20.109 Spring 2016 Module 2 – End of Lecture 1
# System Engineering (March 10th 2016)

Noreen Lyell
Leslie McLain
Maxine Jonas
Jing Zhang(TA)
Leona Samson (Lectures)
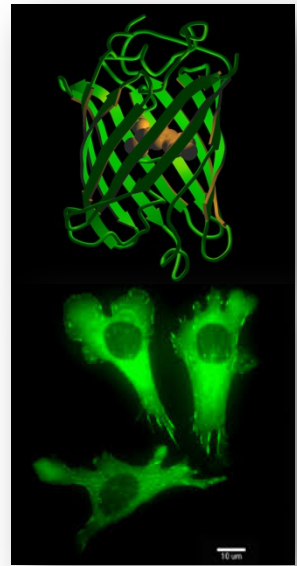Zachary Nagel (help with development) Alex Chaim

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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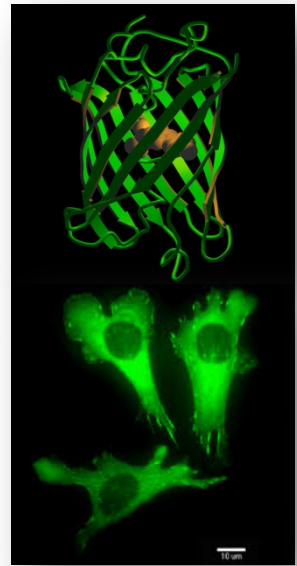
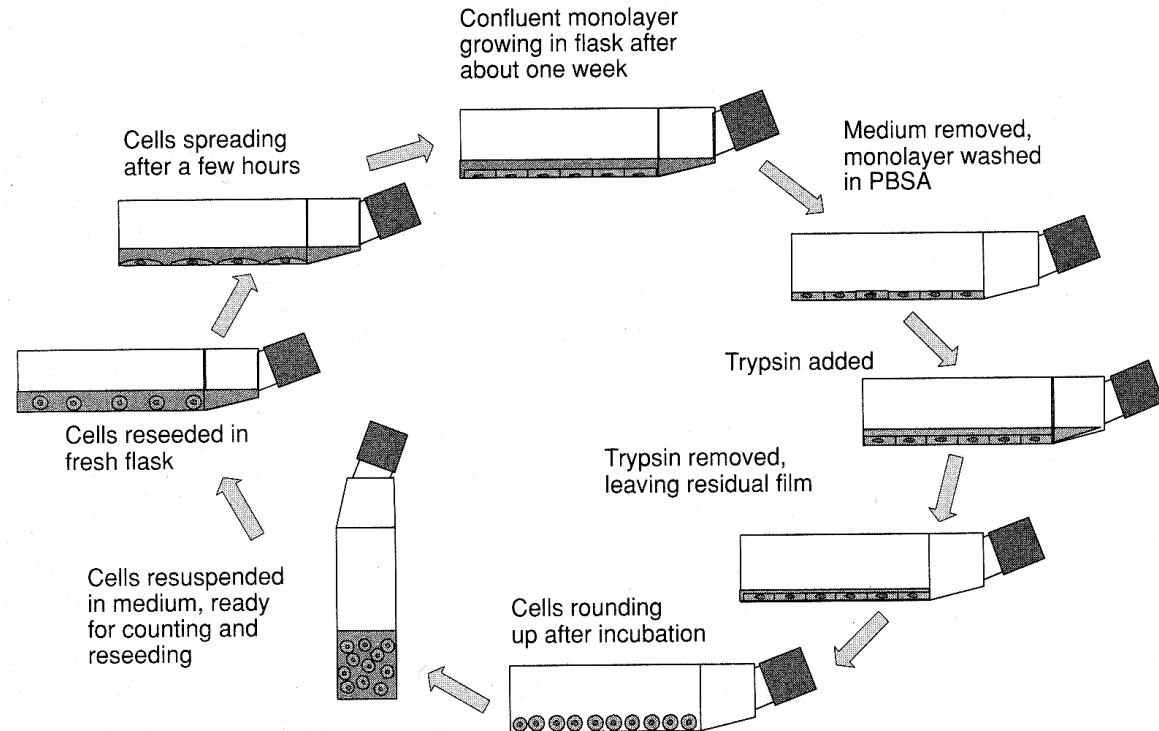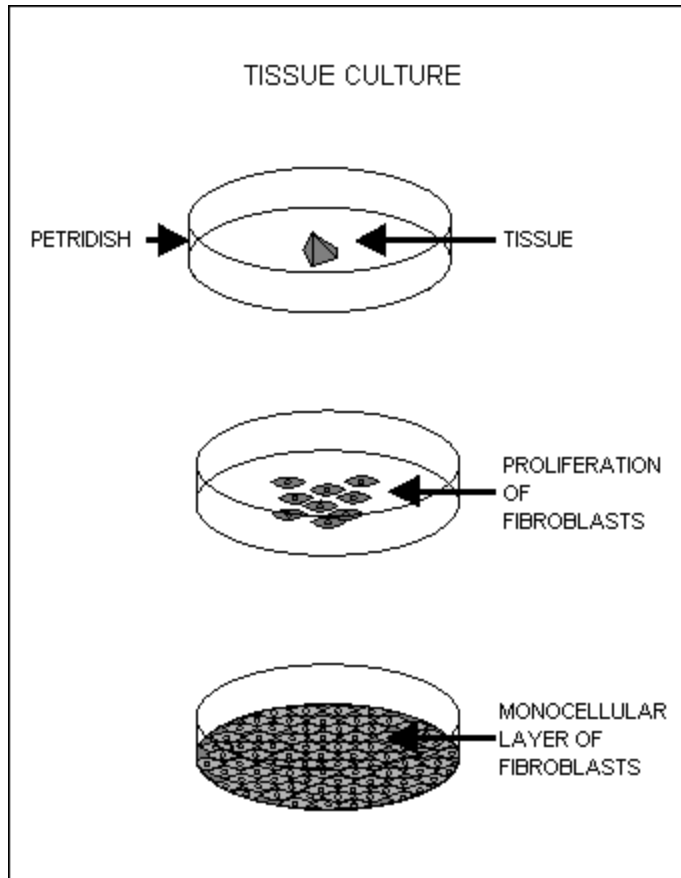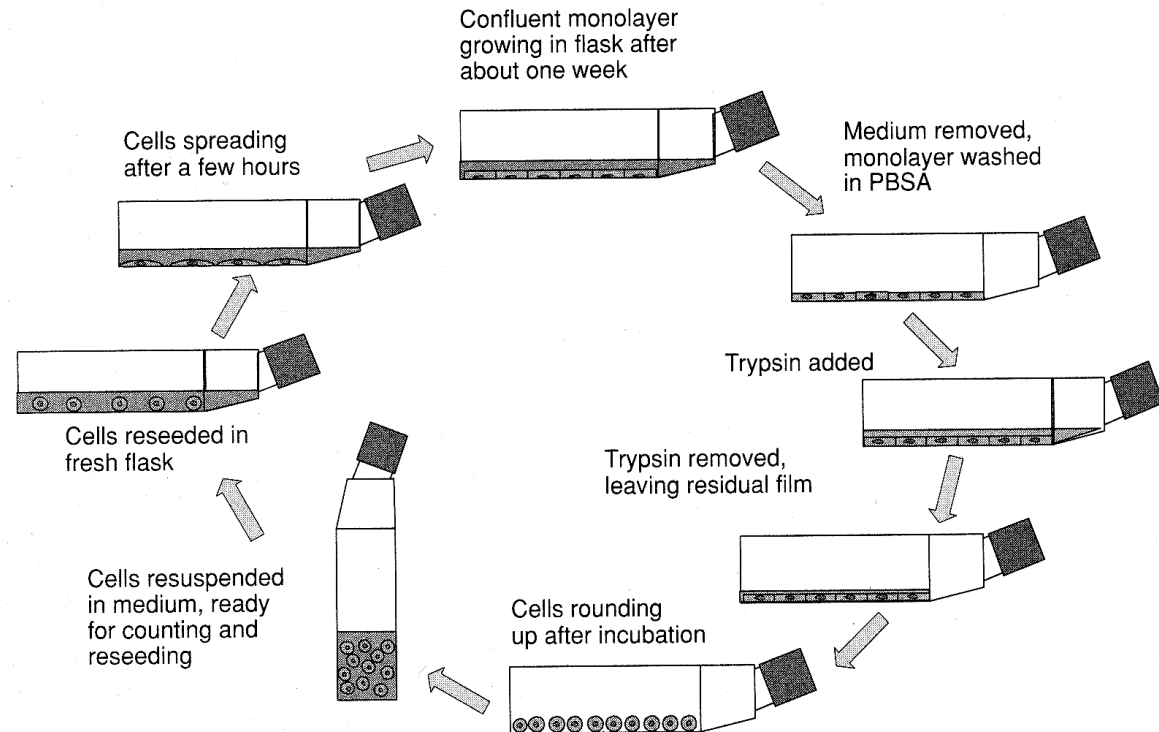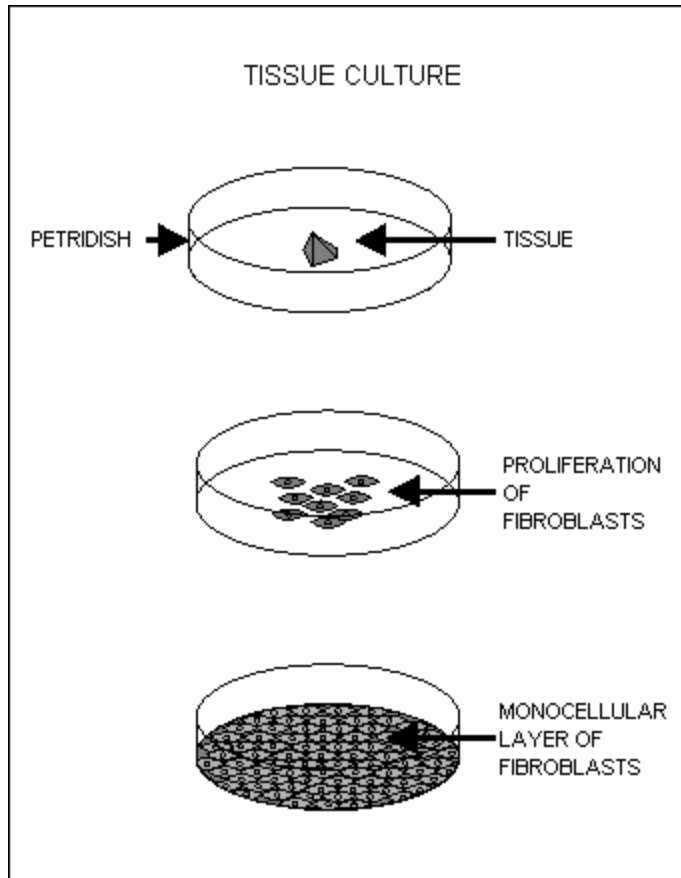- Statistical analysis of biological data

How do you grow mammalian cells?

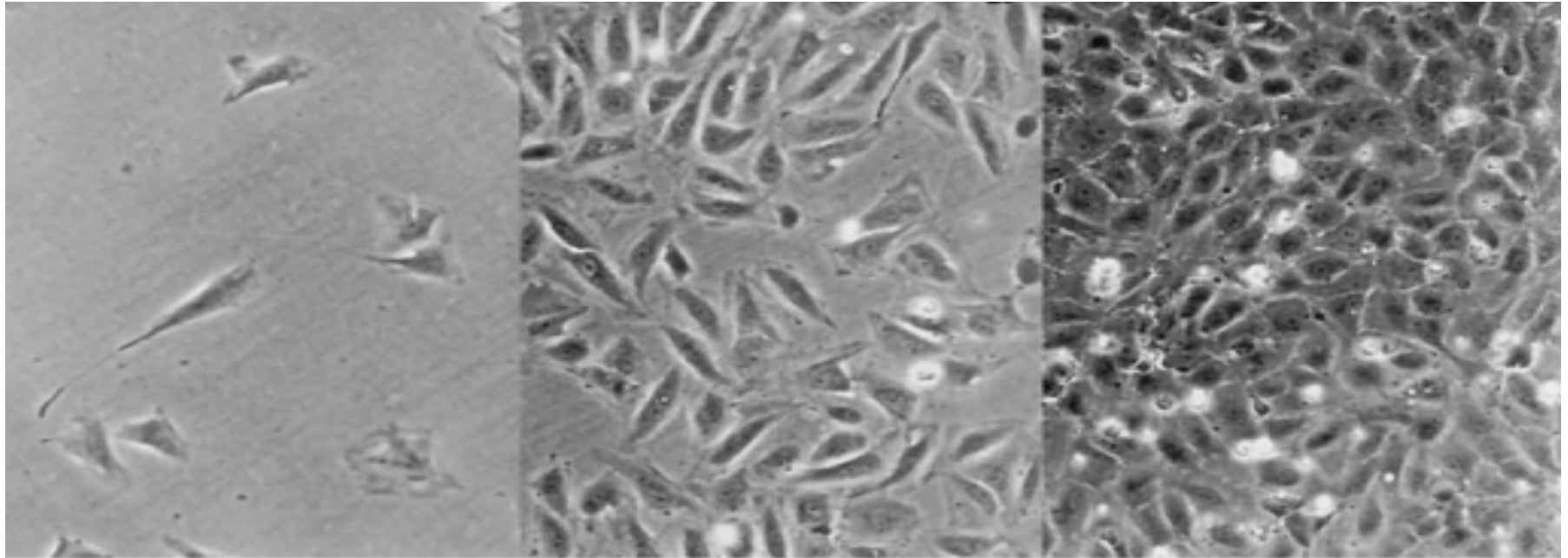From Freshney's "Culture of Mammalian Cells"

How do you grow mammalian cells?

TISSUE CULTURE

PETRIDISH — TISSUE

PROLIFERATION OF FIBROBLASTS

MONOCELLULAR LAYER OF FIBROBLASTS

Confluent monolayer growing in flask after about one week

Medium removed, monolayer washed in PBSA

Cells spreading after a few hours

Trypsin added

Cells reseeded in fresh flask

Trypsin removed, leaving residual film

Cells resuspended in medium, ready for counting and reseeding

Cells rounding up after incubation

"Sub-Culturing"

From Freshney's "Culture of Mammalian Cells"
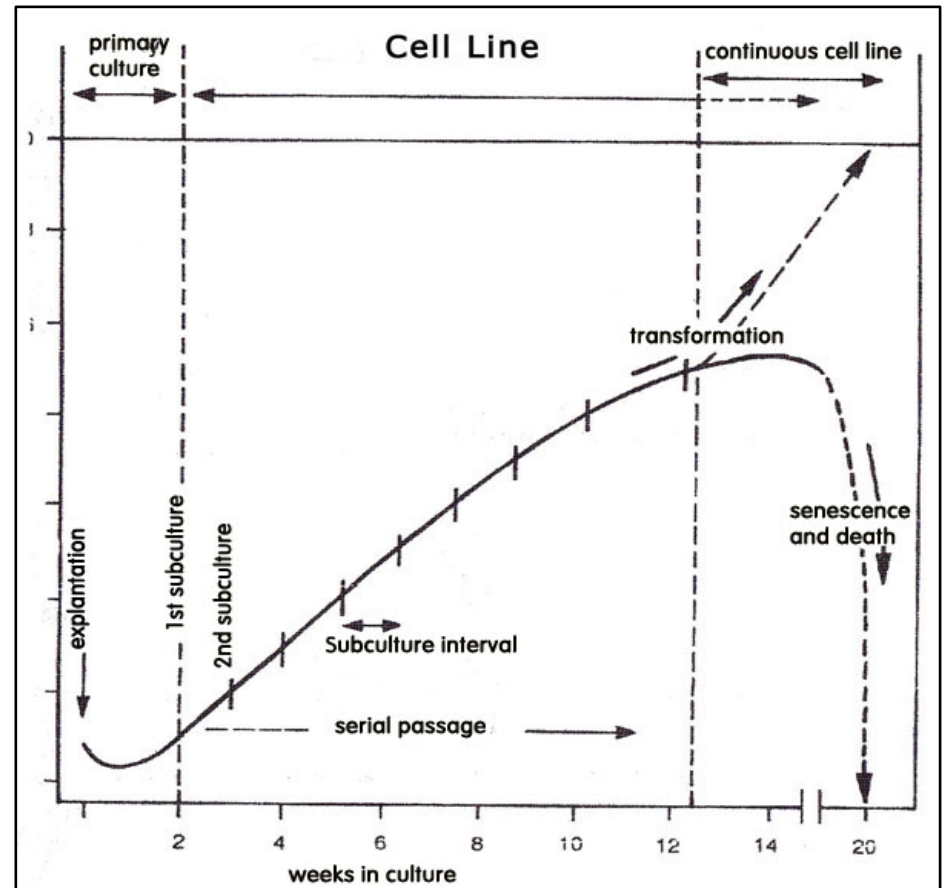
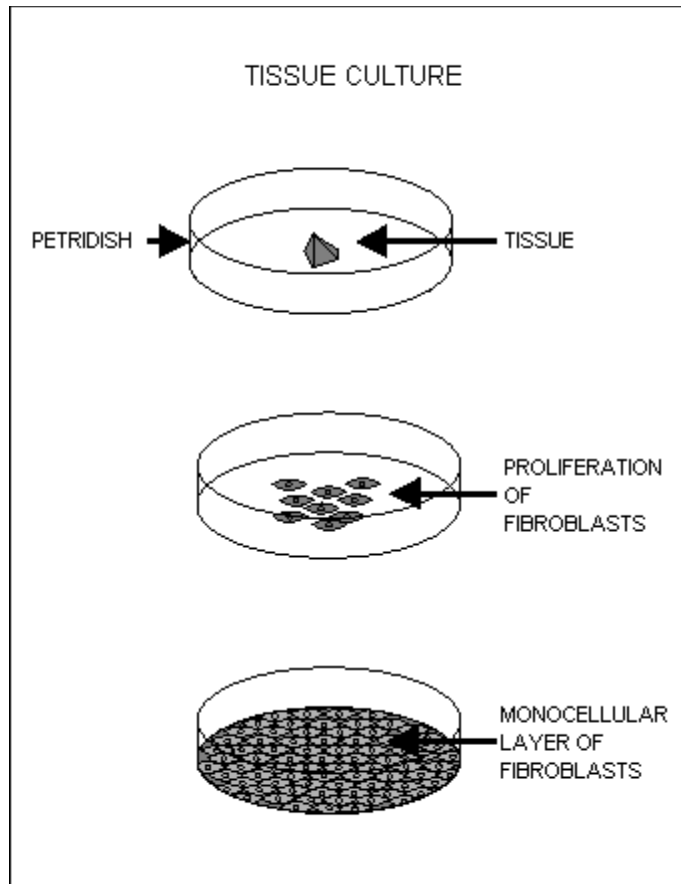# How do you grow mammalian cells?

Just attached after seeded
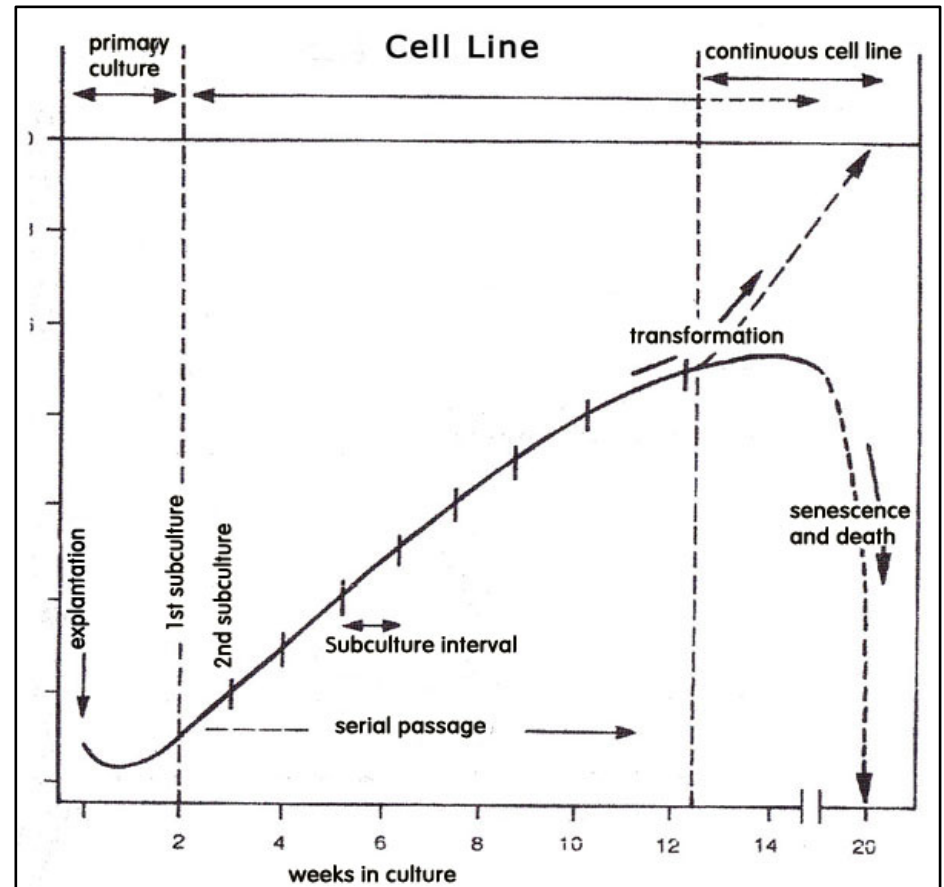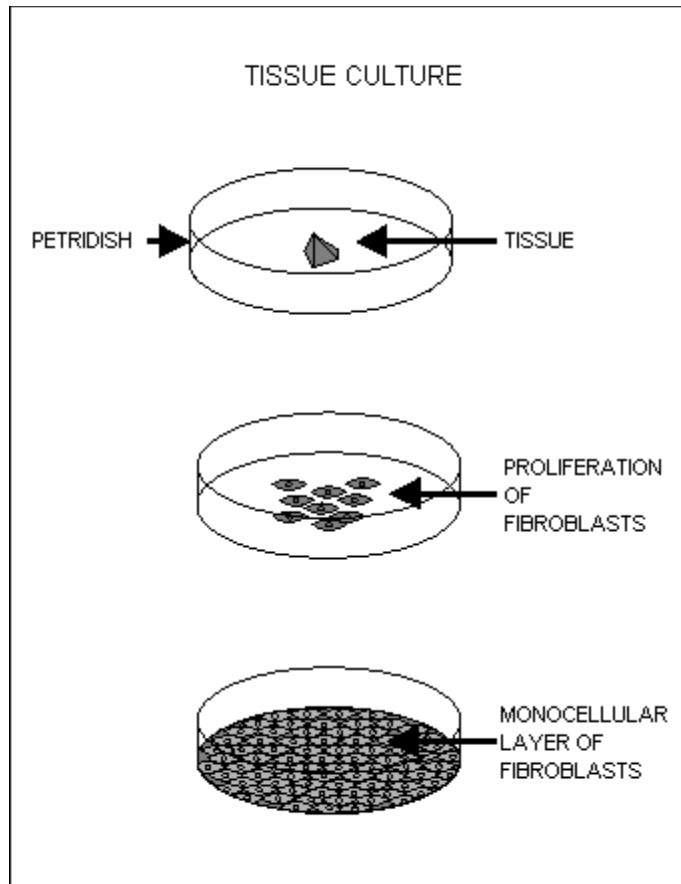
Growing
(Sub-Confluent)

Confluent

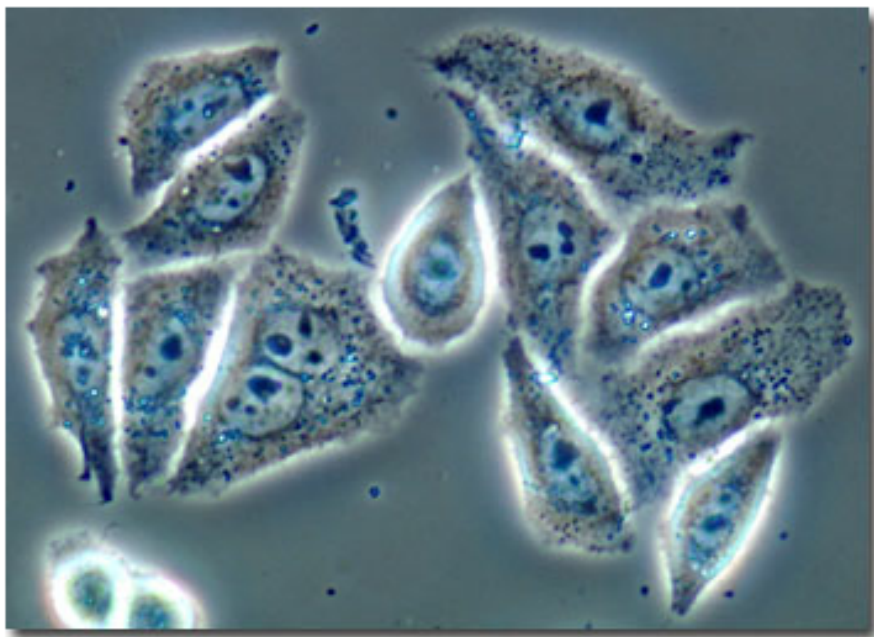# How do you grow mammalian cells?

From Freshney's "Culture of Mammalian Cells"

How do you grow mammalian cells?

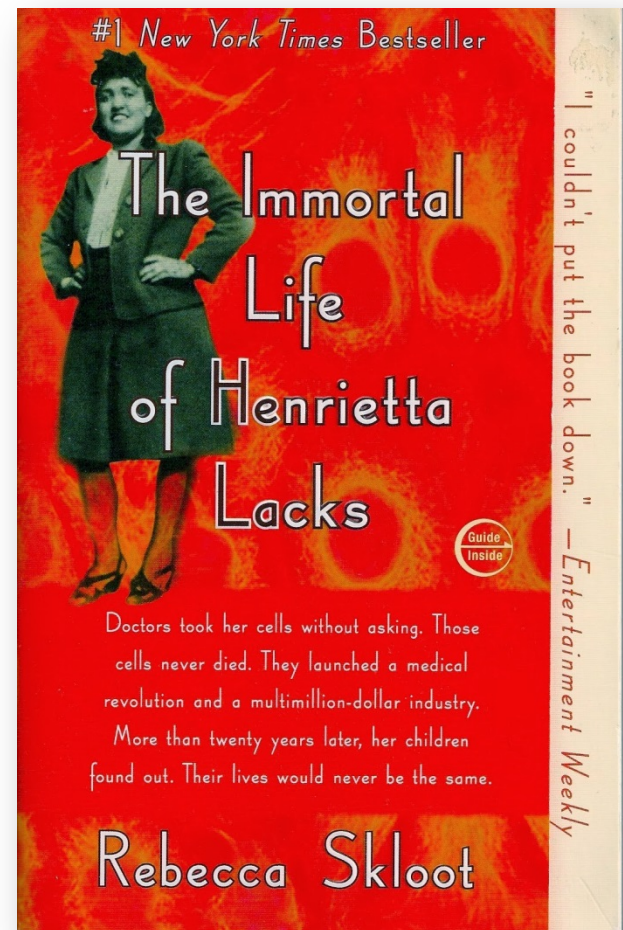This is for normal tissue, what about tumor tissue?

From Freshney's "Culture of Mammalian Cells"

Phase Contrast

HeLa cells from the Nikon microscope web site

HeLa cells have been cultured continuously for scientific use since they were first taken from the ovarian tumor of **Henrietta Lacks** suffering from cervical cancer in the 1950s. They have   been utilized for many purposes, including the development of a polio vaccine, the pursuit of a cure for diseases such as leukemia and cancer, and the study of the cellular effects of drugs and radiation.

Grade IV Glioma Glioblastoma Mulitiforme (GBM)
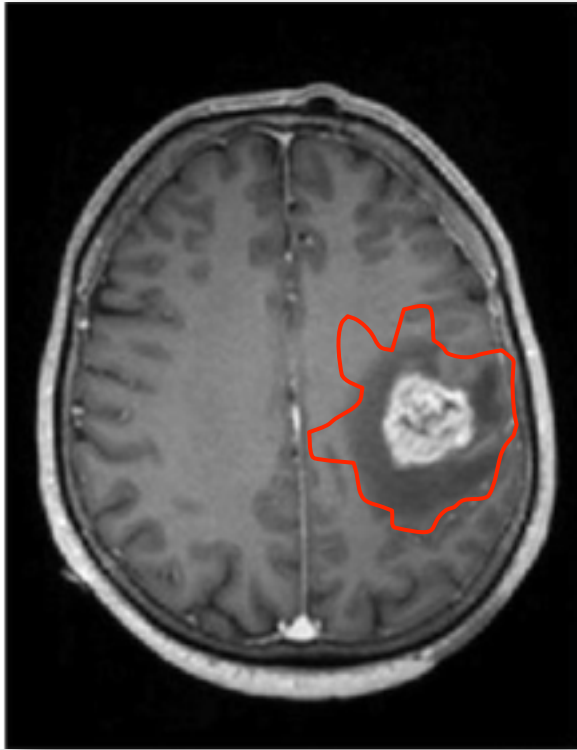
➢ Most common and malignant form of brain cancer

➢ Affects 10,000 people a year in North America alone

➢ Median survival is around 10 months after diagnosis

➢ Highly diffusive tumor type

➢ Treatment consists of surgical resection, followed by radiotherapy and chemotherapy
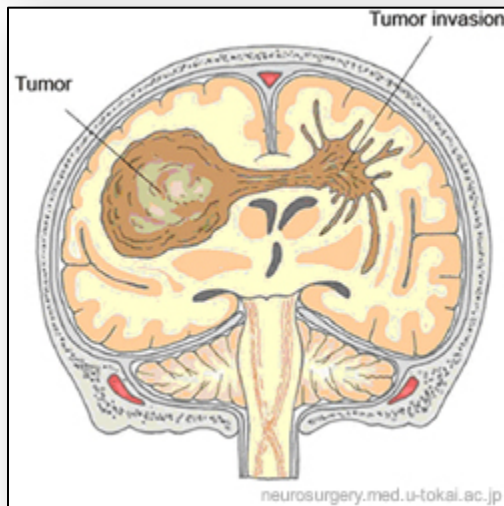
Isolation of Two Cell Lines from a Human Malignant Glioma Specimen Differing in Sensitivity to Radiation and Chemotherapeutic Drugs

M. J. Allalunis-Turner,* G. M. Barron,* R. S. Day III,† K. D. Dobler,† and R. Mirzayans†

*Radiobiology Program, Department of Radiation Oncology, and †Molecular Oncology Program, Department of Medicine, Cross Cancer Institute, Edmonton, Alberta, Canada
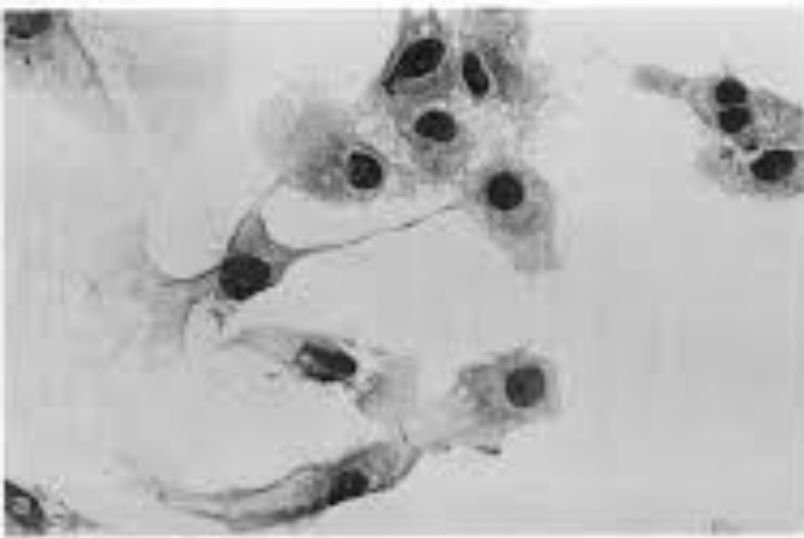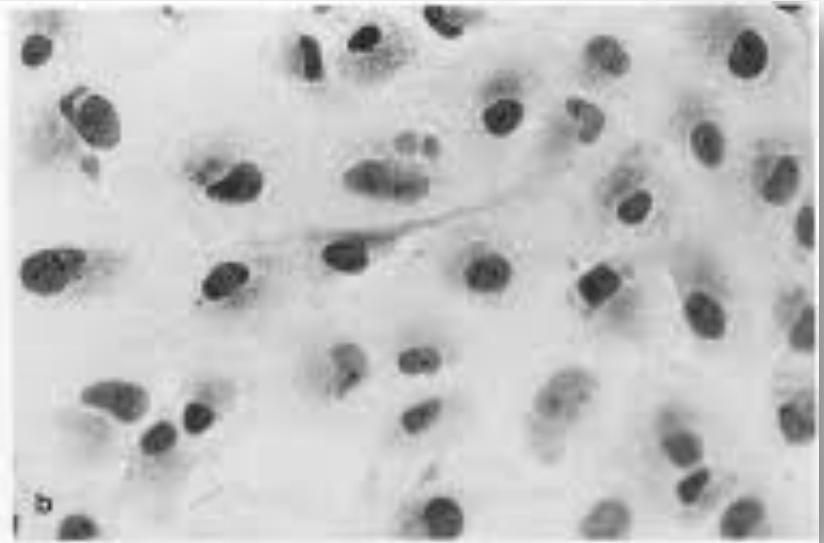
M059J   M059K

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M. J. ALLALUNIS-TURNER,* G. M. BARRON,* R. S. DAY III,† K. D. DOBLER,† AND R. MIRZAYANS†

*Radiobiology Program, Department of Radiation Oncology, and †Molecular Oncology Program, Department of Medicine, Cross Cancer Institute, Edmonton, Alberta, Canada

FIG. 3. The effect of ^{137}Cs γ irradiation on colony-forming ability in M059J (□) and M059K (■) cells. Survival curves were derived from a simple line fit of the data points. Mean values (±SE) derived from five separate experiments are shown.

Isolation of Two Cell Lines from a Human Malignant Glioma Specimen Differing in Sensitivity to Radiation and Chemotherapeutic Drugs

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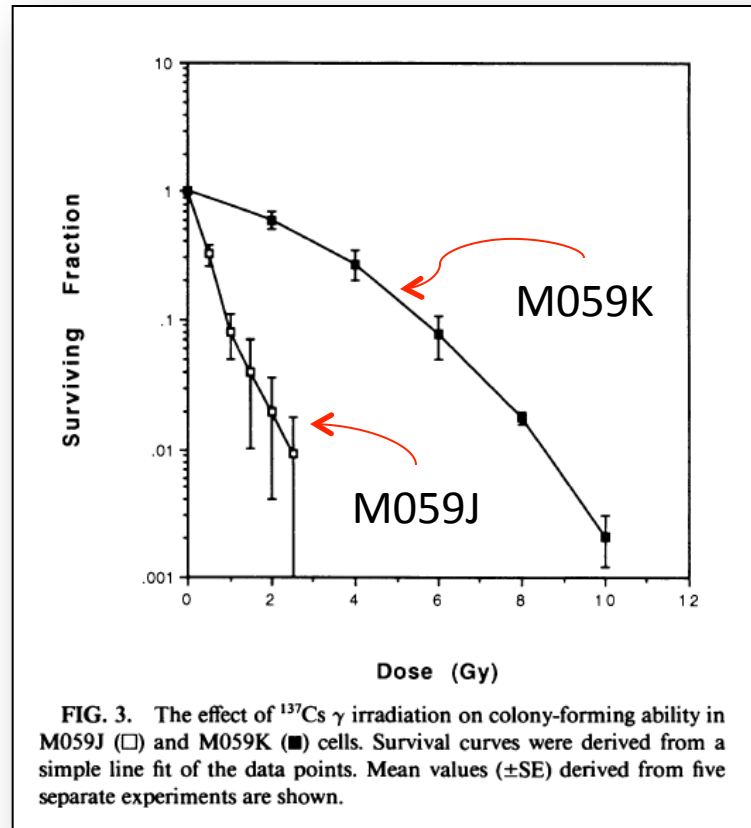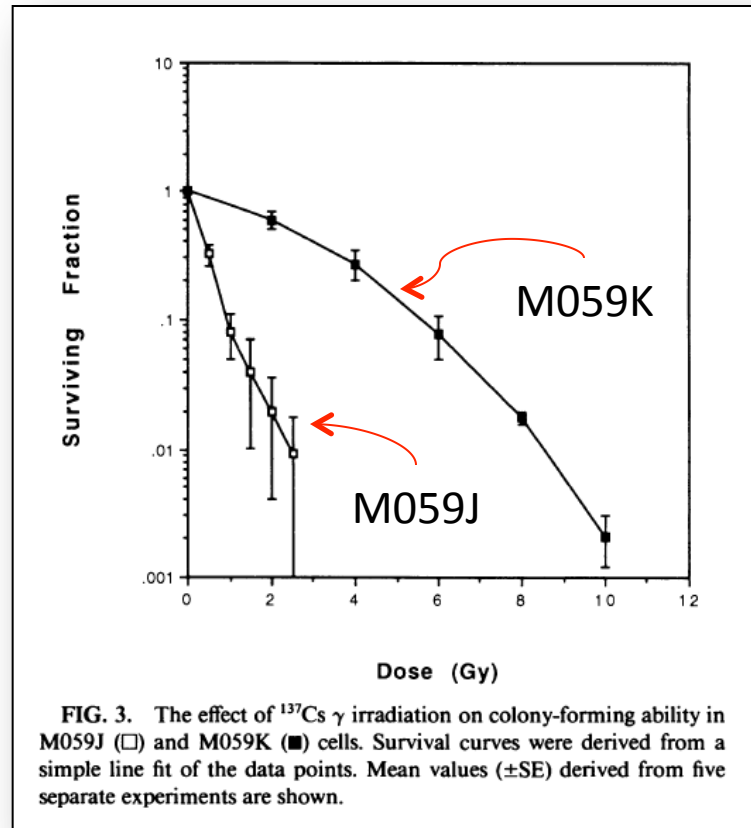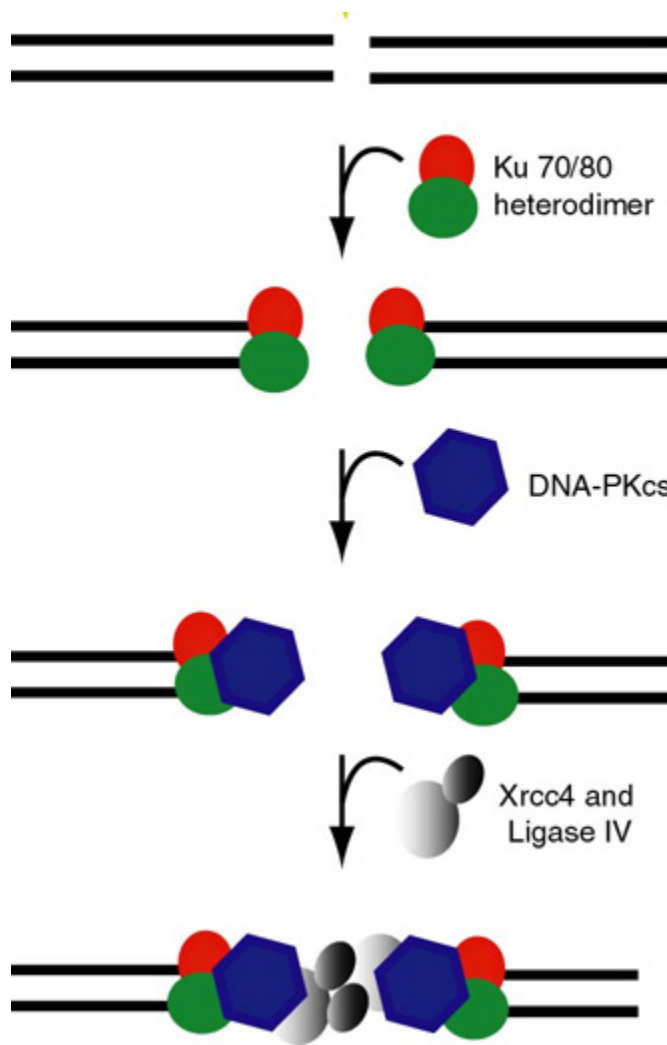
M059K

K = oKay after γ Rays

M059J

J = Junk after γ Rays

FIG. 3. The effect of ^{137}Cs γ irradiation on colony-forming ability in M059J (□) and M059K (■) cells. Survival curves were derived from a simple line fit of the data points. Mean values (±SE) derived from five separate experiments are shown.

Non-Homologous End Joining (NHEJ)

Ku70
Ku80

DNA-PKcs

Xrcc4
Ligase IV

20.109 Spring 2016 Module 2 – Lecture 2
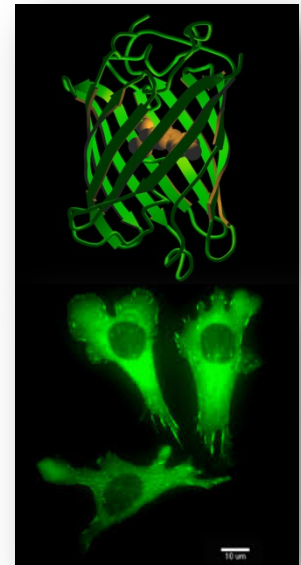# System Engineering (March 10th 2016)

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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?
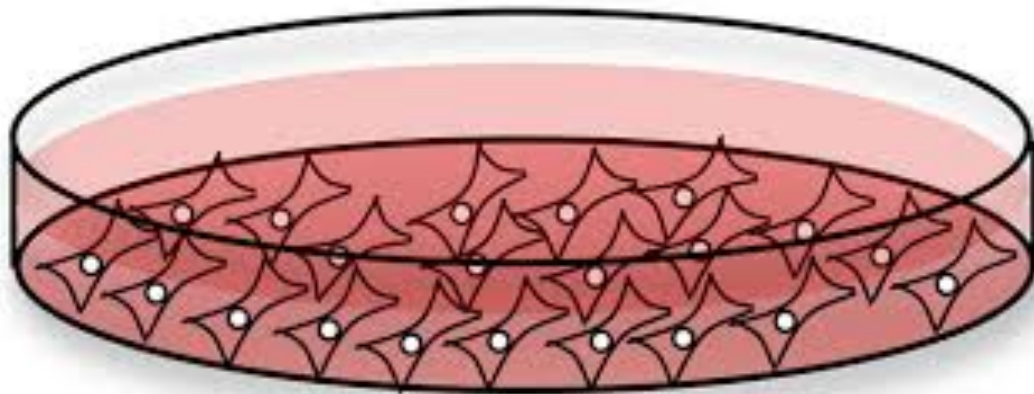

This raises the following questions

- 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?
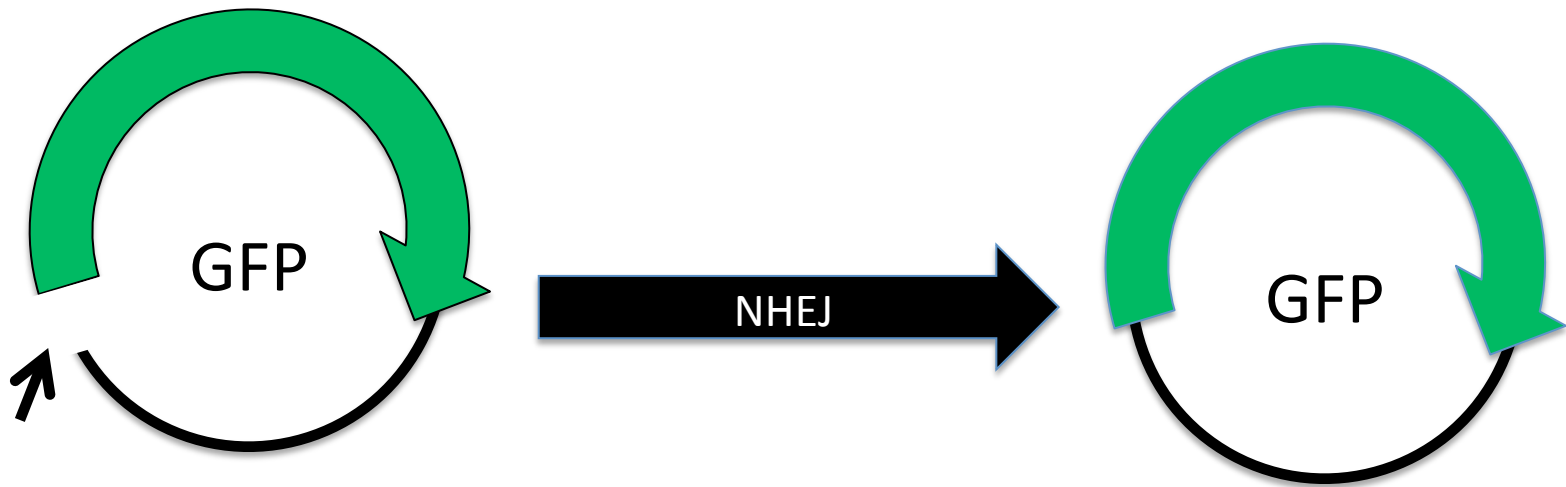
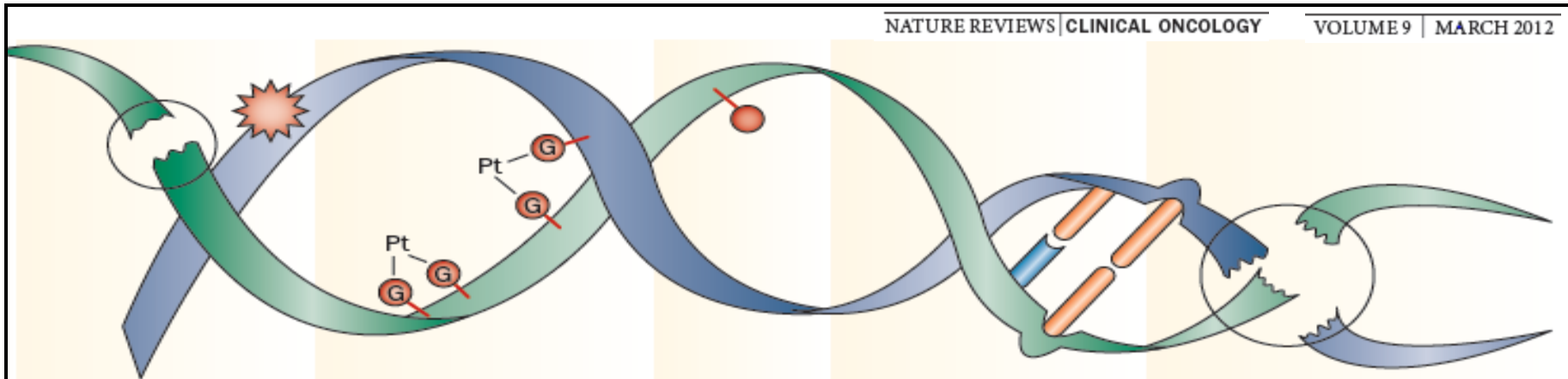Substrate contains a DNA double strand break

Six Major DNA Repair Pathways
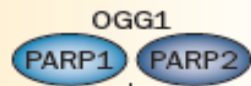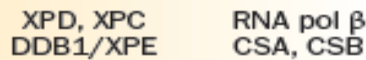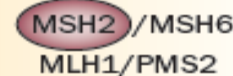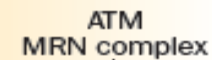
Six Major DNA Repair Pathways
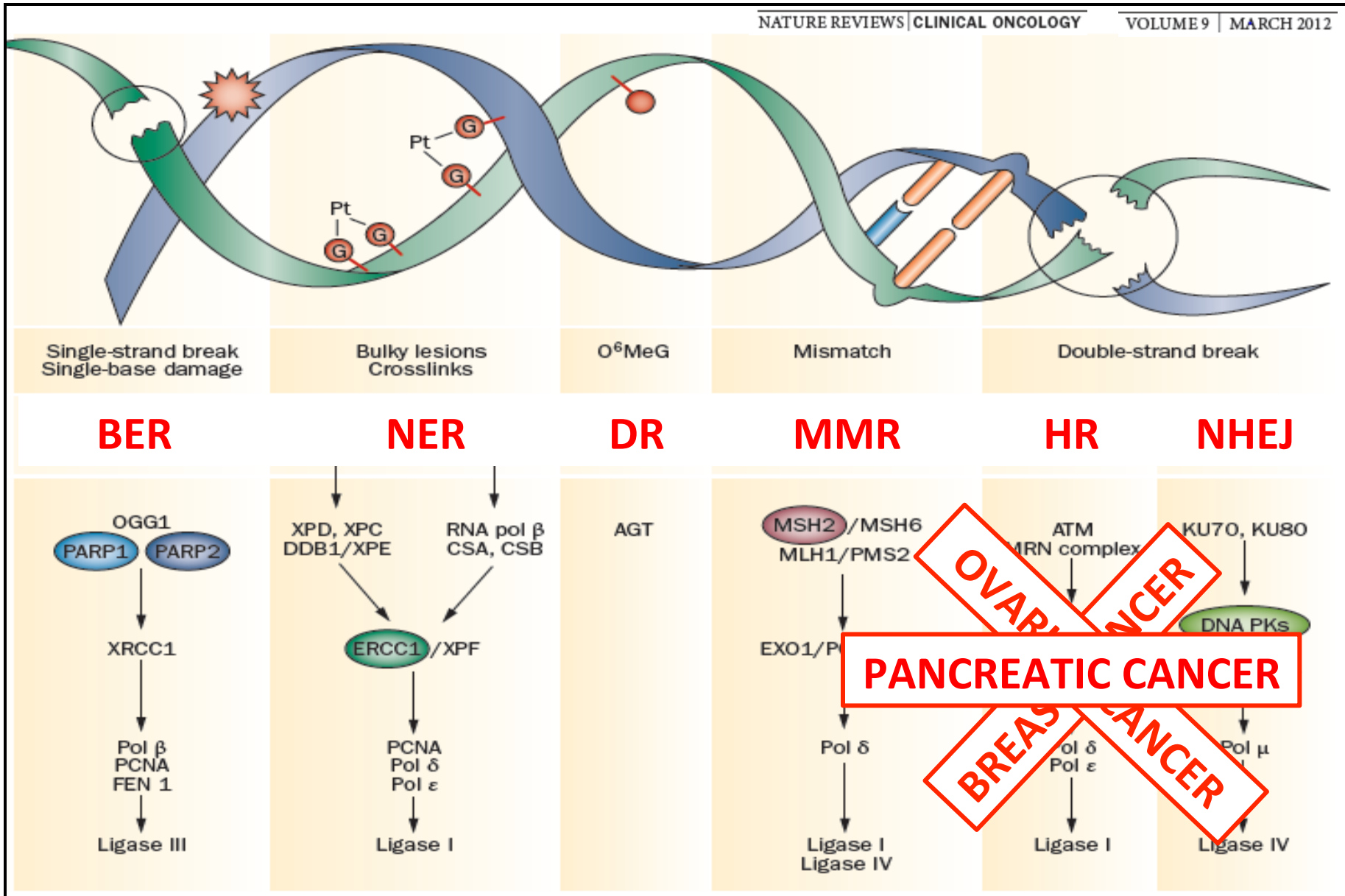
Six Major DNA Repair Pathways
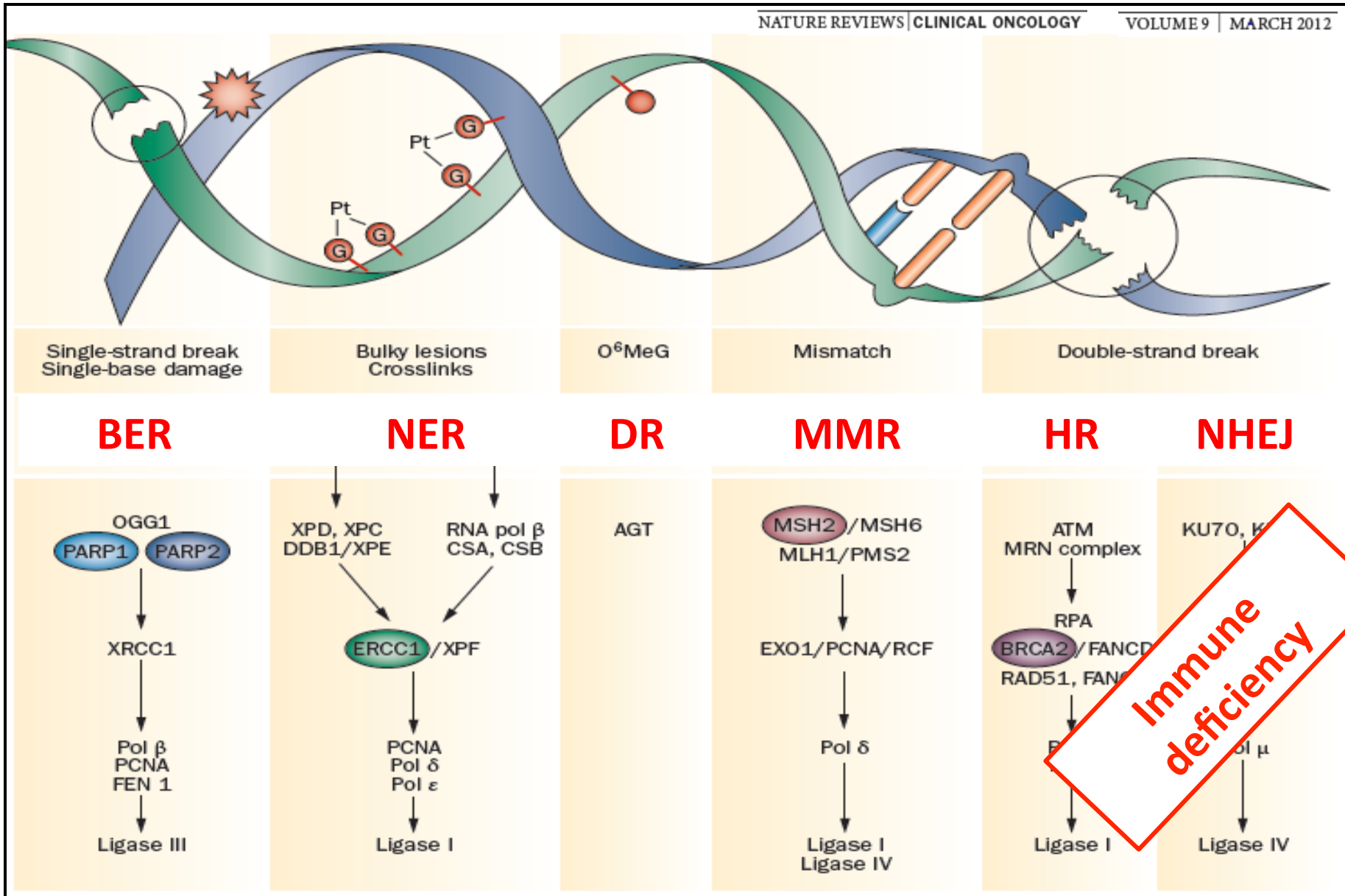
DNA double-strand break repair
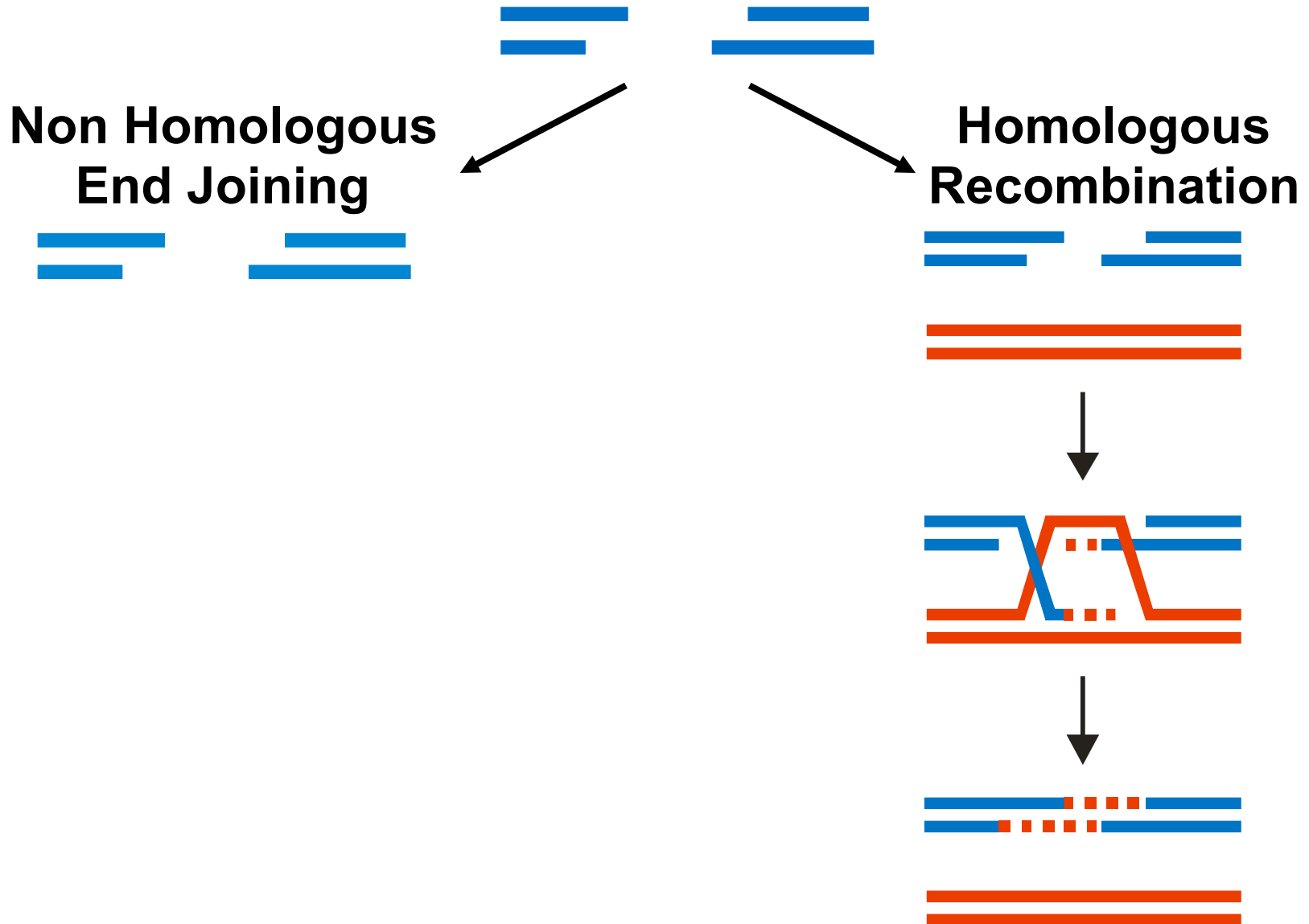
X-rays are extremely good at inducing DNA Double Strand Breaks (DSBs)

Ku80/70

DNA-PKcs

Head / Crown

Kinase domain

Forehead

HEAT repeats

Putative DNA-binding domain

Gap

Putative N-terminus

Ring structure
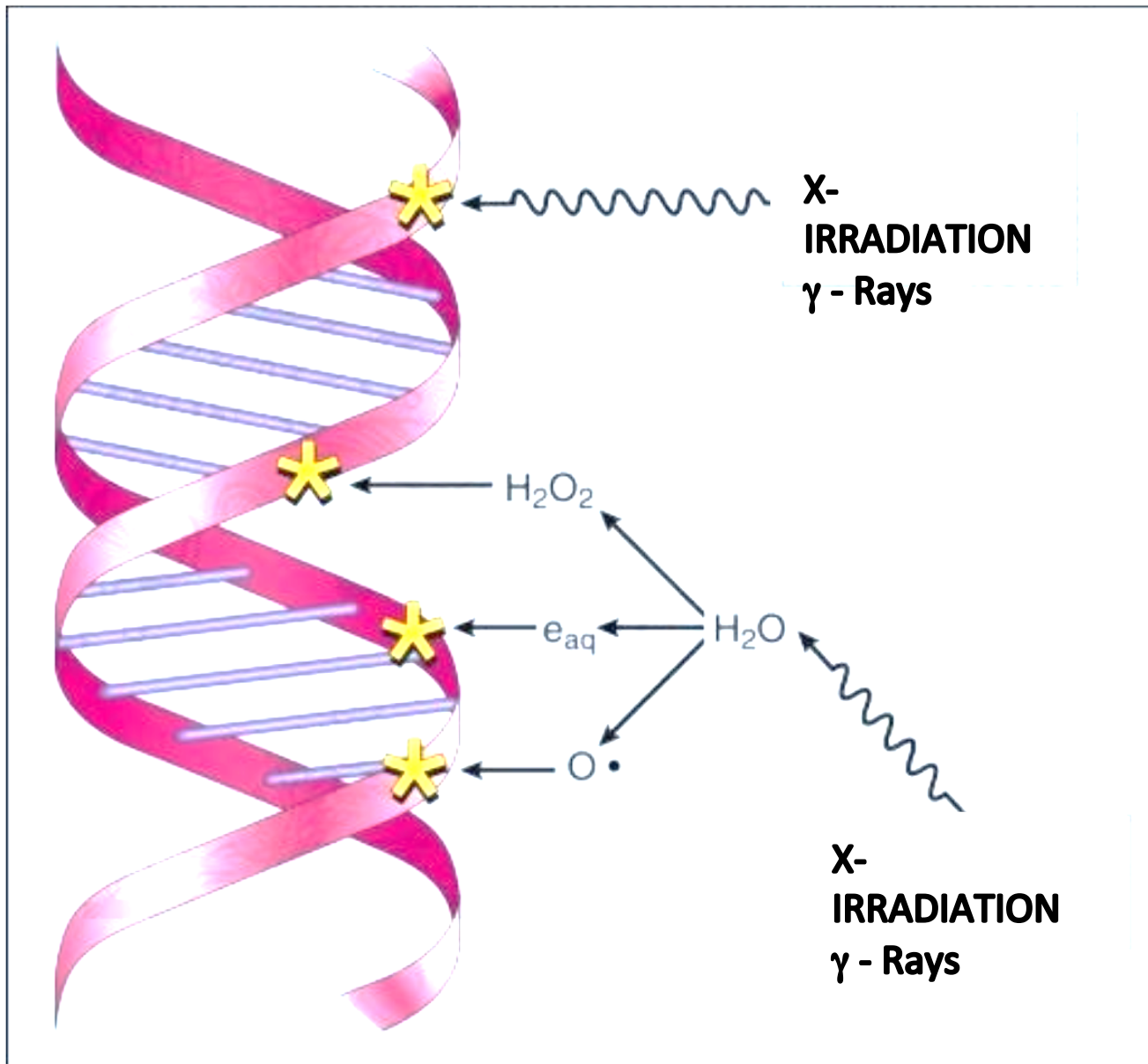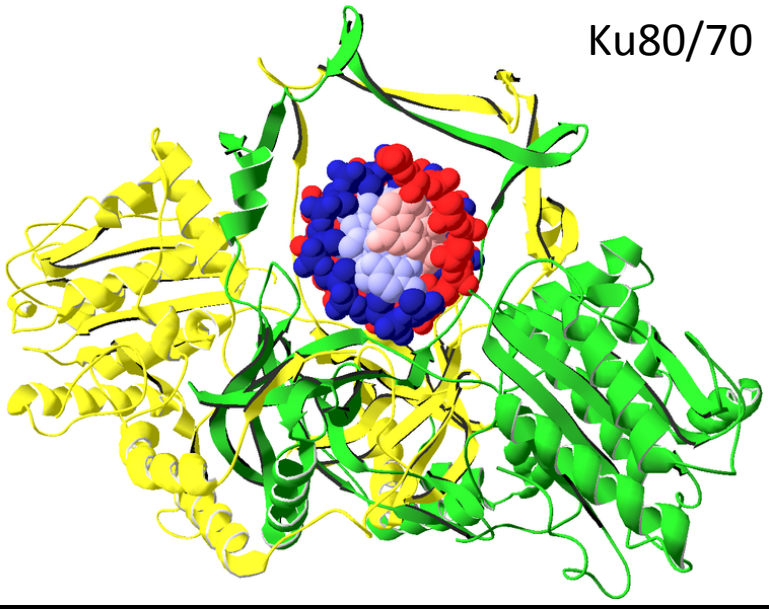
double-strand break in DNA

END RECOGNITION BY Ku HETERODIMERS

ADDITIONAL PROTEINS

PROCESSING OF DNA ENDS

LIMITED REPAIR SYNTHESIS

LIGATION

repaired DNA has generally suffered a deletion of nucleotides

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Absence of p350 Subunit of DNA-Activated Protein Kinase from a Radiosensitive Human Cell Line

Susan P. Lees-Miller, Roseline Godbout, Doug W. Chan,
Michael Weinfeld, Rufus S. Day III, Geraldine M. Barron,
Joan Allalunis-Turner*

Fig. 2. Protein immunoblot analysis of M059J and K cell extracts: Crude extract (10 µg of total protein) was run on 10% acrylamide SDS-PAGE and transferred to nitrocellulose as described (9). The

Immunoblot Analysis

a.k.a.
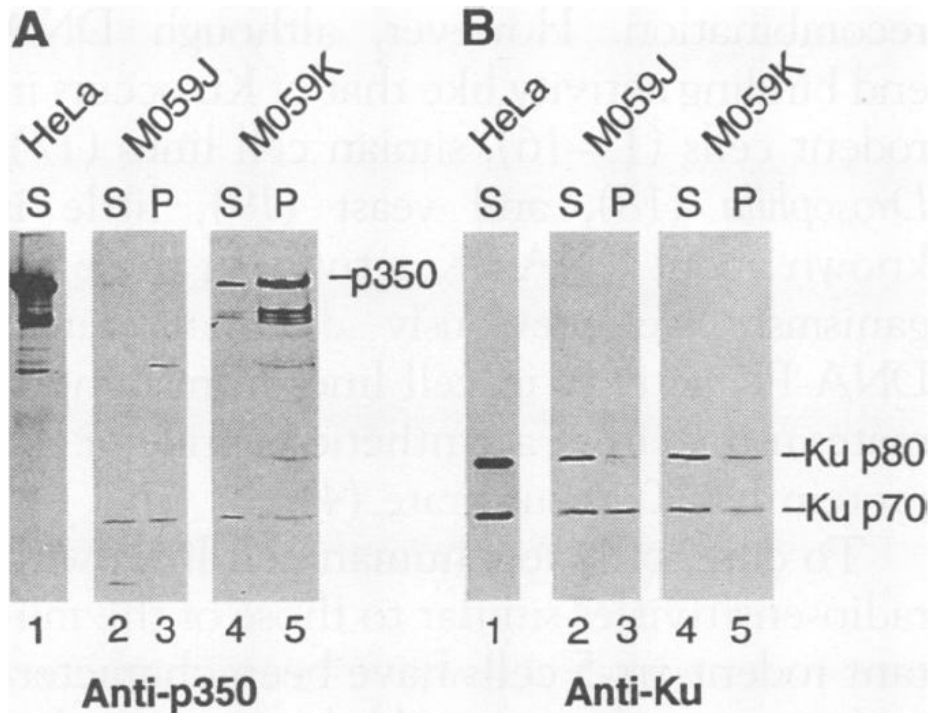
Western Blot Analysis

S = cytoplasmic extract
P = nuclear extract

Prepare Cell Extract for Western Blot (Immunoblot) Analysis

Cell harvesting by centrifugation

Cell pellet is resuspended in lysis buffer

Protein are relesead from cells (yellowish)

cells

Supernatant containing proteins is saved

Estimation of protein concentration

Proteins of all sizes

Cells remainings are centrifuged
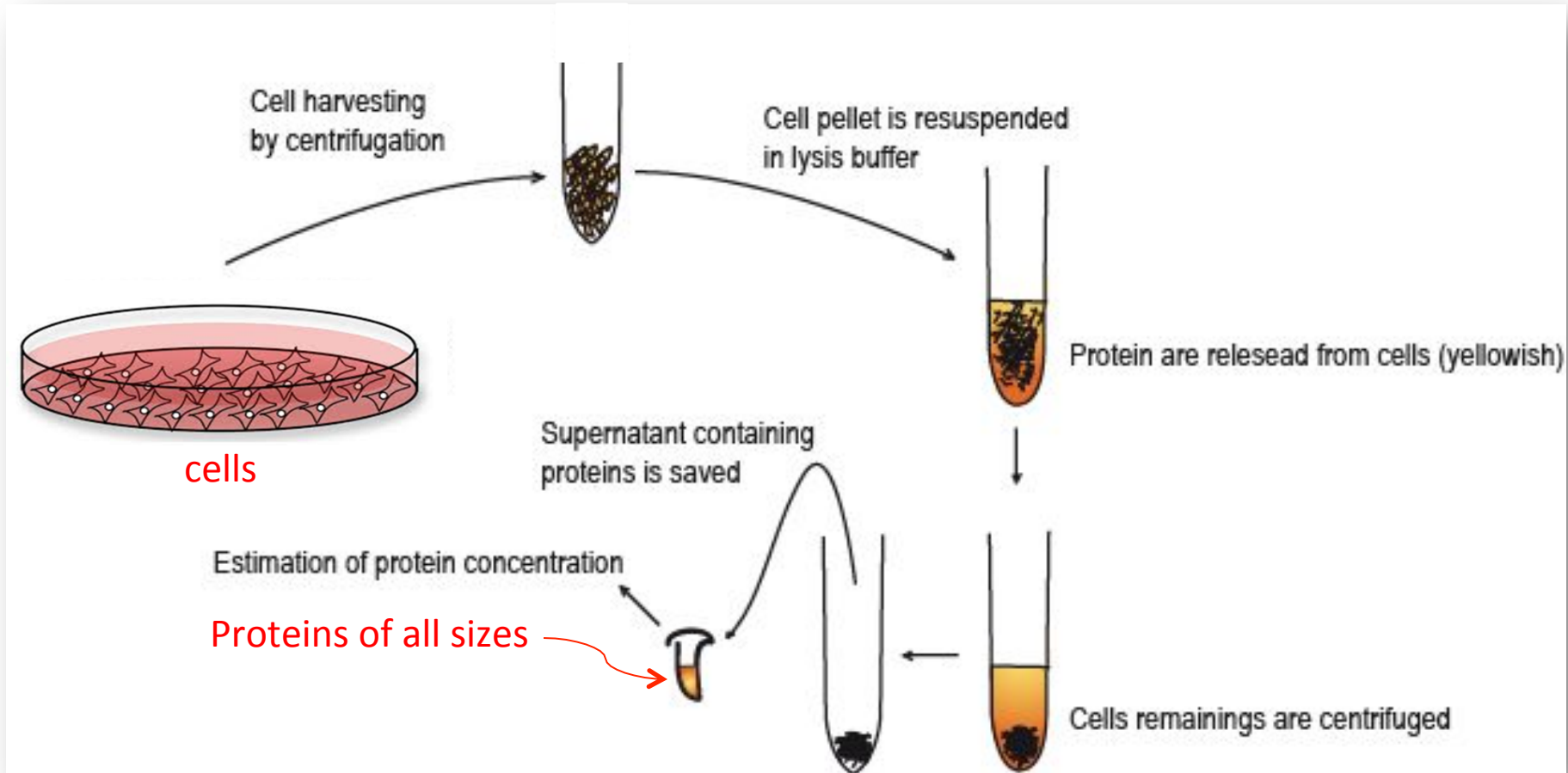
Western Blot or Immunoblot Analysis

Na$^+$ ... O$^-$ ... S ... CH$_3$
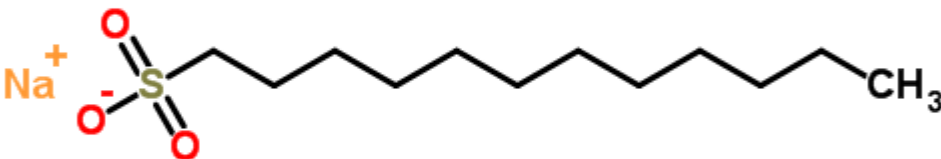
SDS – sodium dodecyl sulfate

Proteins denatured by SDS

BEFORE SDS

charged R-groups

hydrophobic areas

AFTER SDS

SDS to denature proteins plus blue dye – loading buffer

−

Current

+
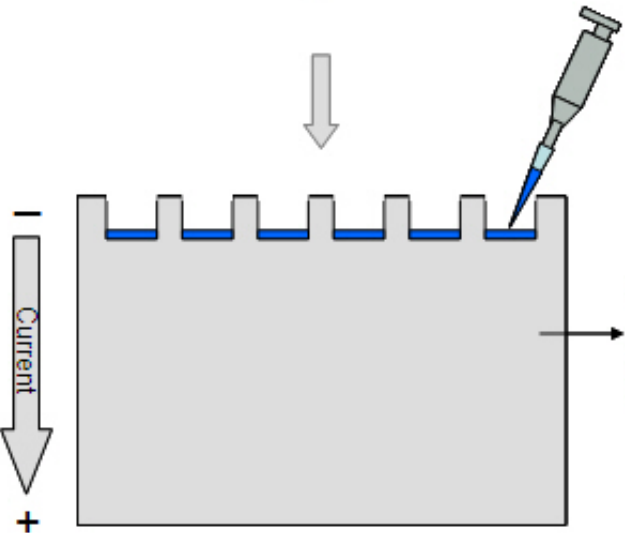
Protein samples along with a protein standard are loaded into wells; gel eletrophoresis is run by electric current.
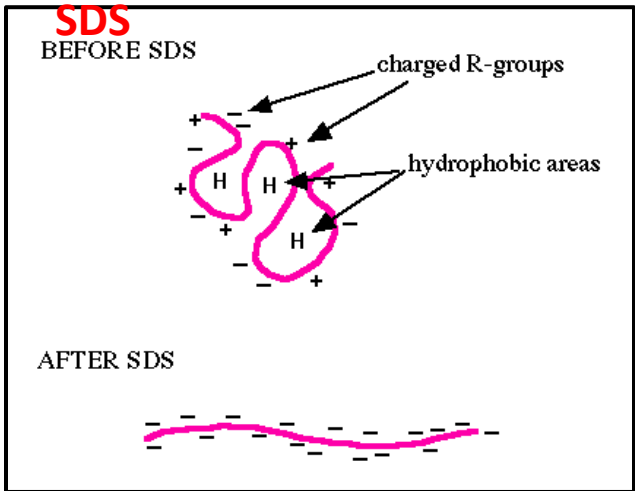
Separating proteins on an SDS polyacrylamide gel

An illustration of an apparatus used for SDS PAGE.

GEL

Protein standard

Proteins of interest → e.g., YFP (your favorite protein)

The protein bands could not be seen without further staining; this figure is only an illustration showing the location of bands.
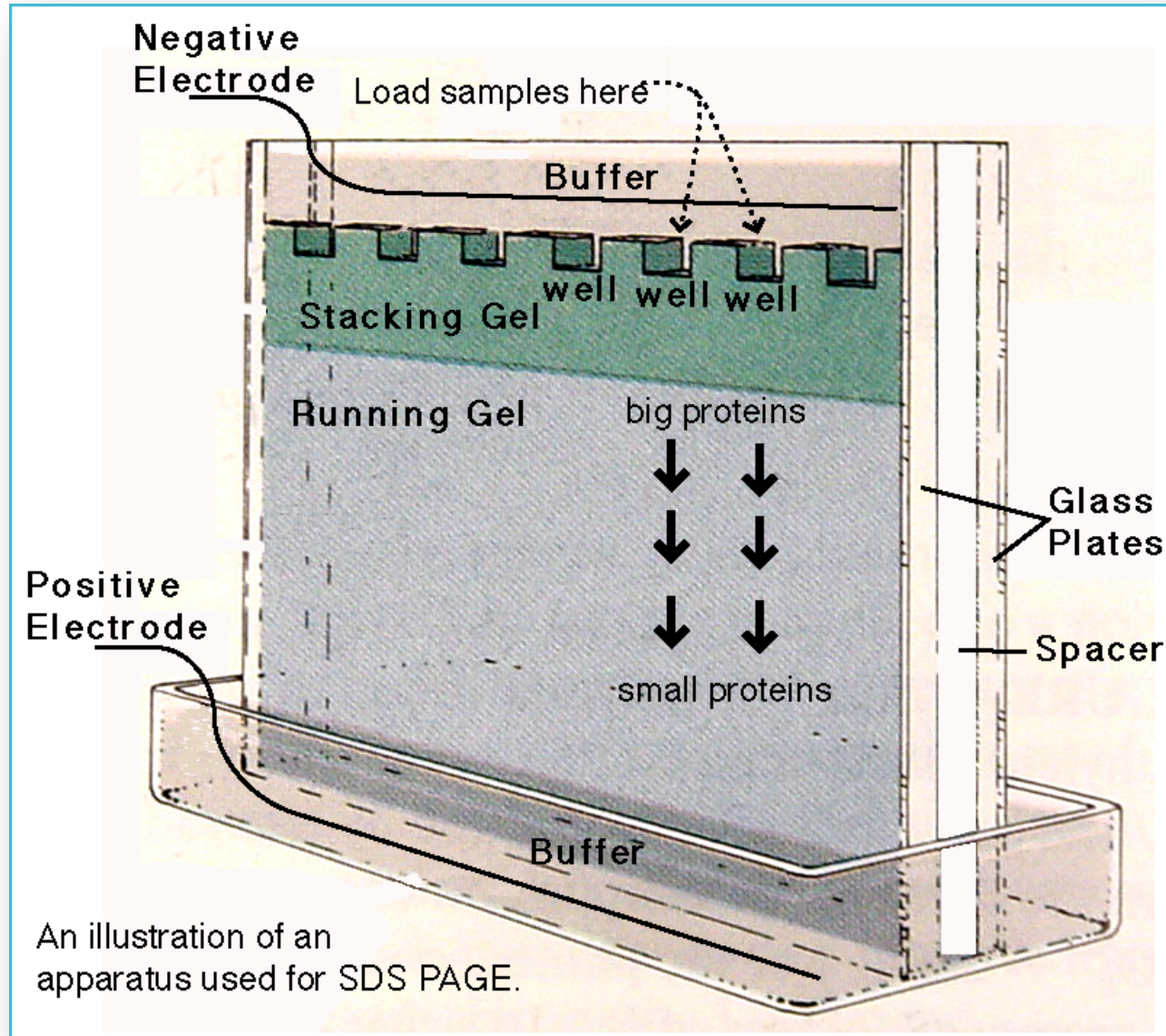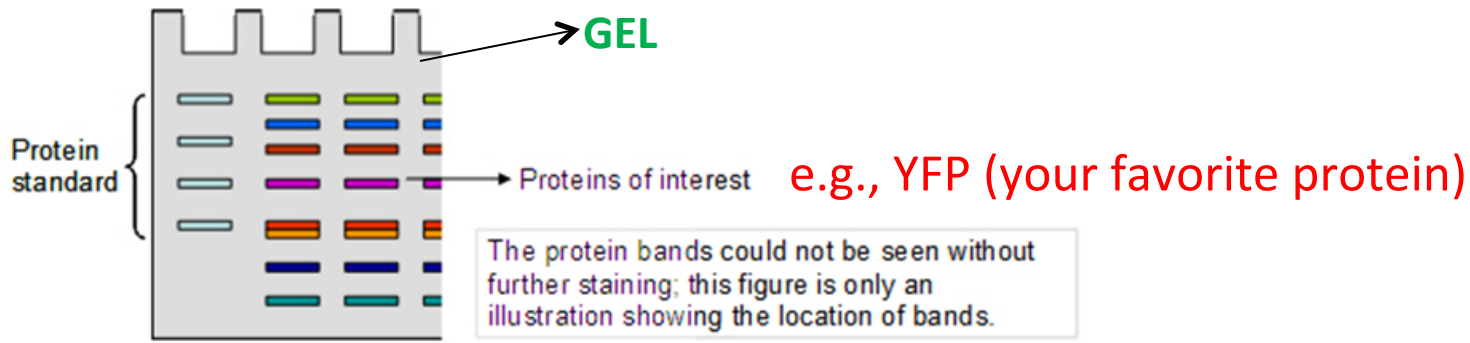
Transfer (Semi-dry transfer as an example)

+ Positive Plate
Sponge
3 Filter Papers
Blotting Membrane
Gel
3 Filter Papers
Sponge
− Negative Plate
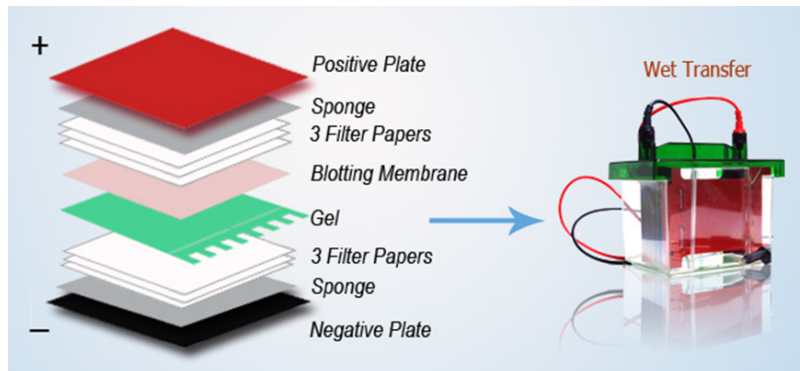
Wet Transfer

The Separated proteins are transferred out of the gel and BLOTTED onto a nitrocellulose or other membrane

Membrane

Protein standard

Proteins of interest → e.g., YFP

The protein bands could not be seen without further staining; this figure is only an illustration showing the location of bands.

This second Ab, with an enzyme linked to it, specifically binds the first Ab

This first Ab specifically binds YFP

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.

chemiluminescent substrates

Light

Protein standard

Exposure to film

Western Blot Analysis

Non-Homologous End Joining (NHEJ)
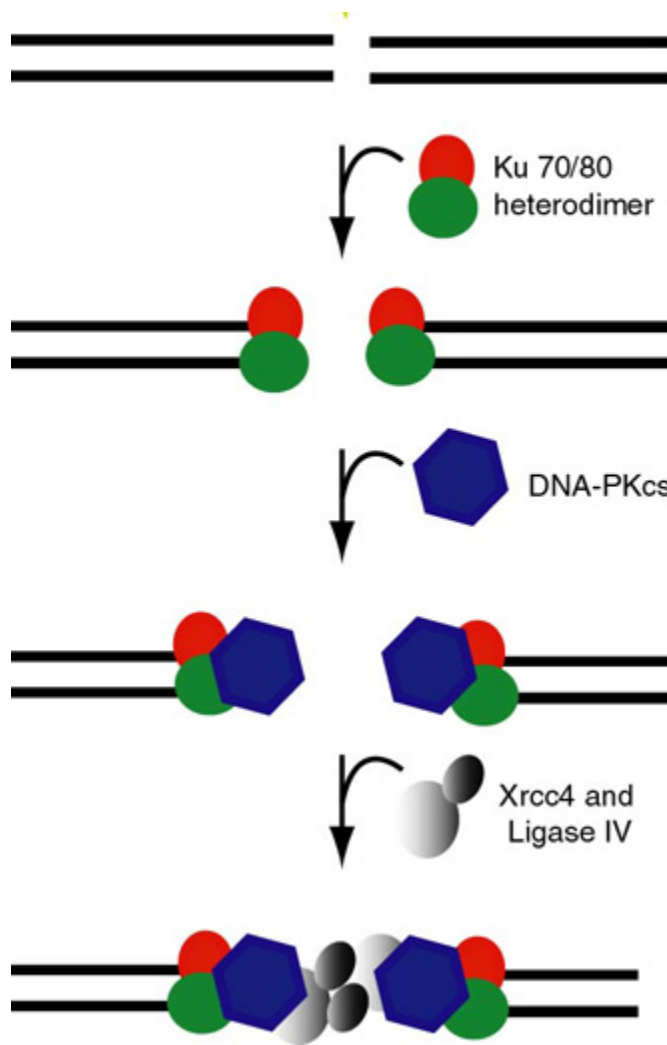
Ku70
Ku80

DNA-PKcs

Xrcc4
Ligase IV

Chinese Hamsters

TISSUE CULTURE

PETRIDISH → ← TISSUE

PROLIFERATION OF FIBROBLASTS

MONOCELLULAR LAYER OF FIBROBLASTS

A
B
ovary
oviduct
uterus
bladder
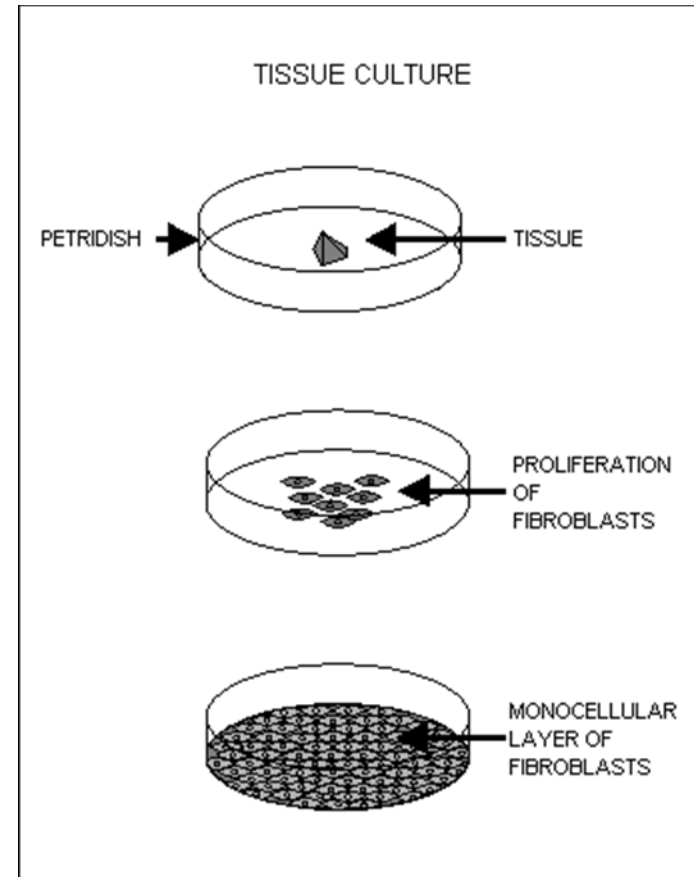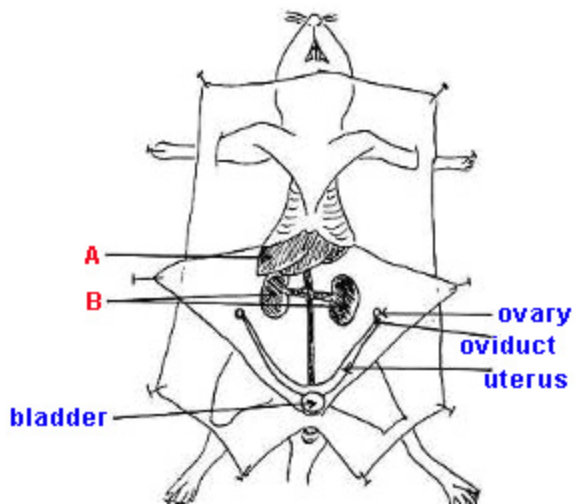
Chinese Hamster Ovary (CHO) cells are immortal – they can grow indefinitely
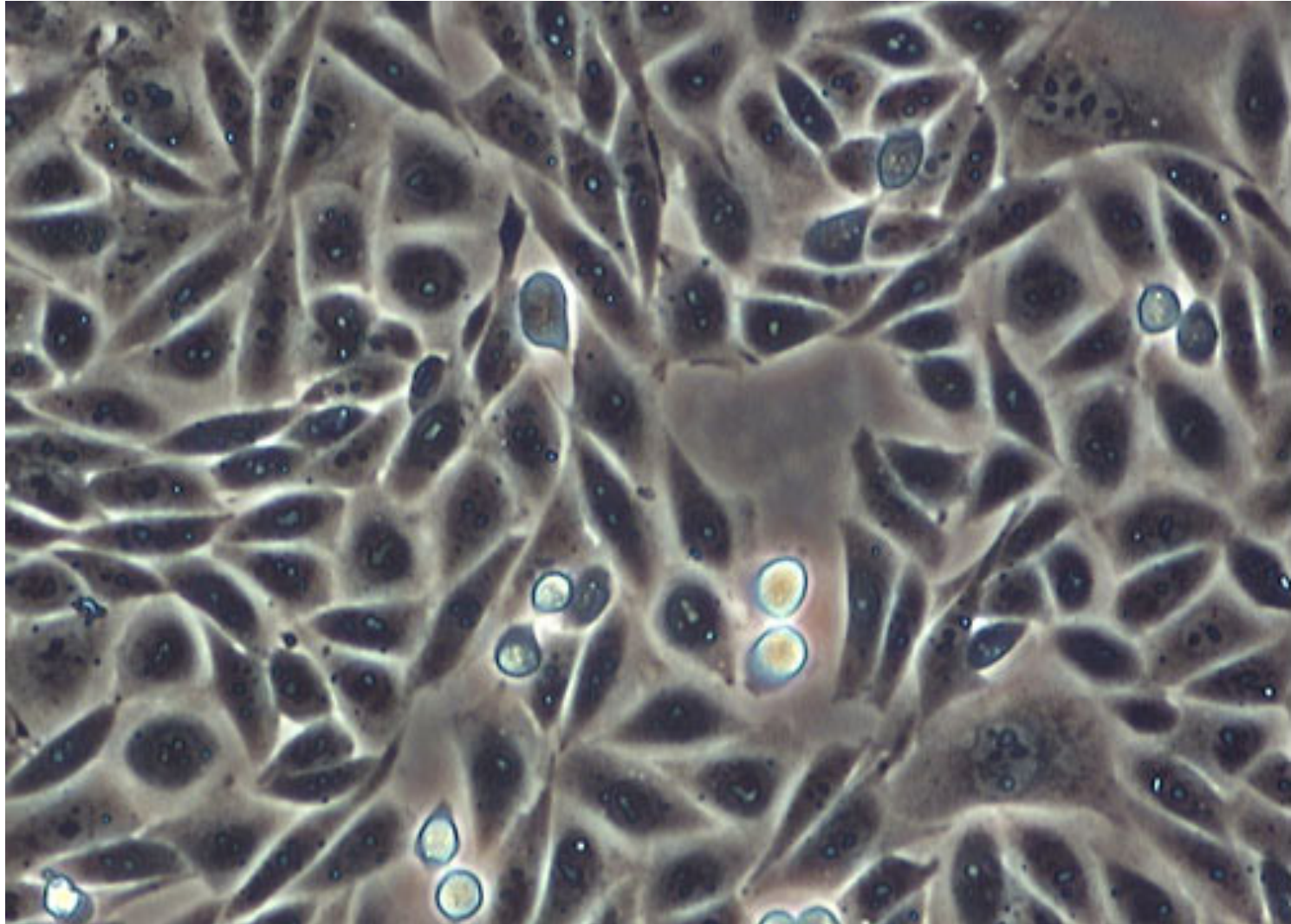
Chinese Hamster Ovary (CHO) cells are immortal – they can grow indefinitely

- Isolating X-ray-sensitive (xrs) CHO cells

- Xrs cells are deficient in NHEJ

- Used these cells to identify human NHEJ proteins

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

> **Forty years ago (1975)**

Age 52   Age 22   Age 25

~3.5 years ago (summer of 2012)

Age 90

Age 60

Age 64
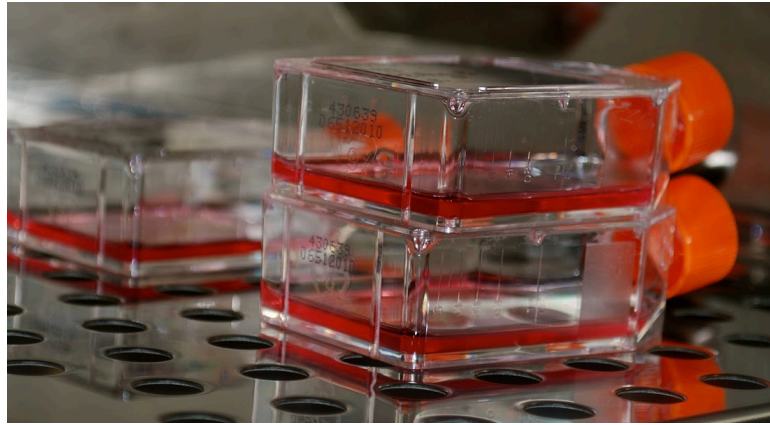
How would *you* go about isolating X-ray resistant cell lines?
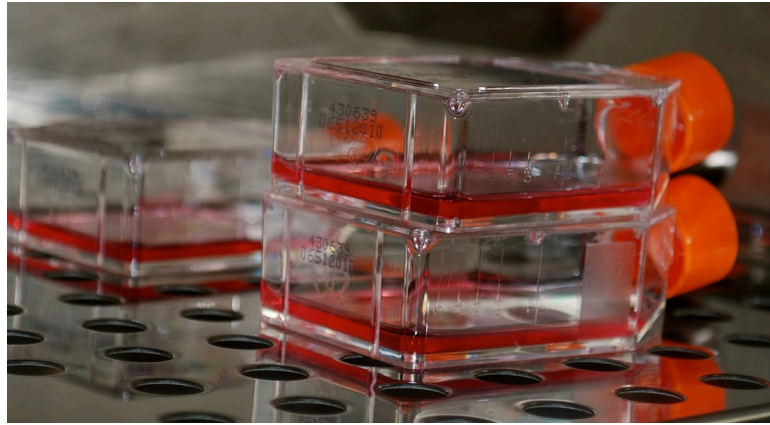
How would *you* go about isolating X-ray sensitive cell lines?

Generate population of CHO cells with a high frequency of mutations

Add potent mutagen

Several million CHO-K1 cells

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})

Con 14day   Con 14day

6TG 30day   6TG 30day
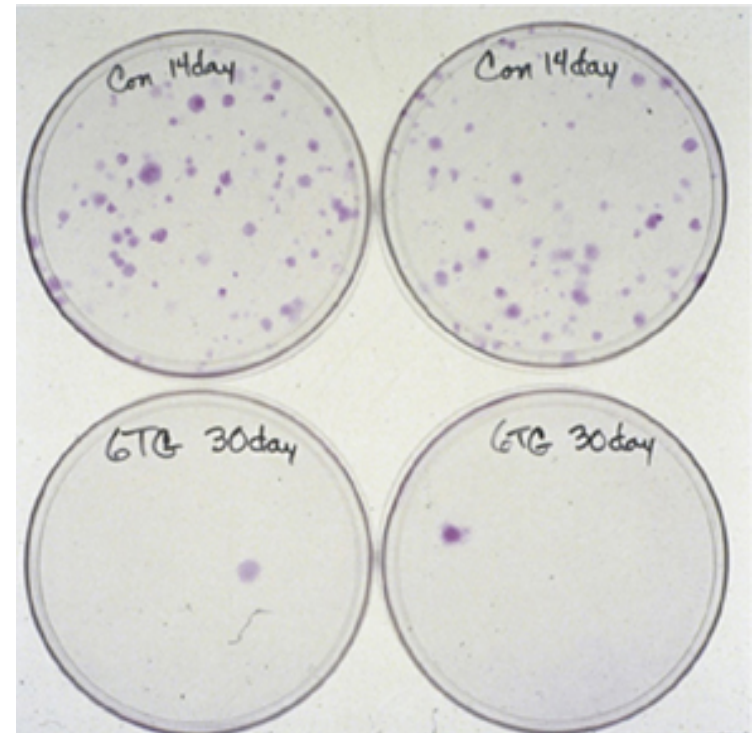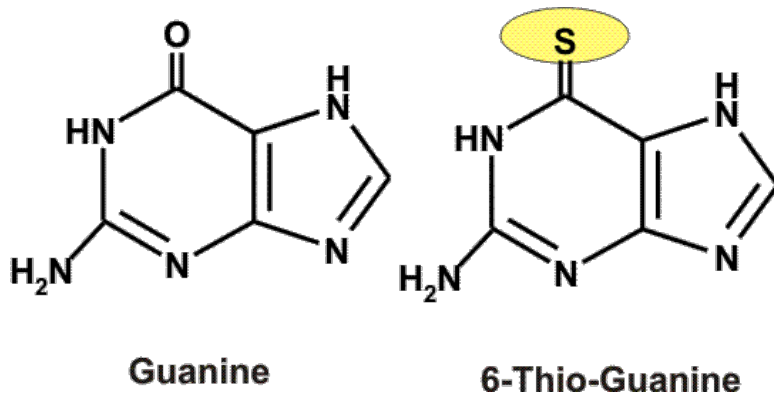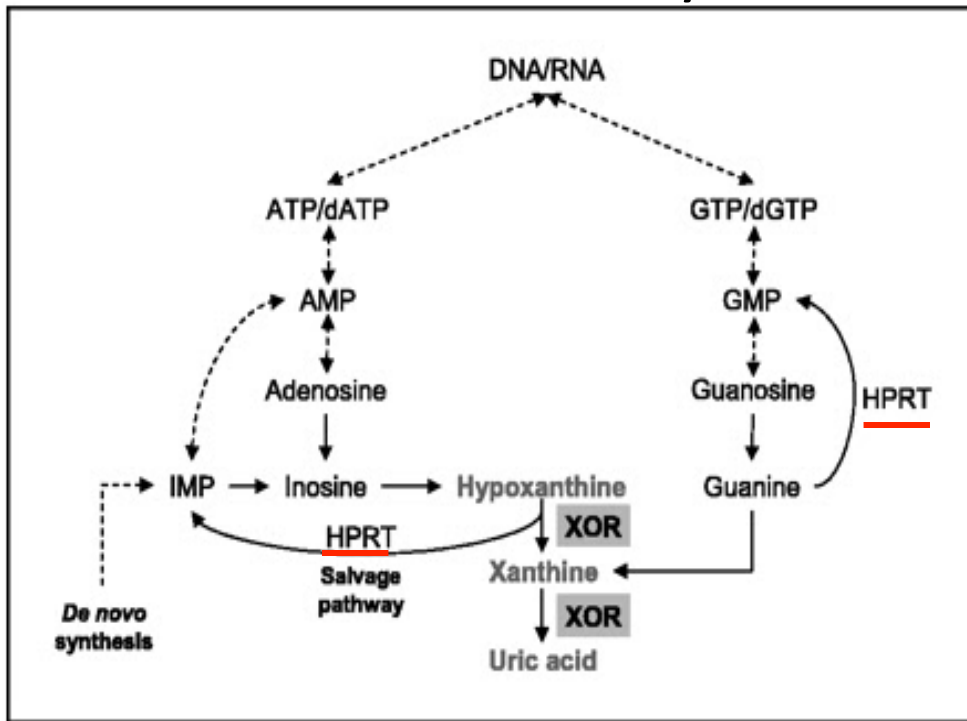
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X-ray-sensitive mutants of Chinese hamster ovary cell line

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)

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

and cross-sensitivity to other DNA-damaging agents

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

6 days after X-RAYS   6 days after No X-RAYS

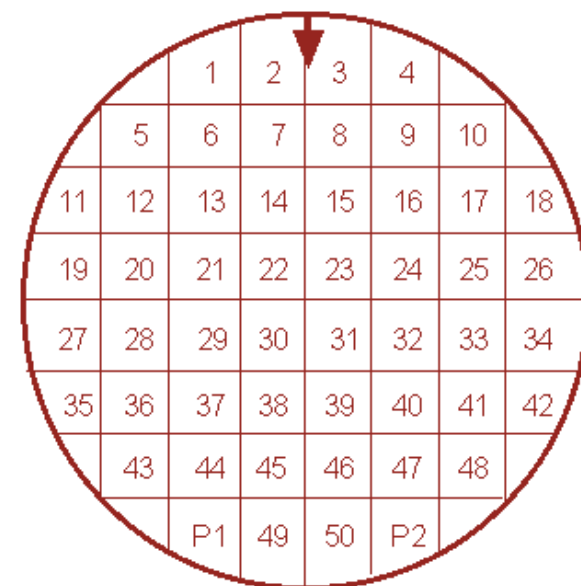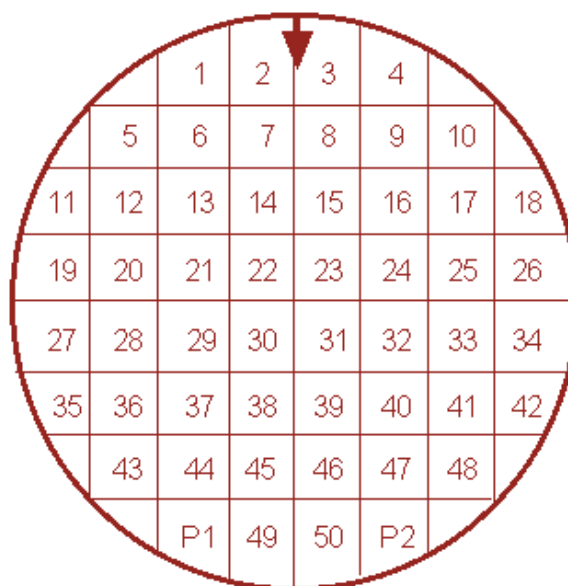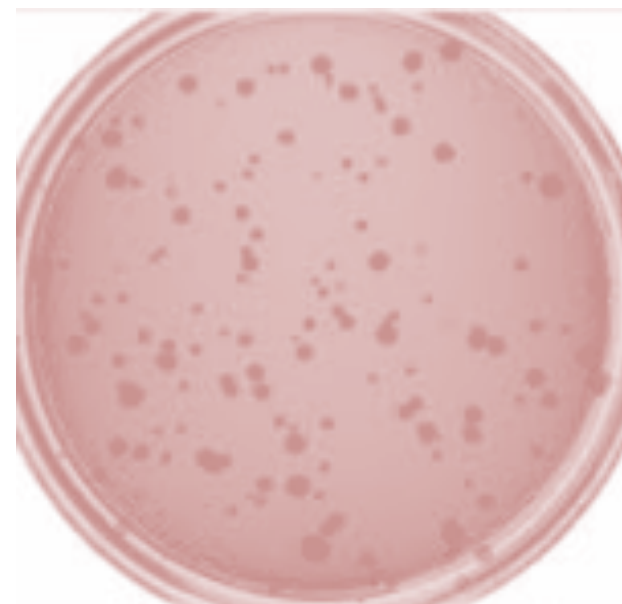# X-ray-sensitive mutants of Chinese hamster ovary cell line

Isolation and cross-sensitivity to other DNA-damaging agents

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

Genetics Division, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA
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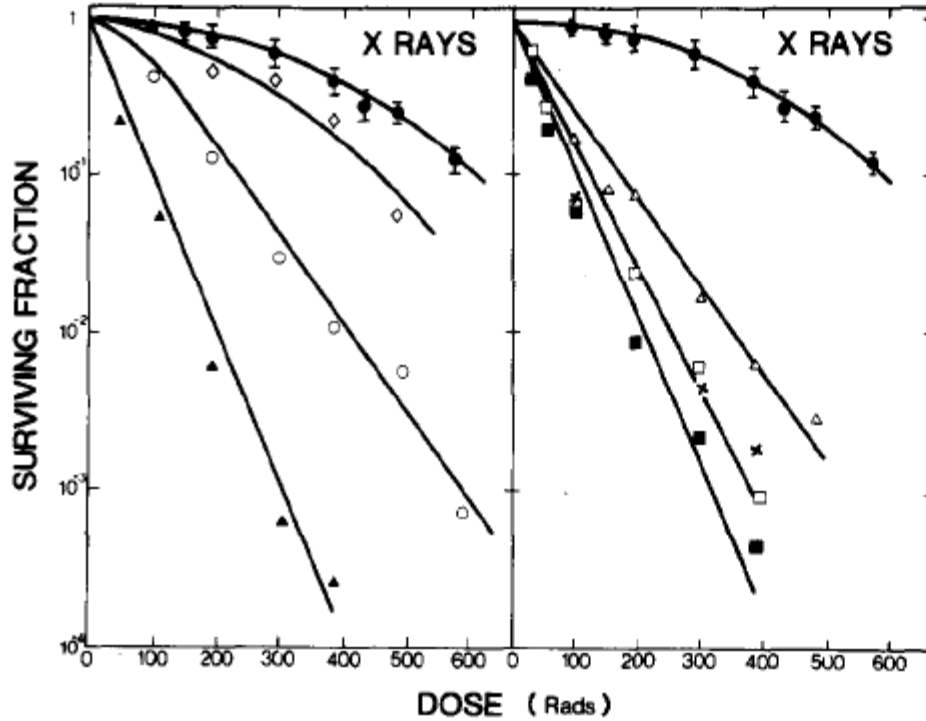
Fig. 1. X-ray survival of CHO-K1 and mutant strains: ●, CHO-K1; □, xrs-1; ○, xrs-2; ◇, xrs-3; △, xrs-4; ▲, xrs-5; ■, xrs-6; ×, xrs-7. The error bars when shown represent one standard deviation. Each survival curve represents the mean of at least three experiments.

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.
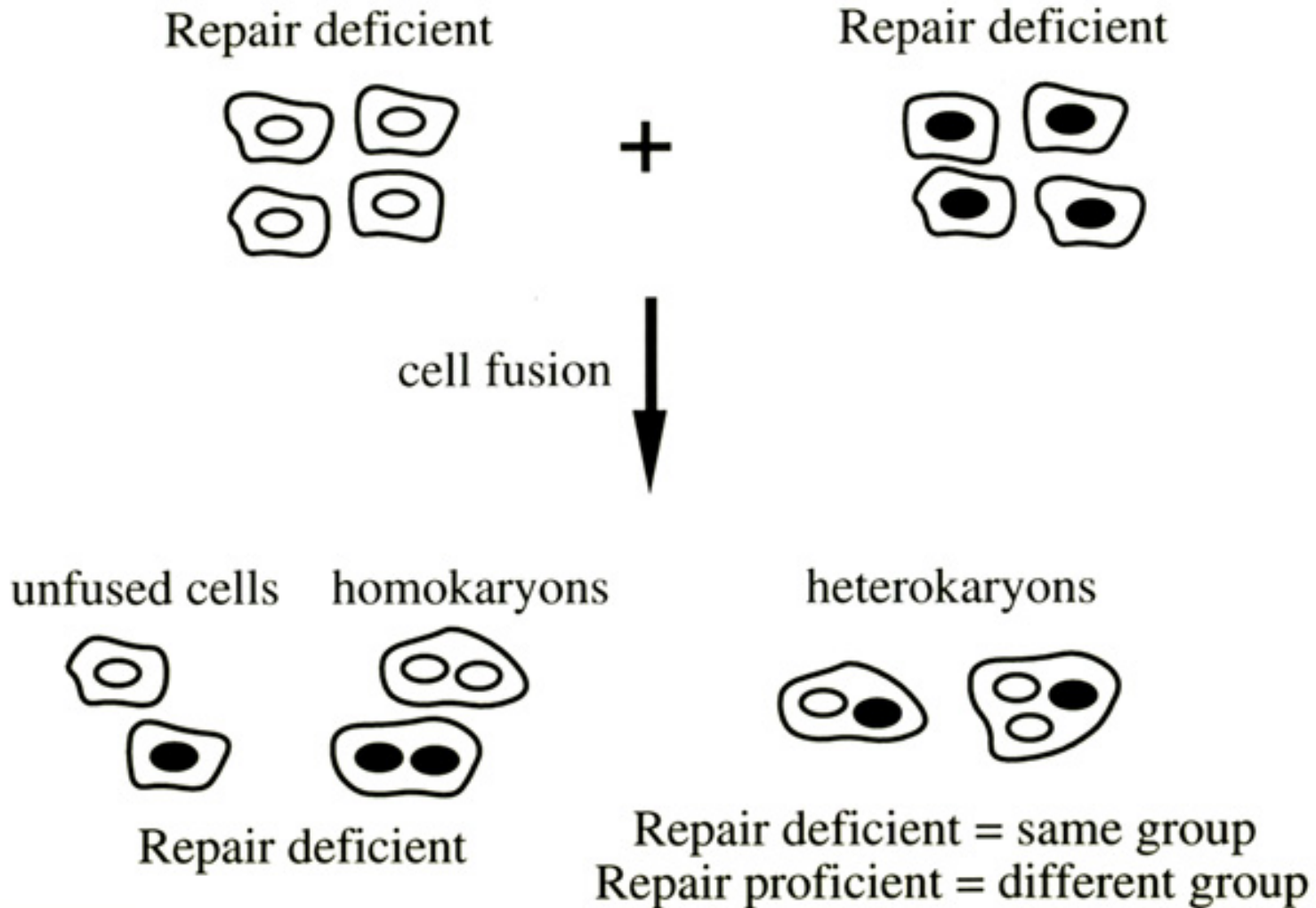
Since the start of my PhD, I have been fascinated by DNA recombination and repair. At that time, the importance of genomic stability was not well appreciated, and at ICRF (now Cancer Research UK) viruses and viral oncogenes were considered to be all important. The work in Cairns's laboratory on DNA repair 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

- 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?

Functional Complementation

Complementation groups

Repair deficient + Repair deficient

cell fusion

unfused cells   homokaryons   heterokaryons

Repair deficient

Repair deficient = same group
Repair proficient = different group

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

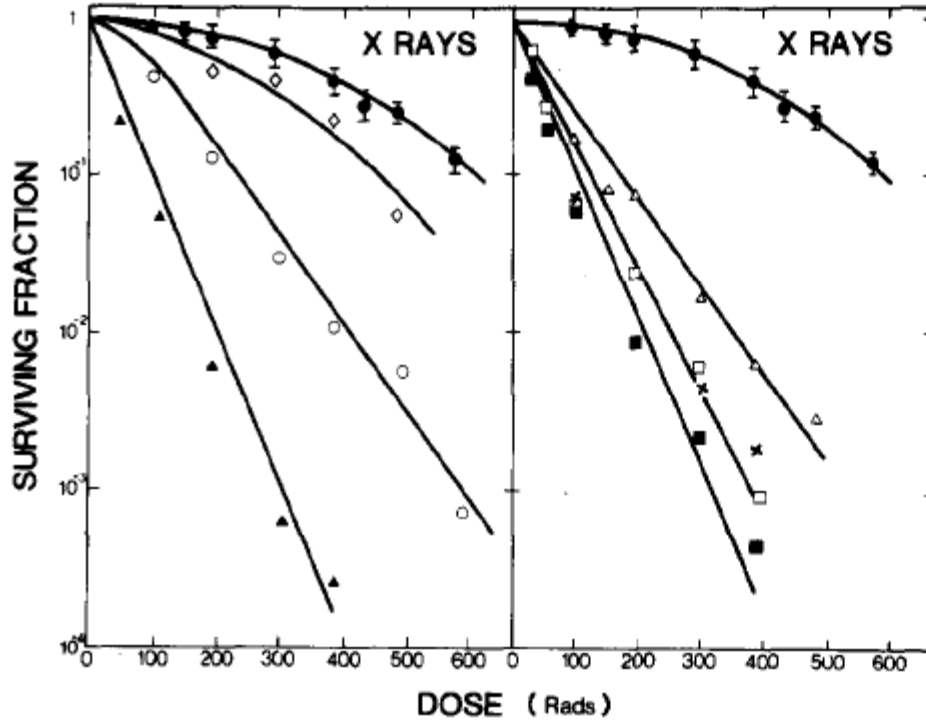*Genetics Division, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA (Great Britain)*

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.

Isolated seven (maybe only 6?) X-ray sensitive clones!!

Fell into different complementation groups

Used to isolate human genes that rescue the X-ray sensitivity

Non-Homologous End Joining (NHEJ)
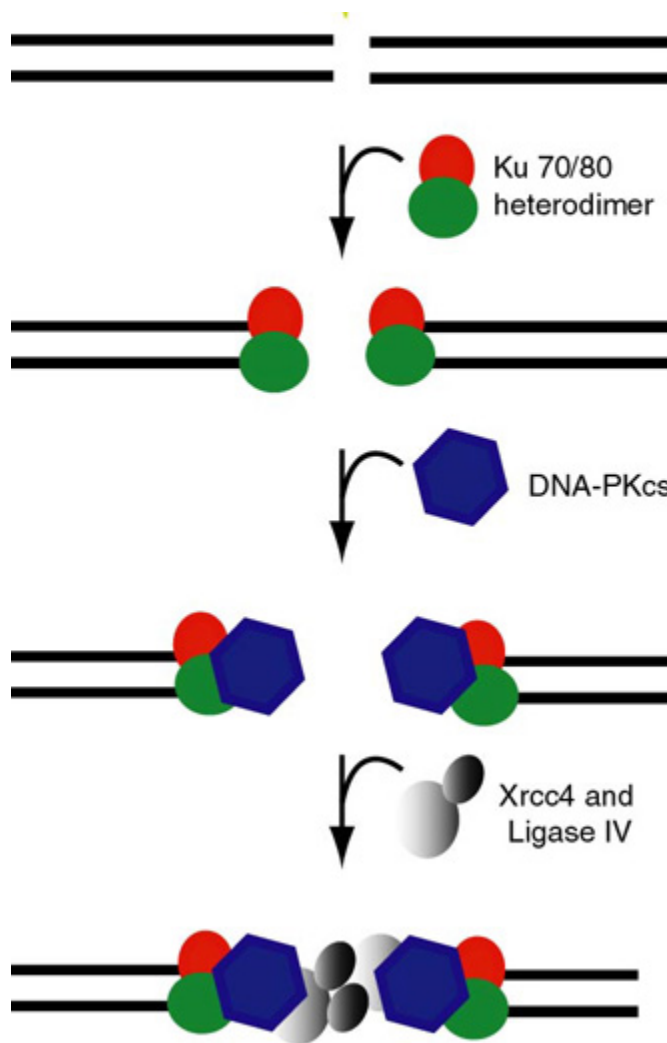
Ku70
Ku80

DNA-PKcs

Xrcc4
Ligase IV

Absence of p350 Subunit of DNA-Activated Protein Kinase from a Radiosensitive Human Cell Line

Susan P. Lees-Miller, Roseline Godbout, Doug W. Chan,
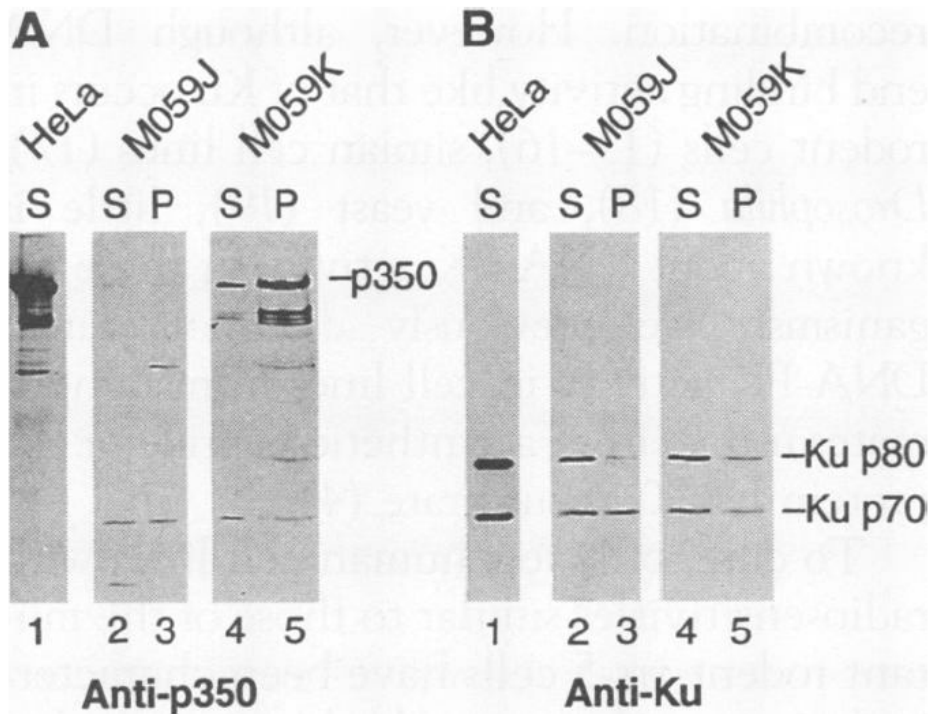Michael Weinfeld, Rufus S. Day III, Geraldine M. Barron,
Joan Allalunis-Turner*

Fig. 2. Protein immunoblot analysis of M059J and K cell extracts: Crude extract (10 µg of total protein) was run on 10% acrylamide SDS-PAGE and transferred to nitrocellulose as described (9). The

Immunoblot Analysis

a.k.a.

Western Blot Analysis

S = cytoplasmic extract
P = nuclear extract

What is the experiment?

How efficiently does DNA repair by the Non Homologous End Joining (NHEJ) pathway act on DNA damage with different topologies?
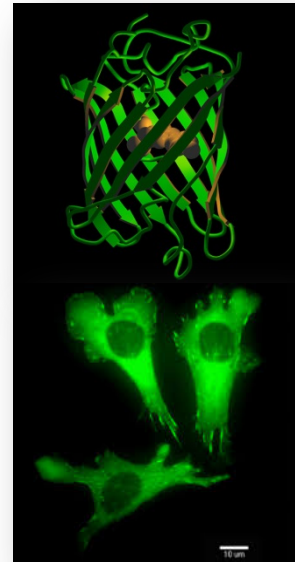
Check list of critical materials to be used:

1. M059K repair proficient cells
2. M059J DNA-PKcs deficient cells
3. Inhibitors of DNA-PKcs
4. Plasmids containing DNA double strand breaks of different topologies.
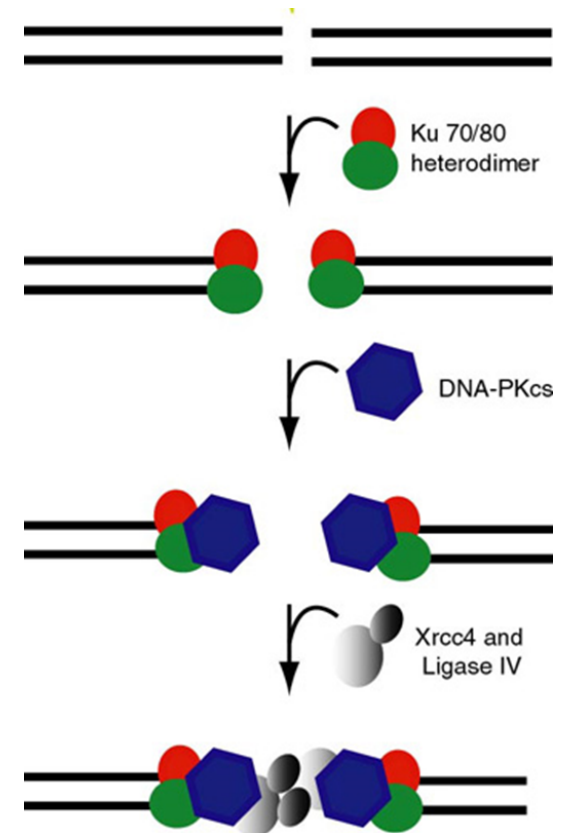
Chinese Hamster Ovary (CHO) cells are immortal – they can grow indefinitely

NEXT LECTURE

- Measuring NHEJ activity
- Using fluorescent proteins to measure biological processes….

20.109 Spring 2016 Module 2 – Lecture 2
System Engineering (March 10th 2016)

Noreen Lyell

Leslie McLain

Maxine Jonas

Jing Zhang(TA)

Leona Samson (Lectures)

Zachary Nagel (help with development) Alex Chaim