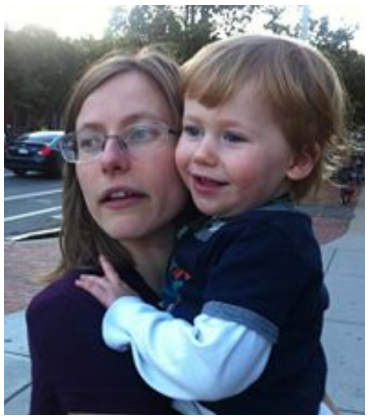
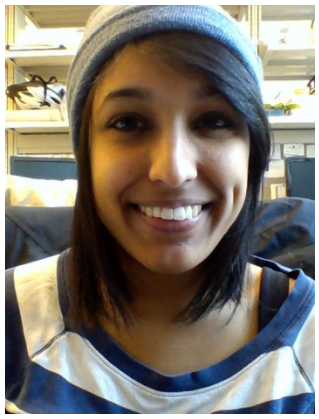
20.109 Spring 2014 Module 2
System Engineering and Protein Foundations



Agi Stachowiak

Shannon Hughes

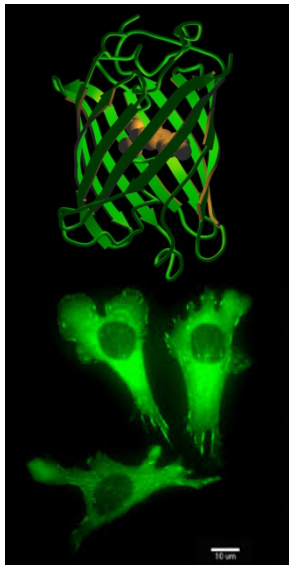
Aneesh Ramaswamy

Suhani Vora (TA)

Leona Samson (Lectures)

Zachary Nagel (help with development)

What experimental question will you ask in Module 2?

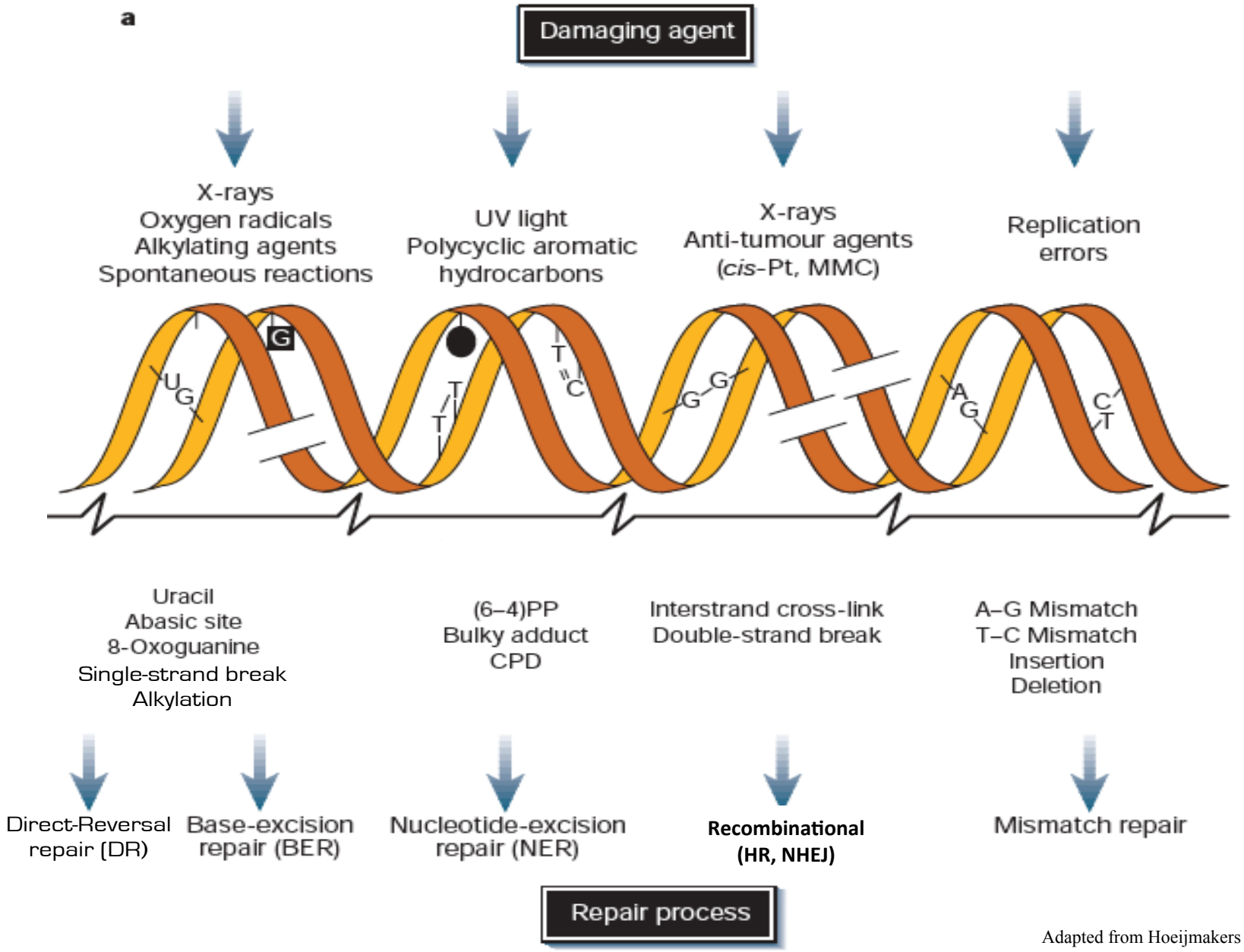


How efficiently does DNA repair by the Non Homologous End Joining (NHEJ) pathway act on DNA damage with different topologies?

This raises the following questions

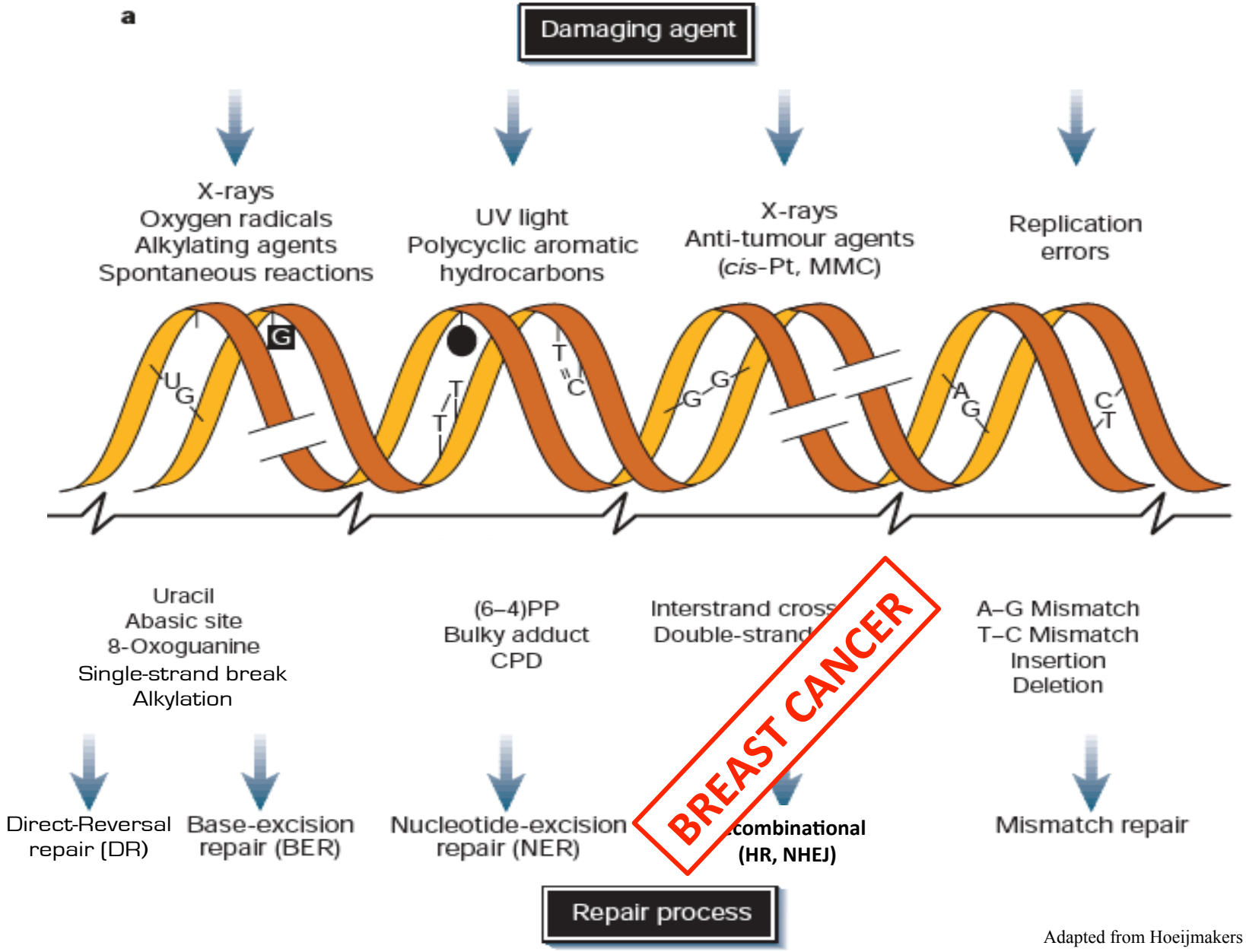
- How does DNA get damaged?
- What is DNA repair?
- Why does DNA repair exist?
- Why do we care about how efficient DNA repair is?
- How does one actually measure DNA repair efficiency?

DNA Damage and Repair



Adapted from Hoeijmakers **2001** *Nature*

DNA Damage and Repair



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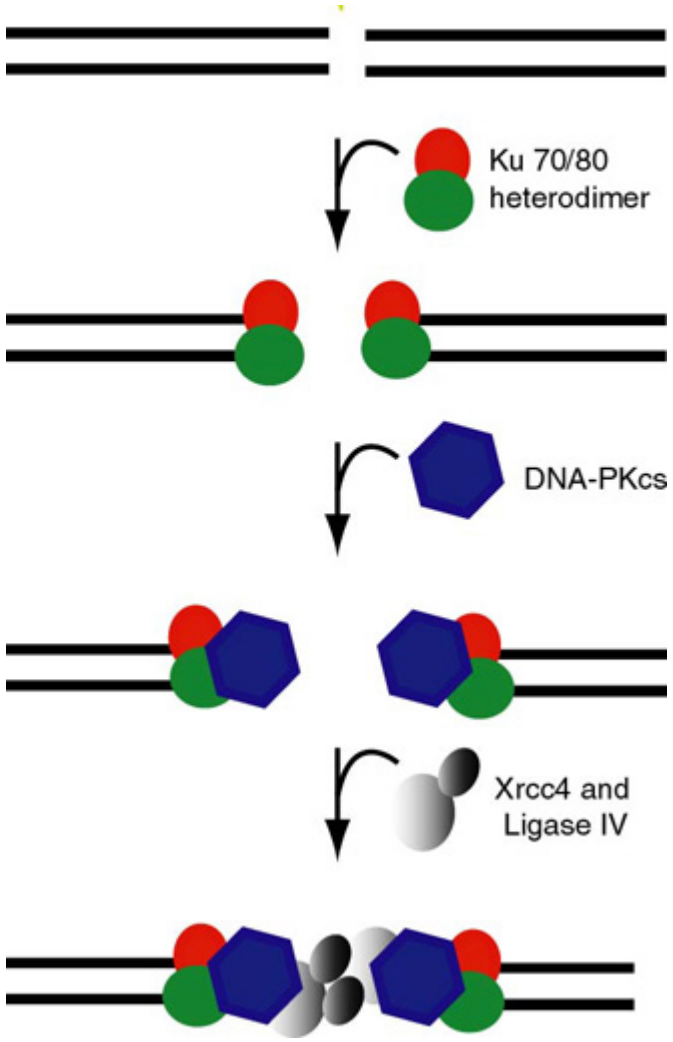
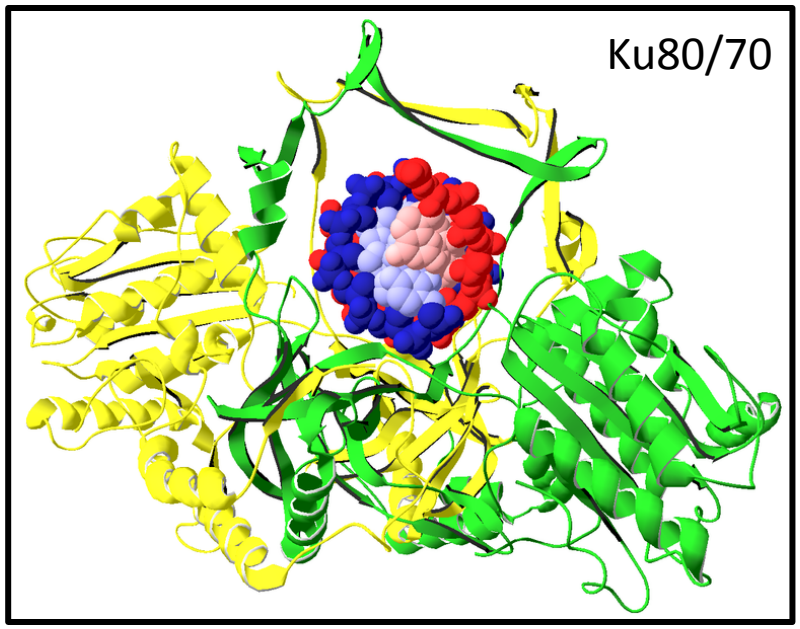
Non-Homologous End Joining (NHEJ)

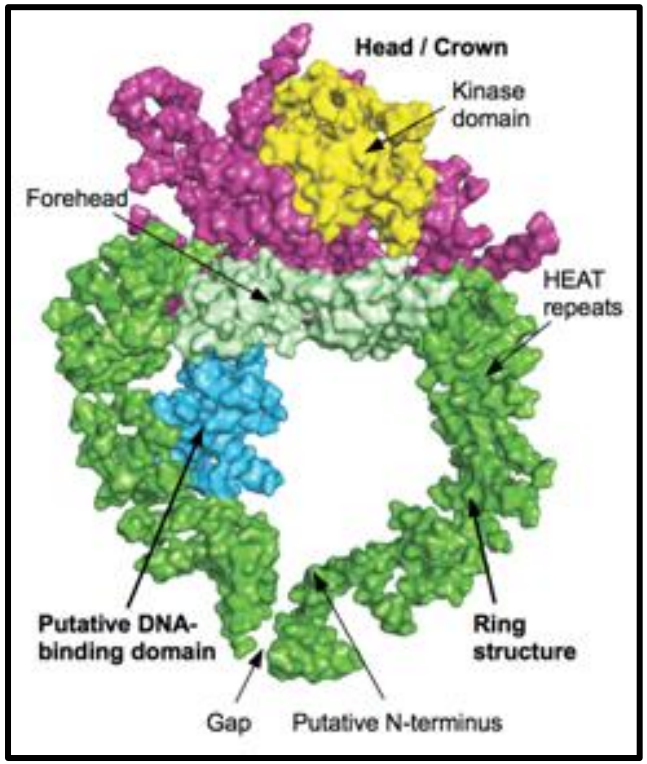


Ku70
Ku80

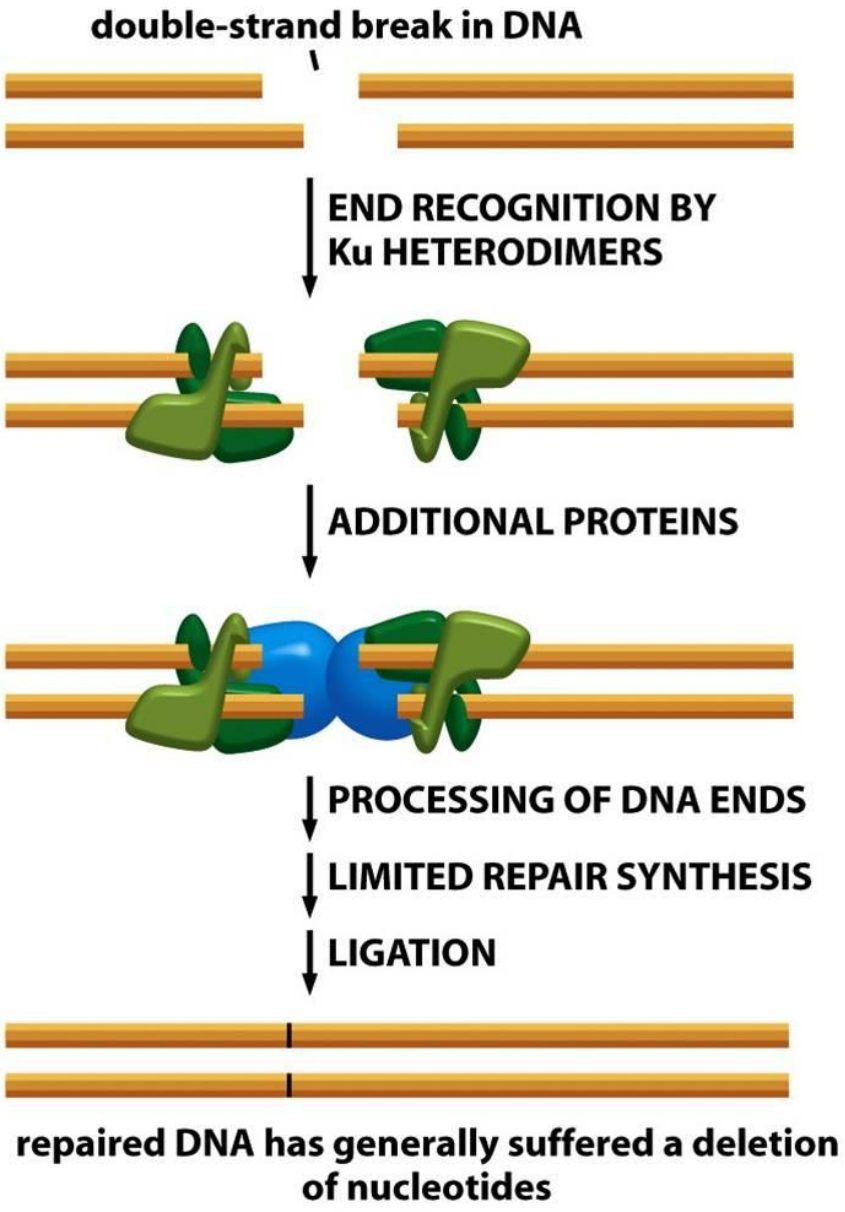
DNA-PKcs

Xrcc4
Ligase IV

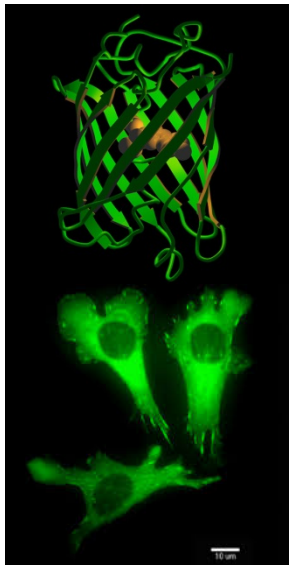

Ku80/70


Head / Crown
Kinase domain
Forehead
HEAT repeats
Putative DNA-binding domain
Ring structure
Gap
Putative N-terminus

DNA-PKcs

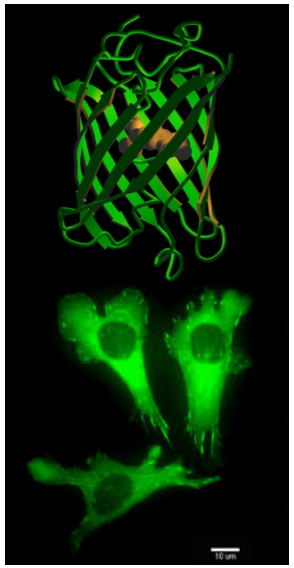

double-strand break in DNA
END RECOGNITION BY Ku HETERODIMERS
ADDITIONAL PROTEINS
PROCESSING OF DNA ENDS
LIMITED REPAIR SYNTHESIS
LIGATION
repaired DNA has generally suffered a deletion of nucleotides

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

Key Experimental Methods for Module 1



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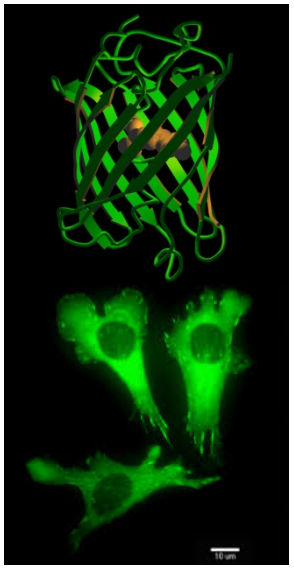
- Statistical analysis of biological data

Chinese Hamster Ovary (CHO) cells are immortal – they can grow indefinitely

NEXT LECTURE

- Isolating X-ray-sensitive (xrs) CHO cells
- Xrs cells are deficient in NHEJ
- Detecting NHEJ proteins by Western
- New ways to measure NHEJ activity
- Using fluorescent proteins to measure biological processes….



Isolating X-ray-sensitive (xrs) CHO cells
Professor Penelope Jeggo

~Forty years ago

Age 52

Age 22

Age 25

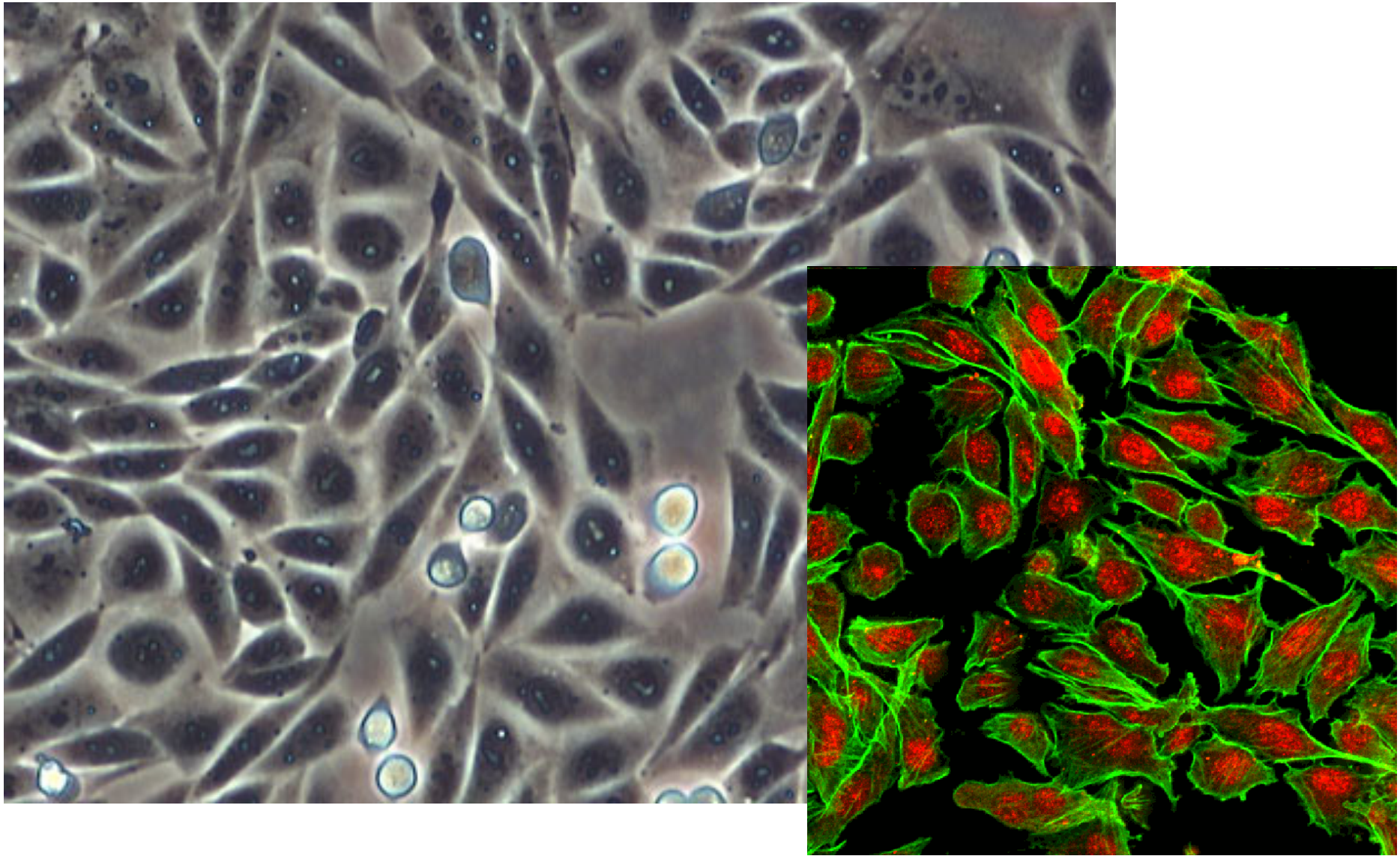
~1.5 years ago (summer of 2012)

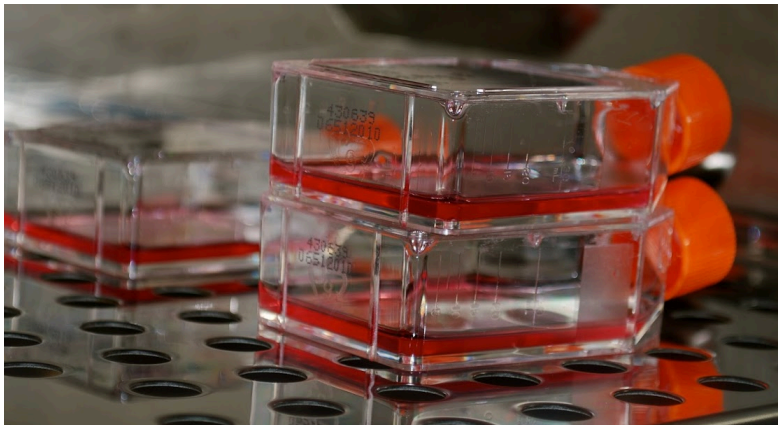
Age 90

Age 60

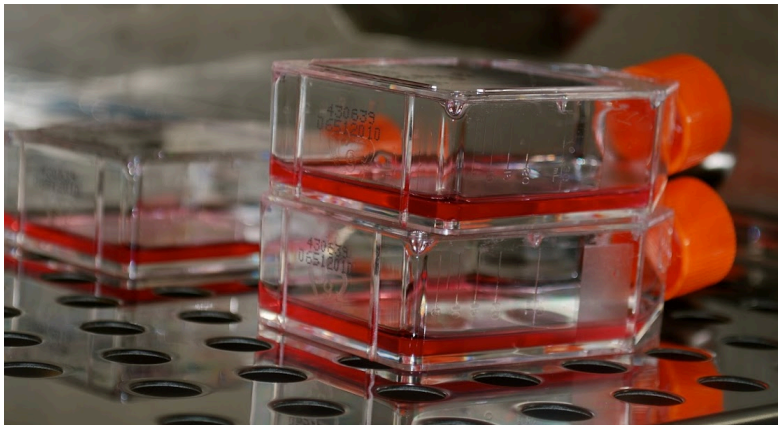
Age 64

Chinese Hamster Ovary (CHO) cells are immortal – they can grow indefinitely





How would you go about isolating X-ray resistant cell lines?



How would you go about isolating X-ray sensitive cell lines?

Generate population of CHO cells with a high frequency of mutations

Several million
CHO-K1 cells

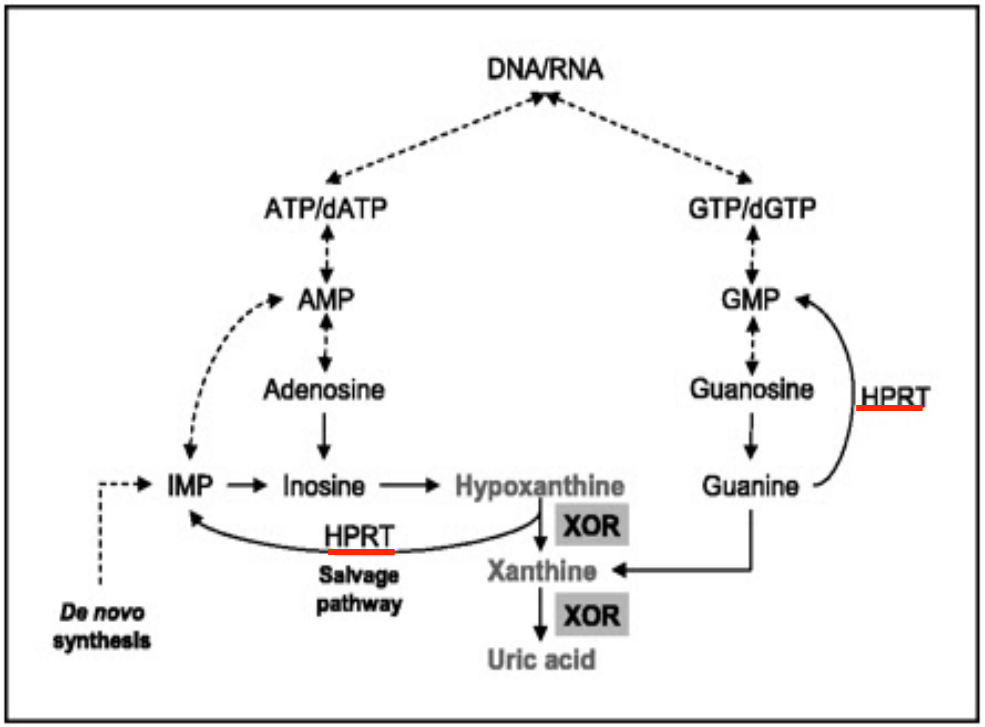
Add potent mutagen

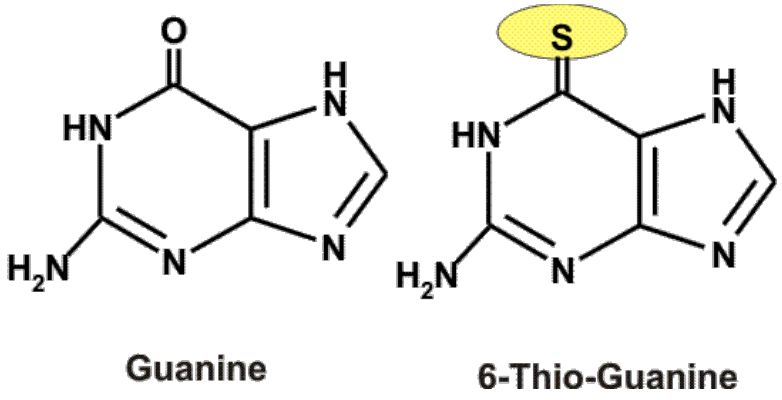
70% cells die.
The 30% of cells that live have a high frequency of mutants with mutations randomly located across the genome

Expand this mutagenized population

Freeze down aliquots of mutagenized cells

Hypoxanthine phoshoribosyl transferase enzyme



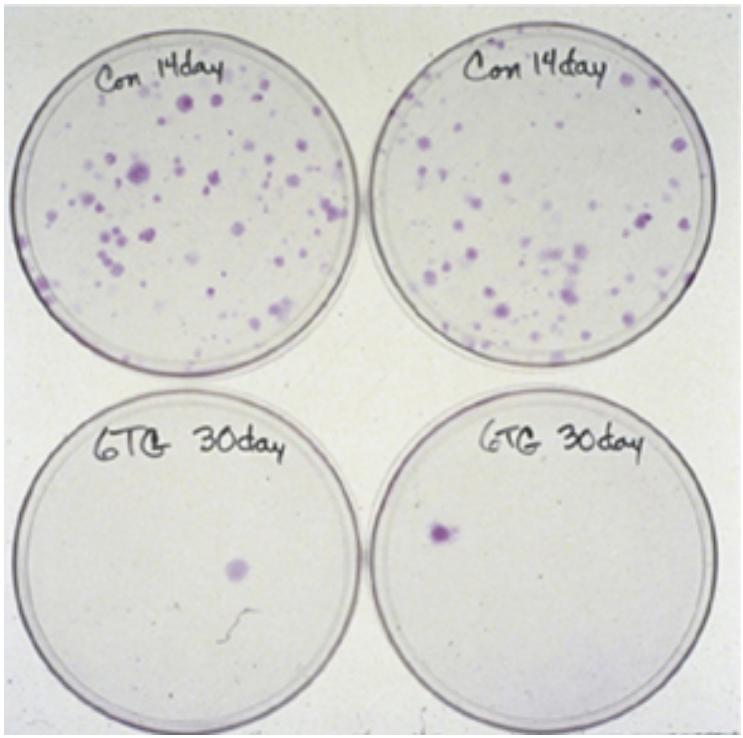


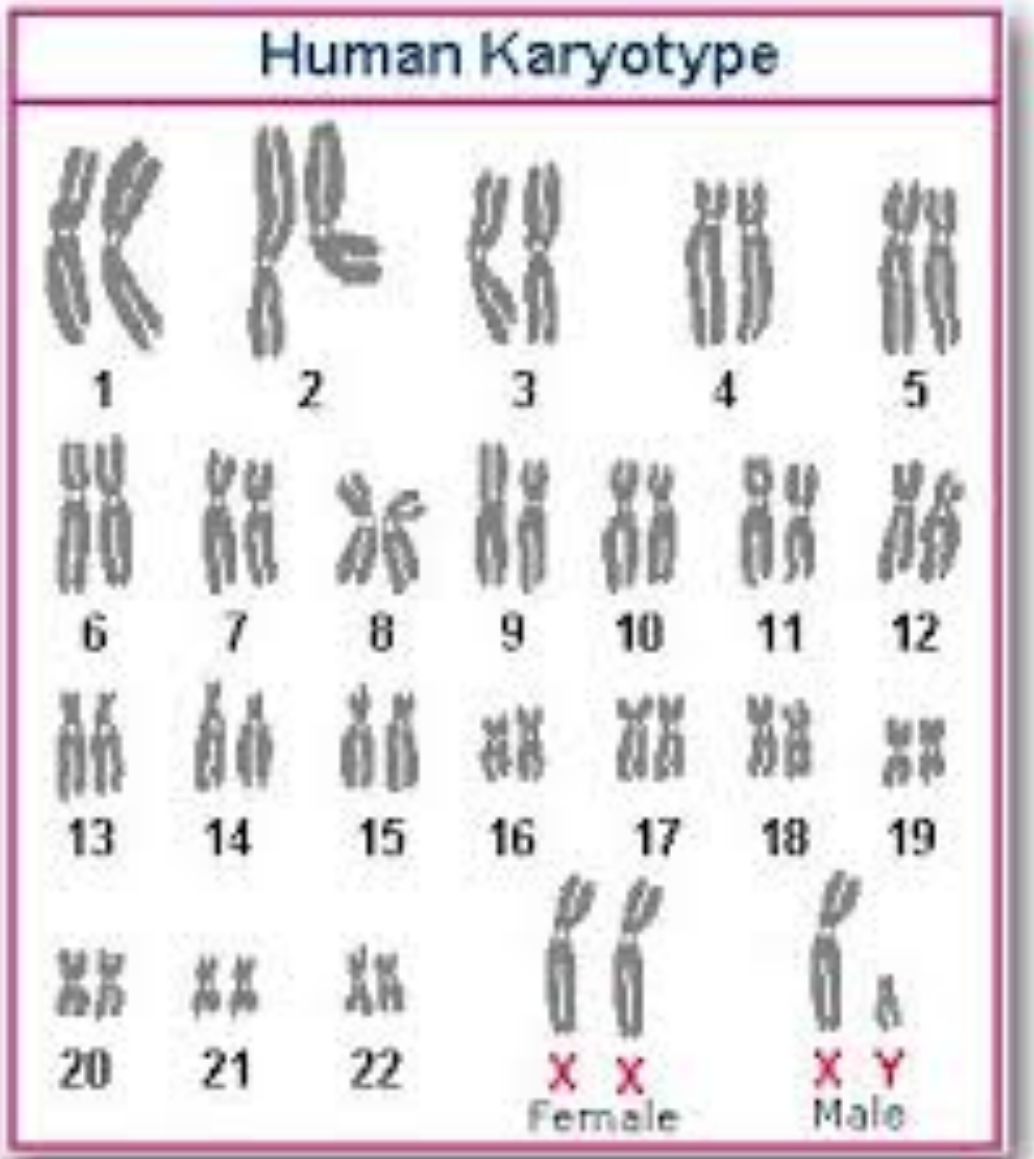
Guanine

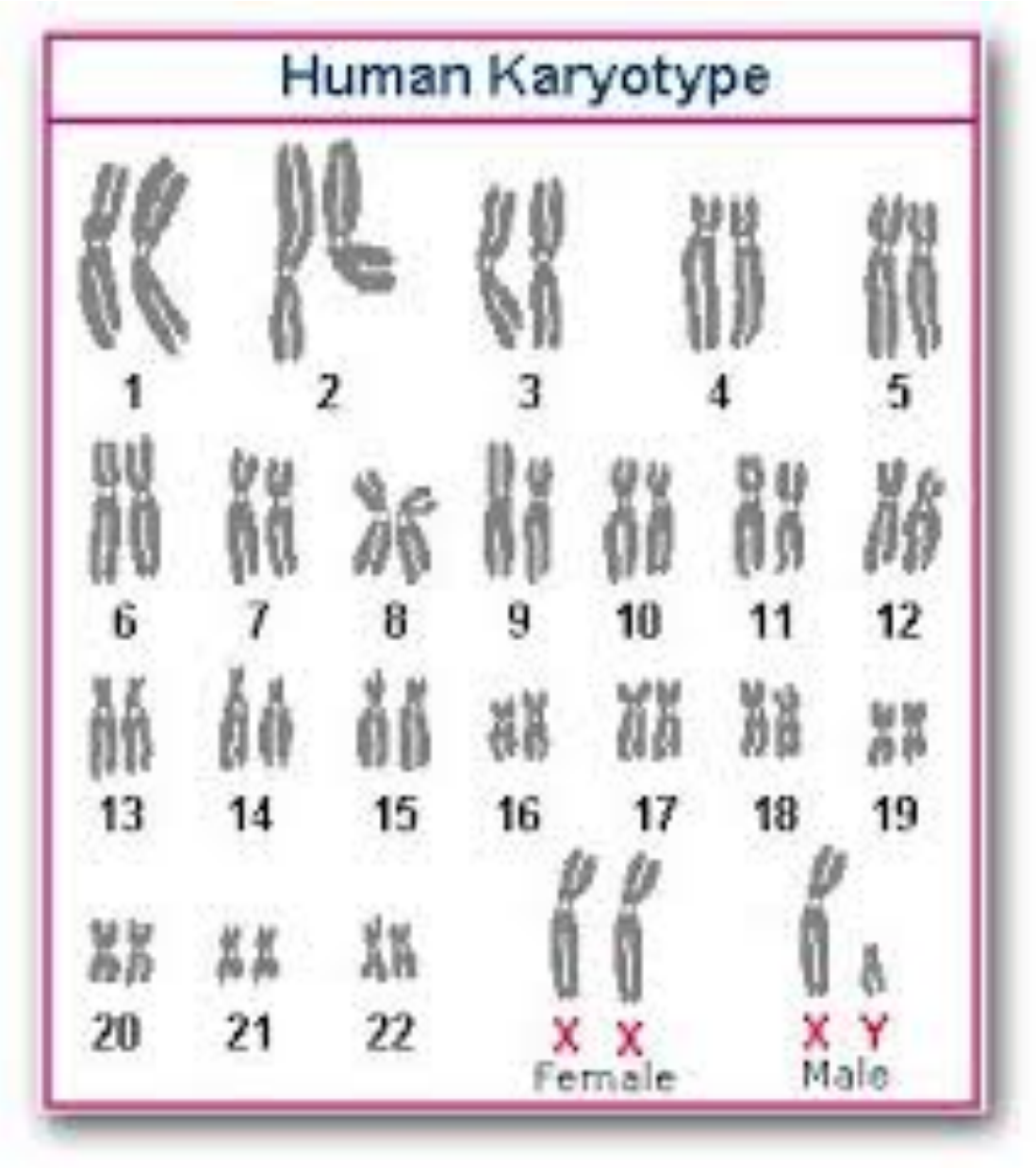
6-Thio-Guanine

Measure the frequency of mutations at a specific gene locus – the *HPRT* gene:

1 in 10^3 or 10^{-3}

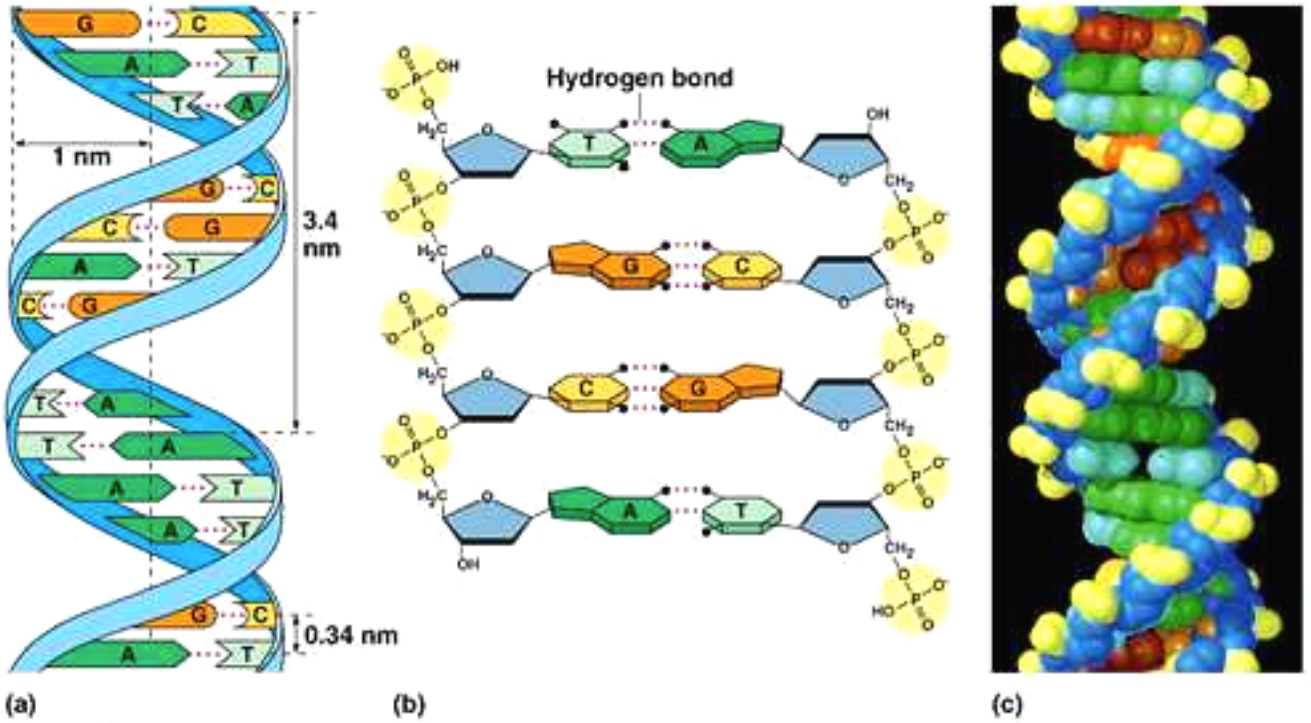


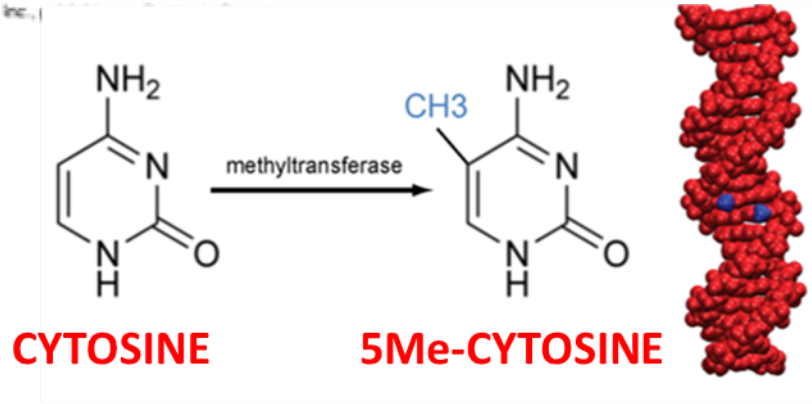

Human Karyotype
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
X X
Female
X Y
Male



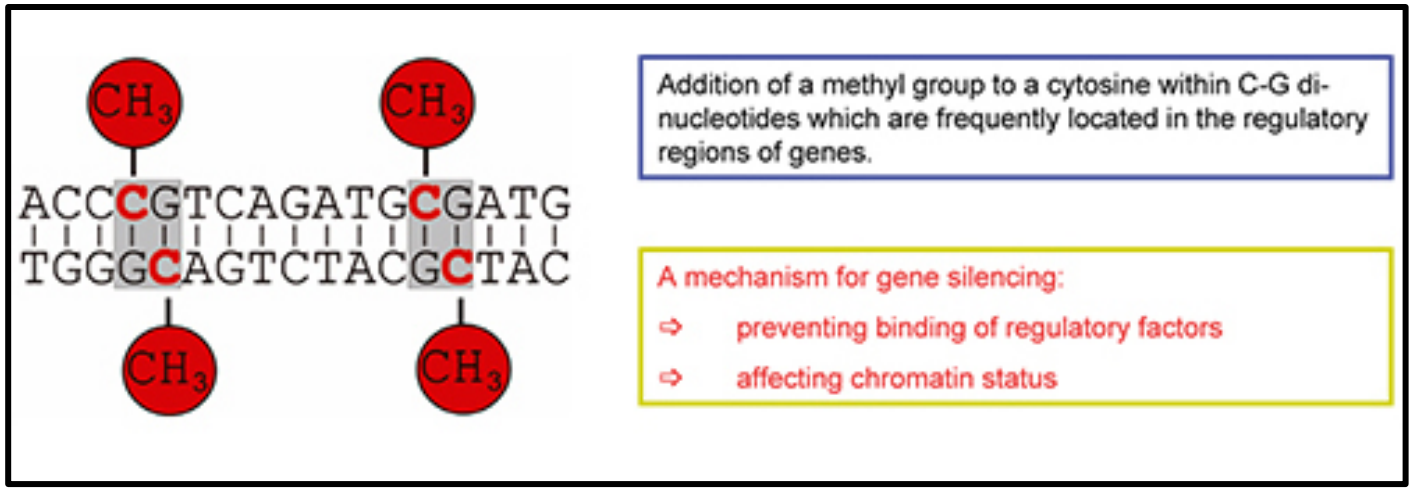
HPRT gene is on the X-chromosome

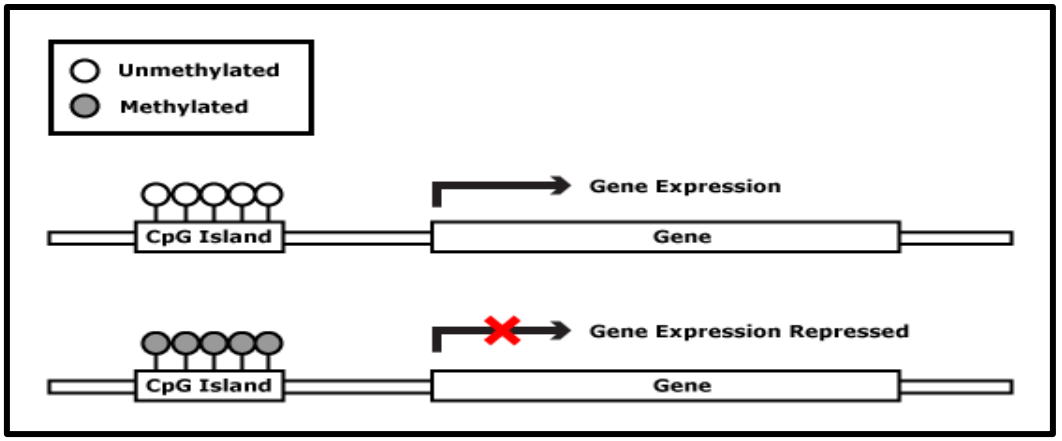
The Structure of DNA

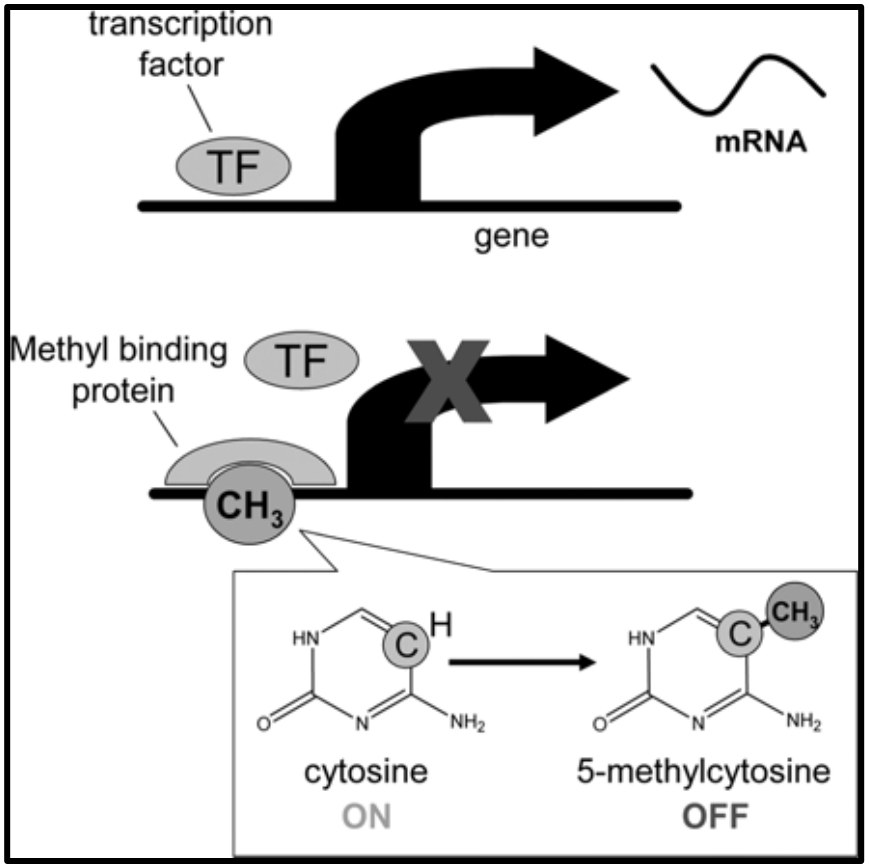


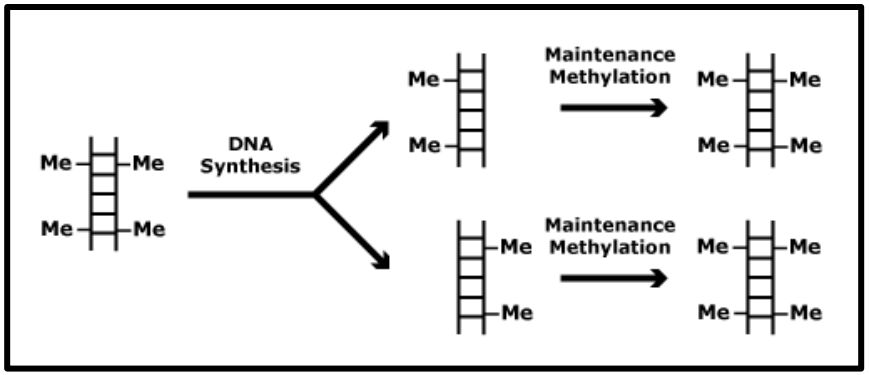


Promoter methylation at CpG silences gene expression





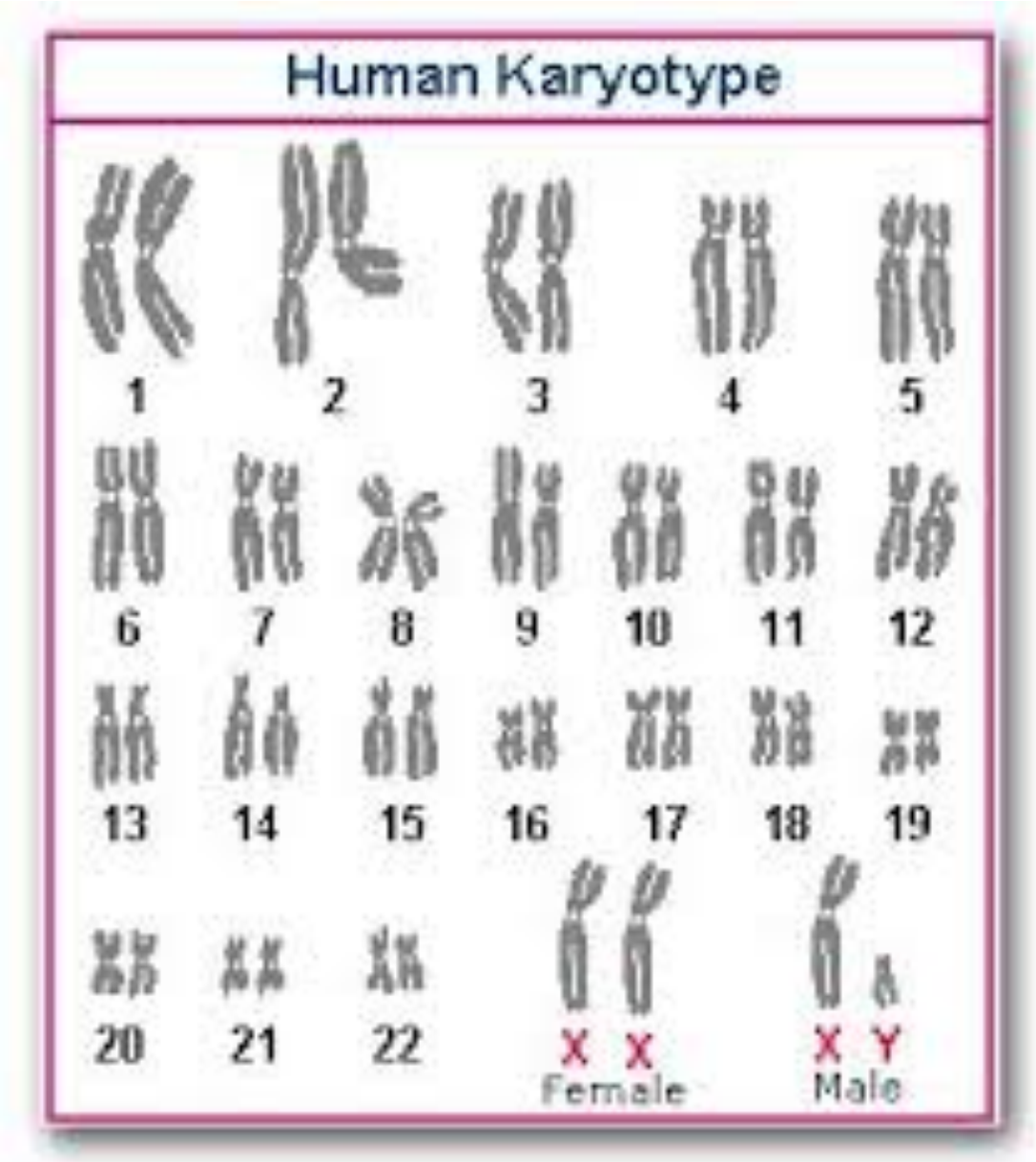




CHO cells happen to have more than the usual amount of CpG methylation

This results in extensive

"FUNCTIONAL HEMIZYGOSITY"



HPRT gene is on the X-chromosome – functional hemizygosity in female cells, actual hemizygosity in male cells

Hope that the genes for repairing X-ray induced damage are also in functionally hemizygous state

Generate population of CHO cells with a high frequency of mutations

Several million
CHO-K1 cells

Add potent mutagen

70% cells die.
The 30% of cells that live have a high frequency of mutants with mutations randomly located across the genome

Expand this mutagenized population

Freeze down aliquots of mutagenized cells

X-ray-sensitive mutants of Chinese hamster ovary cell line

Isolation and cross-sensitivity to other DNA-damaging agents

P.A.Jeggo * and L.M. Kemp

Genetics Division, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA (Great Britain)

(Received 12 April 1983)
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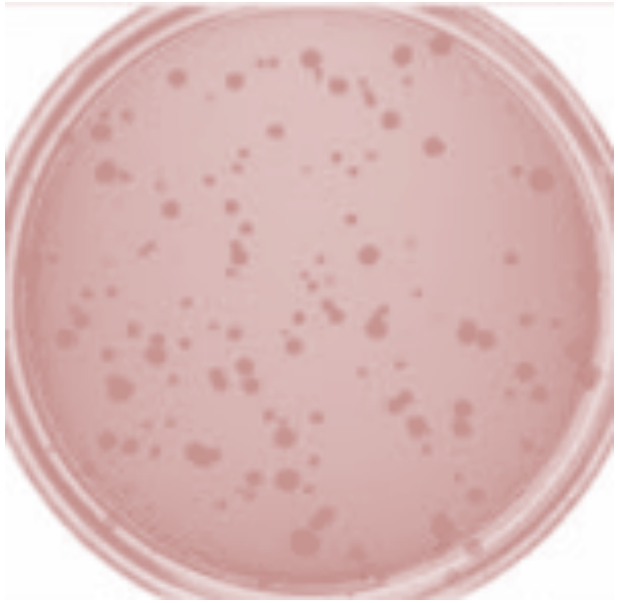

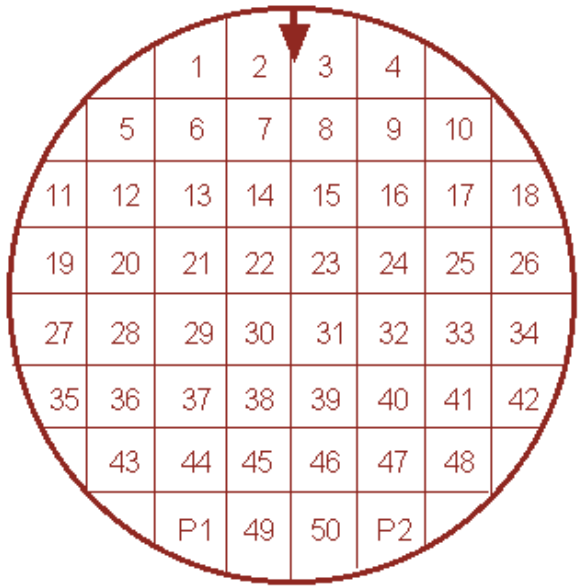

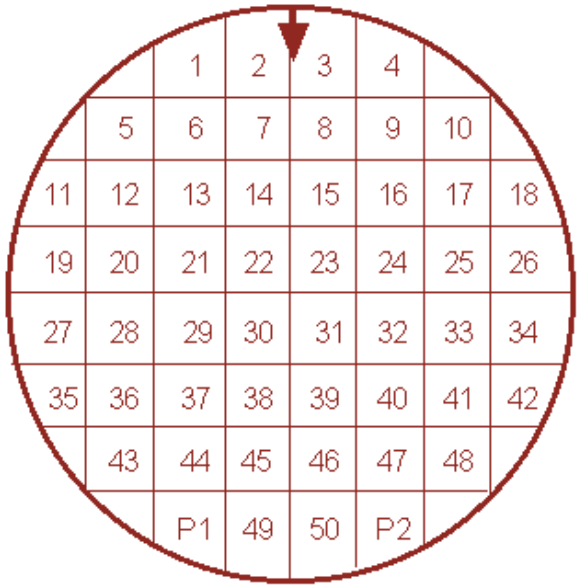

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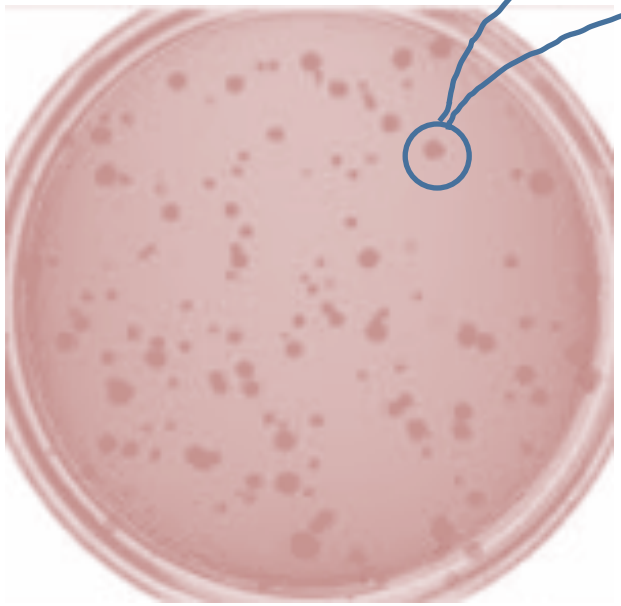

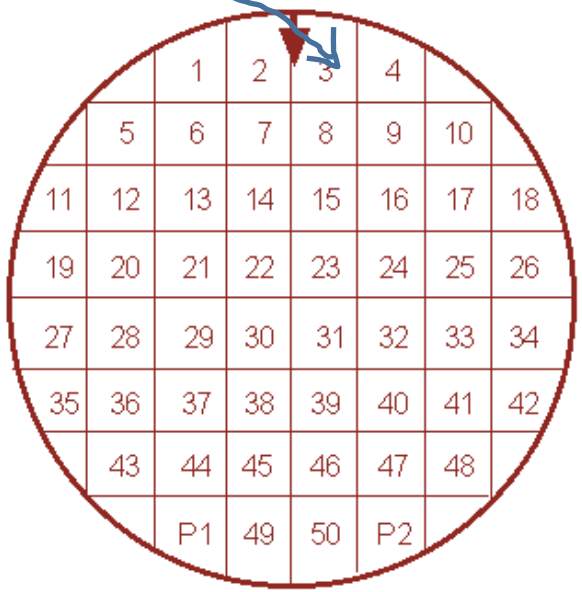

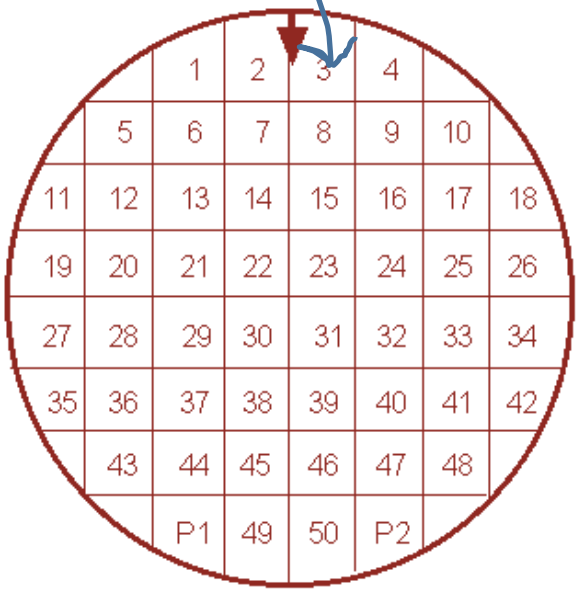

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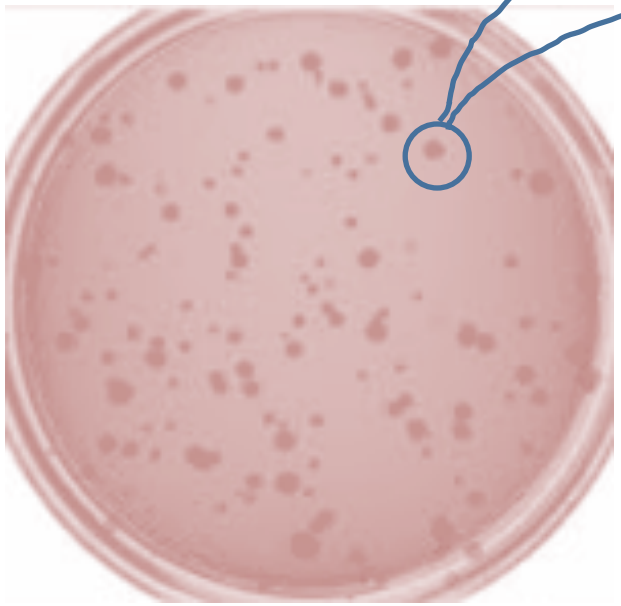
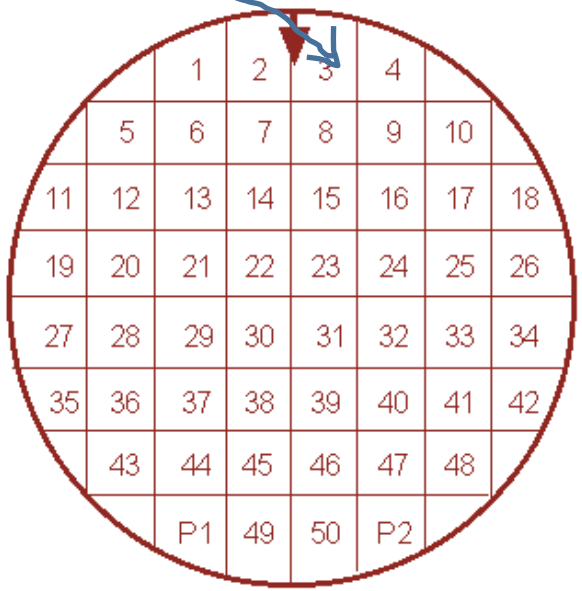
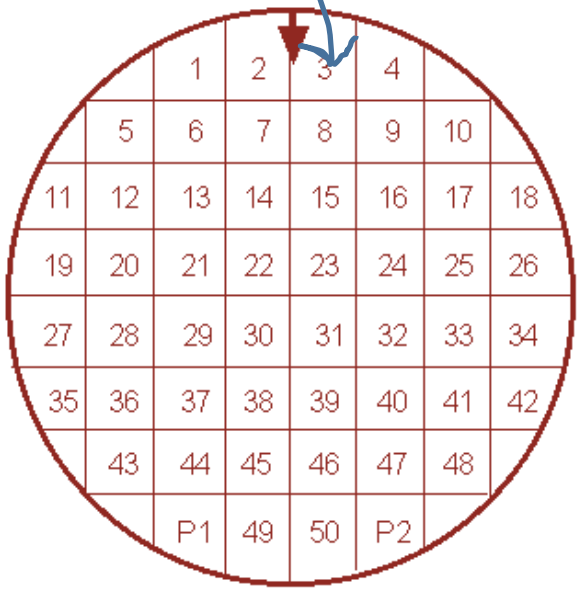




X-RAYS



No X-RAYS

X-ray-sensitive mutants of Chinese hamster ovary cell line

Isolation and cross-sensitivity to other DNA-damaging agents

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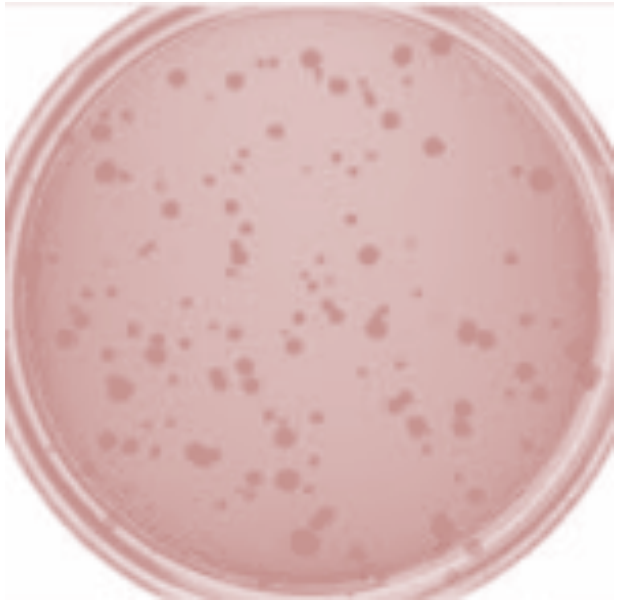
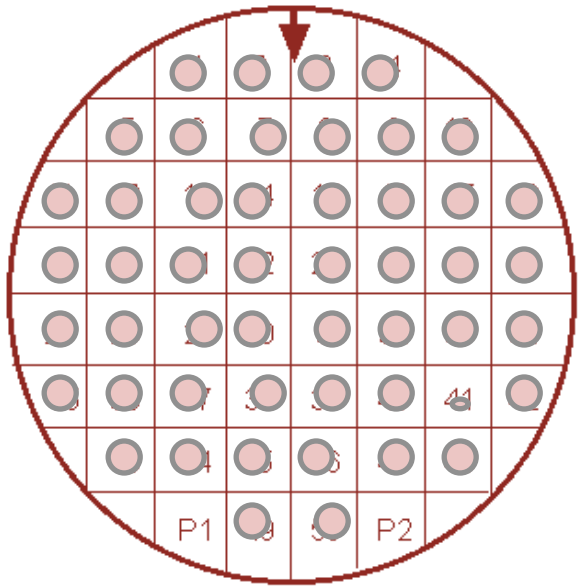
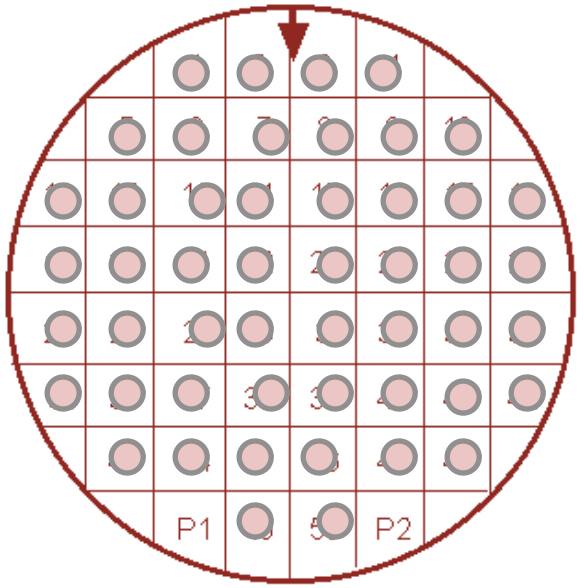




6 days after X-RAYS



6 days after No X-RAYS

X-ray-sensitive mutants of Chinese hamster ovary cell line

Isolation and cross-sensitivity to other DNA-damaging agents

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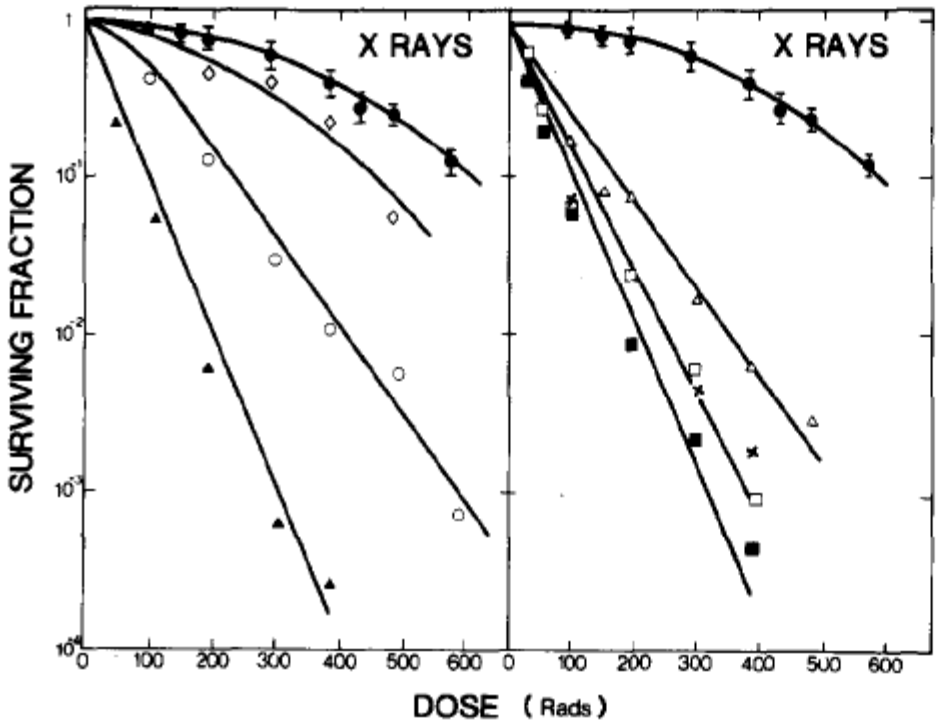


Fig. 1. X-ray survival of CHO-K1 and mutant strains: ●, CHO-K1; □, *xrs-1*; ○, *xrs-2*; ◇, *xrs-3*; △, *xrs-4*; ▲, *xrs-5*; ■, *xrs-6*; ×, *xrs-7*. The error bars when shown represent one standard deviation. Each survival curve represents the mean of at least three experiments.

Tooth-picking and screening
9,000 colonies!!!
Isolated seven (maybe only 6?)
X-ray sensitive clones!!

Women in Cell Science

Penelope Jeggo

Penelope Jeggo

Penny Jeggo was born in Cambridge, England. She obtained a BSc Honours degree in Microbiology at Queen Elizabeth College, University of London in 1970. She then did a PhD in the Genetics Division at the National Institute for Medical Research (NIMR), London, in Robin Holliday's laboratory. Her first postdoctoral position was with John Cairns at the ICRF Mill Hill Laboratory. She then obtained a postdoctoral fellowship with Miroslav Radman at the Université Libre de Bruxelles, Belgium. From there, she returned to the Genetics Division at NIMR as a scientific research officer. In 1989, she moved to the Medical Research Council's Cell Mutation Unit (CMU) at the University of Sussex. In 2001, following closure of the CMU on the retirement of the director, Penny became a founding member of the Genome Damage and Stability Centre (GDSC), a new collaborative research centre established by the University of Sussex and the Medical Research Council.

Penny's research has focused on DNA damage responses and particularly on the repair of DNA double strand breaks (DSBs). She applied the techniques learnt during her early years working with lower organisms to isolate mammalian cell lines sensitive to ionising radiation. Using these cell lines, she characterised the major DSB repair pathway in higher organisms and, in collaborative work, showed that the DSB repair pathway also functions during V(D)J recombination, a critical process during immune development. The cell lines were also pivotal in allowing her to identify the first mammalian genes that significantly contribute to the response to radiation exposure and to V(D)J recombination. The GDSC houses the UK's largest collection of cell lines from patients with damage response disorders. Penny has exploited and extended this resource to identify patients deficient in DSB repair, as well as additional damage response genes. Such studies have provided insights into the role of the damage response pathways in human development and cancer avoidance. She has recently discovered an important connection between the signalling response to DNA damage and the DNA repair machinery, which makes a significant contribution to the response of human cells to ionising radiation. She continues to focus on understanding the basis of human radiosensitivity.

In the interview below, Fiona Watt, Editor-in-Chief of JCS, asks Penny about her experiences as a woman in science.

FMW: *How has your research career impacted on your personal life and vice versa?*

PJ: I consider myself just plain lucky to have chosen a career that continues to excite and motivate me. It is this aspect of a career in science that has impacted upon my personal life in a positive way and provided the raison d'être to endure the hard work and the more difficult challenges. I remember the thrill of doing an undergraduate laboratory project; although my results contributed only the tiniest smidgeon to scientific knowledge, I gained immense satisfaction from it. I was lucky in having two wonderful mentors in my early days of research: Robin Holliday, in whose laboratory I studied for my PhD; and John Cairns, with whom I undertook my first postdoctoral position. In addition to being excellent scientists, they enhanced my ability to enjoy and be excited by science. I believe this is a defining criterion of a great career – if the highs are high enough, the lows can be endured.

As a young postdoc, I was lucky in having a partner who understood the joy I gained from laboratory life. Though not a research scientist, and certainly not motivated in the same way as me, he supported my needs and achievements, and encouraged my independence. Finally, he found his own job satisfaction, which resulted in us commuting between Germany and England for a couple of years.

Enjoying science as I did, and taking the opportunity to do a postdoctoral fellowship outside the UK with Miroslav Radman, I didn't worry too much about starting a family. But as my thirties progressed, the motherhood desire set in and new excitement entered my life. Tragically, my partner, who had endured

Penny Jeggo with her son, Matthew, taken in the Grange Gardens at Lewes, East Sussex around 1990.

sympathy pains during the course of my pregnancy, was diagnosed with colon cancer not long after our baby was born and died within a year. I felt let down by cancer research as a career and I might well have quit, had it not been for the support of wonderful colleagues and friends. But I recovered the fire, and before long the thrill of a good result was sustaining me through the difficult times of being a single parent in a demanding career.

A wonderful aspect of science is the ability to form friendships around the world. A few years after the death of my husband, I had the opportunity to re-establish myself and make new friends by undertaking a sabbatical in the USA. This was of huge personal benefit. Although it becomes increasingly tiring, I still love to travel and meet with my international friends and colleagues.

Since the start of my PhD, I have been fascinated by DNA recombination and repair. At that time, the importance of genomic stability was not well appreciated, and at ICRF (now Cancer Research UK) viruses and viral oncogenes were considered to be all important. The work in Cairns's laboratory on DNA repair was only tolerated as a concession that some basic research had to be undertaken. Those of us in the field were convinced, however, that the maintenance of genomic stability was central to cancer avoidance. I still feel thrilled by the recent conversion of many scientists to appreciate the importance of my field!

I rarely felt guilty about the limitations that a research career places on

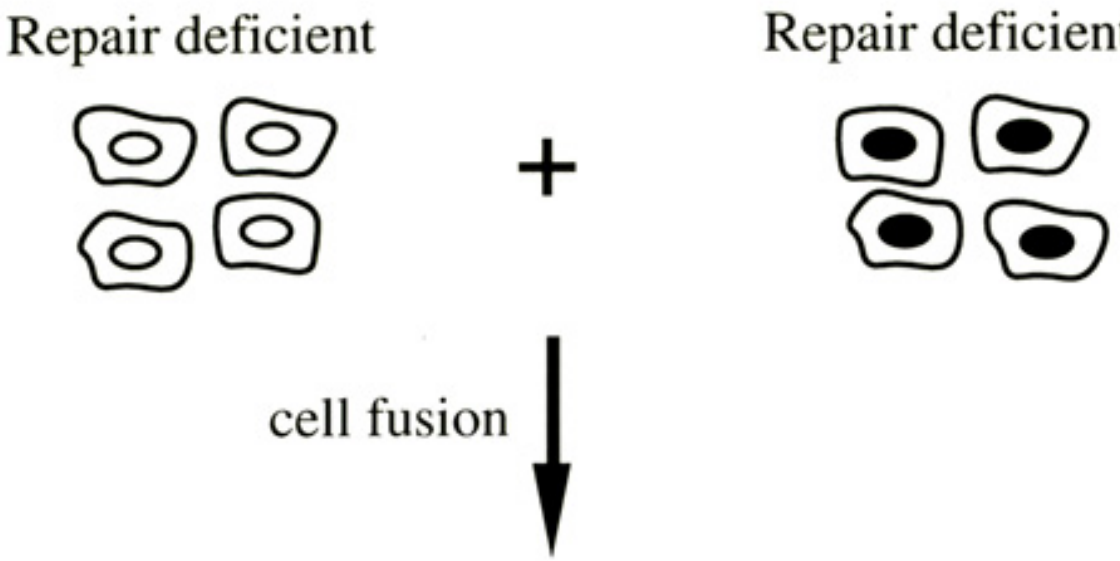
Watt F M J Cell Sci 2004;117:5459-5460

Journal of Cell Science jcs.biologists.org

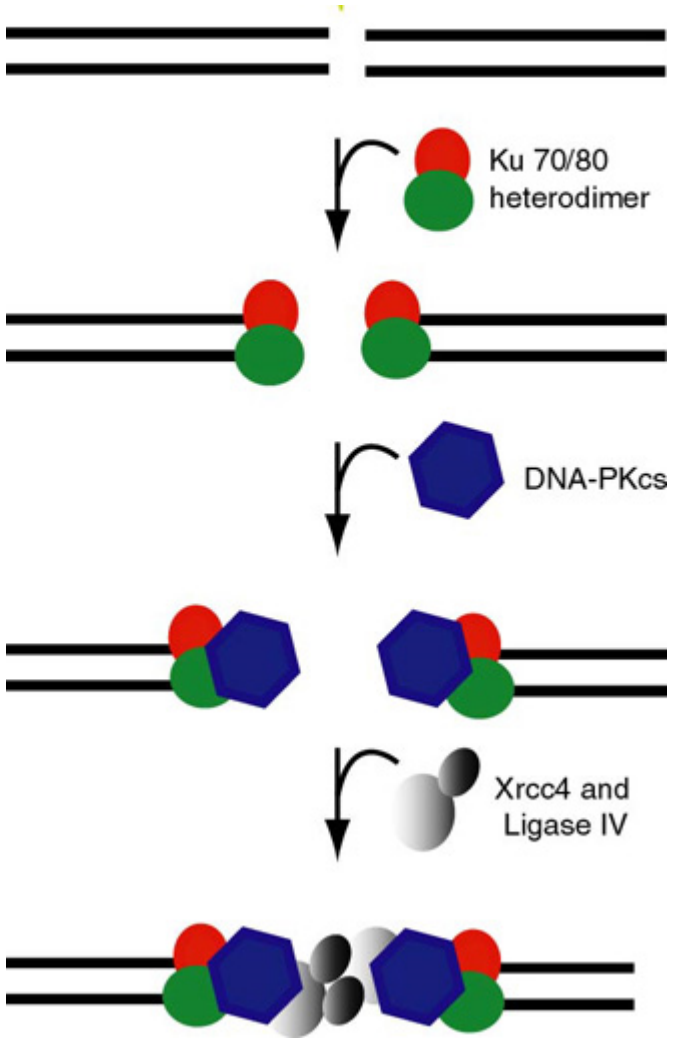
- You now have 6 or 7 mutant cell lines with similar phenotype

- How do you figure out if the cell lines have mutations in the same genes, or in different genes?

Complementation groups



Non-Homologous End Joining (NHEJ)



Ku70
Ku80

DNA-PKcs

Xrcc4
Ligase IV

What is the experiment?

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

Check list of critical materials to be used:

1. Wild type CHO-K1 cells
2. CHO xrs6 cells, deficient in Ku80 protein
3. C401 inhibitor of DNA-PKcs
4. Plasmids containing DNA double strand breaks of different topologies.

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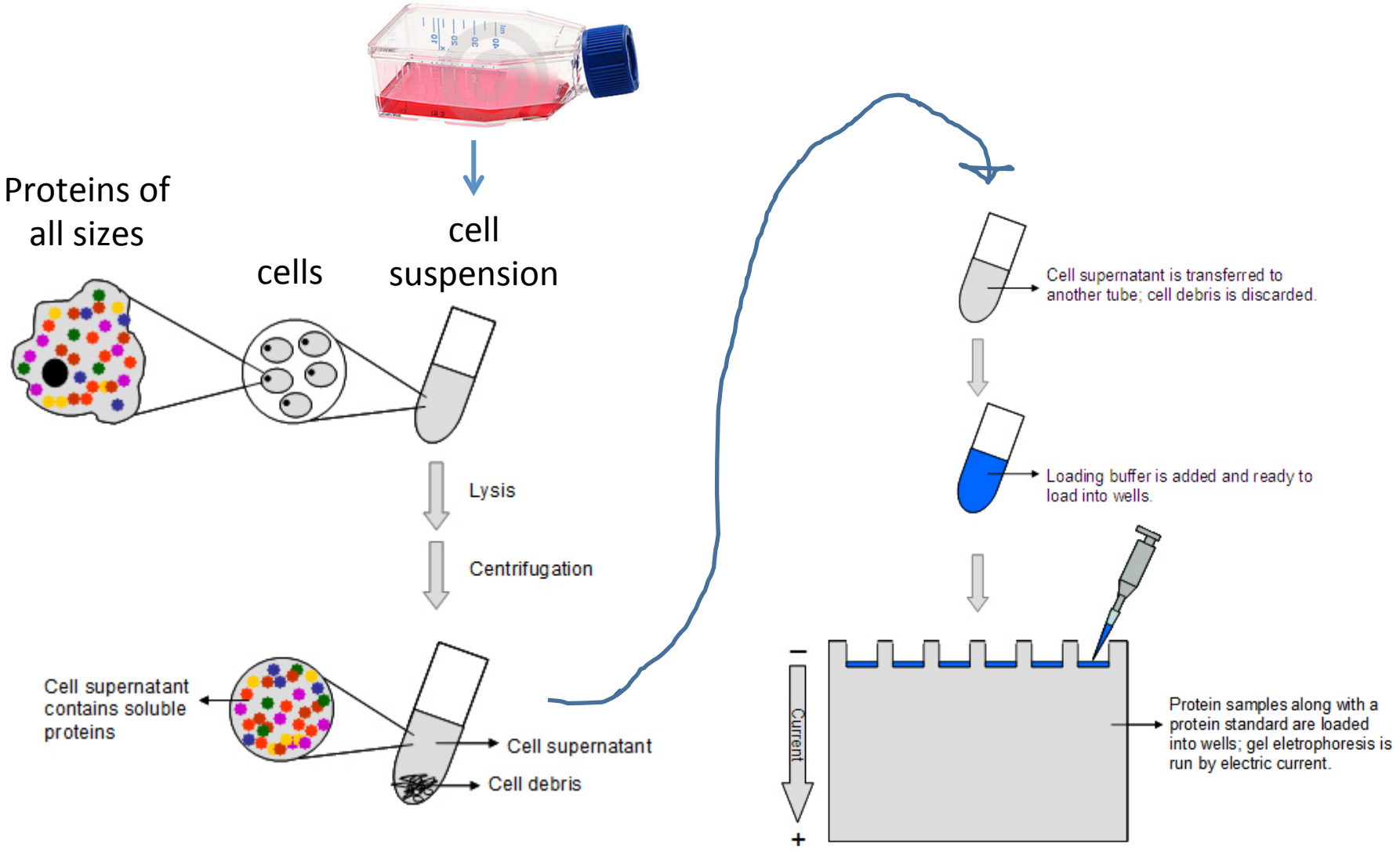
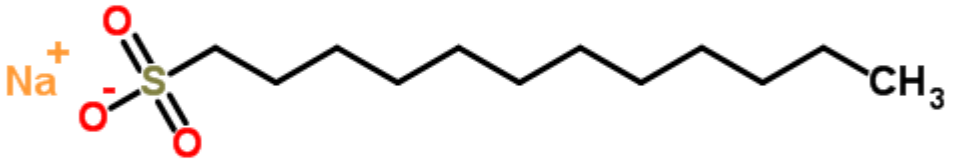
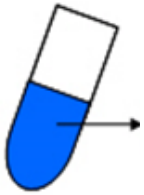
Western Blot or Immunoblot Analysis



Separating proteins on an SDS polyacrylamide gel

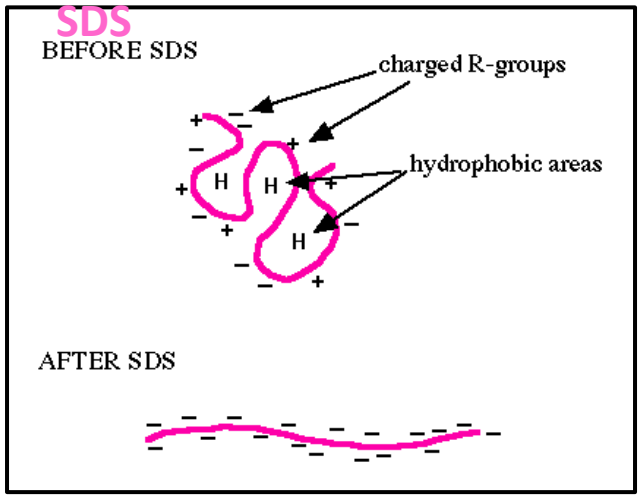


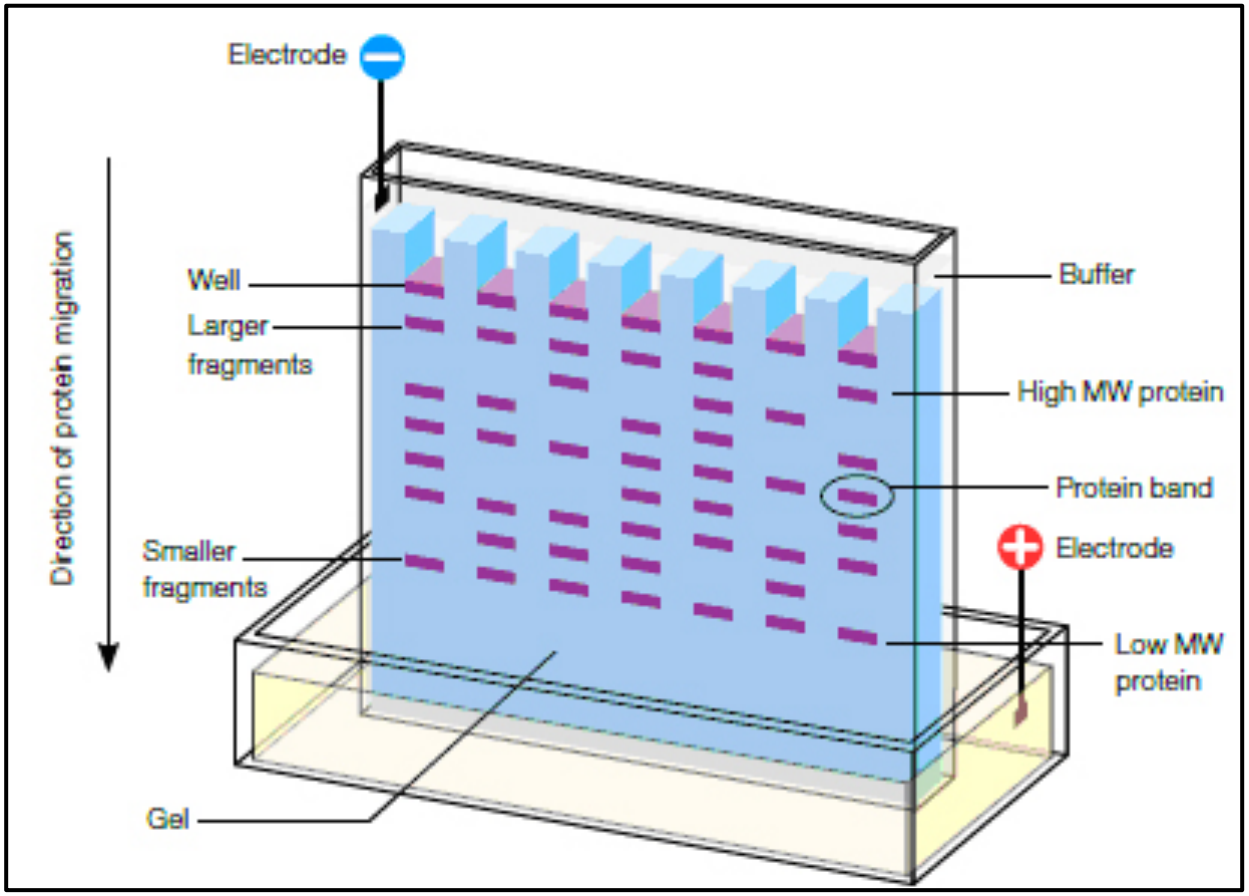
SDS – sodium dodecyl sulfate

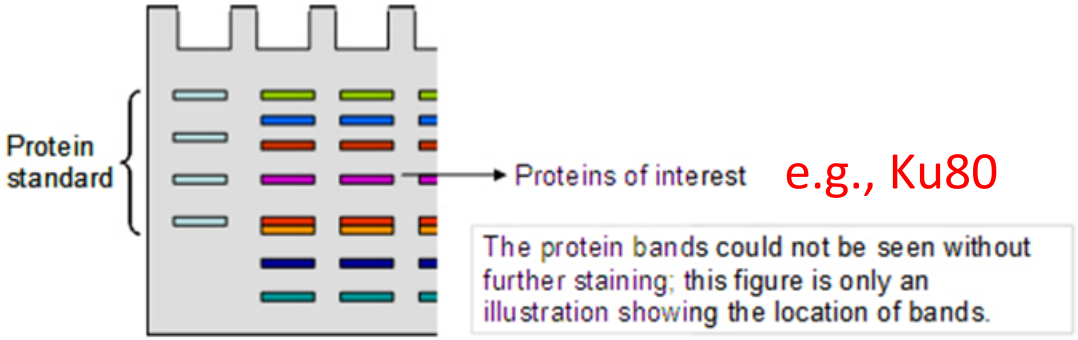


SDS to denature proteins plus blue dye – loading buffer

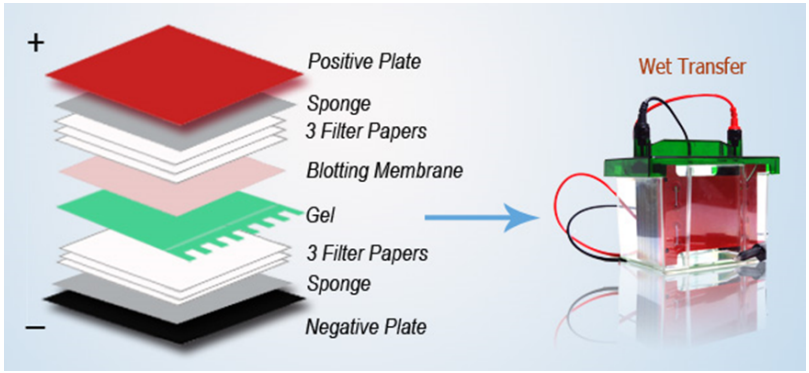
Proteins denatured by SDS

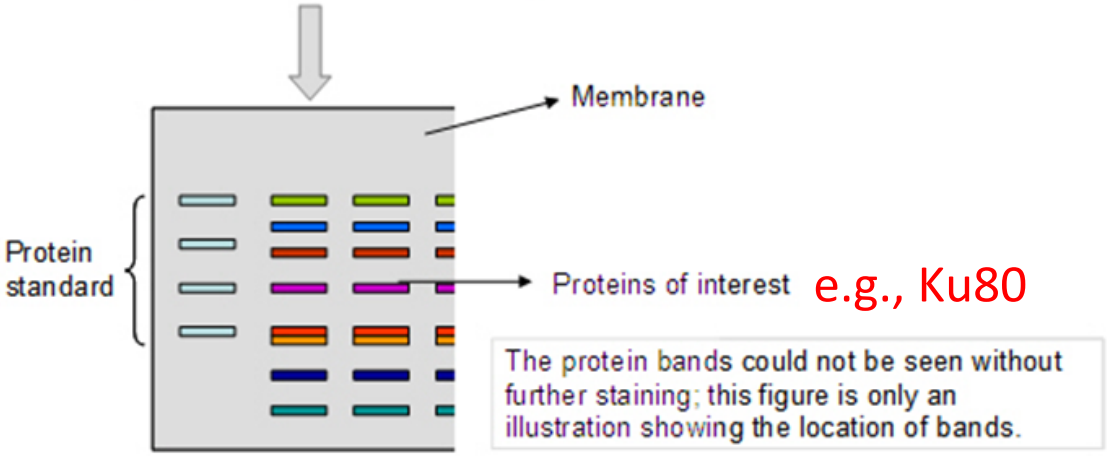


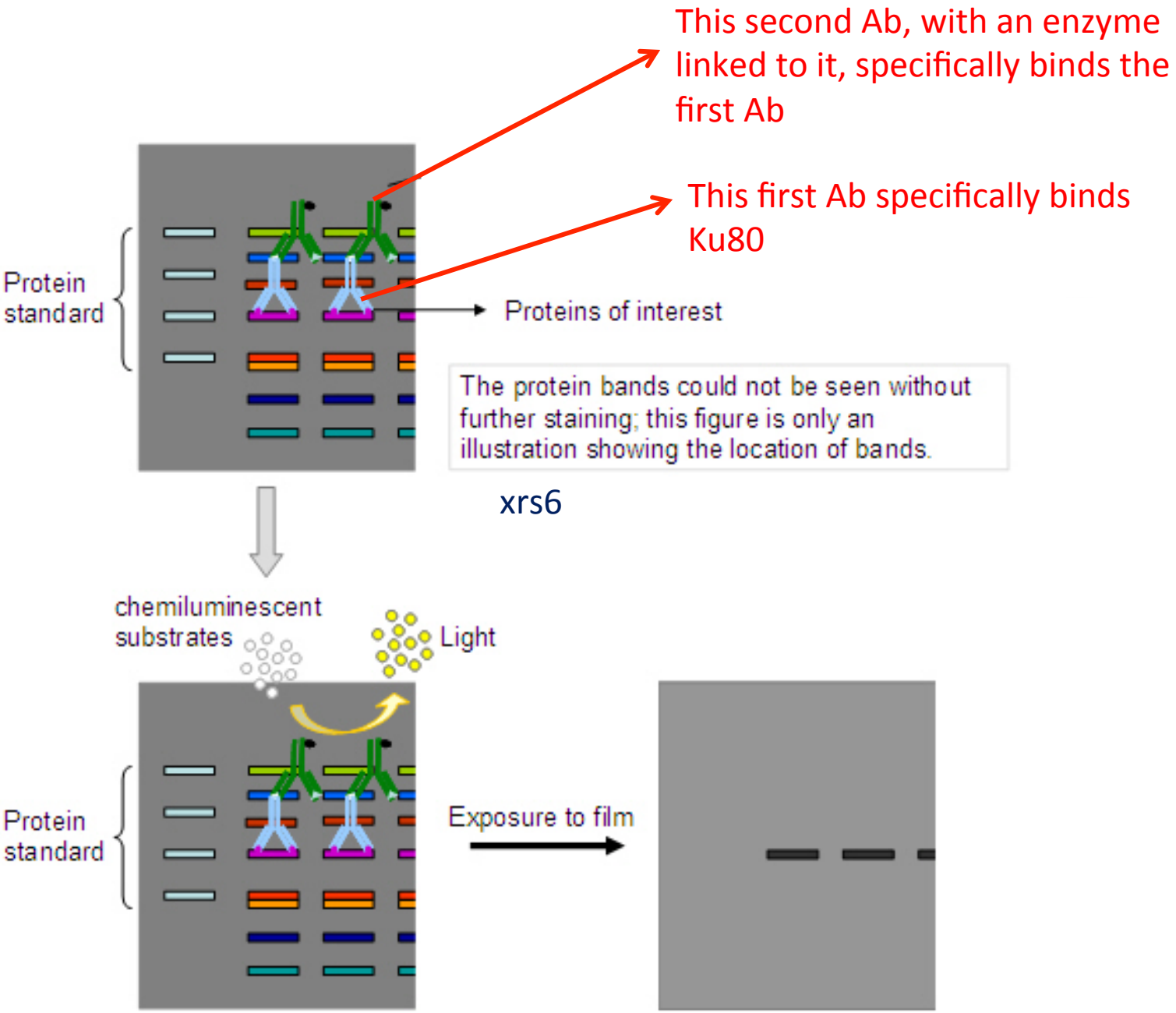




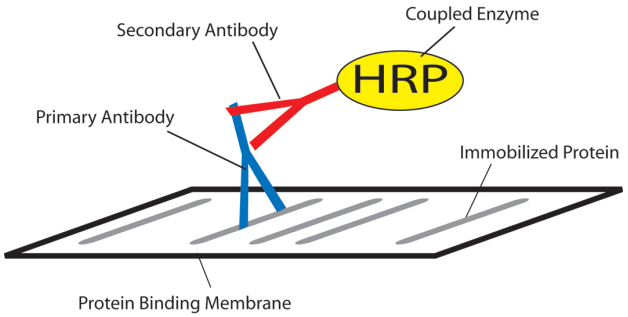
Transfer (Semi-dry transfer as an example)

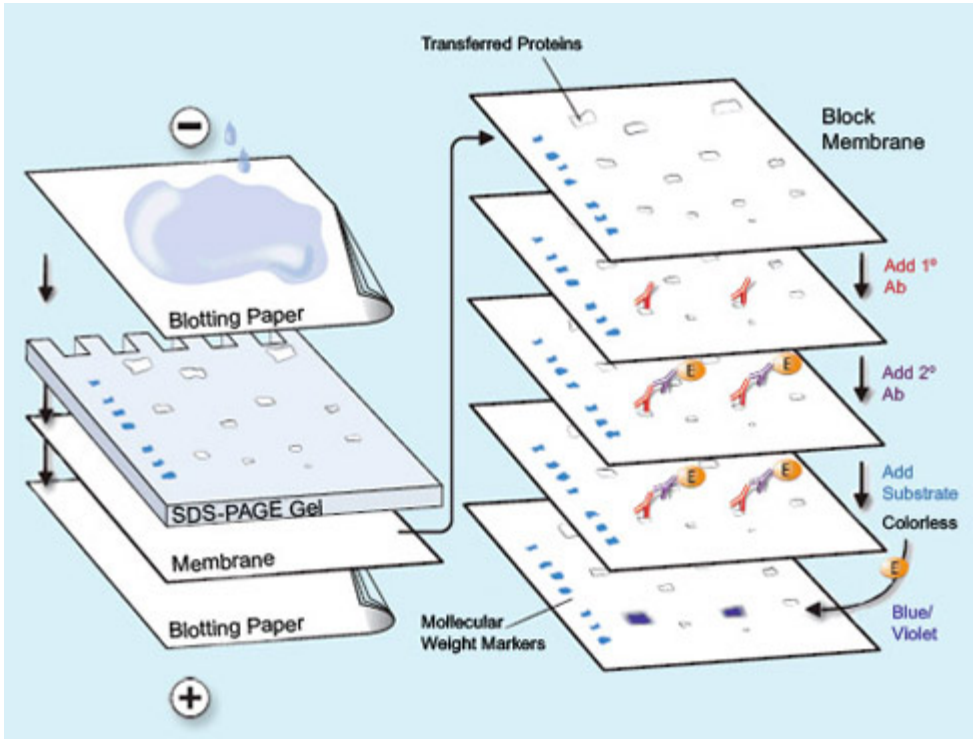





This second Ab, with an enzyme linked to it, specifically binds the first Ab
This first Ab specifically binds Ku80
Protein standard
Proteins of interest
The protein bands could not be seen without further staining; this figure is only an illustration showing the location of bands.
xrs6
chemiluminescent substrates
Light
Protein standard
Exposure to film

Horseradish Peroxidase





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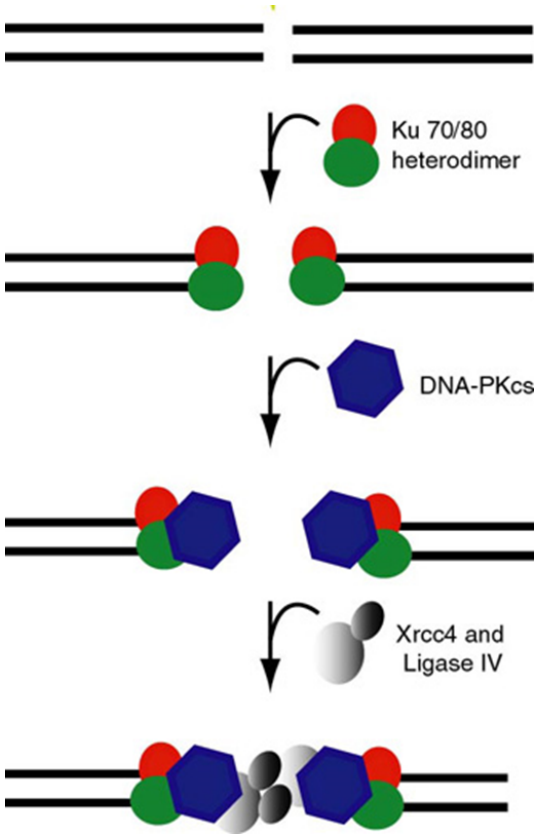
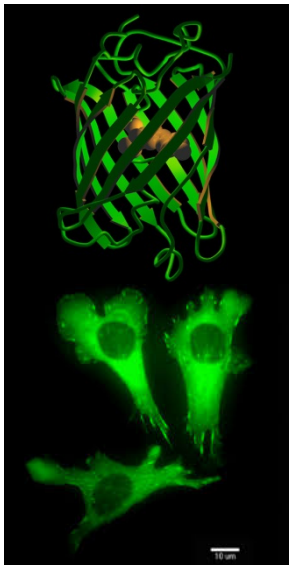


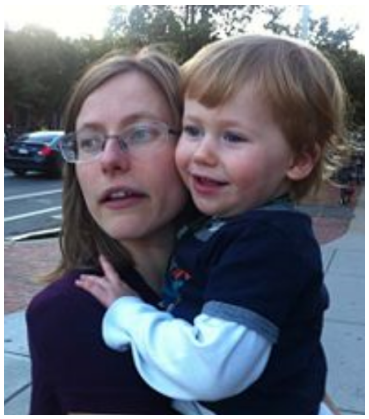
Chinese Hamster Ovary (CHO) cells are immortal – they can grow indefinitely

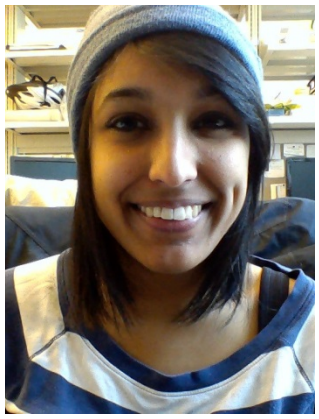
NEXT LECTURE

- Isolating X-ray-sensitive (xrs) CHO cells
- Xrs cells are deficient in NHEJ
- Detecting NHEJ proteins by Western
- Measuring NHEJ activity
- Using fluorescent proteins to measure biological processes….



20.109 Spring 2014 Module 2
System Engineering and Protein Foundations



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Suhani Vora (TA)

Leona Samson (Lectures)

Zachary Nagel (help with development)