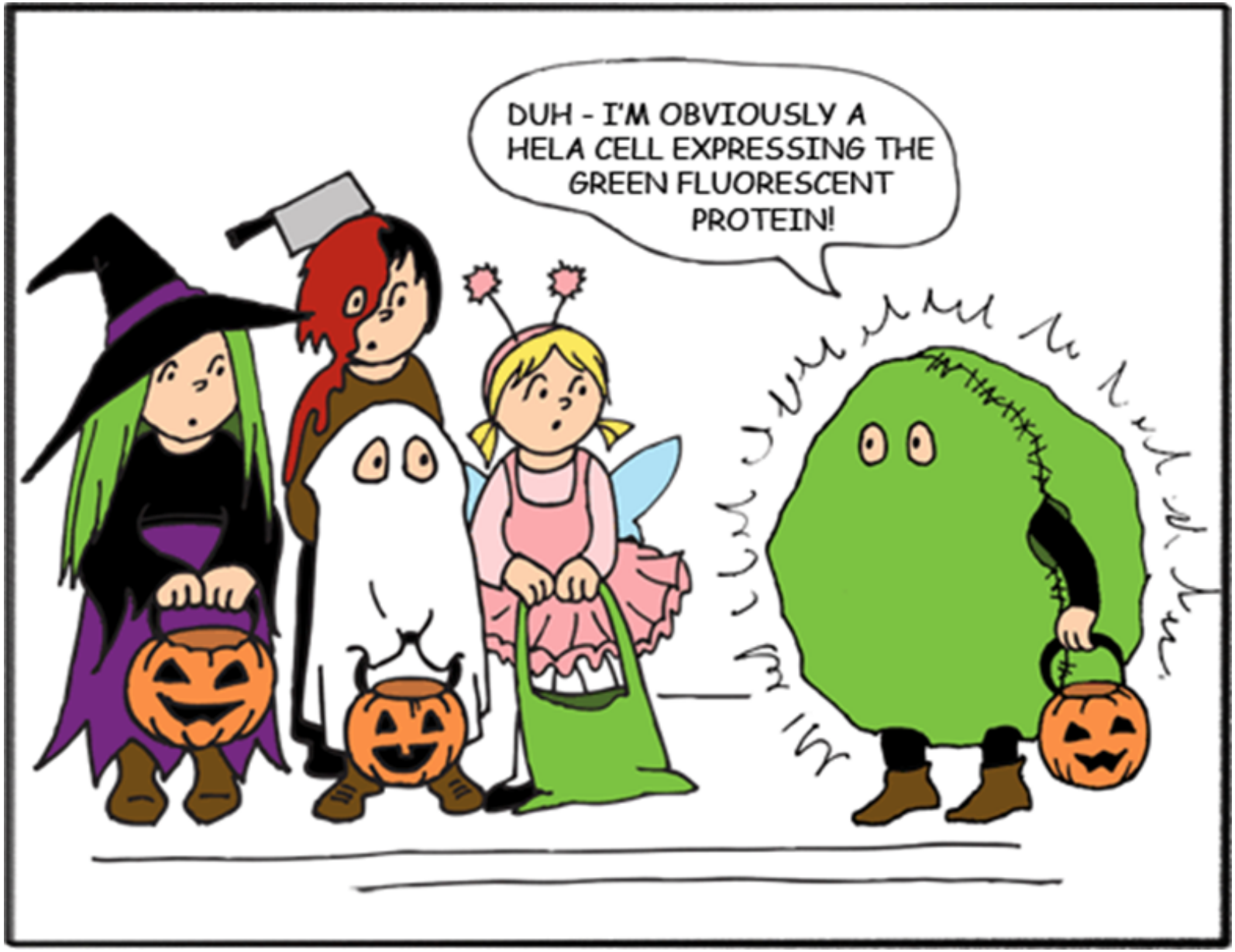




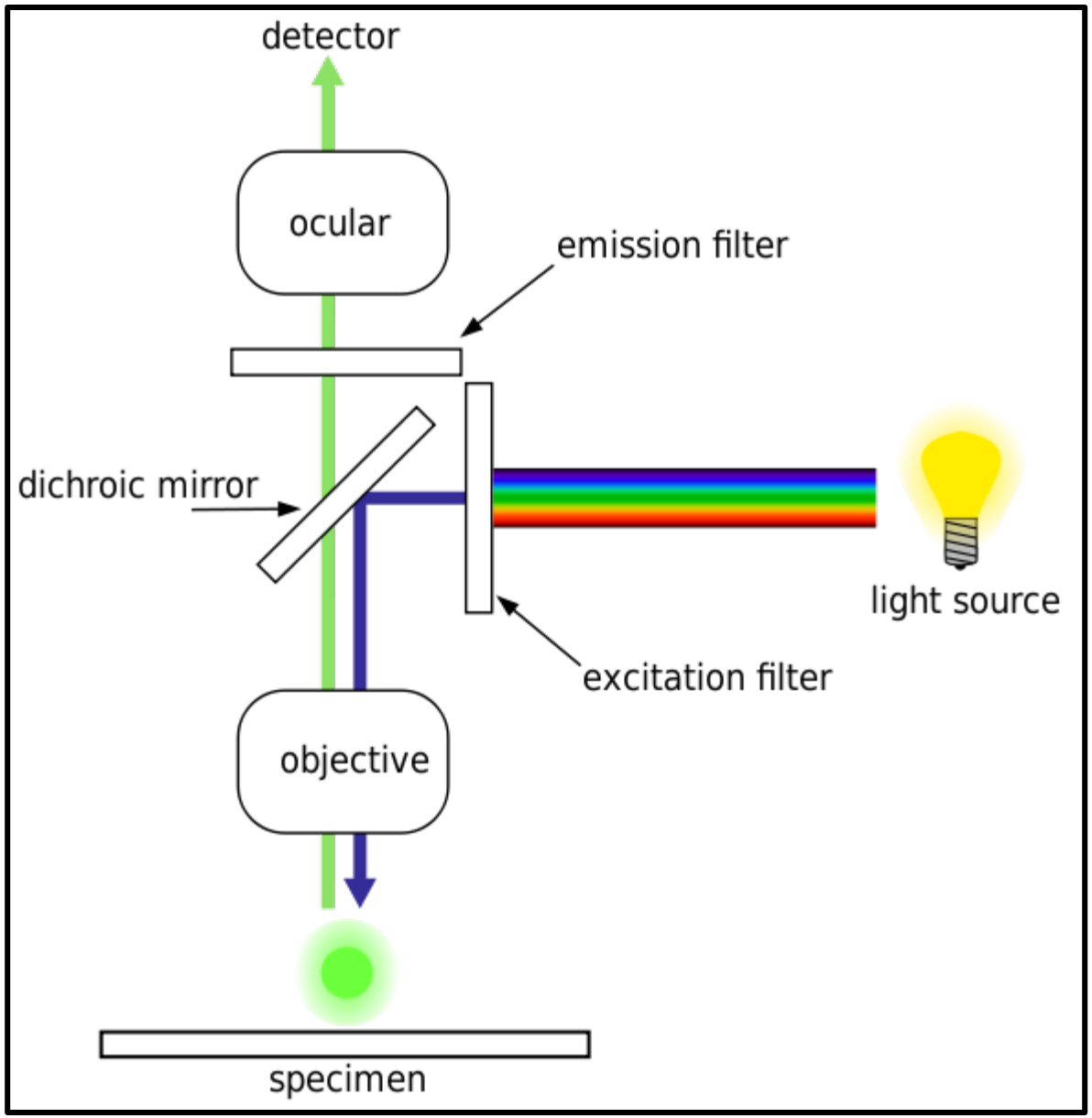
DNA – Blue

GFP - Green

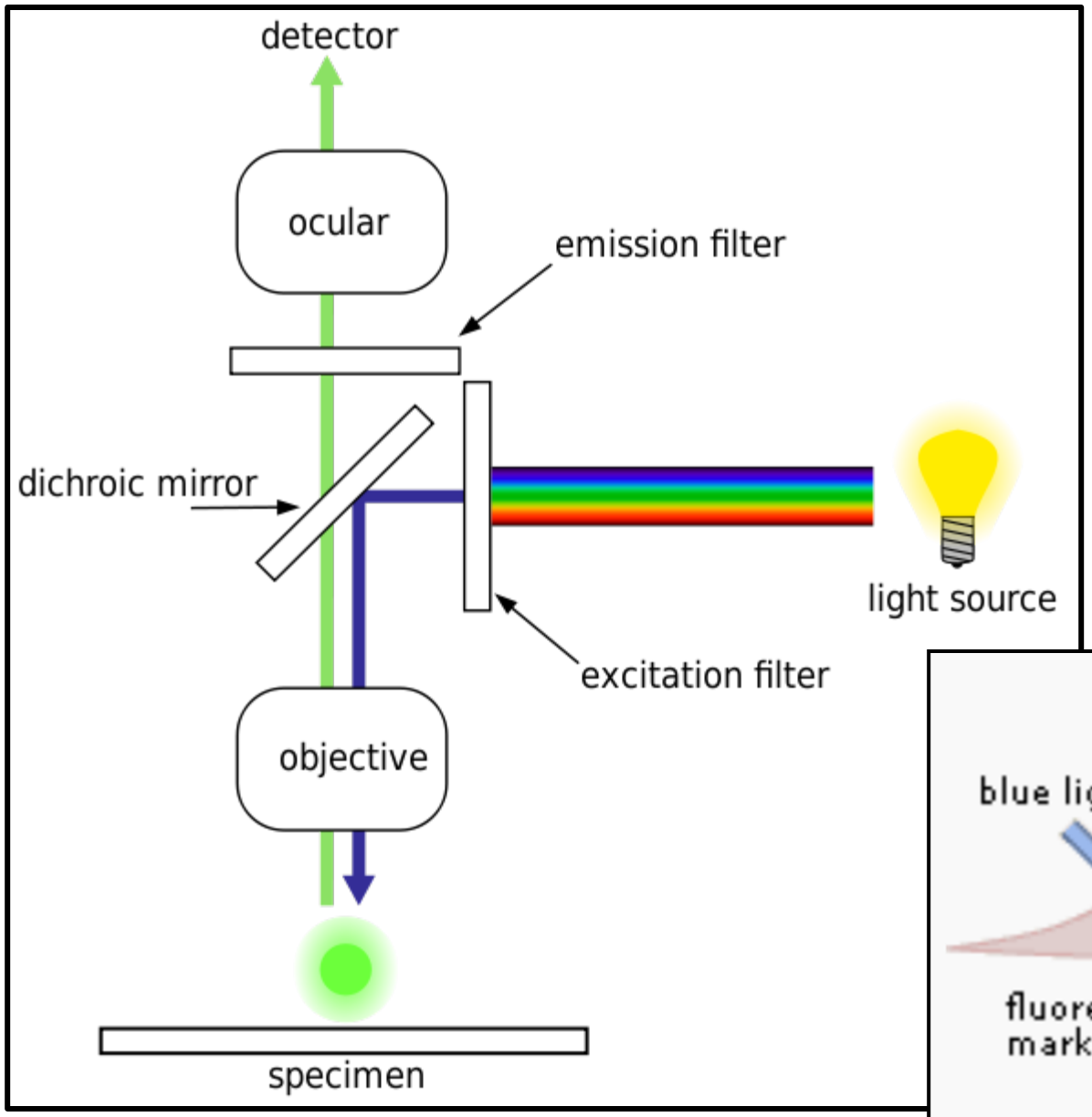
Next Halloween Costume??

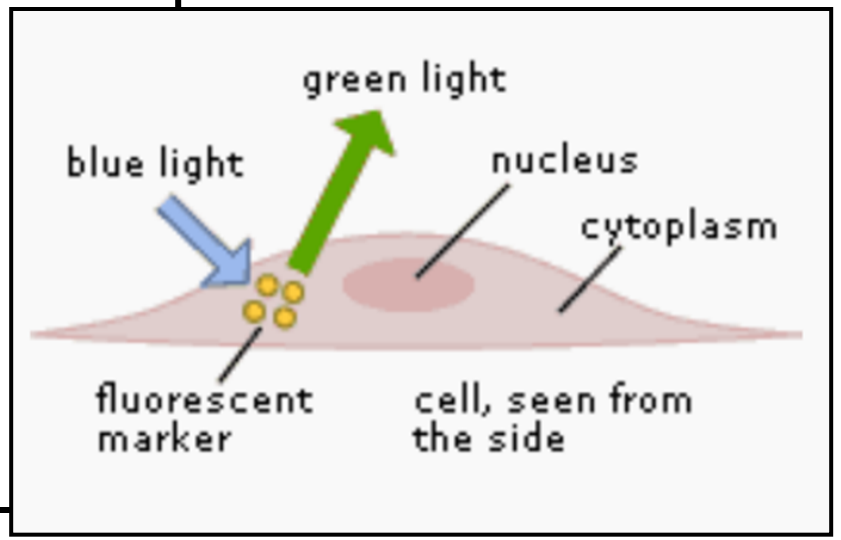


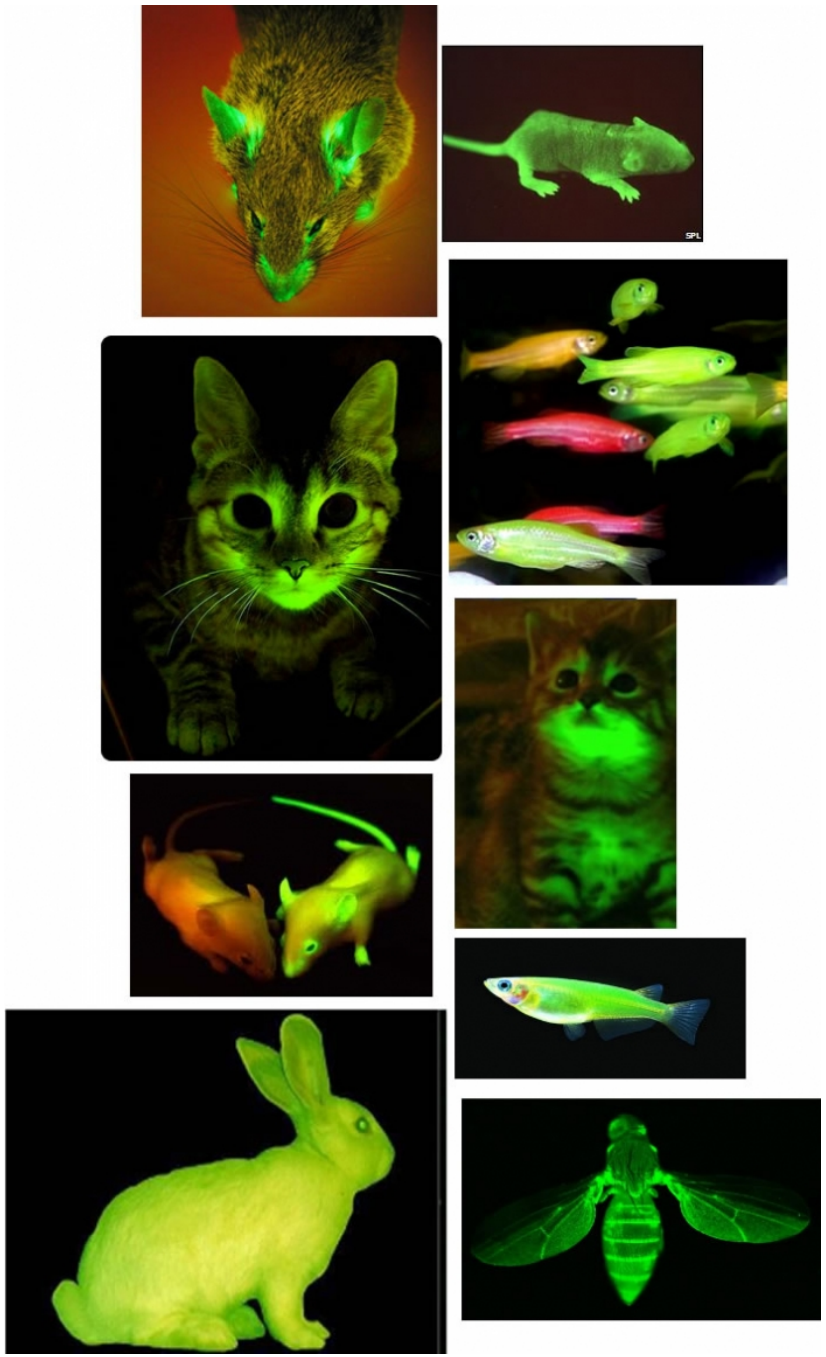
Fluorescence Detection

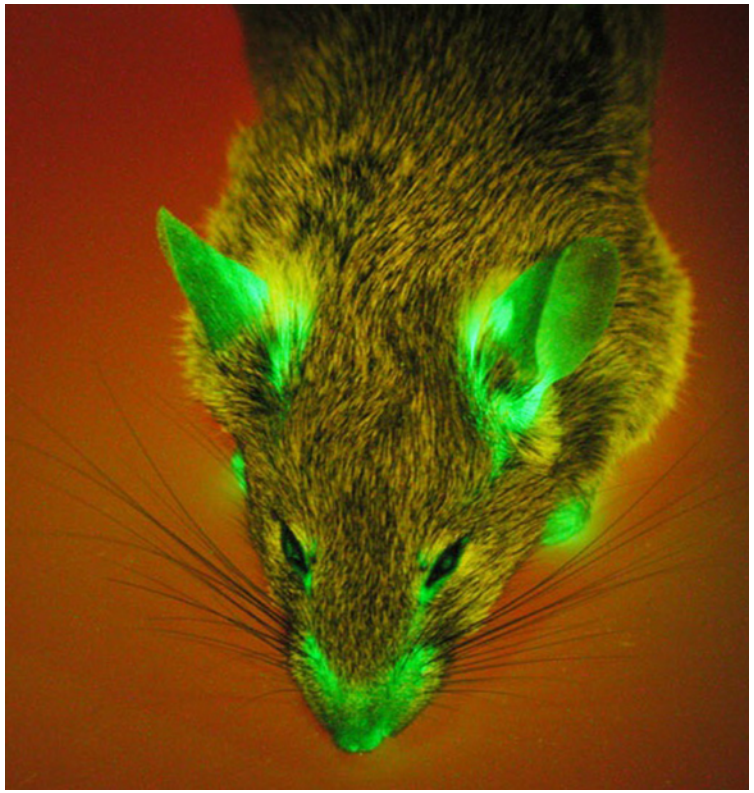


Fluorescence Detection









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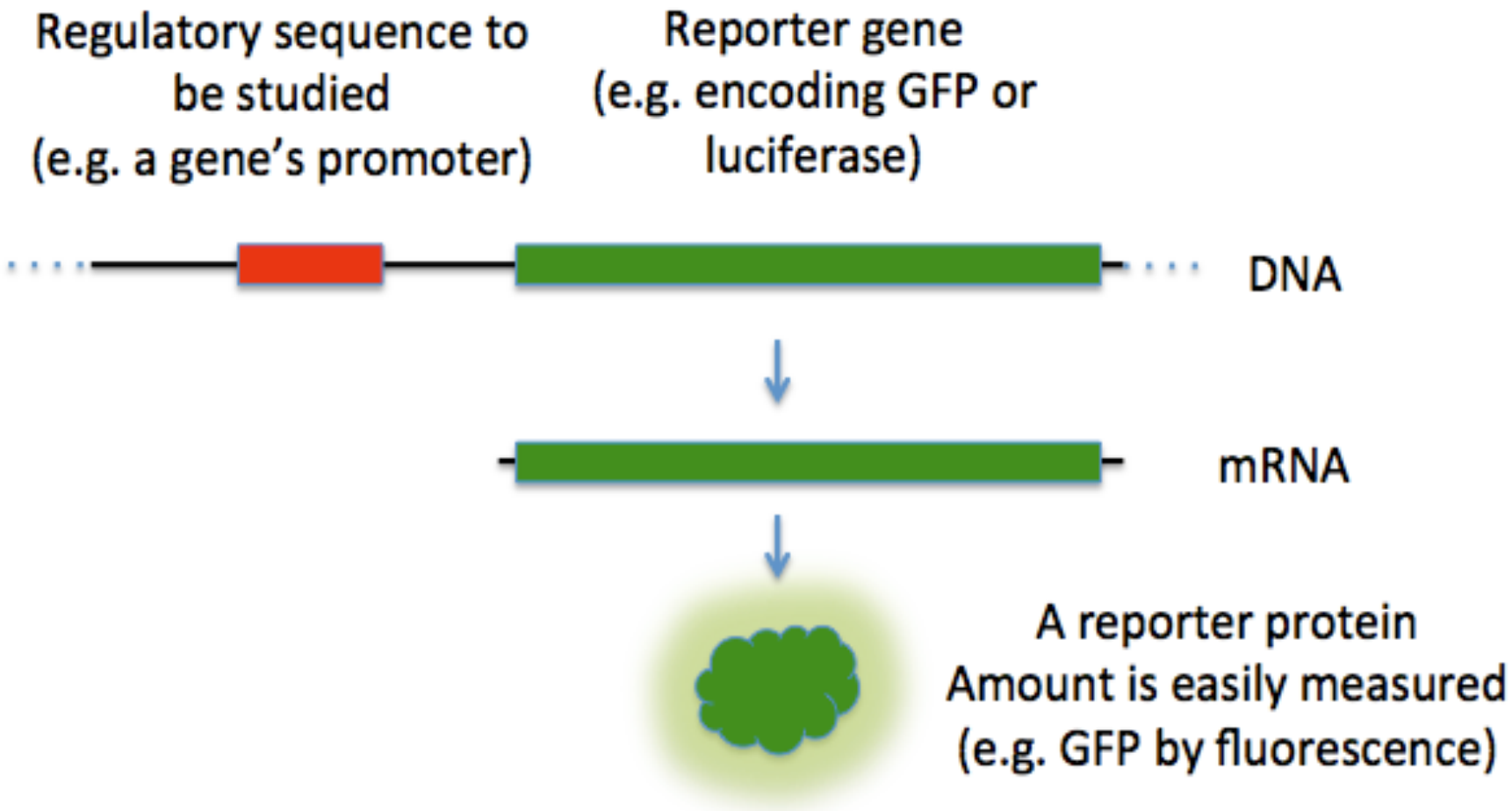
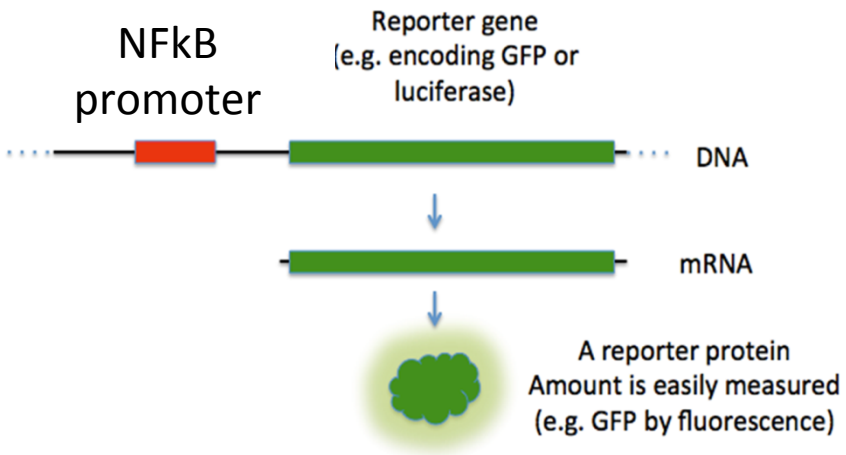
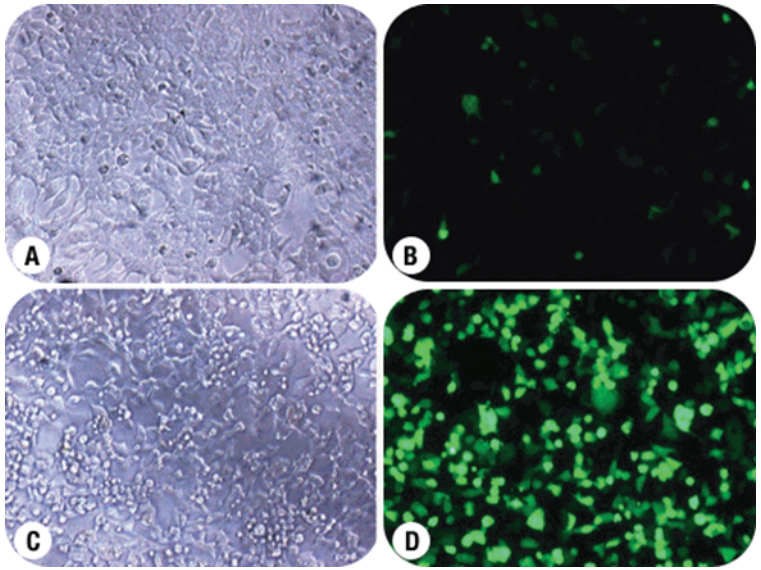
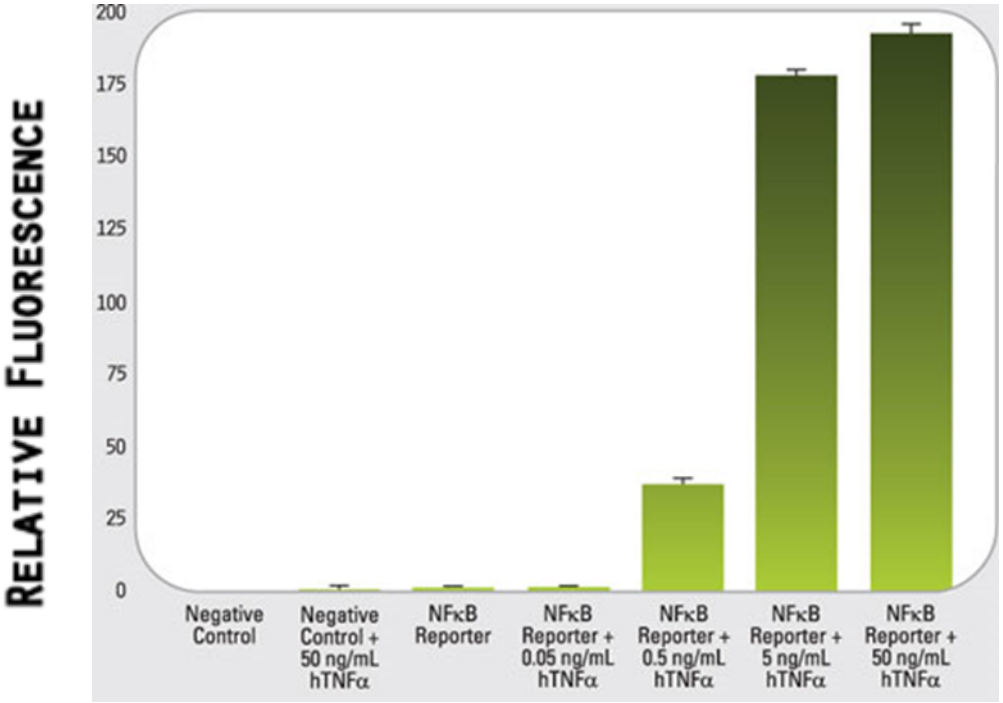




Fluorescent Protein Reporters are often used to report the amount of transcription from a specific promoter





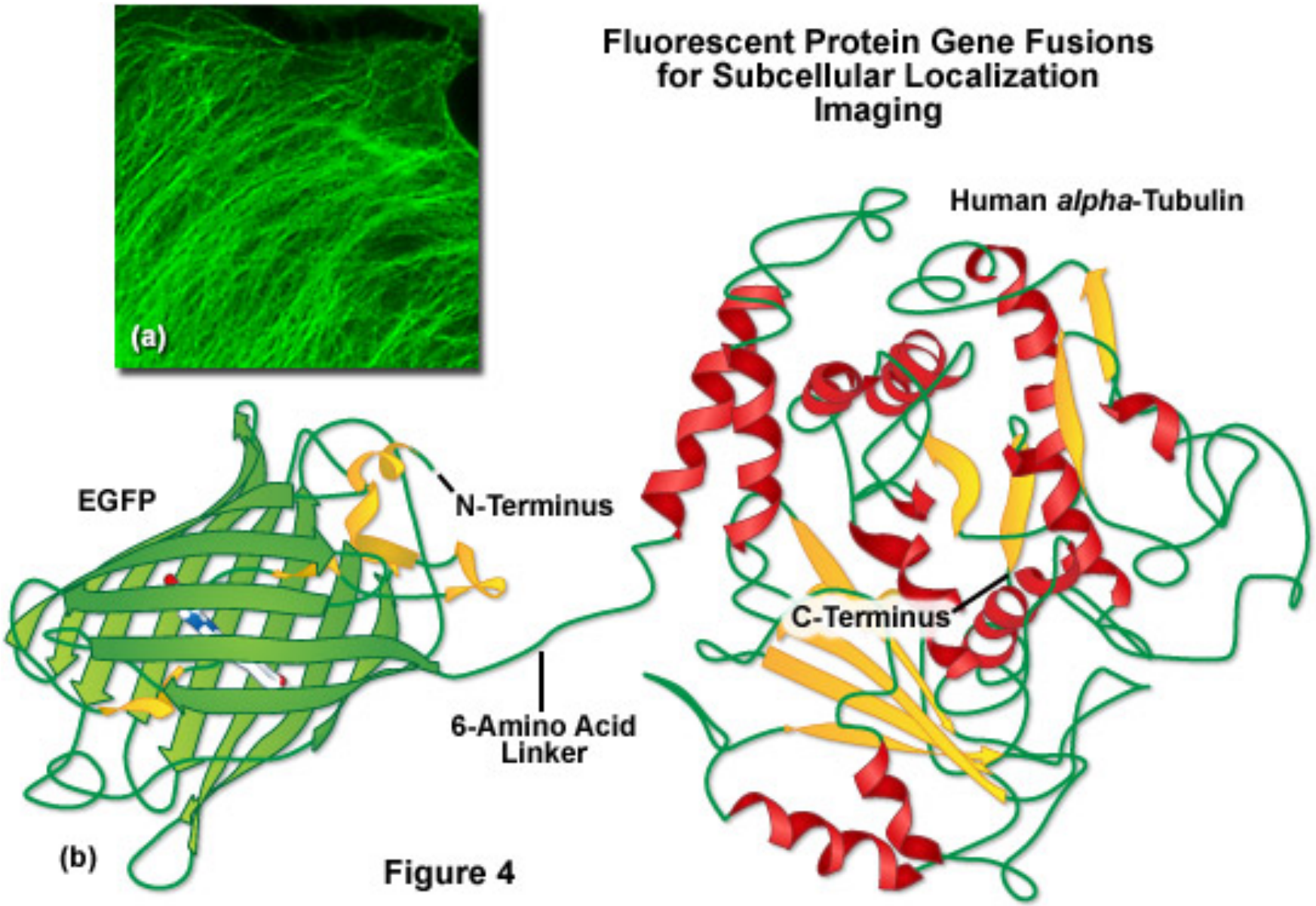
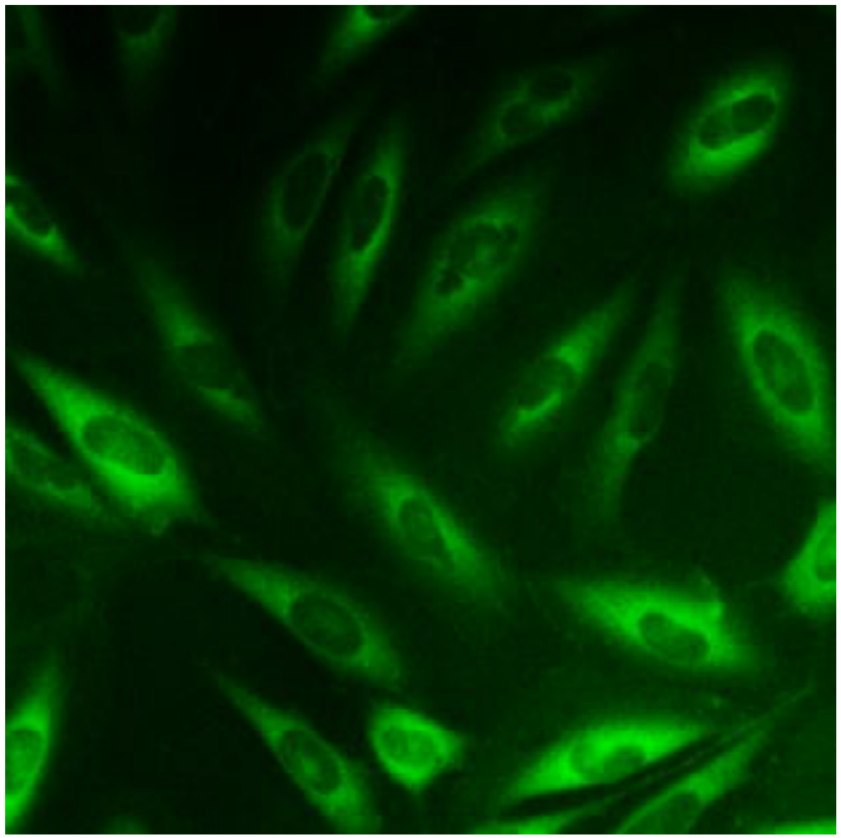
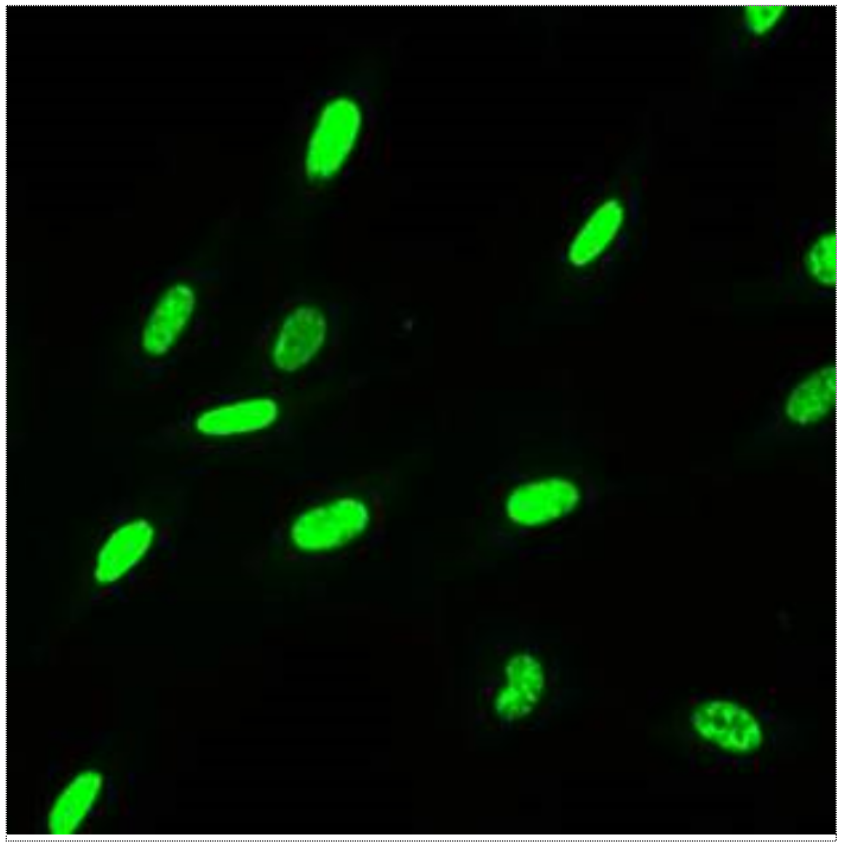

Fluorescent Protein Gene Fusions
for Subcellular Localization
Imaging
(a)
Human *alpha*-Tubulin
EGFP
N-Terminus
C-Terminus
6-Amino Acid
Linker
(b)

Figure 4

DNA Damage Induced NFKB Signalling



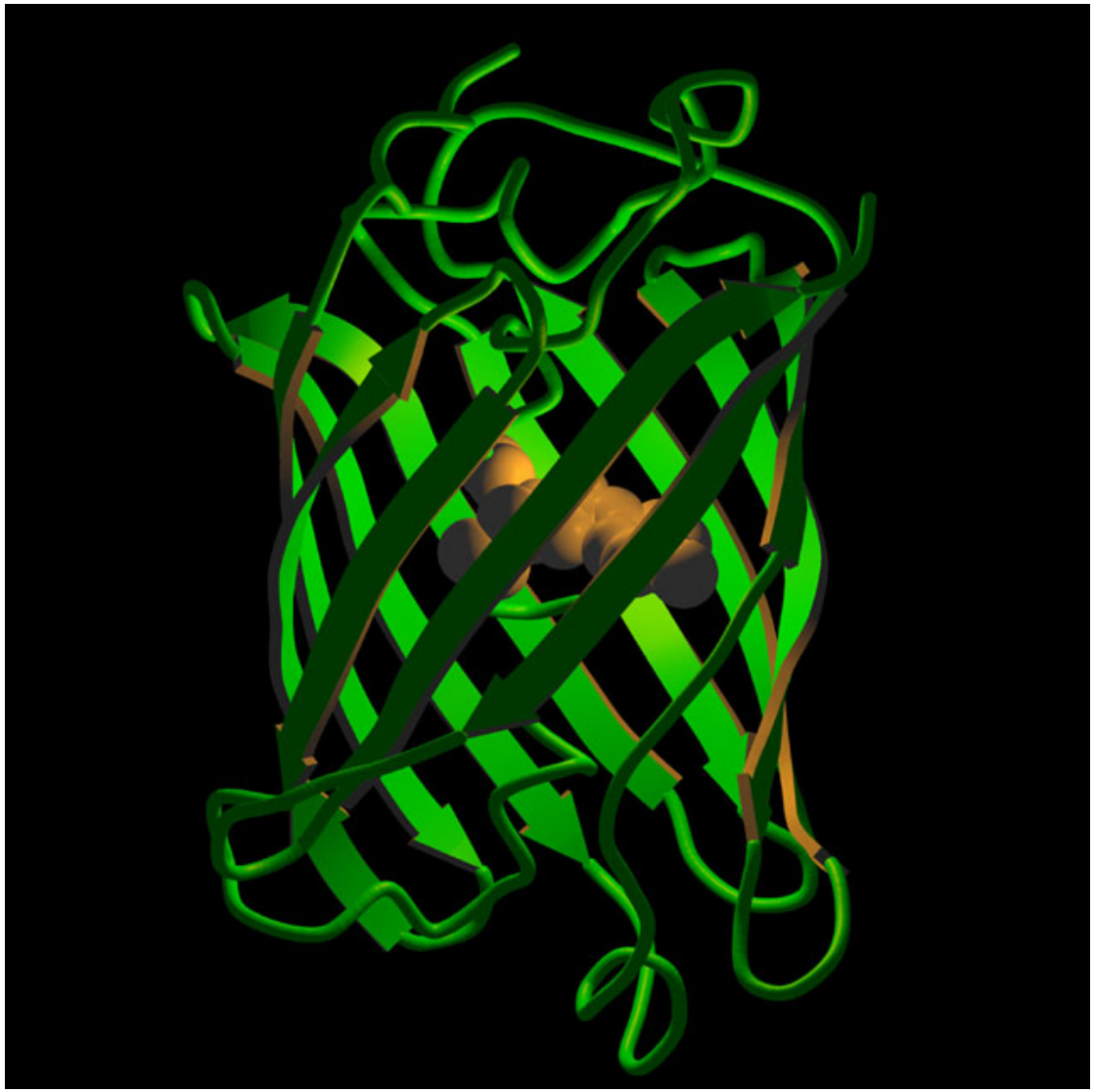
inactive NFKB



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

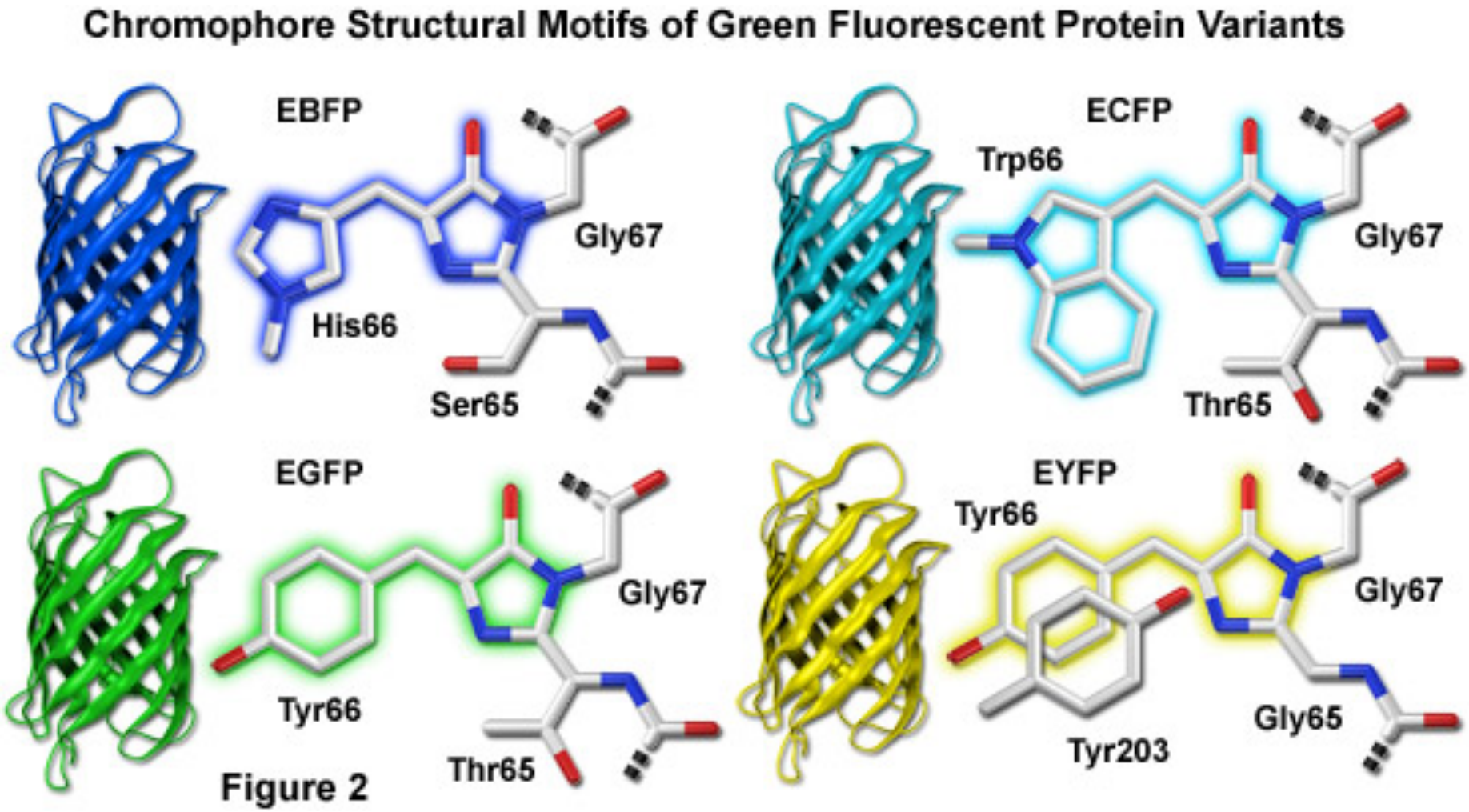
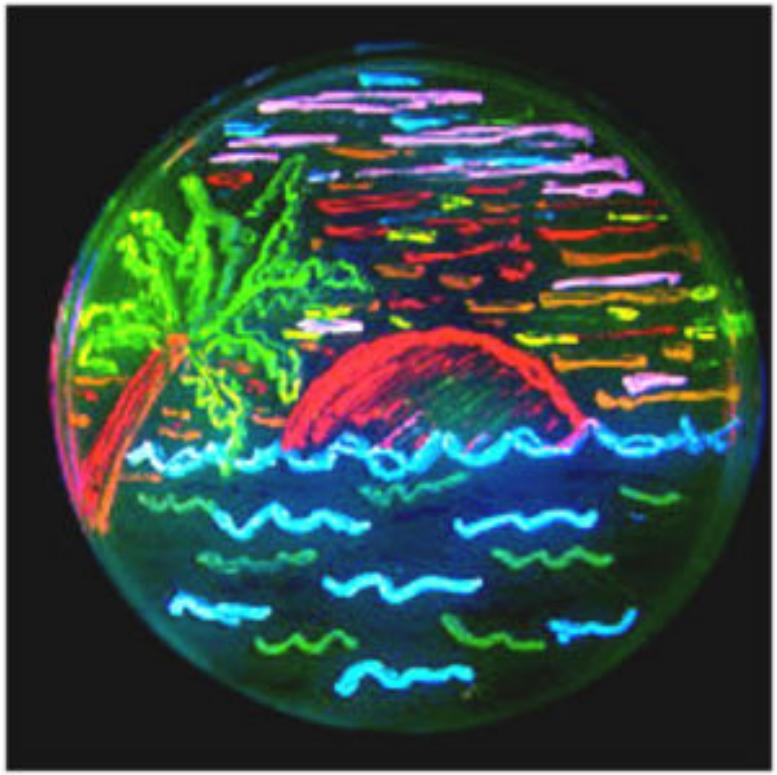


Figure 2

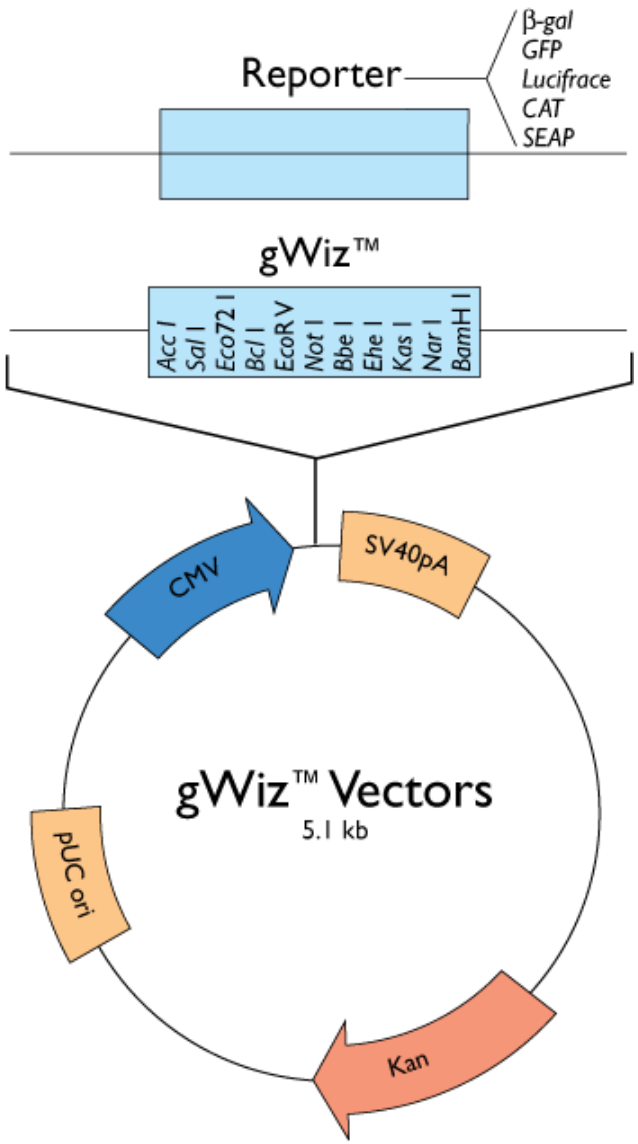
Mushroom Coral

Fluorescent Bulb Anemone (Entacmaea quadricolor)



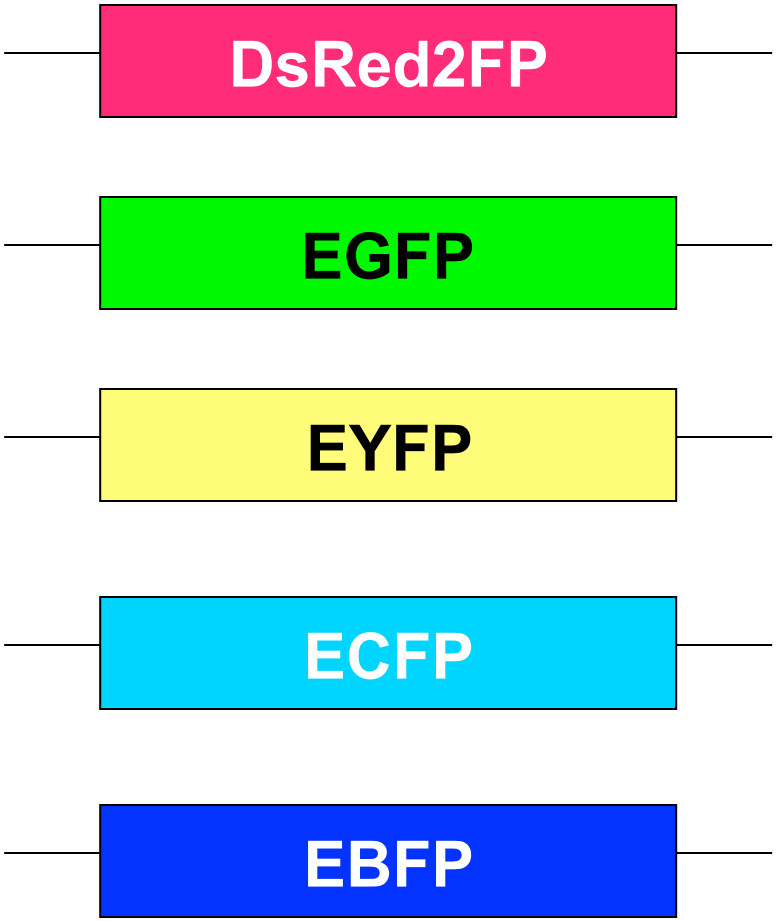
The diversity of fluorescent proteins and genetic mutations is illustrated by this San Diego beach scene drawn with living bacteria expressing 8 different colors of fluorescent proteins.

Reactivation of damaged DNA – multiplexed

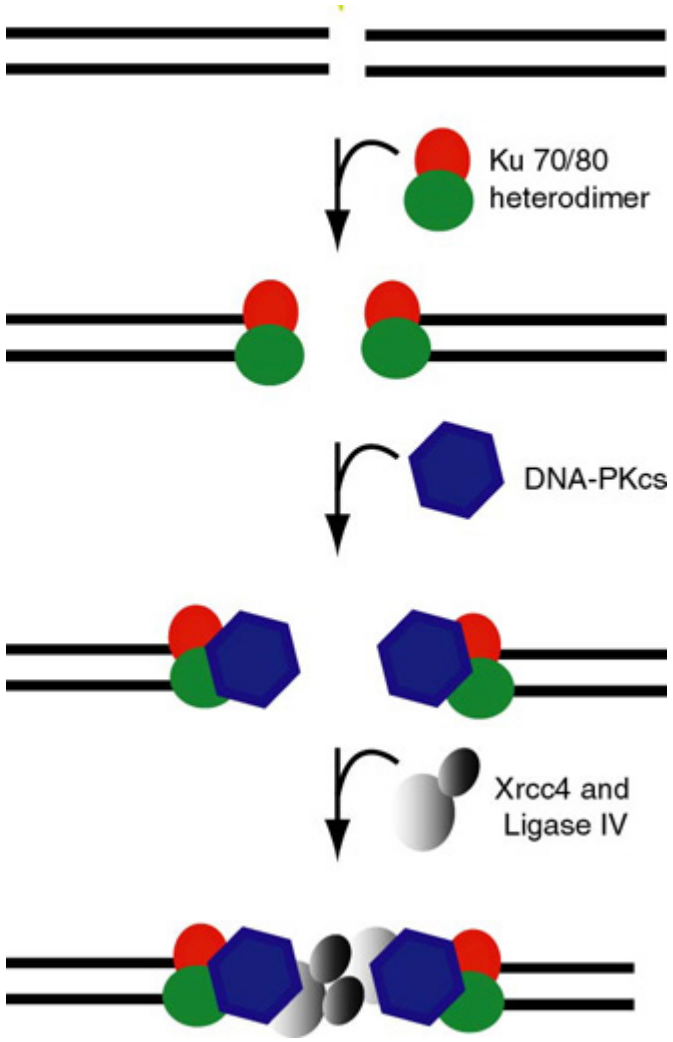


http://www.genlantis.com/corp/images/gWiz_vectmap.gif

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



Non-Homologous End Joining (NHEJ)

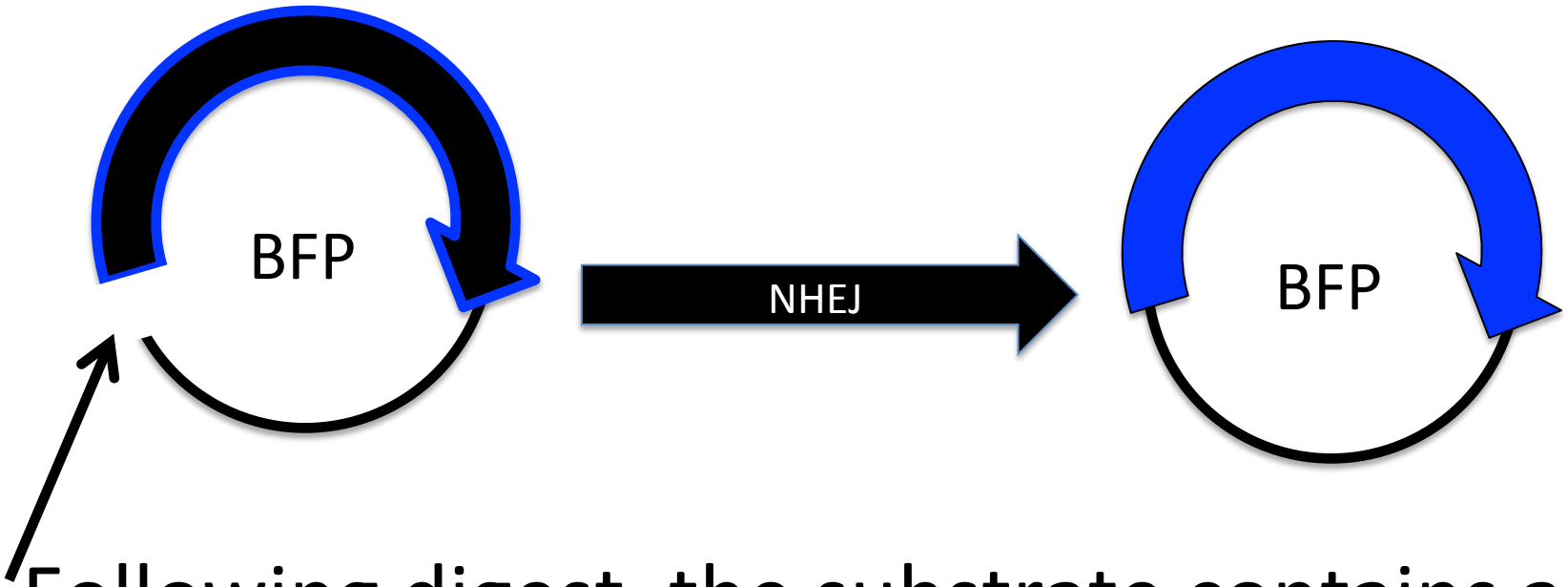


Ku70
Ku80

DNA-PKcs

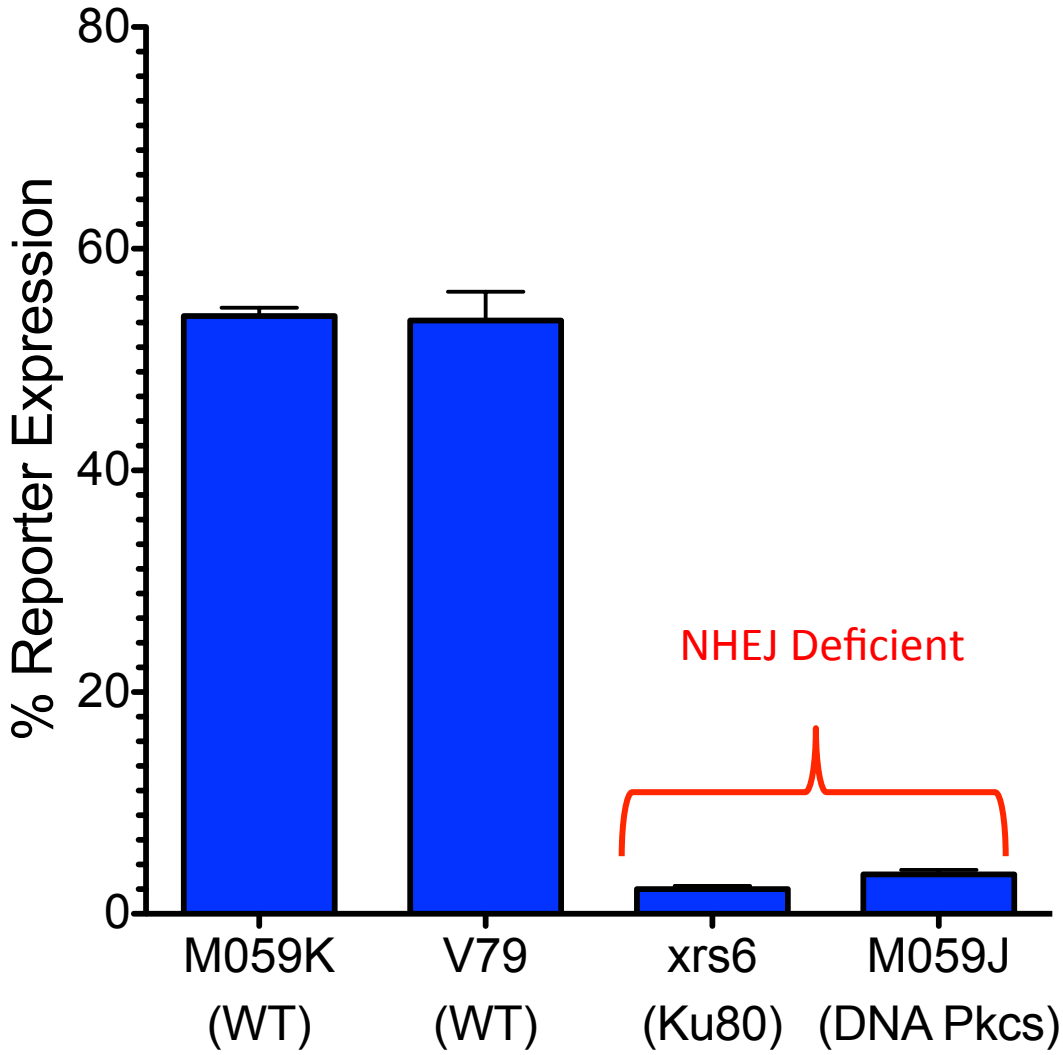
Xrcc4
Ligase IV

Basis for the fluorescent reporter assay:

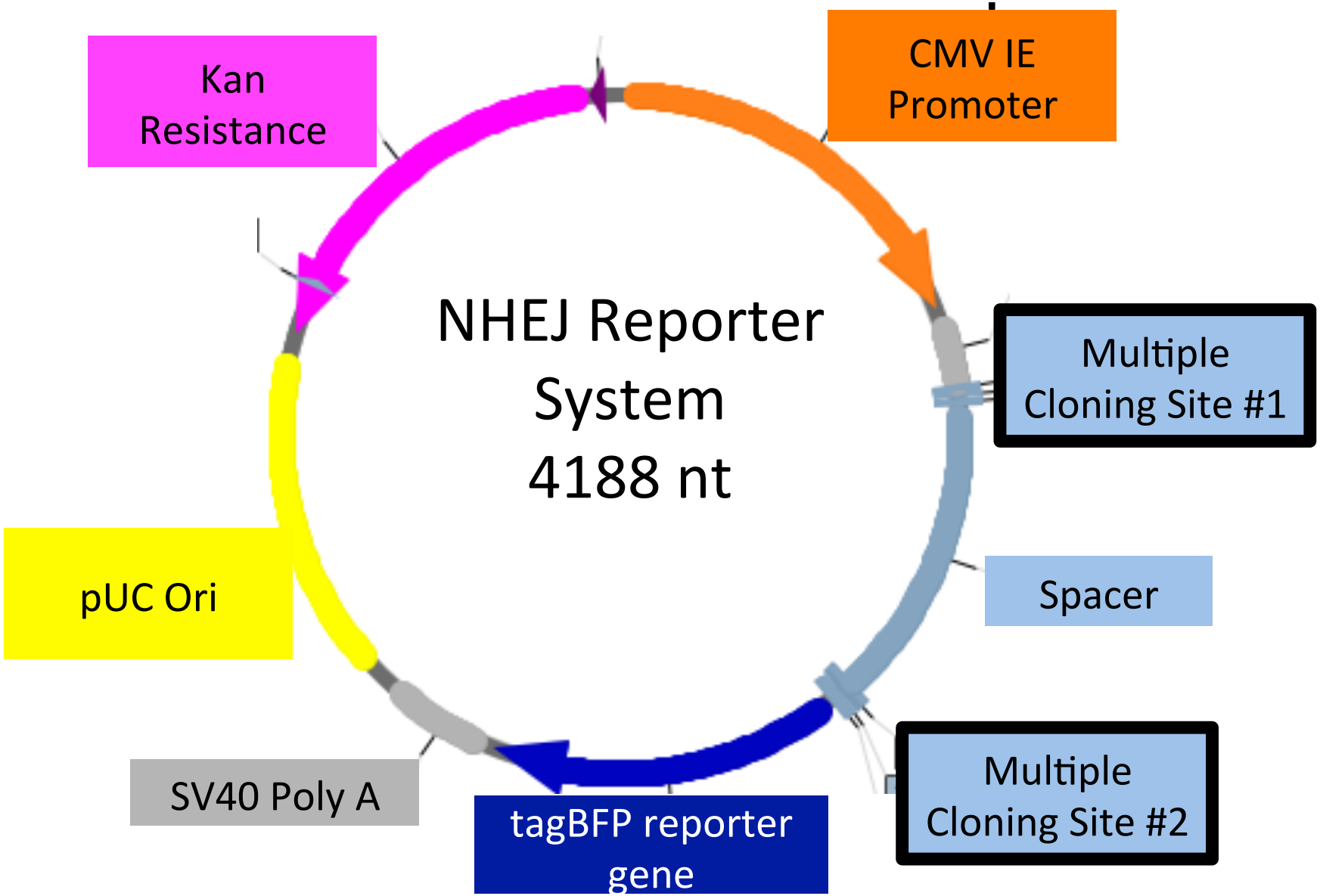


Following digest, the substrate contains a DSB in the 5’ UTR that prevents fluorescent reporter expression

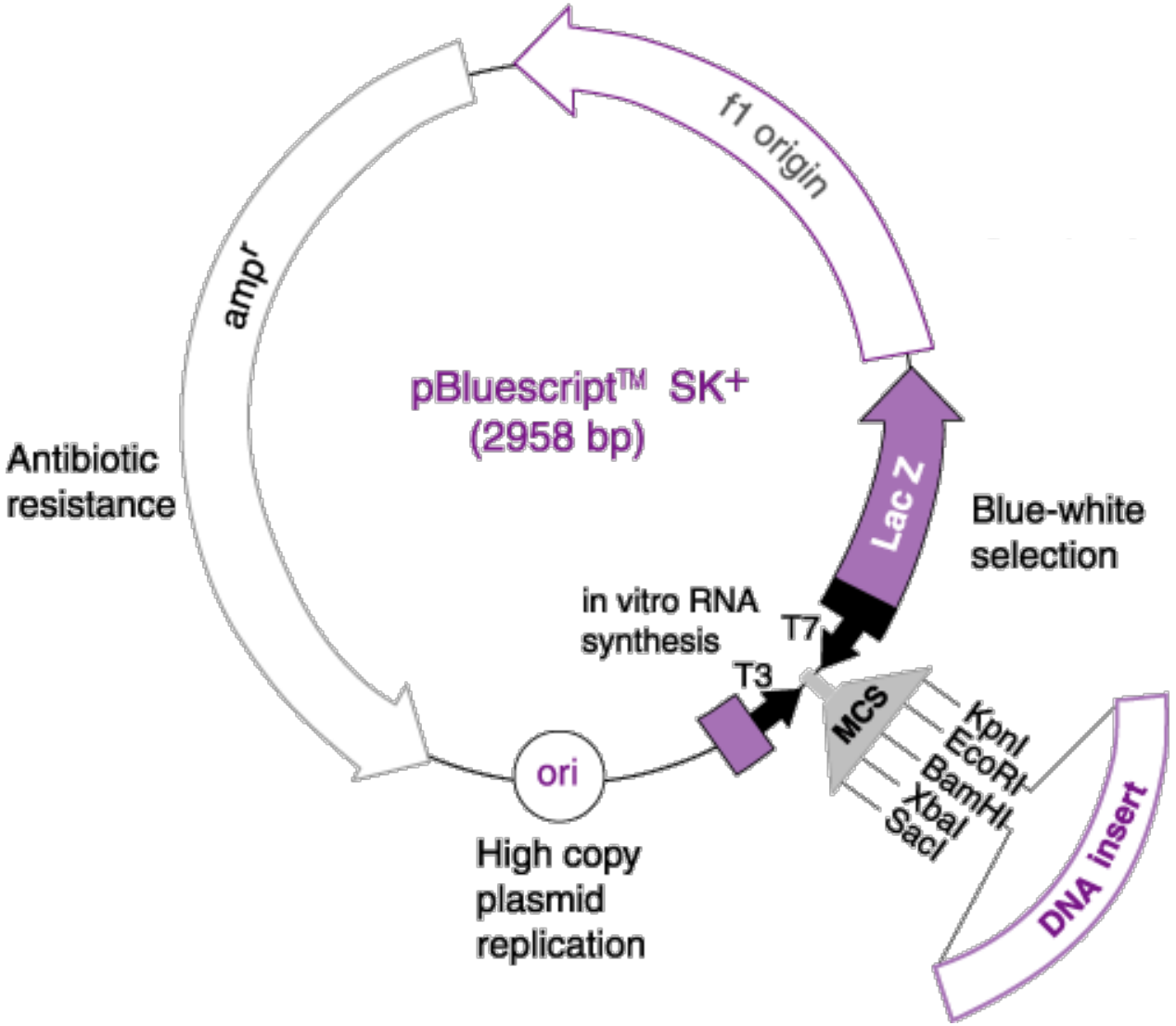
NHEJ HCR in WT and NHEJ defective cells at 18 hours post-transfection:



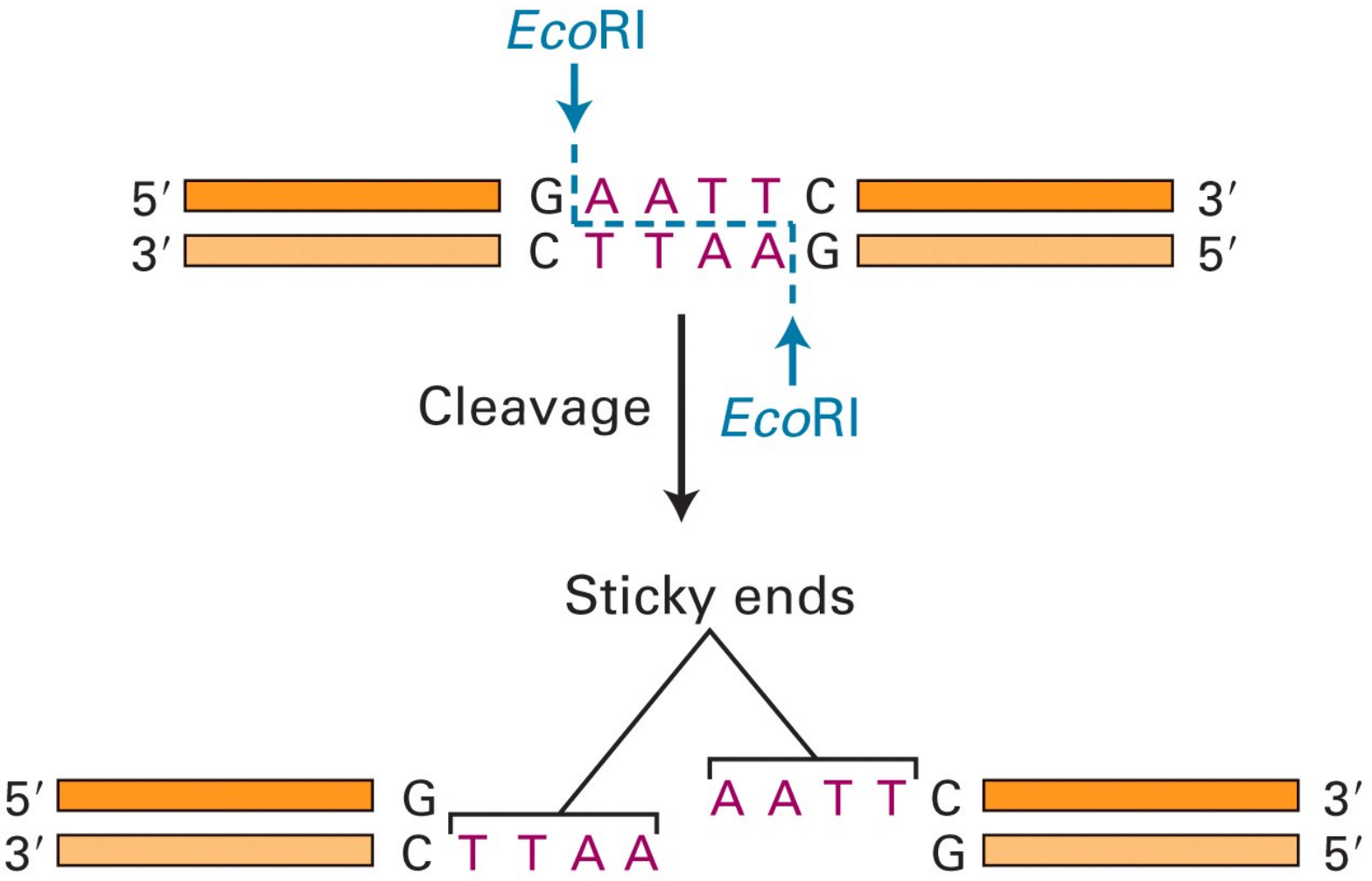
Overall Structure of the Reporter:



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.

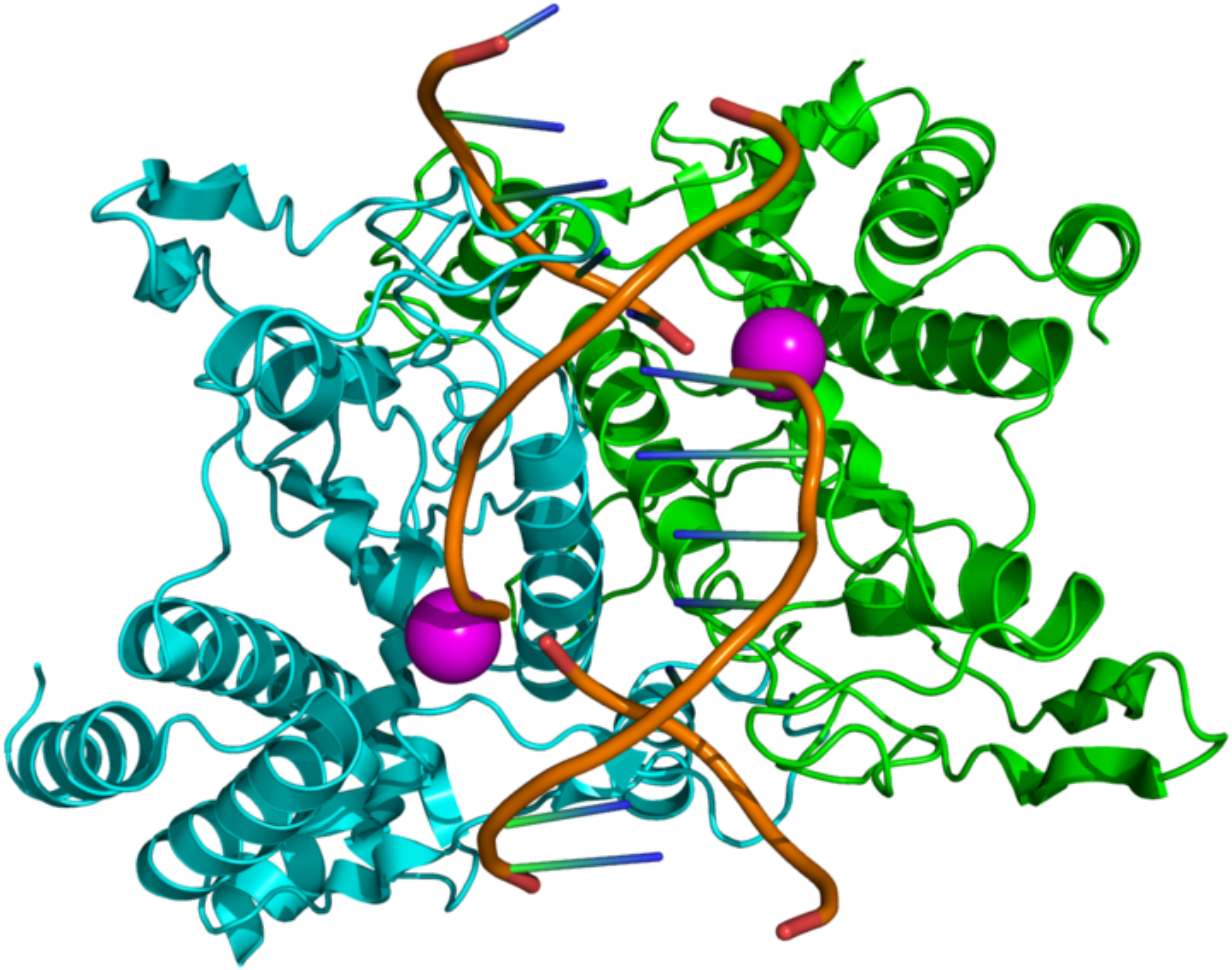
Flee to me, remote elf.

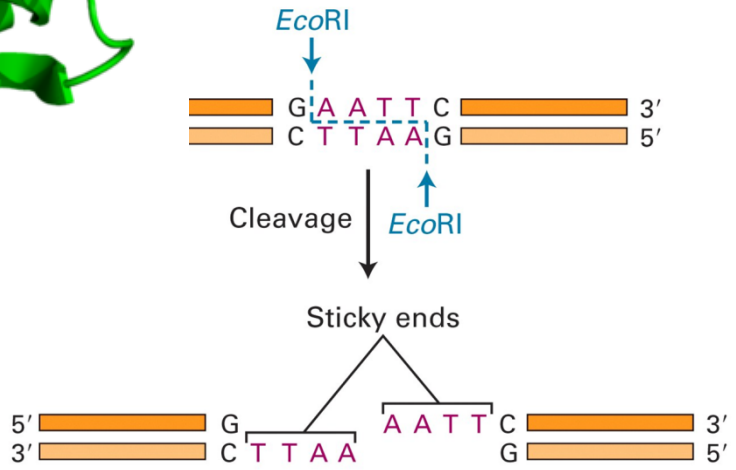
Gnu dung.

Lager, Sir, is regal.

Tuna nut.

What is a Restriction Enzyme?

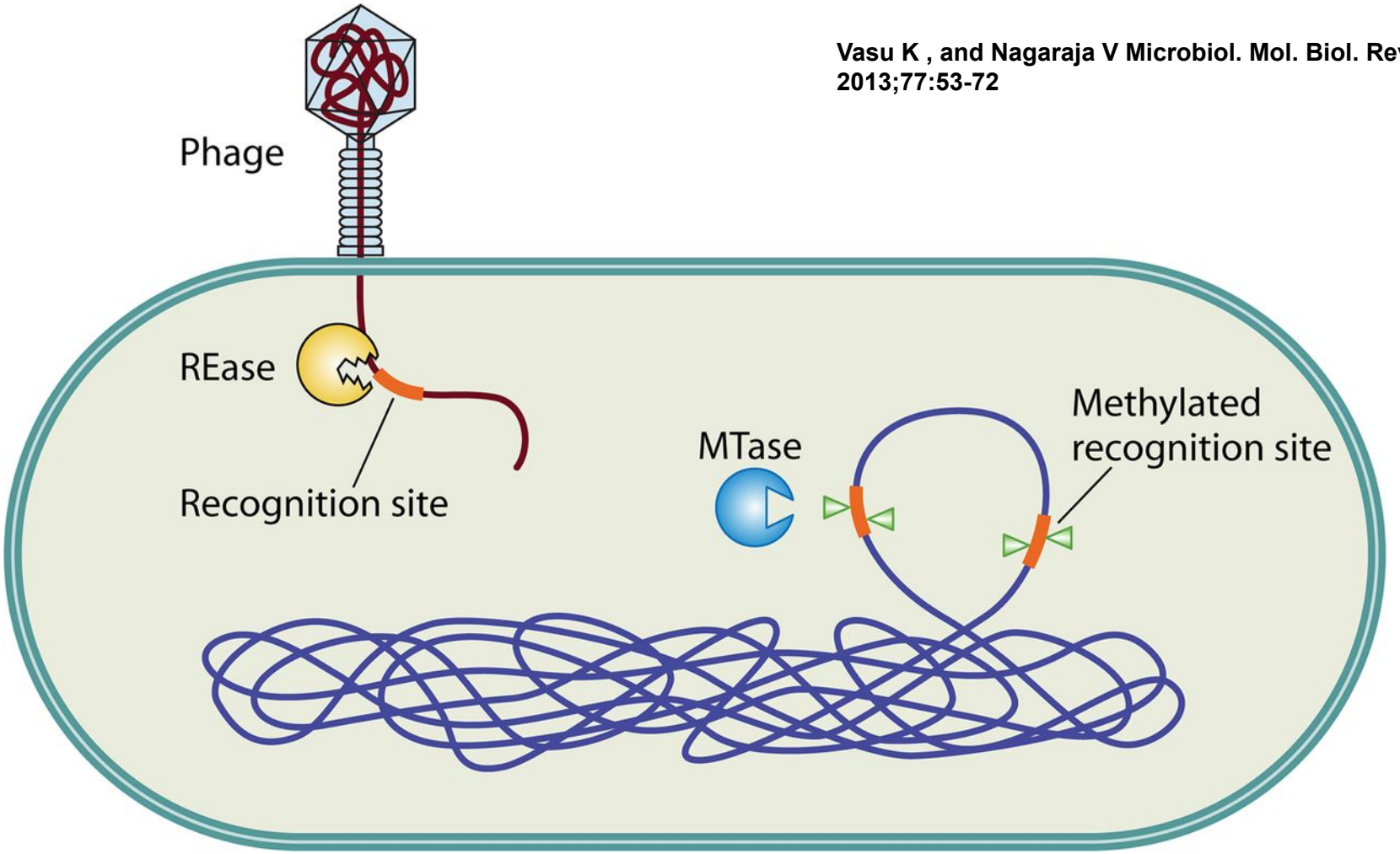




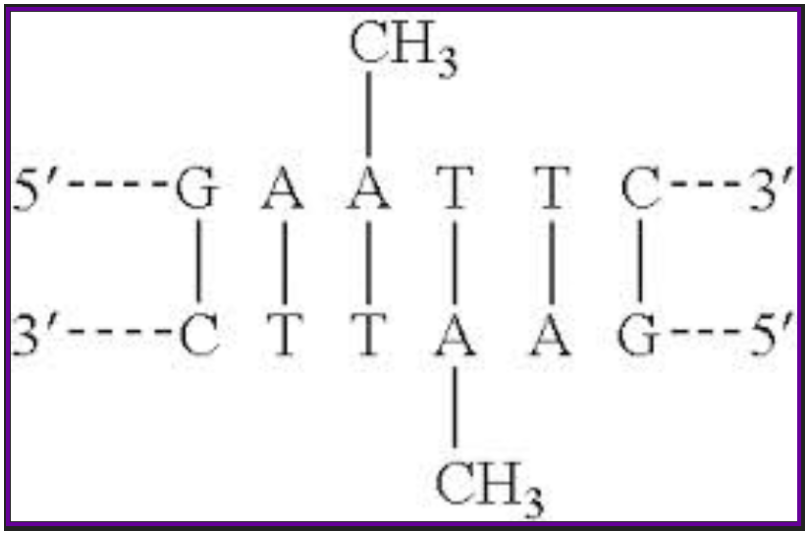
Why do Restriction Enzymes exist?

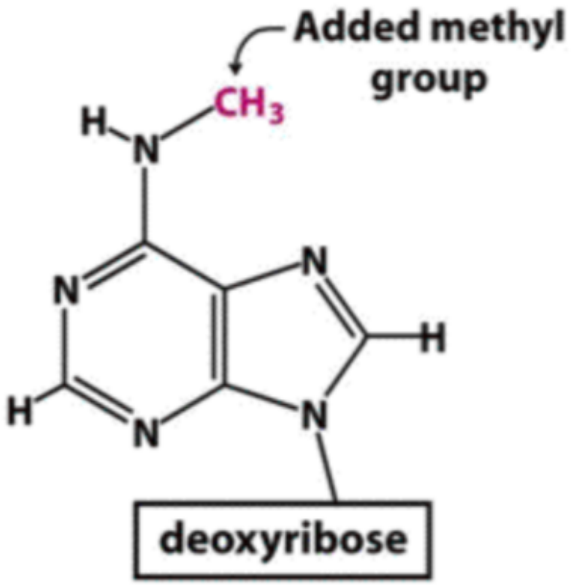
Restriction-modification (R-M) systems as defense mechanisms.

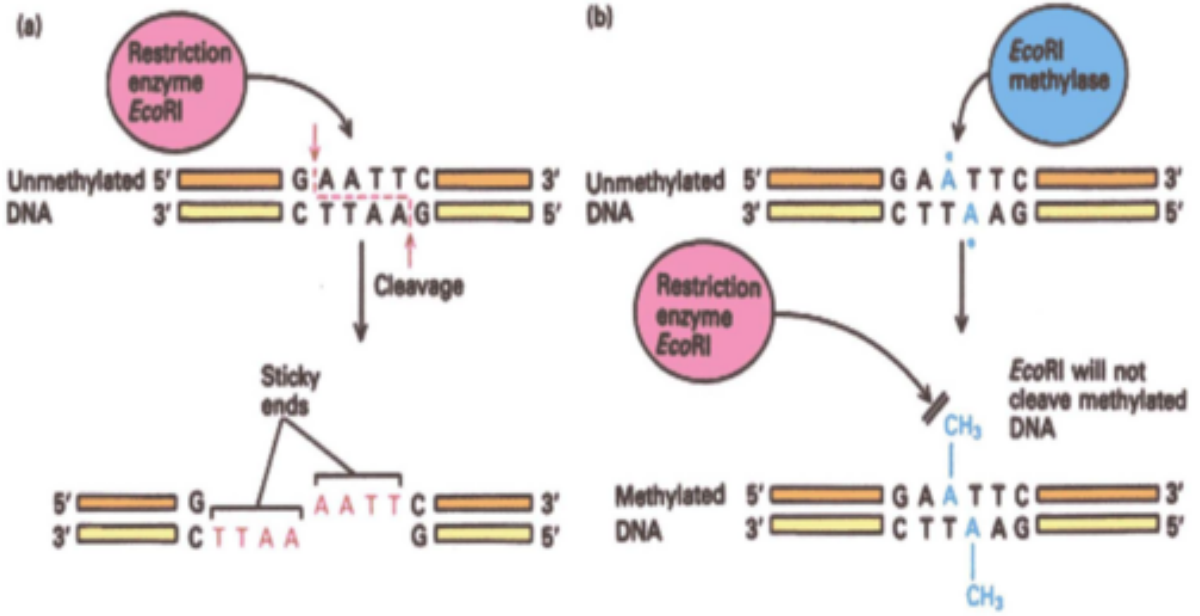
Vasu K , and Nagaraja V Microbiol. Mol. Biol. Rev. 2013;77:53-72



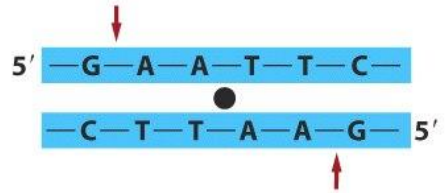
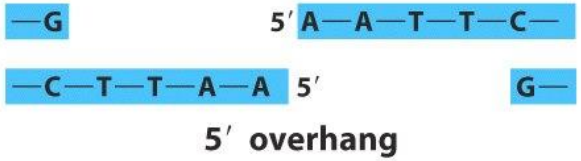
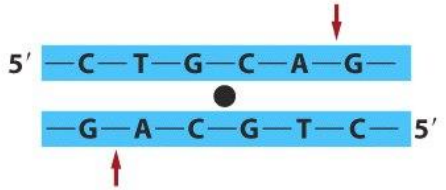
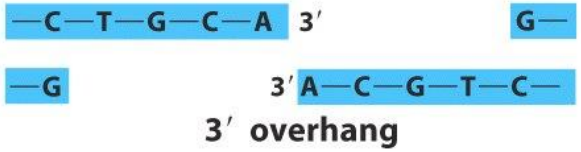
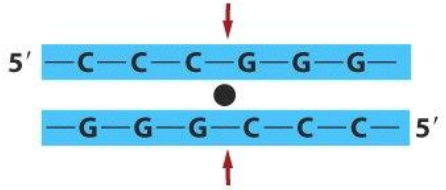
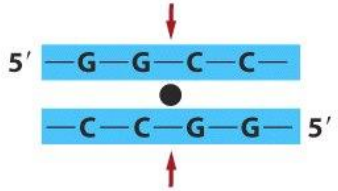
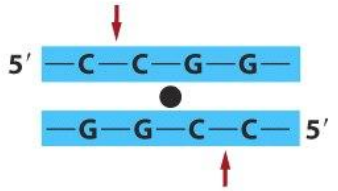
R-M systems recognize methylation status of incoming foreign DNA, e.g., phage genomes. Methylated sequences are recognized as self, while recognition sequences on the incoming DNA lacking methylation are recognized as nonself and are cleaved by the restriction endonuclease (REase). The methylation status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

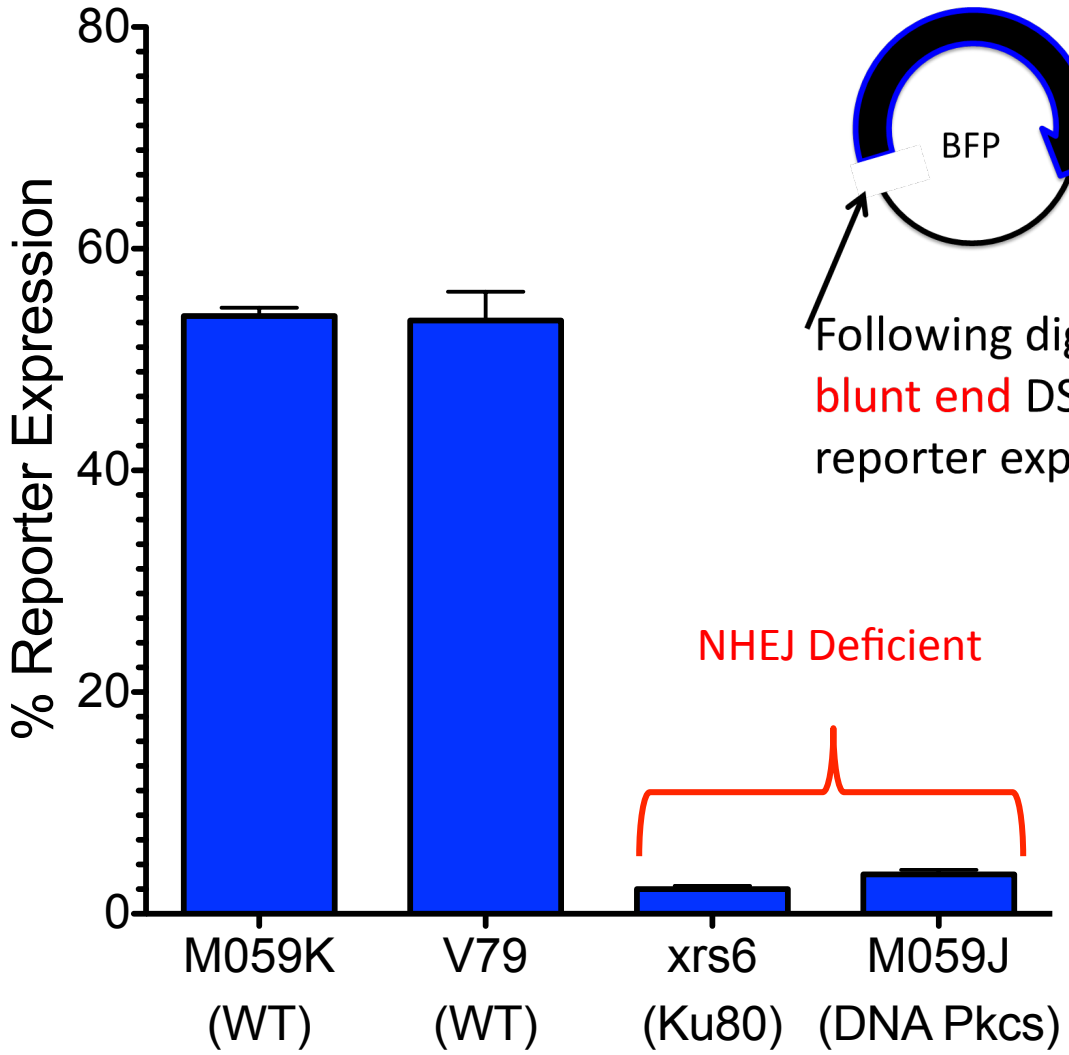

Added methyl group
CH3
H
N
N
N
N
H
H
N
N
deoxyribose

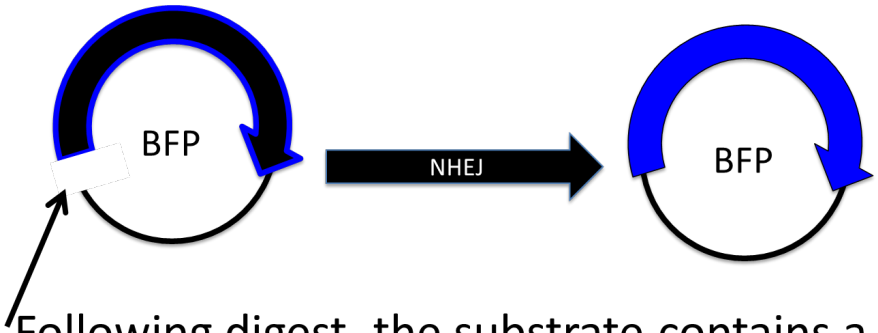

(a)
Restriction enzyme EcoRI
Unmethylated DNA
5′ G A A T T C 3′
3′ C T T A A G 5′
Cleavage
Sticky ends
5′ G A A T T C 3′
3′ C T T A A G 5′
(b)
EcoRI methylase
Unmethylated DNA
5′ G A A T T C 3′
3′ C T T A A G 5′
Restriction enzyme EcoRI
EcoRI will not cleave methylated DNA
CH3
Methylated DNA
5′ G A A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

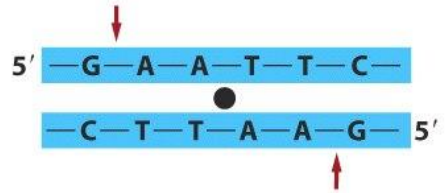
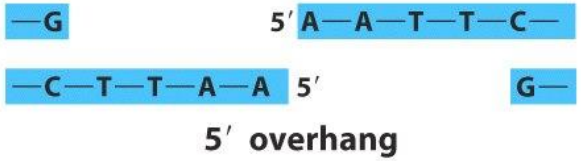
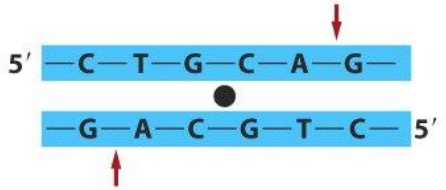
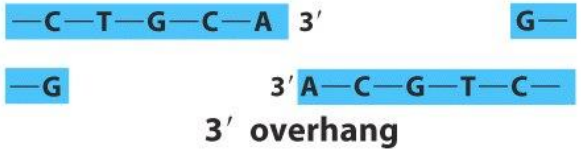
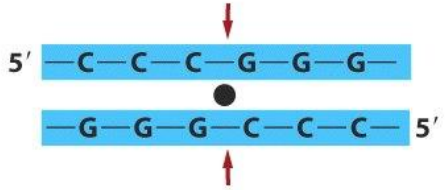
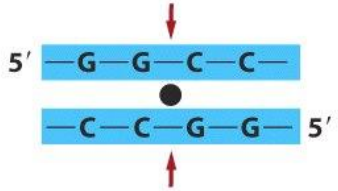
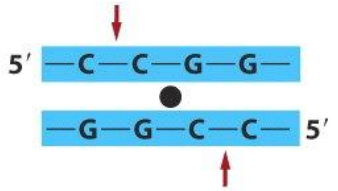
NHEJ HCR in WT and NHEJ defective cells at 18 hours post-transfection:





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: