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Workshop on Biomedical Applications of High Energy Ion Beams

Co-sponsored by: ICGEB and University of Surrey

12-16 February 2007

Venue:
Adriatico Guest House Giambiagi Lecture Hall
ICTP, Trieste, Italy

Non Targeted Effects

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Biomedical Applications of High Energy Ion Beams

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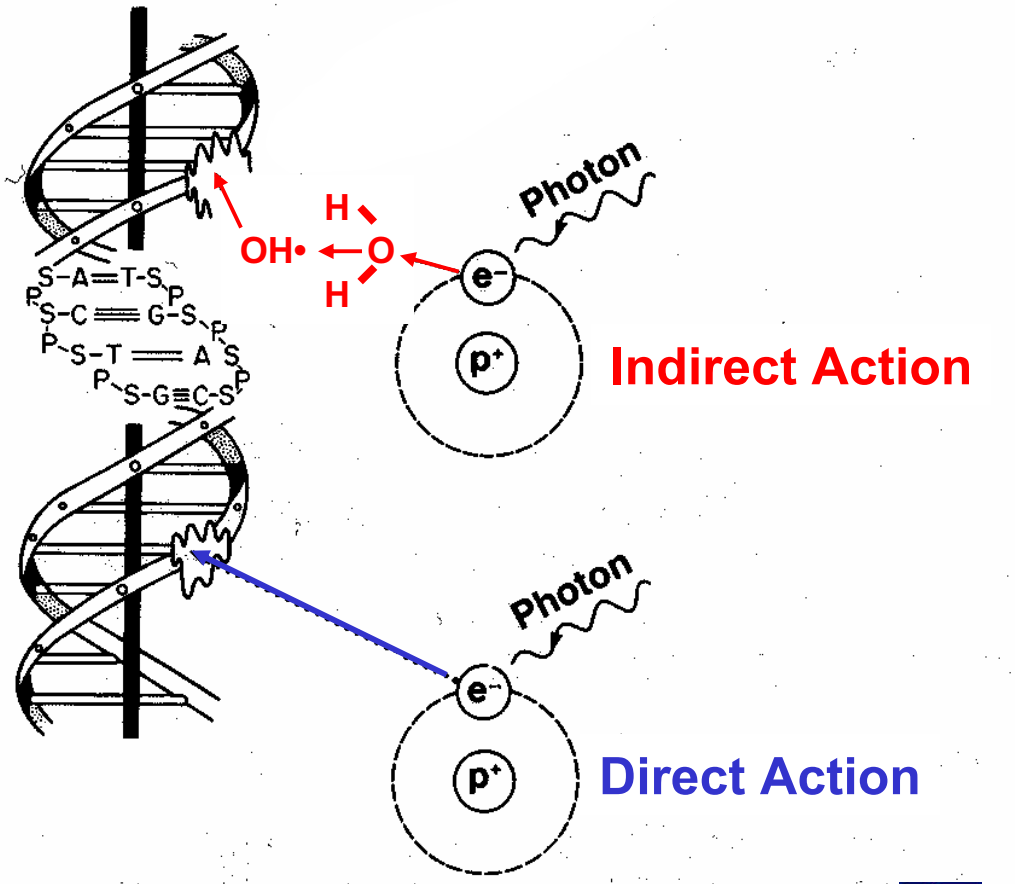
Non-targeted radiation effects

- Classical Radiation Model of DNA damage
- Definition of Non-targeted effects
- Bystander responses
- Bystander and radiation risk
- Genomic instability and cancer
- Radiation-induced genomic instability
- Other non-targeted responses
- Summary

Radiation Interactions with DNA

- Radiation can interact directly with the DNA
  - Direct Effect
- Radiation can interact with other molecules to produce free radicals which can diffuse and damage DNA
  - Indirect effect
- The main source of free radicals is hydroxyl radicals (OH•) produced by ionisation of water
- For X-rays about 70% of DNA damage is produced by the indirect effect from OH• radicals



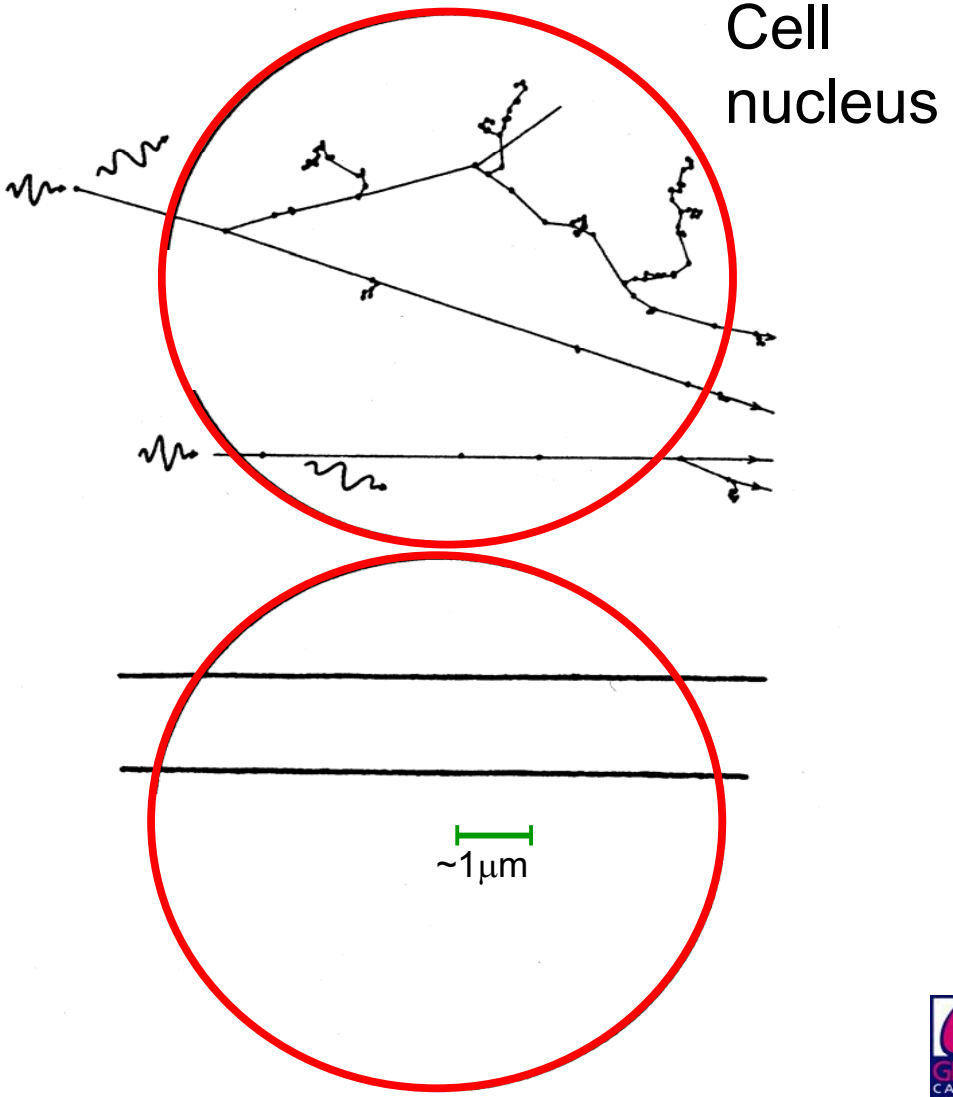
Radiation track structure

Different types of radiation deposit their energy in different patterns in the cell nucleus

Sparsely ionising
Low LET

γ-rays / X-rays

1 Gy corresponds to
10^5 ionisations in
~ 1000 tracks



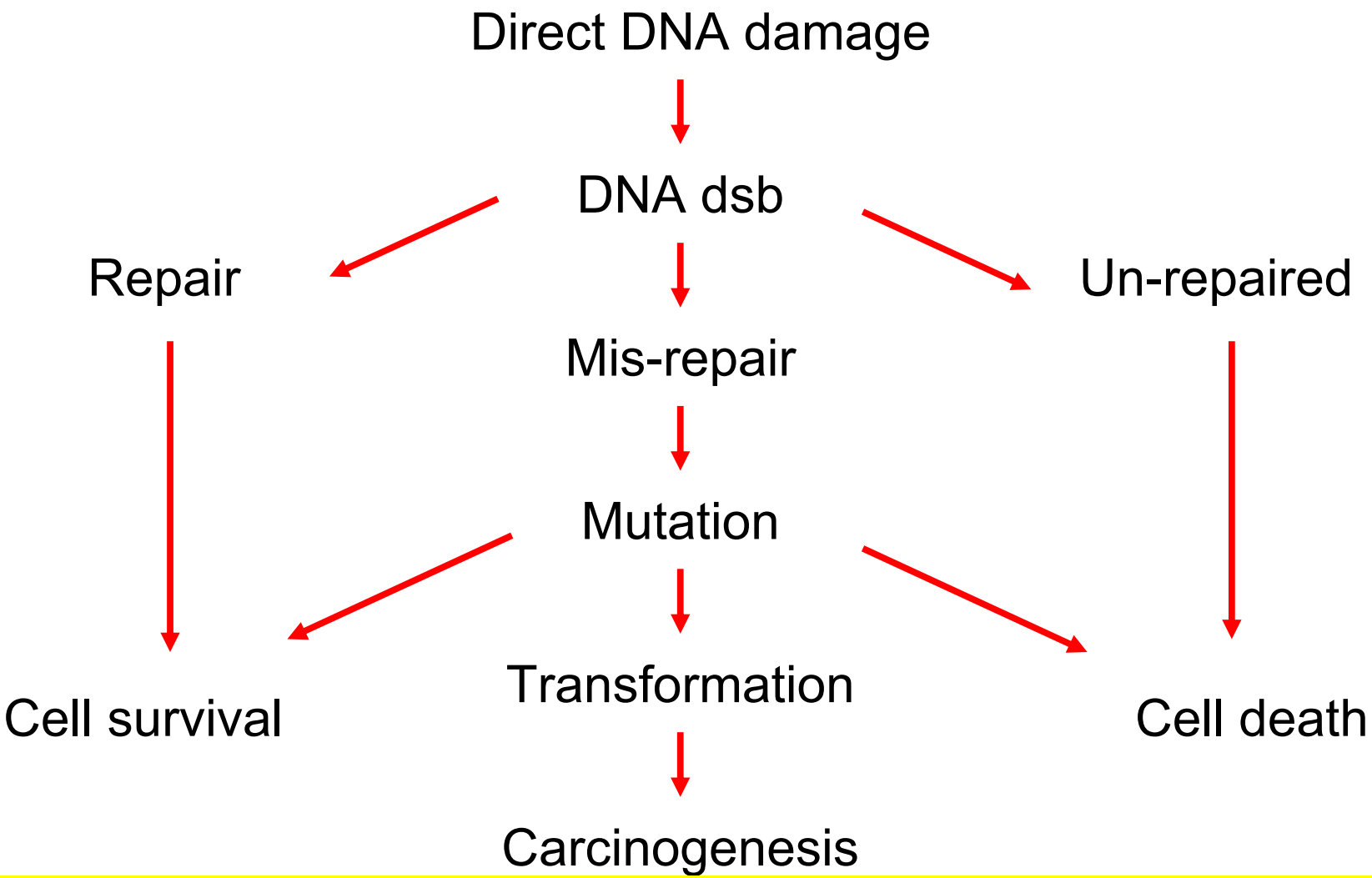
Densely ionising
High LET

α-particles

1 Gy corresponds to
~ 4 tracks

LET = linear energy transfer (keV/μm)

Classical Model



Biological consequences of radiation exposure are due to direct DNA damage

Non-(DNA) targeted effects

Responses which do not follow the standard model of biological effect in direct proportion to energy deposited in nuclear DNA

- Bystander responses
  - response of neighbours of irradiated cells
- Genomic instability
  - increased rate of acquisition of alterations in genome
- Adaptive response
  - Pre-treatment with a low priming dose leads to protection against a second challenging dose
- Gene induction
  - gene expression under conditions where no direct DNA damage
- Low dose hypersensitivity
  - deviations from LQ model at low doses
- Inverse dose-rate effect
  - Increasing effect with decreasing dose-rate

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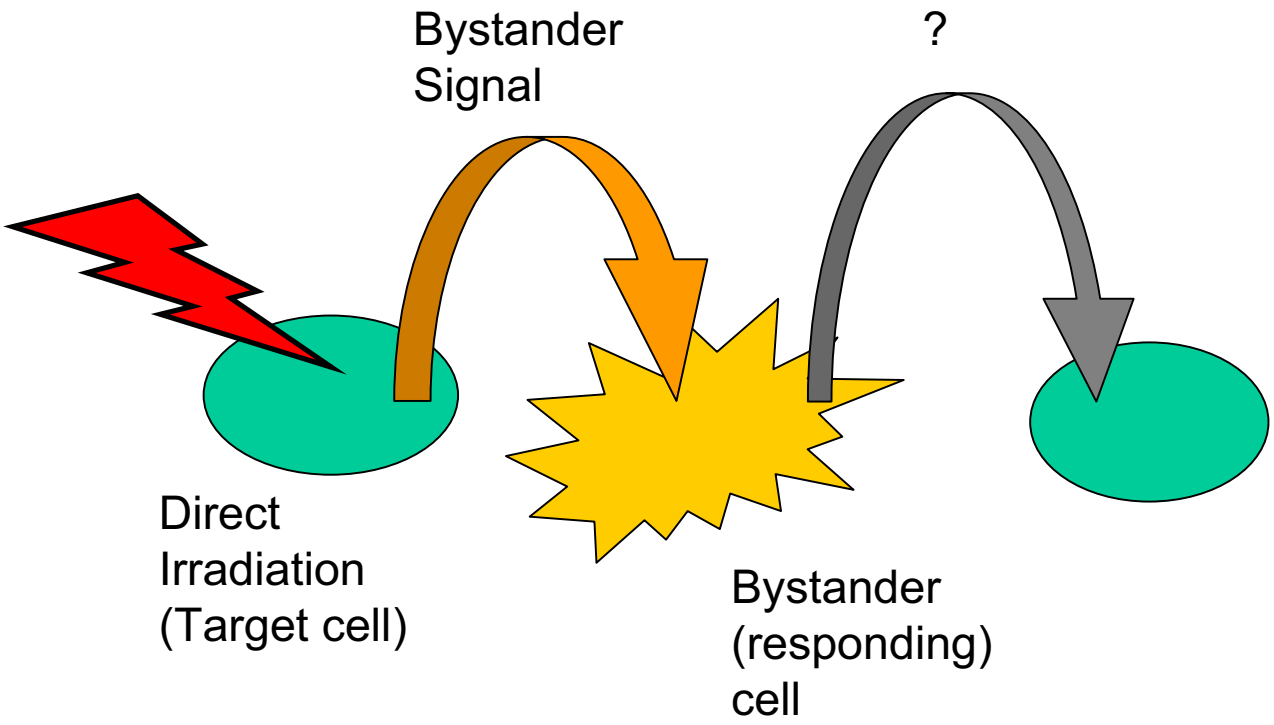
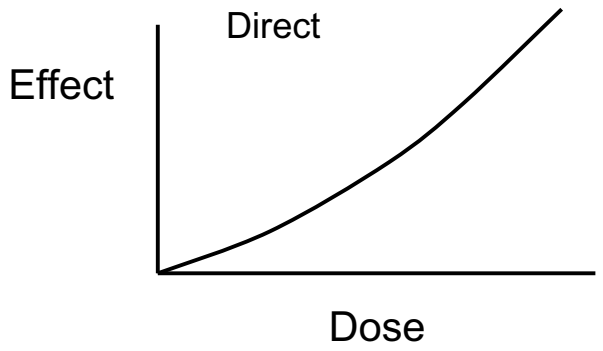
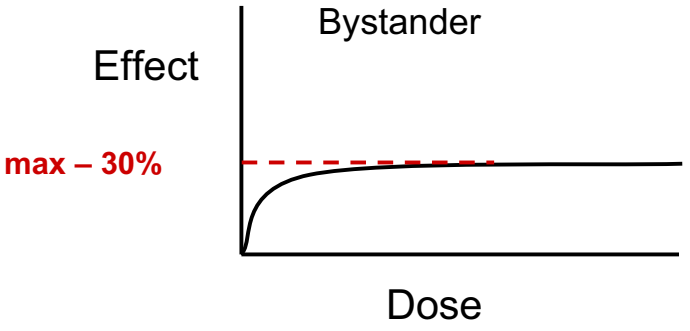
Radiation induced bystander response – when cells respond to their neighbour(s) being irradiated



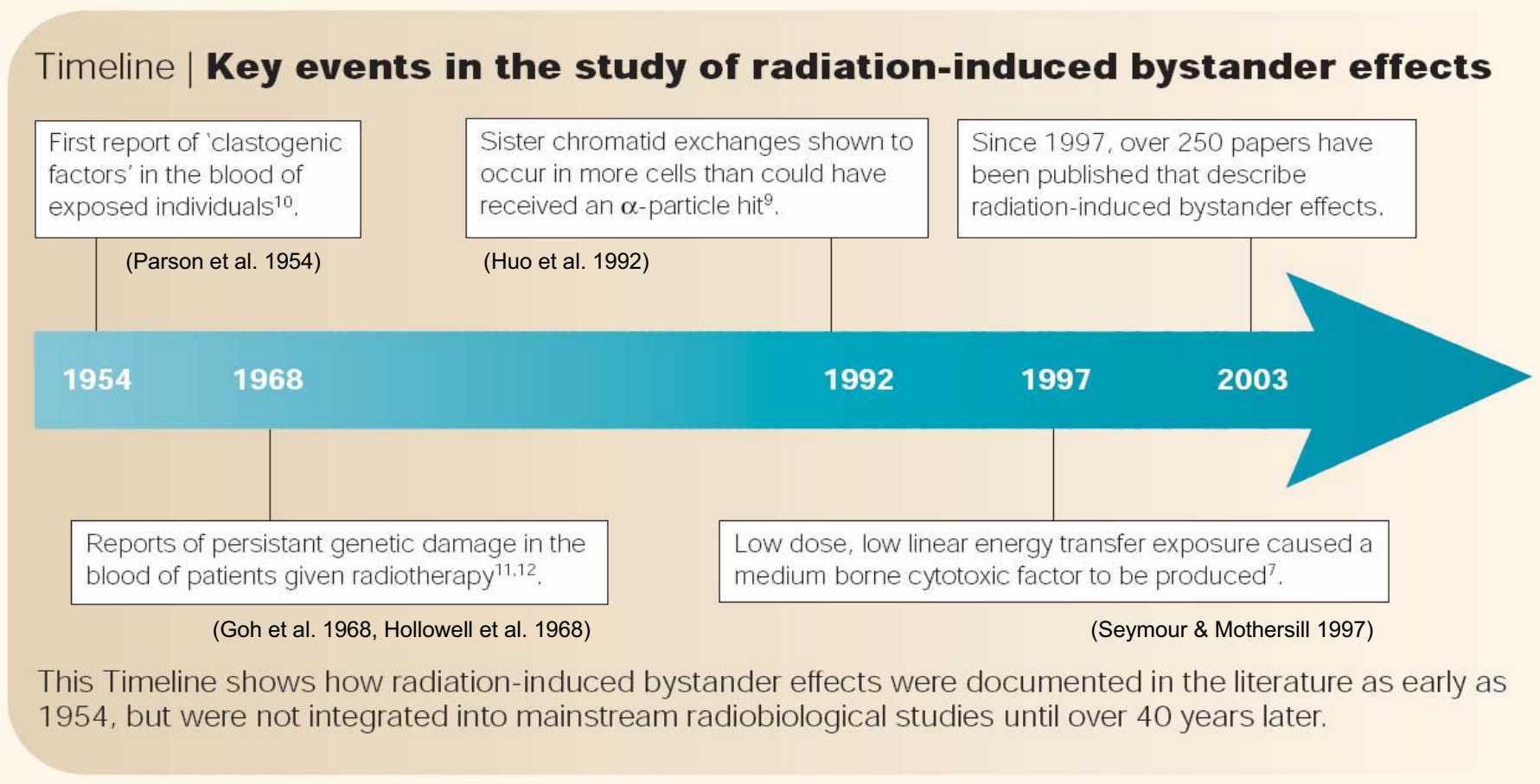
In vitro studies of bystander effects





- Significant at low doses (<<1 Gy)
- Observed in a range of cell types
- Observed for a range of end-points
  - Cell killing, apoptosis, chromosomal damage, mutation, transformation
- Several mechanisms involved
  - Direct cell-cell communication
  - Release of factors into the medium
- Bystander signals
  - Reactive oxygen and nitrogen species
  - cytokines
  - calcium

Radiation induced bystander effects



Mothersill & Seymour, Nature Reviews Cancer 4:158, 2004

Experimental approaches for studying bystander responses

- Medium transfer
  - From irradiated to non-irradiated cells
- Co-culture
  - Membrane inserts, double Mylar dishes
- Low fluence ion sources
  - α-particles
- Shielding
  - Grids, partial physical shielding
- Microbeam approaches
  - Charged particle, electrons, soft X-rays

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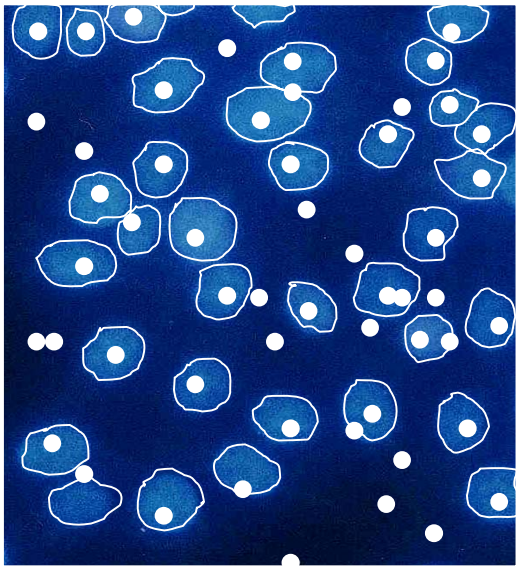
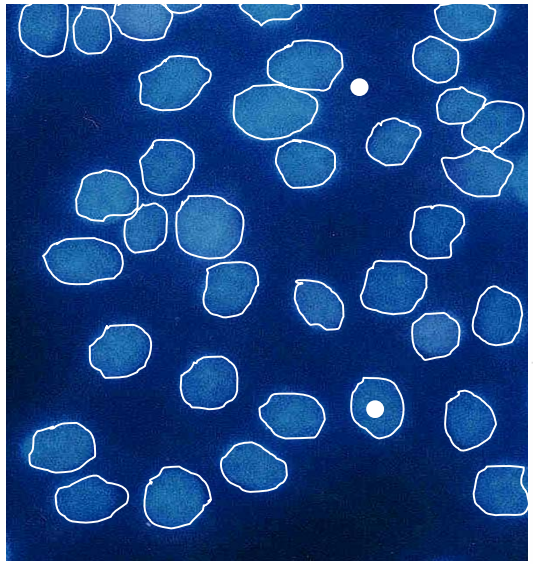
Experimental evidence for bystander responses

- Low fluence particle sources
  - α-particles
  - Nagasawa and Little, 1992

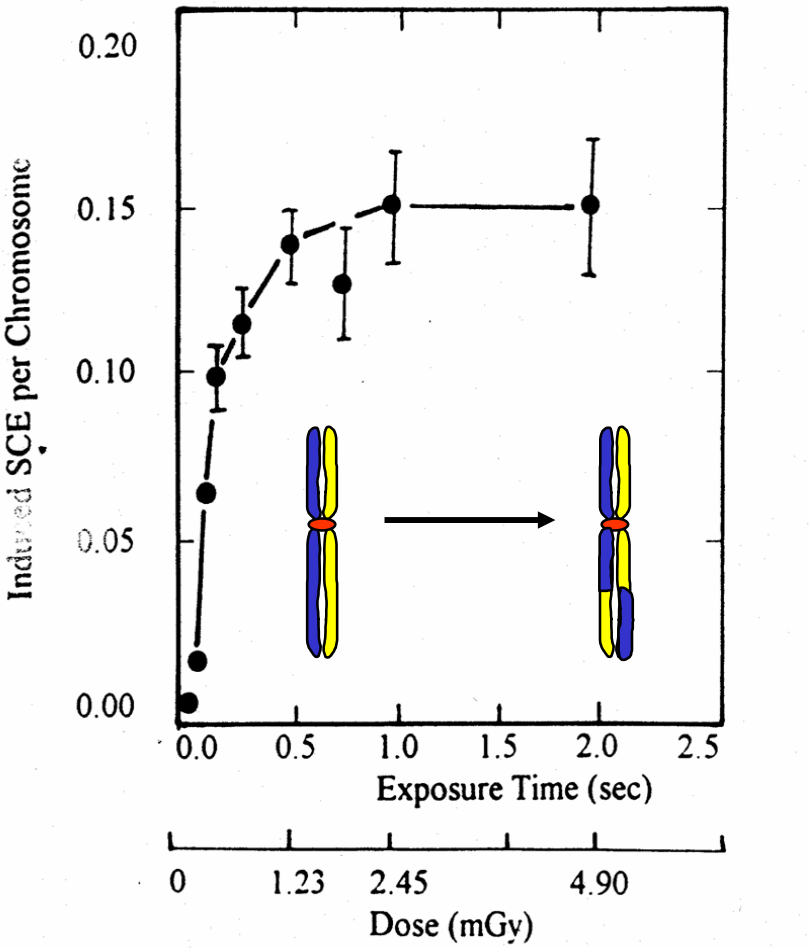


100% cells irradiated



<1% cells irradiated

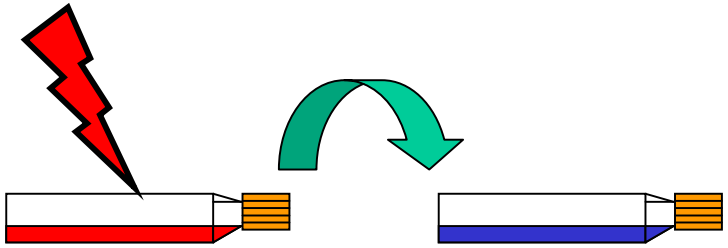
- 30% of the cells showed sister chromatid exchanges (chromosome changes)



Nagasawa, H. and Little, J. B., 1992, *Cancer Res.*, **52**, 6394

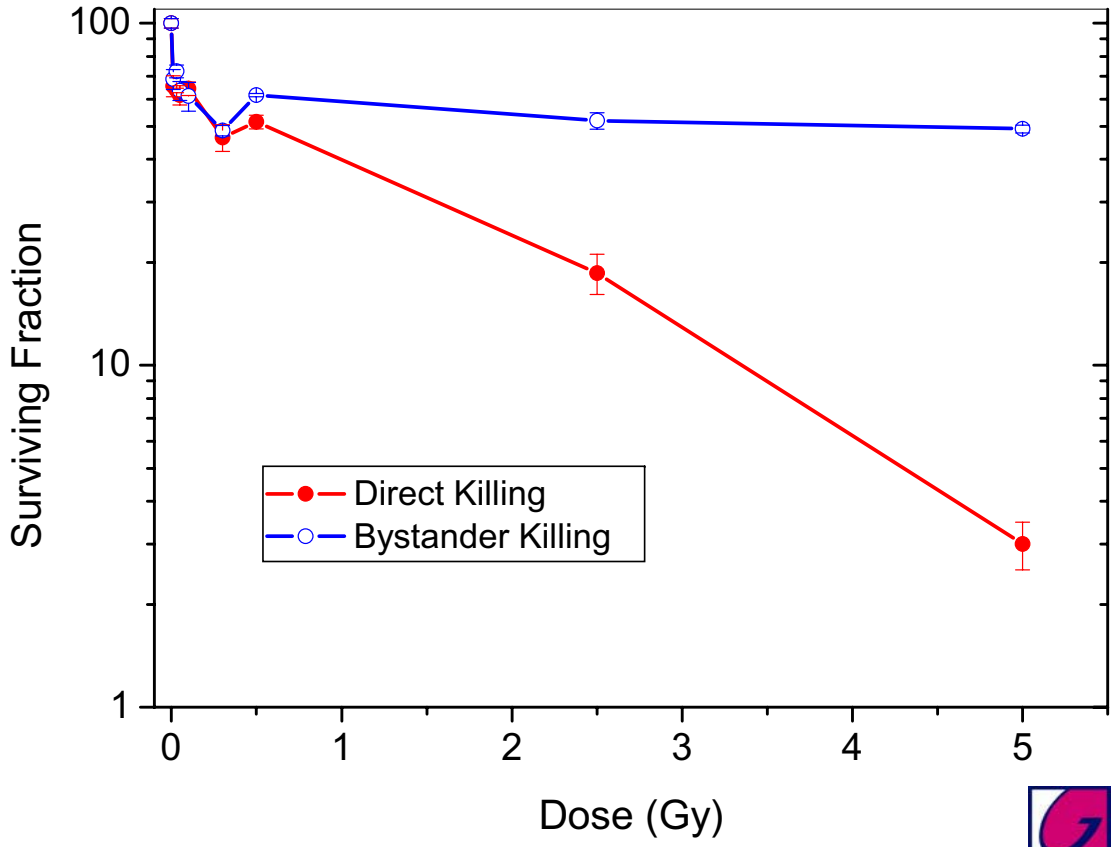
Experimental evidence for bystander responses

- Medium transfer
  - From irradiated to non-irradiated cells
  - Mothersill and Seymour, 1997



Cell culture medium filtered then transferred from irradiated to non-irradiated cells

Seymour and Mothersill, 2000
Radiat. Res., **153,** 508-511



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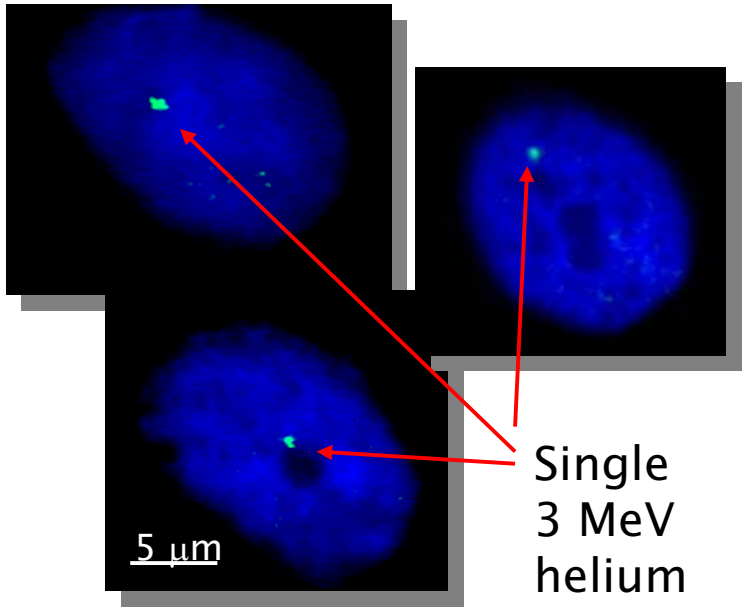
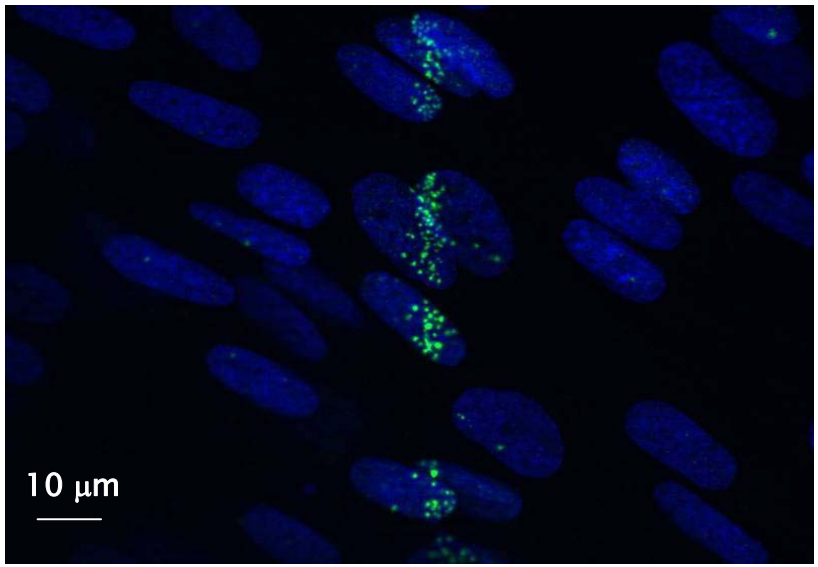
DNA damage in microbeam targeted human fibroblasts

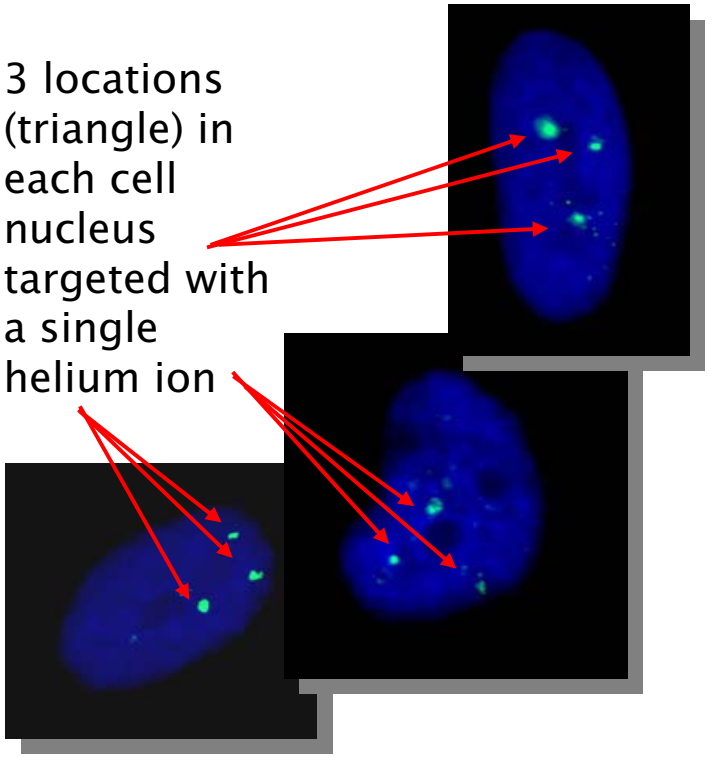


Single 3 MeV helium ion



5 helium ions per μm

3 locations (triangle) in each cell nucleus targeted with a single helium ion



1 helium ion delivers 100 mGy equivalent to 4 – 6 dsb

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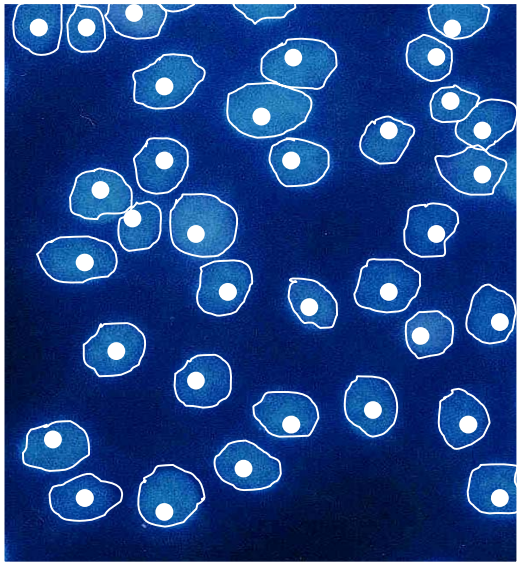
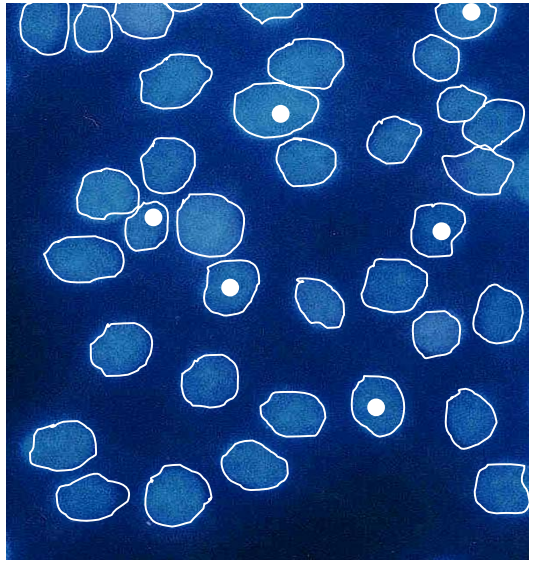
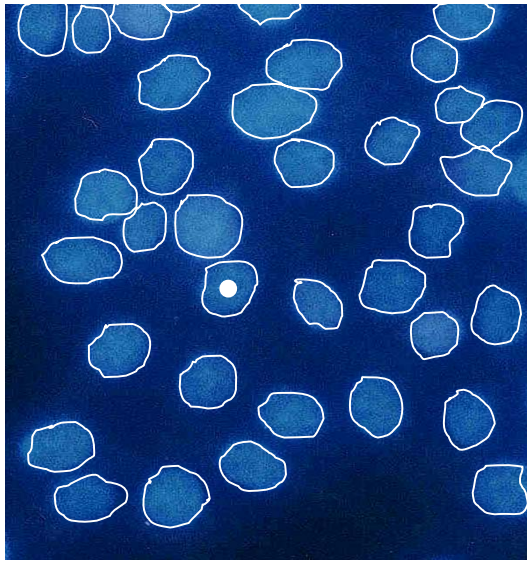
Ion-microbeam bystander studies



100% cells



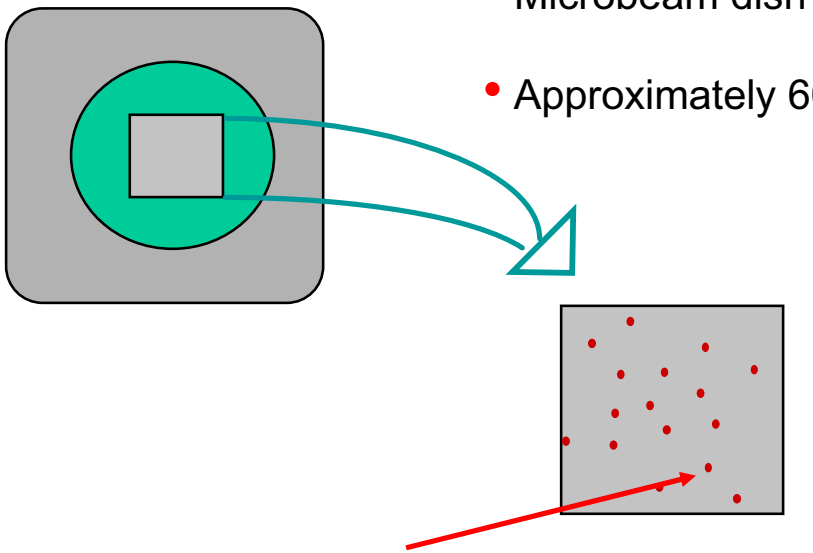
20% cells



Single cell

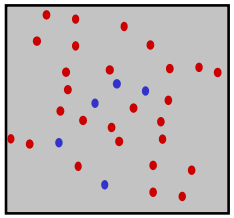
Cell dish scanned and all cells located
Different percentages irradiated with individual ions
Selected at random if less than 100%

Microbeam bystander experiments



- Microbeam dish with 1 region (10 x 10 mm)
- Approximately 600 - 800 cells (G_1) seeded in each area

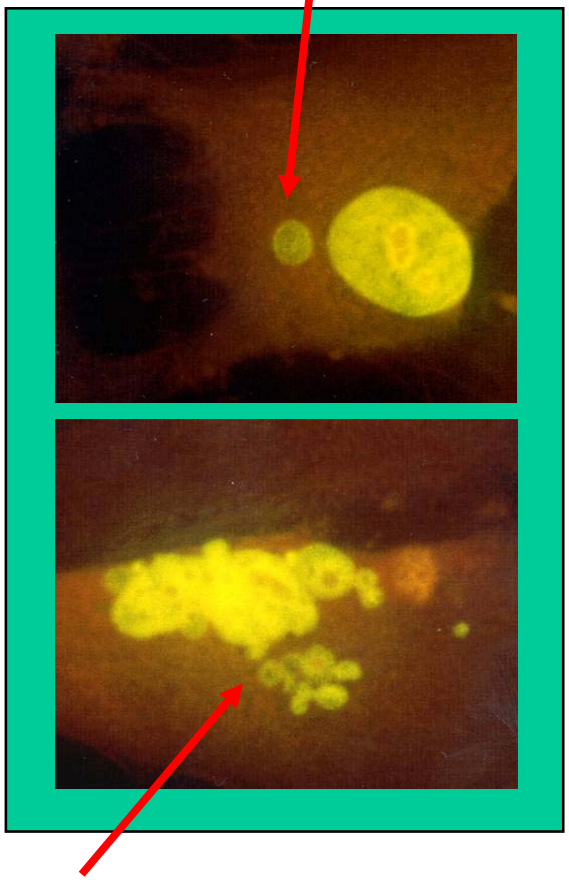
- 1 cell in area located and exposed to 0 - 15 particles



- Dish scored 3 days later for damaged cells

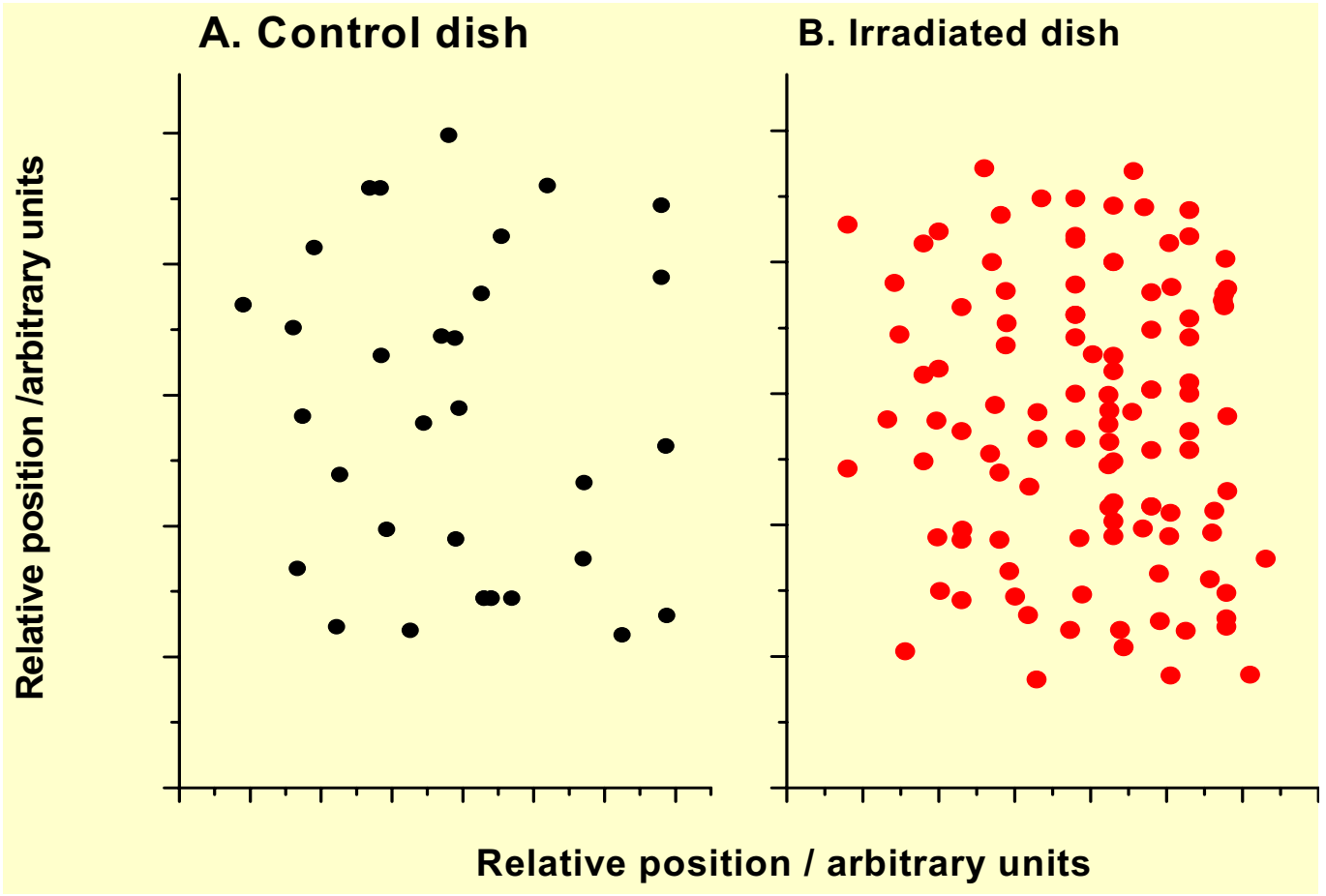
Distribution of damaged cells

Fragment of chromosome (micronucleus)

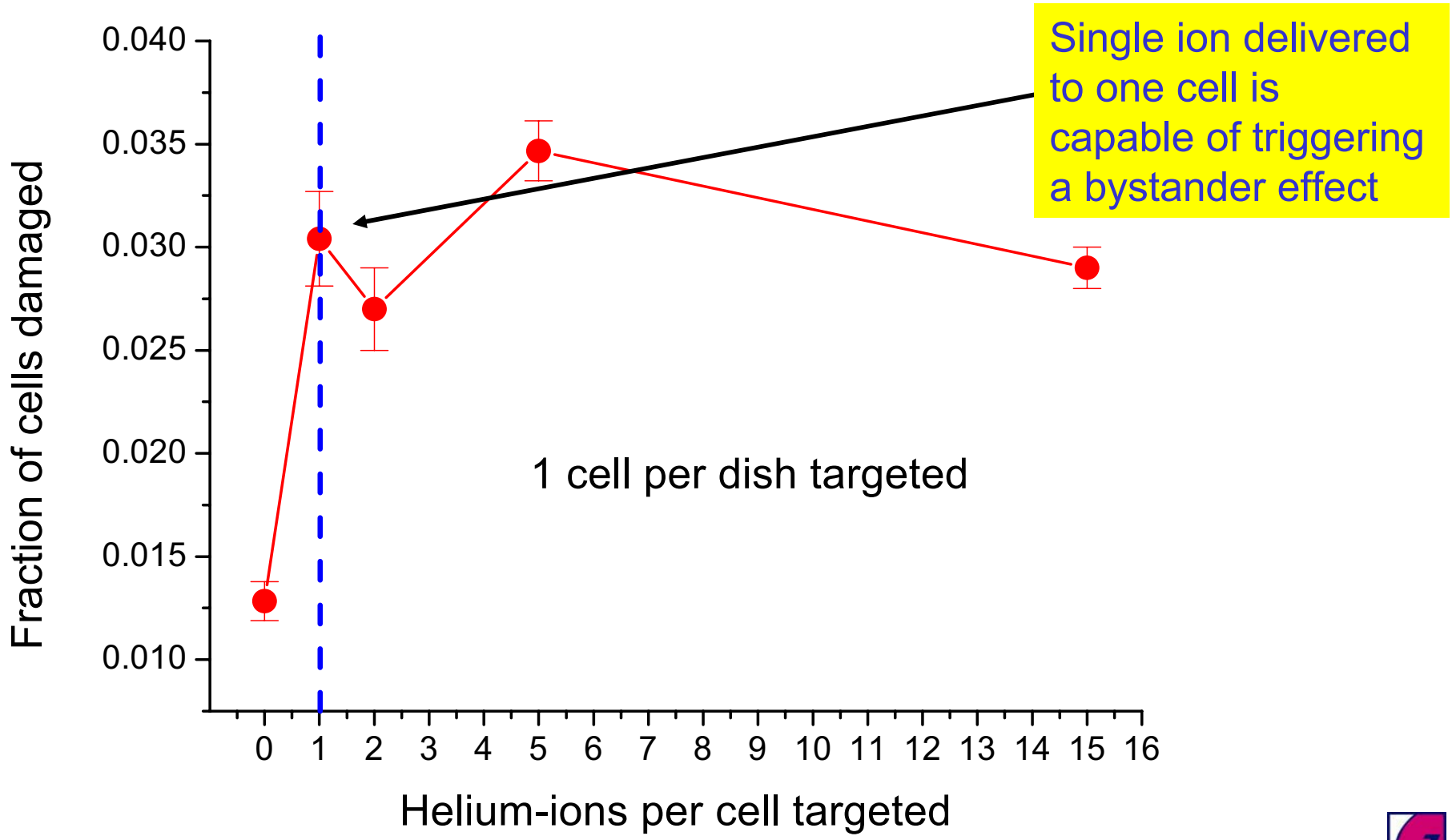


Heavily damaged cell nucleus

Single cell irradiated with microbeam but multiple cells damaged



Bystander damaged cells in human fibroblasts

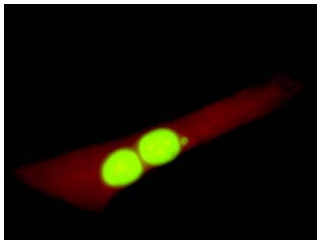


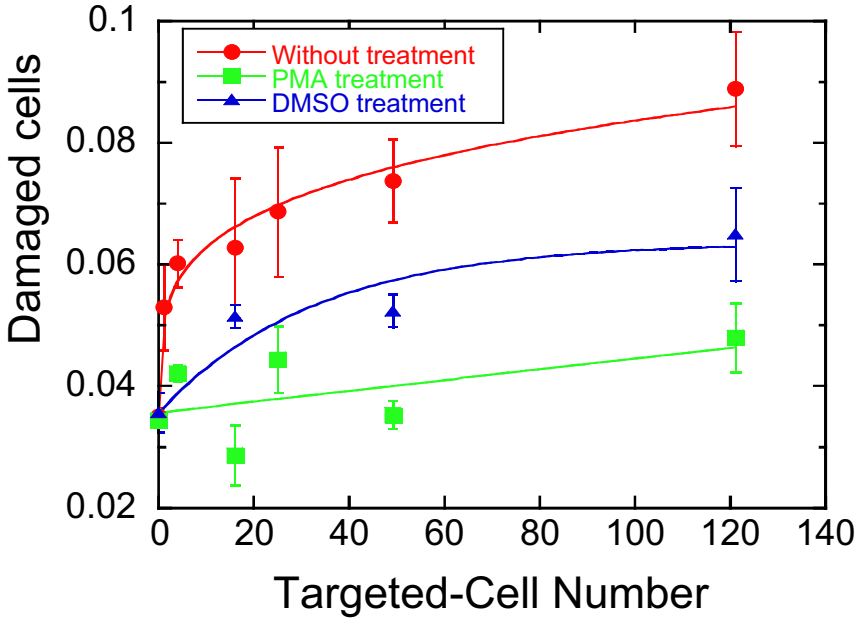
Belyakov *et al.,* 2001, *Brit. J. Cancer,* **84,** 674-679

Microbeam bystander studies with heavy ions

Confluent human fibroblasts
JAERI heavy ion microbeam

^{40}Argon ions (~1260keV/μm)





PMA prevents direct cell – cell communication

DMSO removes damaging free radicals in the medium

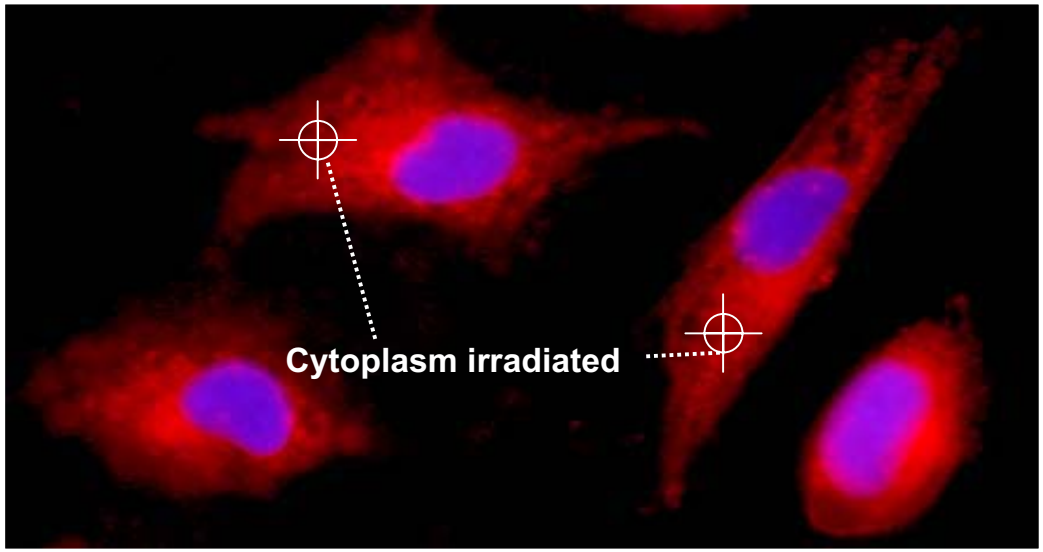
Direct cell – cell communication important

Shao *et al.*, (2003) *FASEB J.* 17:1422-1427

Targeted Studies – microbeam ions

- 1 or 10 tumour cells within 1200 cells targeted with individual helium ions

- Particles delivered to
  - Centre of nucleus
  - Cytoplasm 9μm away from nucleus
  - Control 9μm away from nucleus or cytoplasm



- Micronuclei scored 48 hours later

Where are the targets for initiating a bystander signal?

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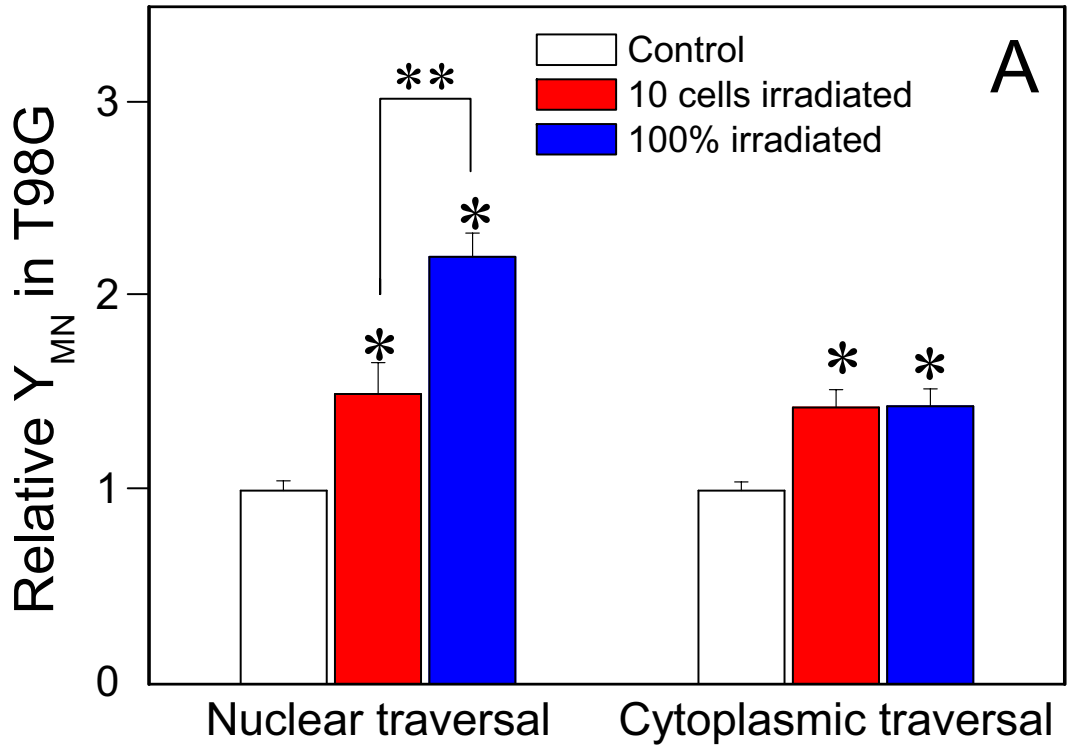
Targeted studies in fibroblasts and tumour cells

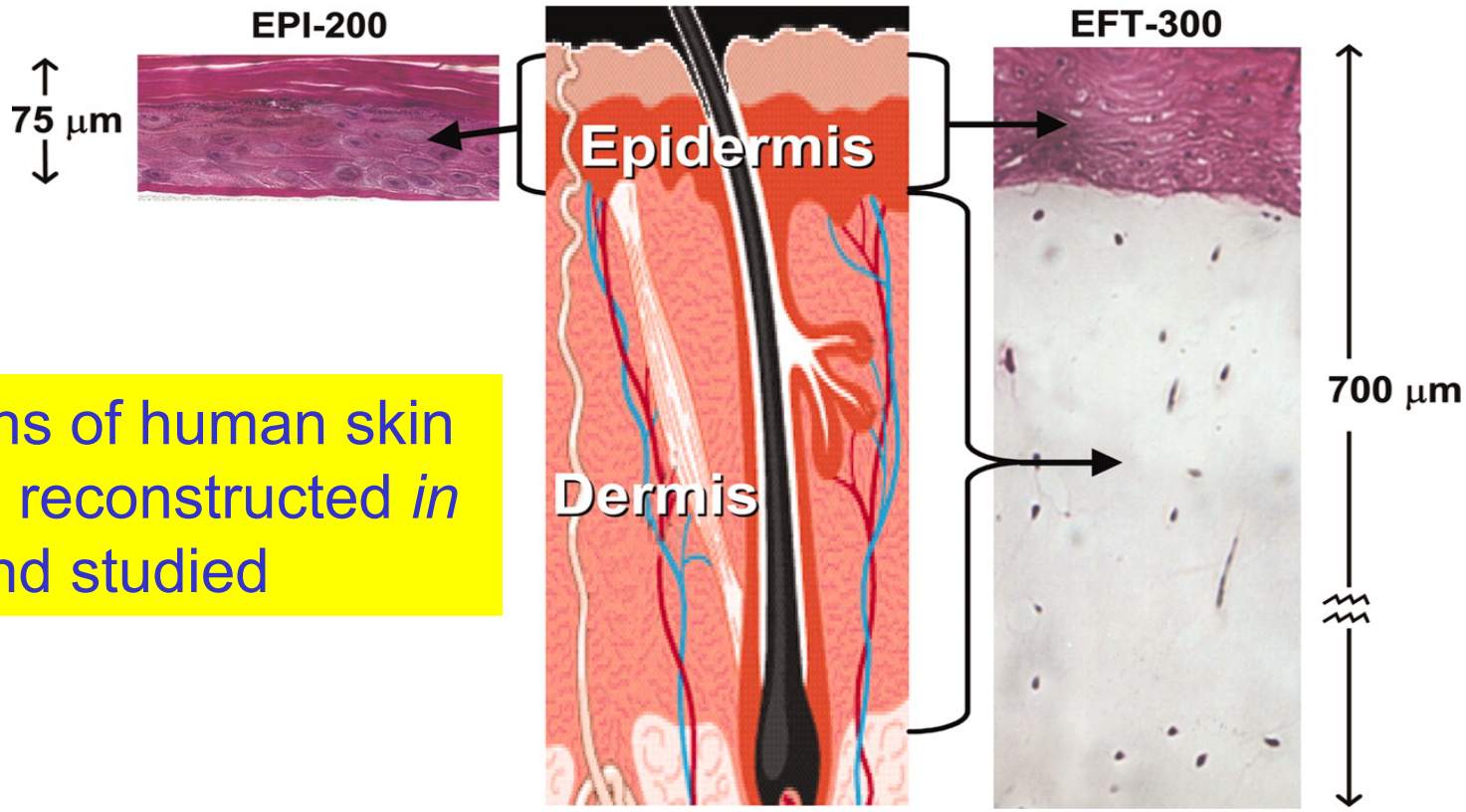
- 10 cells or 100% irradiated with 1 helium-3 ion
- Cell irradiated through nucleus or cytoplasm only
- Cytoplasm effect independent of number of cells targeted



Direct DNA damage not required to produce a bystander response

Shao *et al 2004*

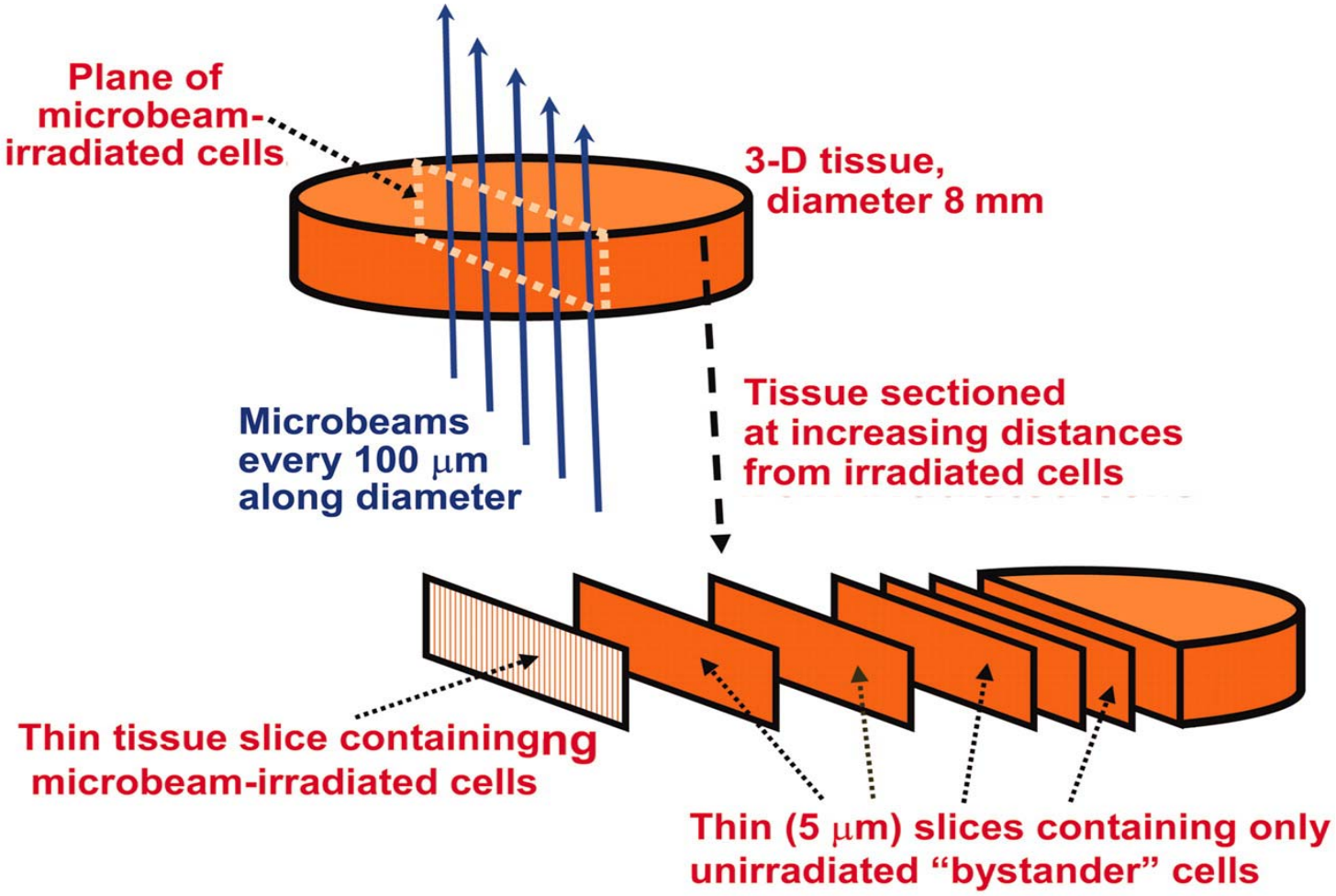
Bystander effect – Skin reconstruct model



Sections of human skin can be reconstructed *in vitro* and studied

Belyakov, Oleg V. et al. (2005) Proc. Natl. Acad. Sci. USA 102, 14203-14208

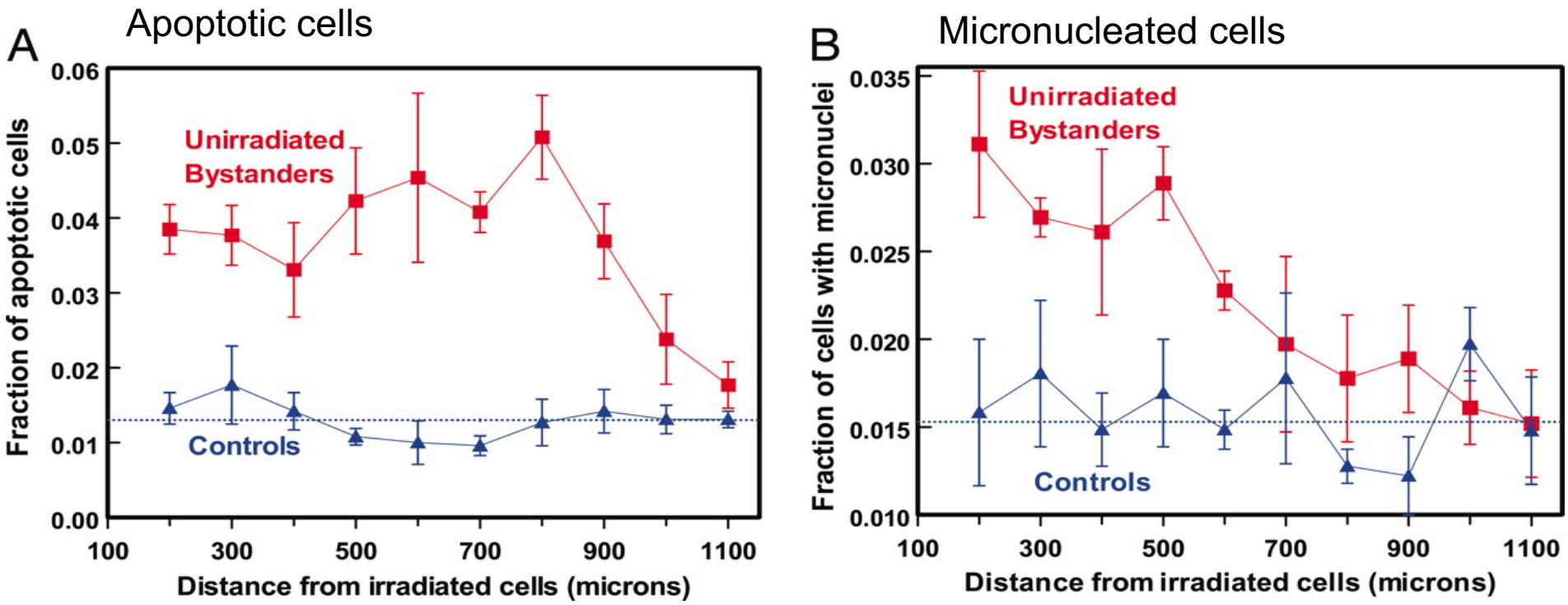
Bystander effect – tissue targeting



Tissues can be locally irradiated with a microbeam

Belyakov, Oleg V. et al. (2005) Proc. Natl. Acad. Sci. USA 102, 14203-14208

Bystander effect – range in tissue



- Bystander response observed in 3-D tissue
- Range of up to 1 mm from exposed cells

Belyakov, Oleg V. et al. (2005) Proc. Natl. Acad. Sci. USA 102, 14203-14208

Evidence for bystander responses *in vivo*

- In radiotherapy there is evidence for effects outside the radiation fields (Abscopal effects)
  - Distant bystander effects?
    - Kaminski *et al.*, 2005
- Localised irradiation *in vivo* shows evidence for bystander responses
  - Irradiation of base of rat lung leads to damage in apex (TGF-β)
    - *Khan et al., 1998*
  - Irradiation of mouse leg leads to p53 dependent tumor volume reduction at remote site
    - *Camphausen et al., 2003*

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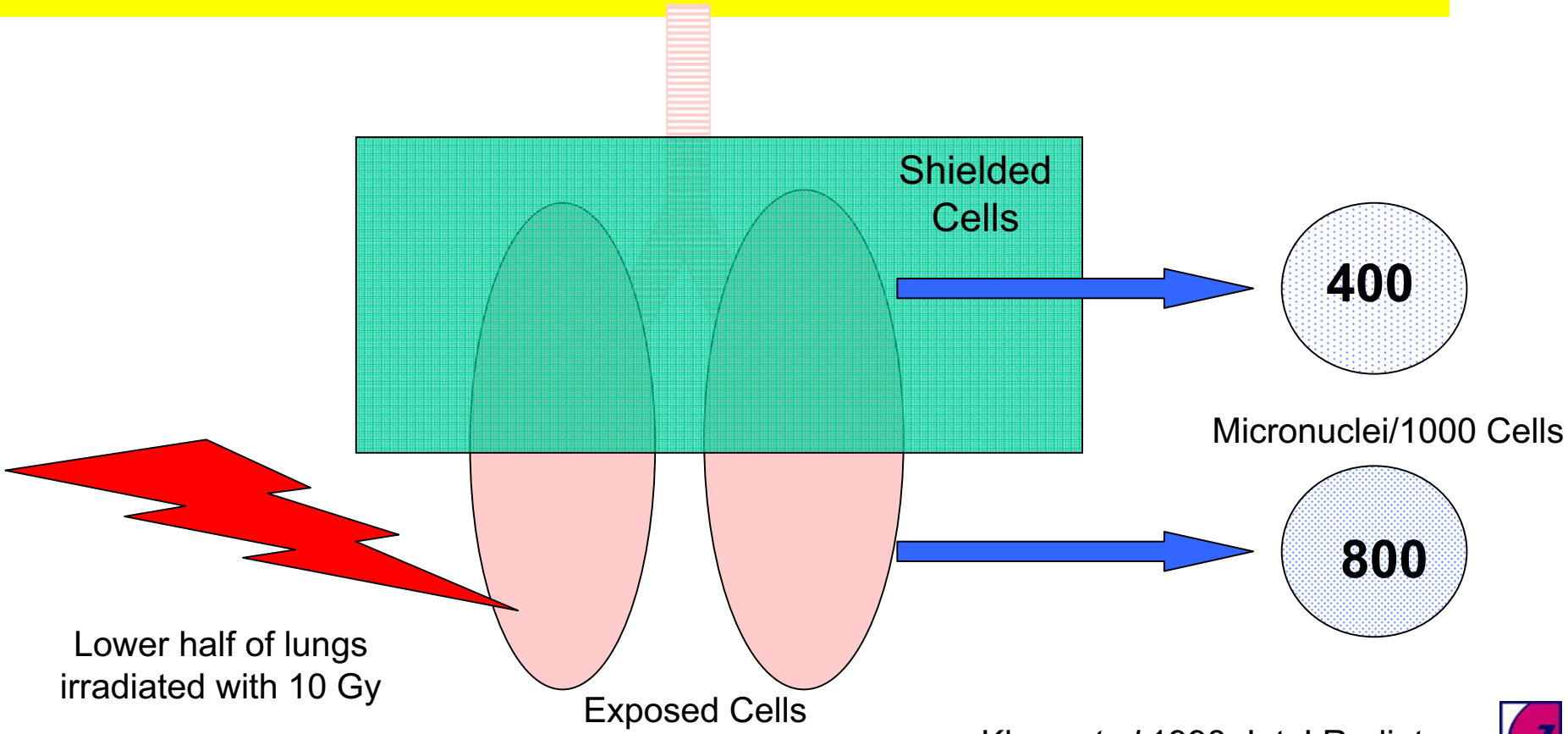
Bystander effects *in vivo* radiation-induced micronuclei in lung

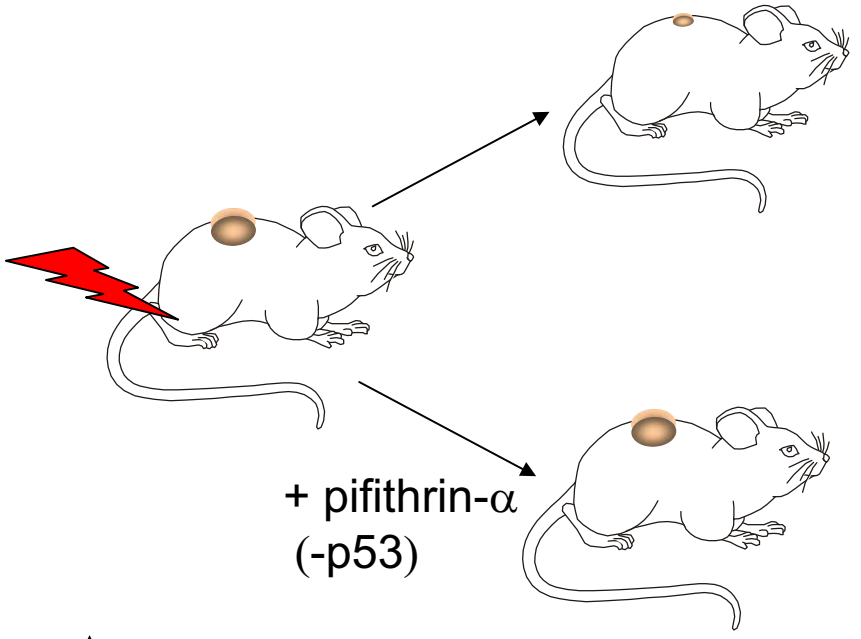
- Lung cells shielded from direct irradiation show increased chromosomal damage
- Long range communication within the lung

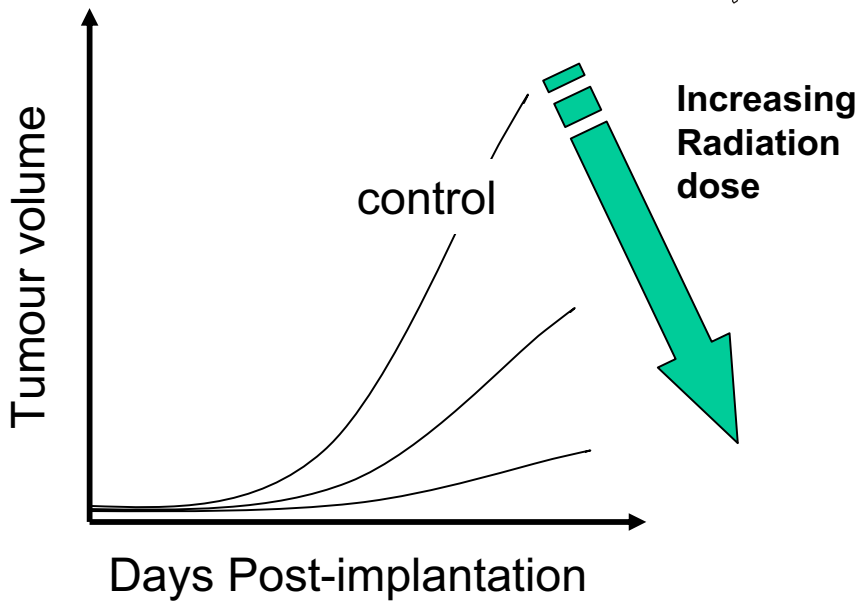


Khan *et al* 1998, Int J Radiat Oncol Biol Phys, **40,** 467-76.

Long range effects *in vivo*

- Mice irradiated on leg, tumour transplanted on back
- Dose-dependent *reduction* in tumour volume
- *Relevance to abscopal effects and therapy?*

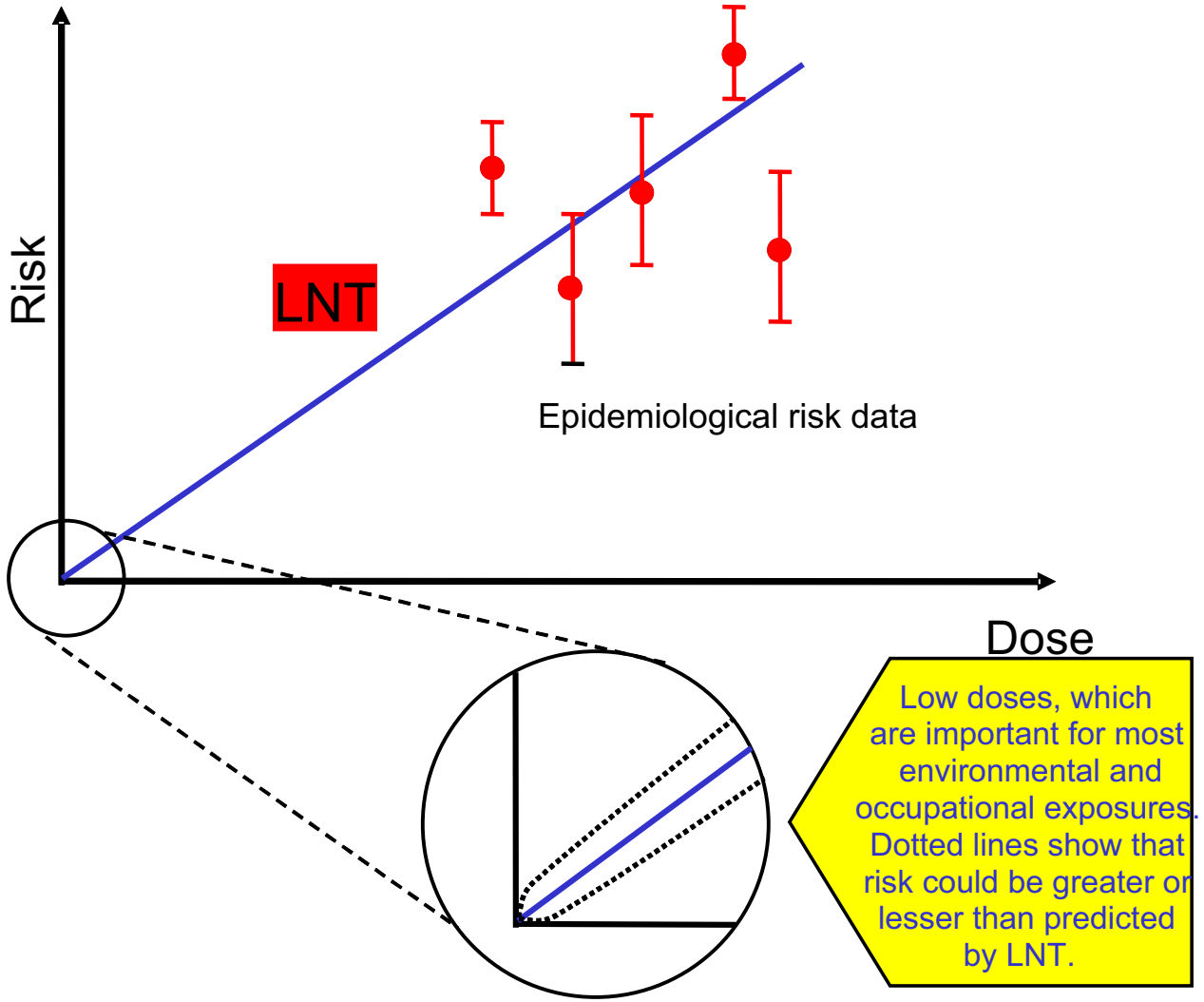




Camphausen *et al.,* 2003 *Cancer Res,* **63,** 1990-1993

Non-targeted responses and radiation risk

- Cancer risk from ionising radiation exposure at low dose is calculated by extrapolation from high dose exposure (Atomic Bomb survivors)
- A linear no-threshold model (LNT) is used
- Evidence for bystander responses being both damaging and protective have been observed in experimental systems



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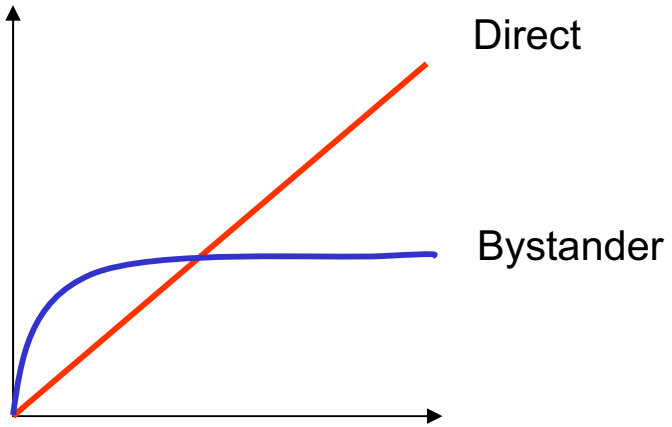
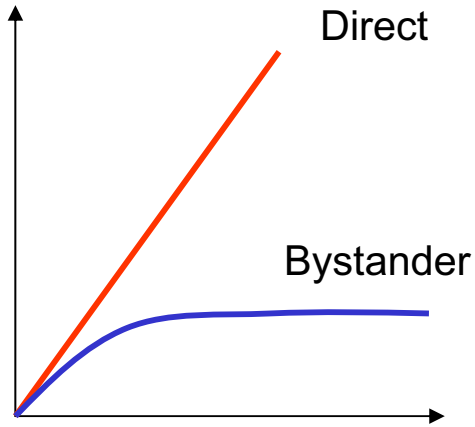
Do bystander responses contribute to radiation risk?

- Transformation data in C3H 10T$^{1/2}$ cells (Brenner, *et al.,* 2002) and mutation data in A_L human-hamster hybrid cells (Zhou *et al.,* 2002) predict additional risk.

- Modelling studies predict little influence in the process of radon-induced lung carcinogenesis (Little and Wakeford, 2001).



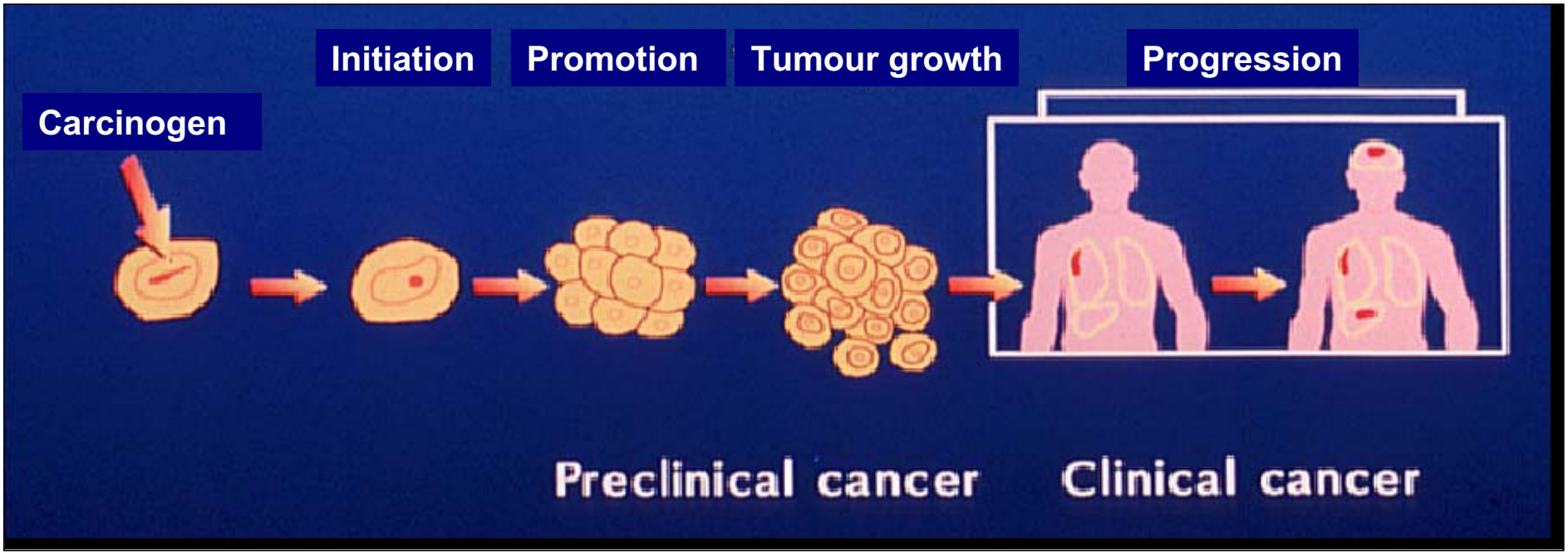


If bystander effects are damaging process they could *increase* risk at low dose and if they are protective processes the could *decrease* risk

Key Points – bystander responses

- Low dose effect – saturates at high doses
- Observed for a range of endpoints
- Factors released into the medium or direct cell-cell communication involved
- Can involve damaging or protective responses
- May impact on radiation risk at low dose and use of radiation in therapeutic approaches

Cancer is a multistage process



Progression involves significant instability leading to tumour formation

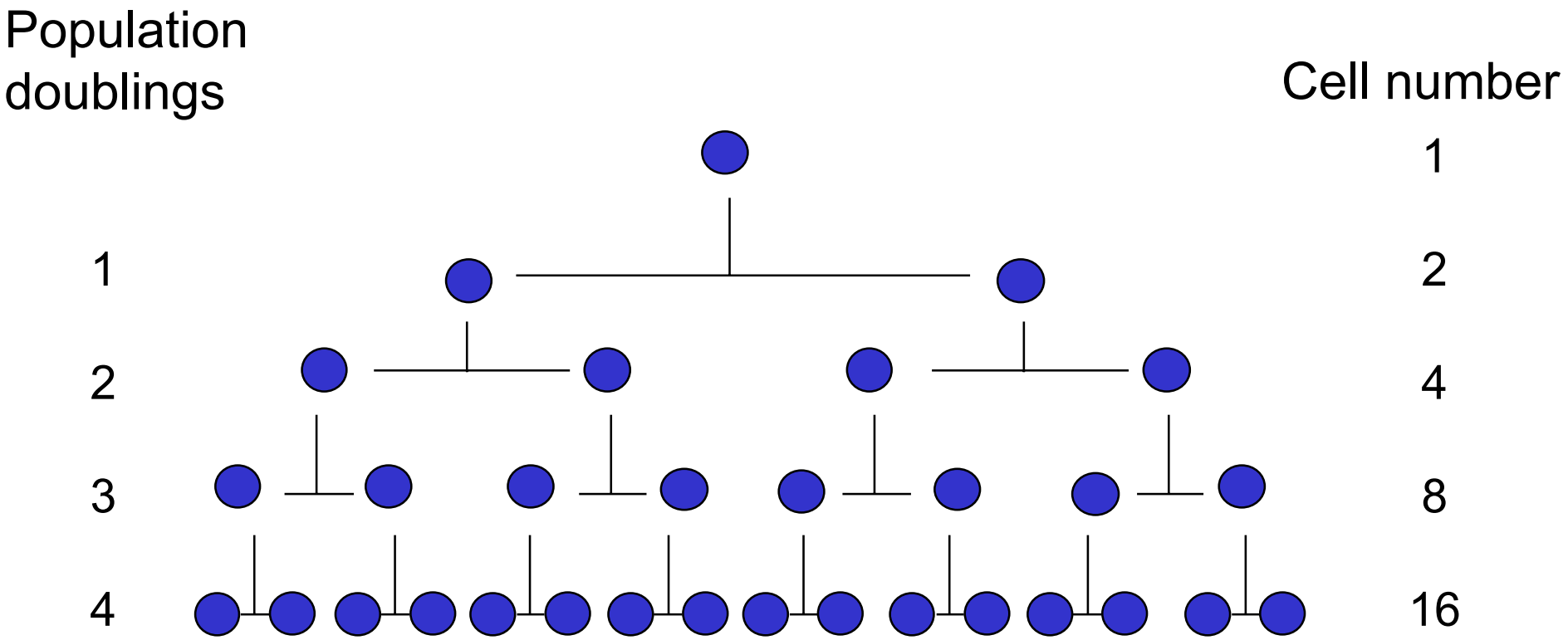
Genomic Instability and Cancer

- Cancer is a multistage process: An accumulation of genetic defects in surviving cells eventually result in a group of cells with cancerous attributes

- Operationally divided into:
  - Initiation – damage to a target cell
  - Promotion – amplification of the effect (cell proliferation)
  - Progression – cell proliferation / genetic instability

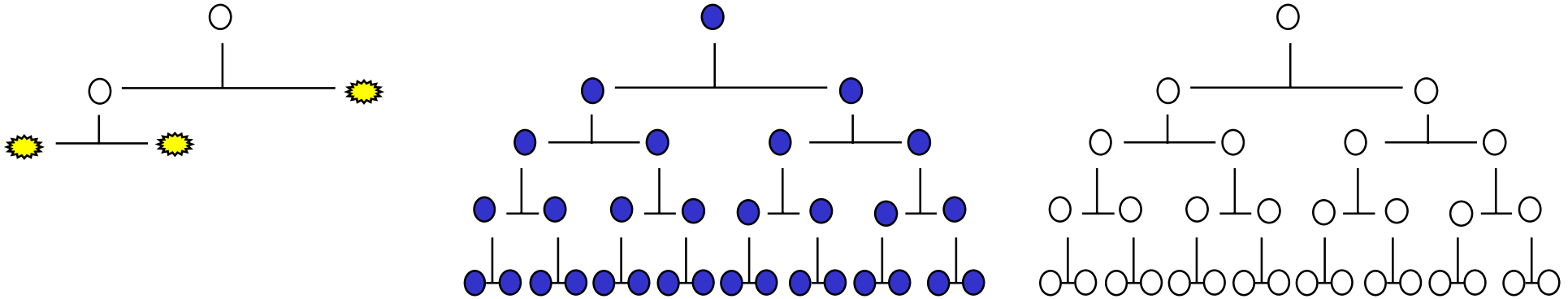
- Genomic / genetic instability is defined as an increased rate of acquisition of alterations in the genome

Control population



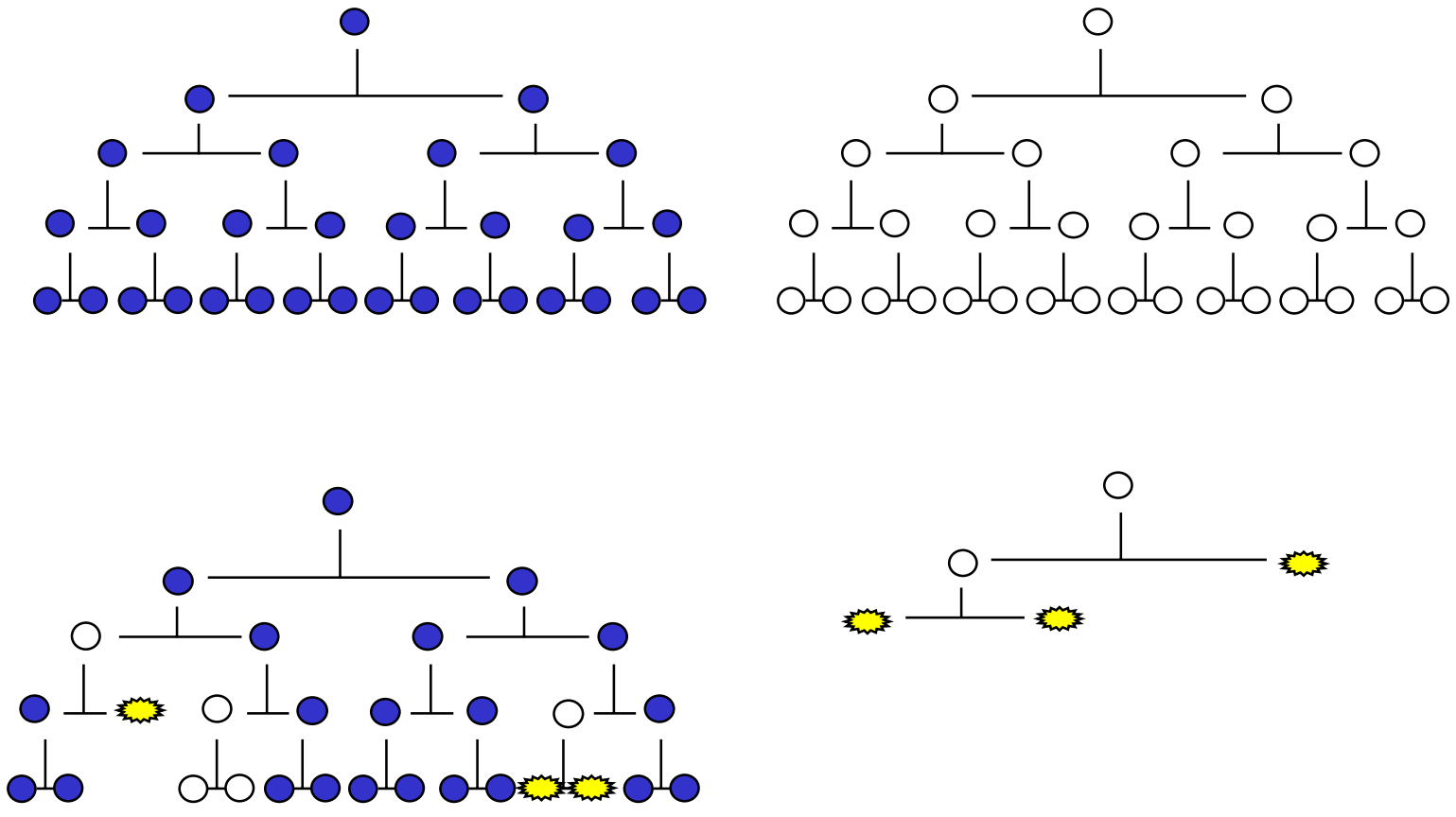
Clonal response - each cell identical to original cell/clone

Irradiated - clonal damage



Normal cell
Mutated cell
Dead cell

Irradiated - instability



- Radiation-induced genomic instability leads to delayed, non-clonal effects

Acute cell survival

- Puck and Marcus, 1956 – developed to measure radiation killing in tumour cells *in vitro*
- Measures reproductive death
- Cells allowed to divide for several generations until visible colonies
- 50 cells per colony taken as arbitrary cutoff for viability
- For radiation, cells undergo several rounds of division to form abortive colonies
- For radiation, heterogeneity in colony sizes observed

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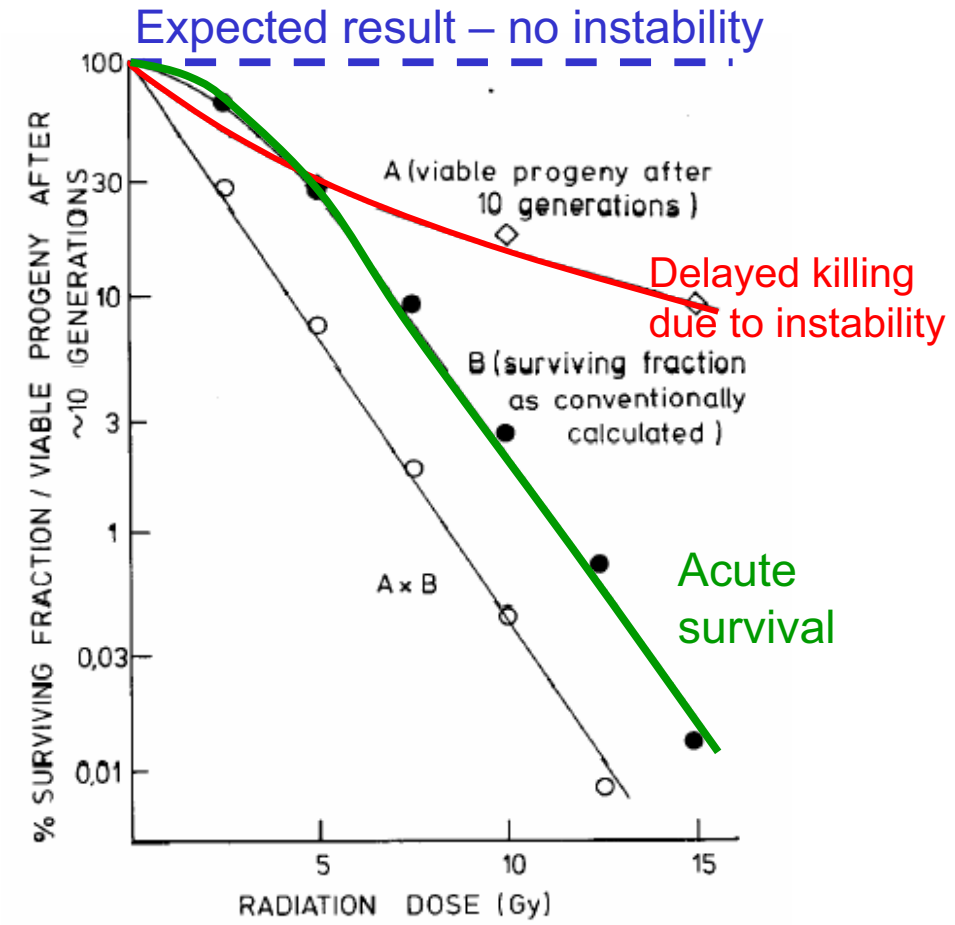
Instability – lethal mutations

- Irradiated cells allowed to form colonies and surviving fraction calculated.
- Individual cells isolated from these colonies and cultured for a further 10 generations before survival measured.
- Delayed cell killing classified as lethal mutations.



Mothersill, C. and Seymour, C., 1997, *International Journal of Radiation Biology,* **71**, 751-8.

Measurement of DNA mutations

- After irradiation, DNA damage not correctly repair leads to mutations

- Different types of mutations
  - Point mutation – 1 or more base pairs substituted
  - Partial deletion – part of the gene missing
  - Total deletion – all of the gene deleted

- A common assay is to measure these in a single gene
  - Hypoxanthine-guanine phosphoribosyltransferase
  - Located on X-chromosome (single copy in male cells)
  - Catalyses the conversion of guanine and hypoxanthine to corresponding nucleoside -5'-monophosphates
  - 6-thioguanine when added to cells incorporated into DNA and toxic if HPRT functional

- Spontaneous mutation rate very low - ~ 1 in 10^6 cells

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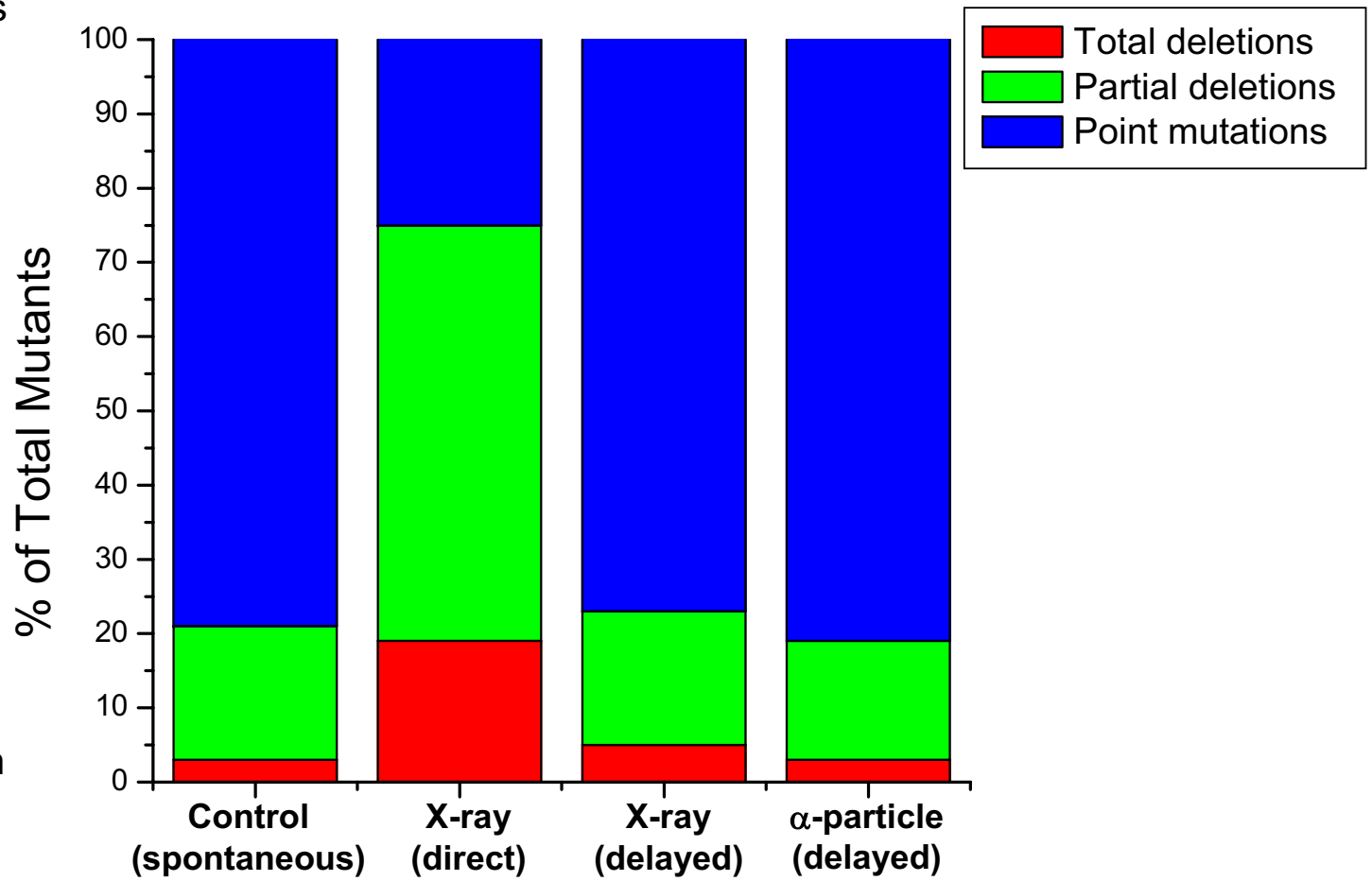
The spectra of mutations produced by instability is different from direct damage

- Measurement of mutations at the HPRT gene in hamster cells
- Mutation measured immediately after irradiation (direct) or 25 population doubling times later (delayed)
- Direct mutations produced by X-rays are predominantly deletions
- 10% of population 25 population doublings later showed increased mutation frequency 100 -10,000 fold above background
- Spectrum of delayed mutations similar to spontaneous mutations

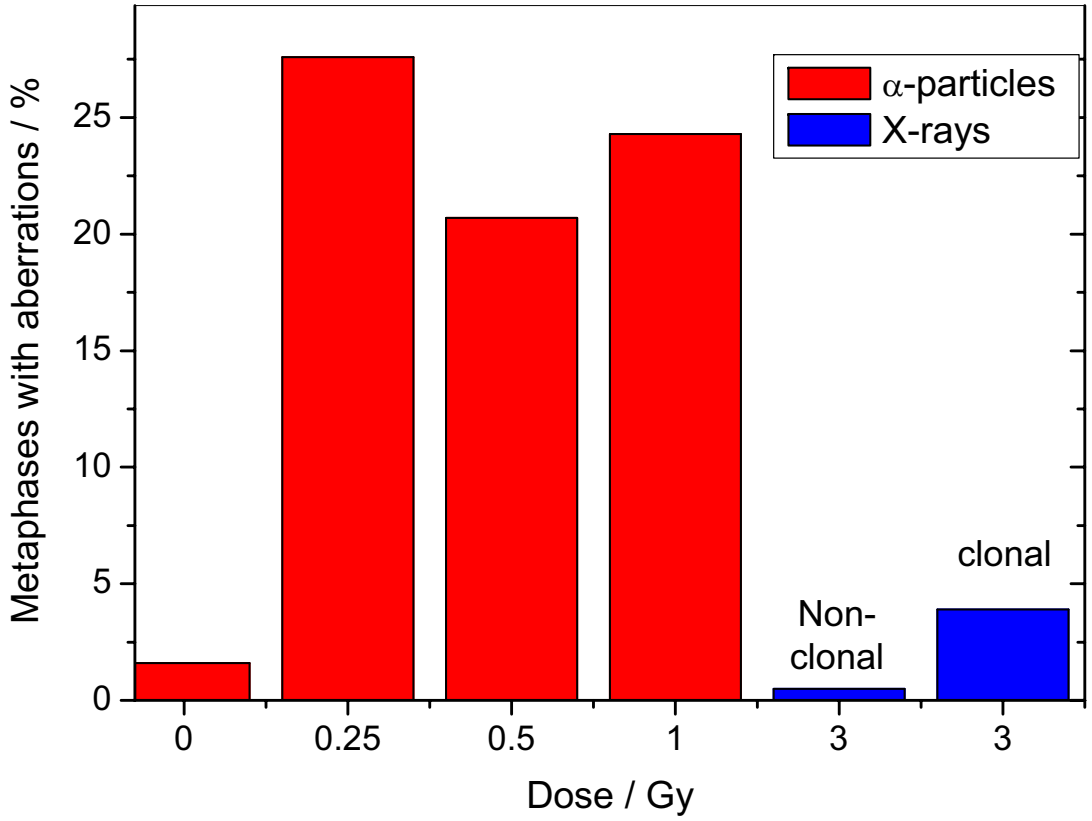


Little, J. B., Nagasawa, H., Pfenning, T. and Vetrovs, H., 1997, *Radiation Research,* **148**, 299-307.

Radiation-induced genomic instability – radiation type

Studies in haemopoietic stem cells

- Precursors of white and red blood cells
- Target cell population for leukaemia
- Chromosomal aberrations in mouse stem cells measured 12-13 population doublings after irradiation

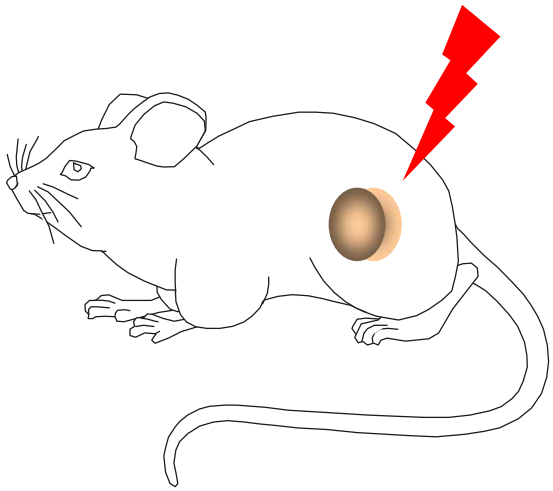


- Highly dependent on radiation type (quality)
- Significant at low doses
- No dose response
- Aberrations were non-clonal

Kadhim, M. A., Macdonald, D. A., Goodhead, D. T., Lorimore, S. A., Marsden, S. J. and Wright, E. G., 1992, *Nature,* **355**, 738-40.

Genomic instability *in vivo*

- Mouse fetuses irradiated at the zygote (2 cell stage) with 2Gy of X-rays
- Skin biopsies obtained at day 19 of gestation and propagated *in vitro*
- Chromosome aberrations measured
- Developmental abnormalities observed
- Increased levels of chromosome and chromatid aberrations



Pampfer, S and Streffer, C. 1989, *Int. J. Radiat. Biol.* **55,** 85-92

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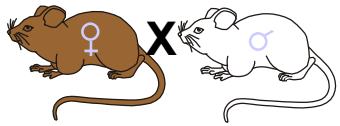
Evidence for a genetic component to instability

CBA/H  Sensitive

DBA/2  Sensitive

C57BL/6  Resistant

C57BL/6 x CBA/H  Resistant

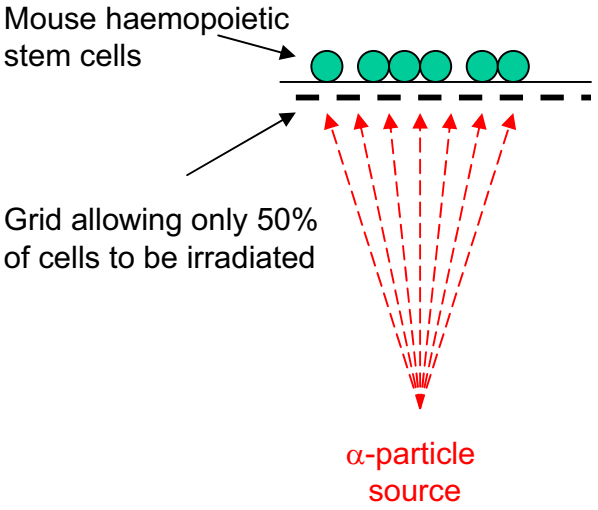
C57BL/6 x DBA/2  Resistant

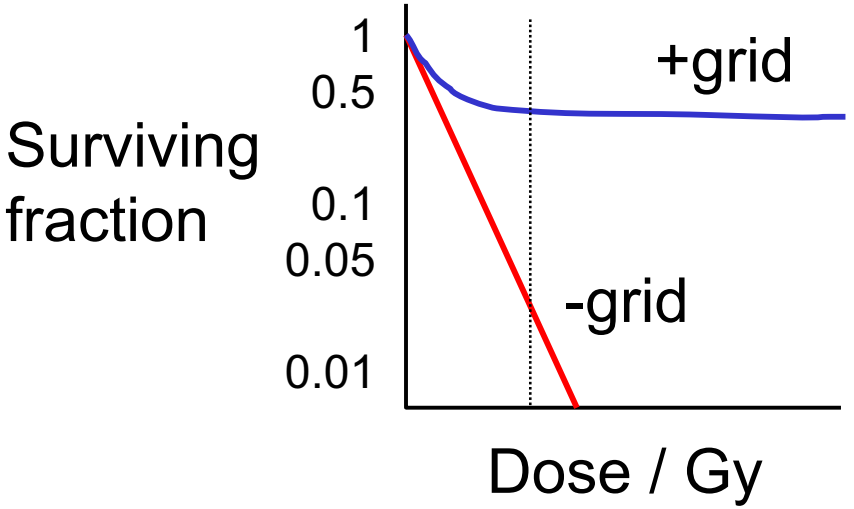
Watson, GE, Lorimore, SA, Clutton, SM, Kadhim, MA ,Wright, EG (1997) *Int J Radiat Biol,* **71:**497-503

Genotype of bone Marrow cells	Metaphases exhibiting chromosomal instability (%)	
CBA/H	50/413	(12.1)
DBA/2	35/335	(10.5)
C57BL/6	11/312	(3.5)
(C57BL/6 x CBA/H) F1	7/191	(3.7)
(C57BL/6 x DBA/2) F1	16/465	(3.4)

- Genomic instability transmissible from generation to generation *in vivo*
- Mouse strains resistant to the genomic instability phenotype are dominant in crosses
- Persisting oxidative stress observed in sensitive strains

Bystander and instability may be related





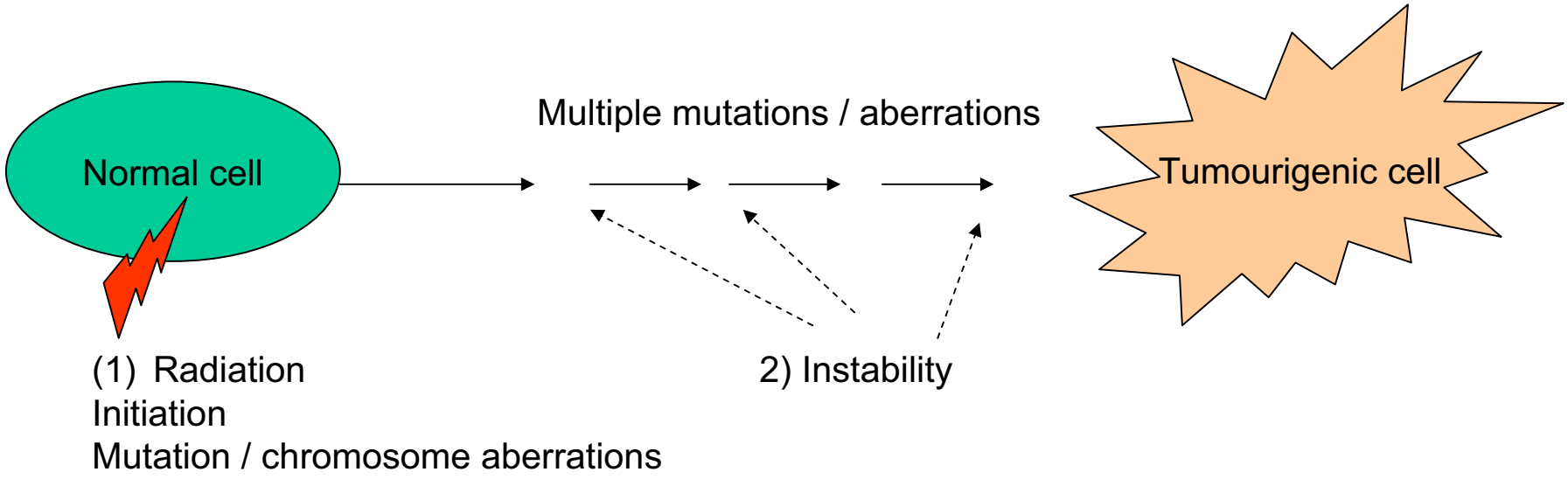
α-particle irradiation dose (Gy)	Surviving Fraction	Mean number of aberrations per cell (%)	
Control	1.00	36/662	(7.0)
1Gy	0.01	137/1009	(22.0)
1Gy + grid	0.58	115/871	(21.0)

- The level of instability is similar despite the presence of the grid shielding 50% of the targeted cells

Lorimore, SA, Kadhim, MA, Pocock, DA, Papworth, D, Stevens, DL, Goodhead, DT, Wright, EG (1998), *Proceedings of the National Academy of Sciences* **95:**5730-5733.

Bystander effects and carcinogenesis

Multistage Cancer Model



- Bystander effects can lead to mutations
- Bystander effects may be related to instability

Key points – genomic instability

- Genomic instability is defined as an increased rate of acquisition of alterations in the genome
- Genomic instability is involved in carcinogenesis
- Many effects of radiation are clonal in origin, radiation-induced instability is non-clonal
- Instability observed up to 50 generations after exposure
- Also observed *in vivo* with a genetic component
- Spectra of damage induced is different from direct damage

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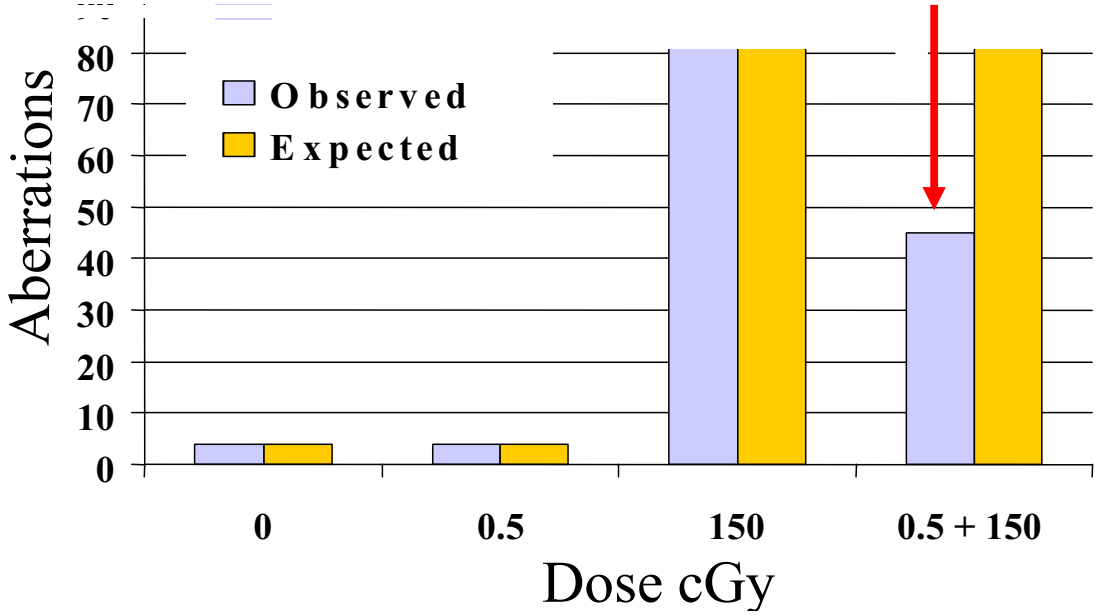
Adaptive responses

- Pre-treatment with a low dose leads to protection from a subsequent high dose
- Observed in a range of cells and tissues
- Observed for a range of endpoints
- DNA repair pathways involved

Adaptive Response – *in vitro*

- Pre-treatment with low dose (< 10cGy)
- 24 hours incubation
- Challenge with high dose (> 0.5Gy)

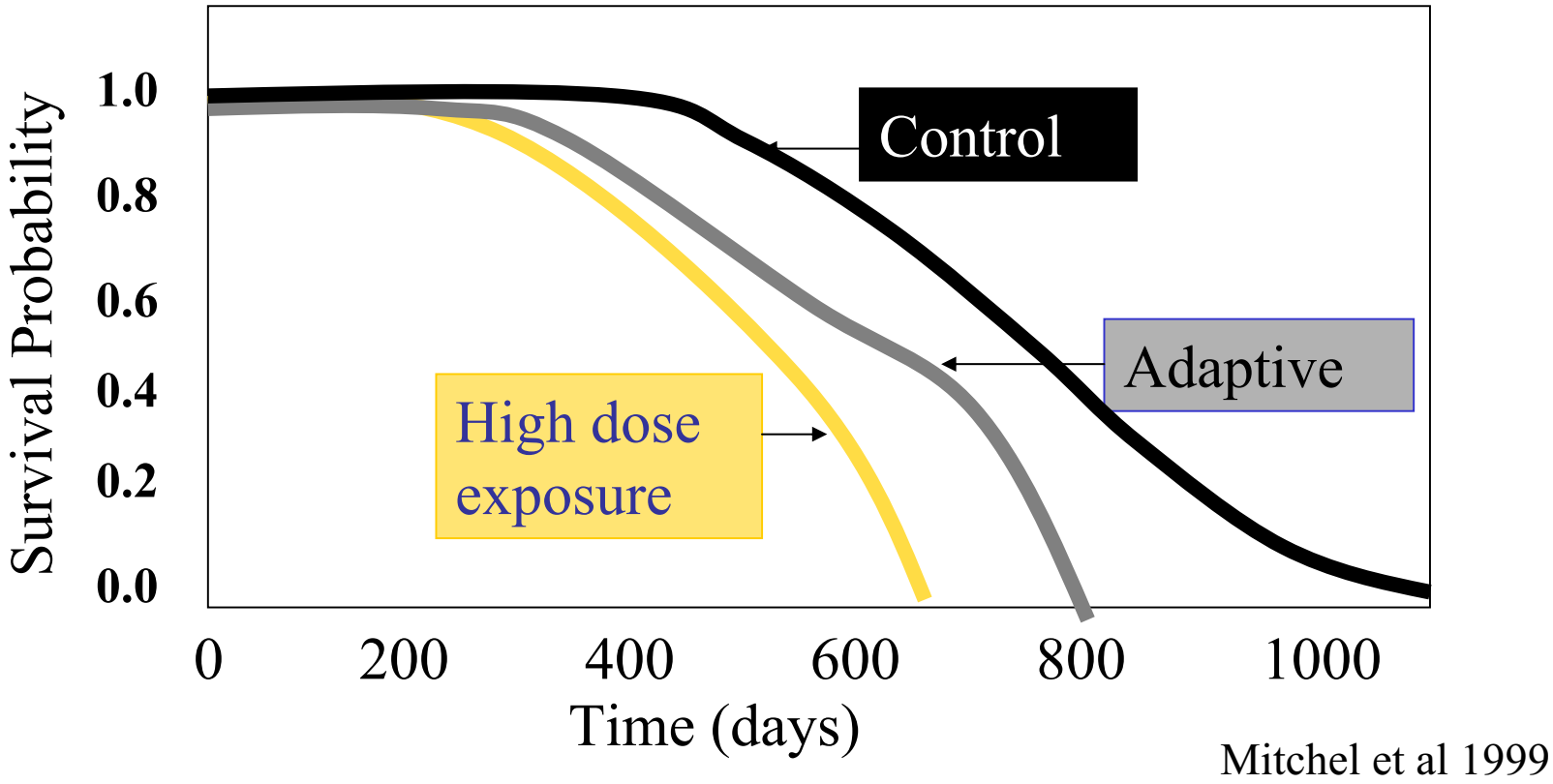
Human lymphocytes exposed to X-rays
Aberrations scored after irradiation



Shadley & Wolff, 1987,Radiat Res., 111, 511

Pre-treatment with a low dose leads to protection from a subsequent high dose

Adaptive Responses *in vivo*



Mitchel et al 1999

Mice that were exposed to 10 cGy of radiation before the large dose of radiation, developed leukemia later and lived longer than the mice that received only a large dose of radiation.

Hormesis

- The word "hormesis" is derived from the Greek word "hormaein" which means "to excite"
- Predicts that agents may be beneficial at low concentration, but harmful at higher
- The theory that small doses of radiation can induce beneficial biological processes and are healthful

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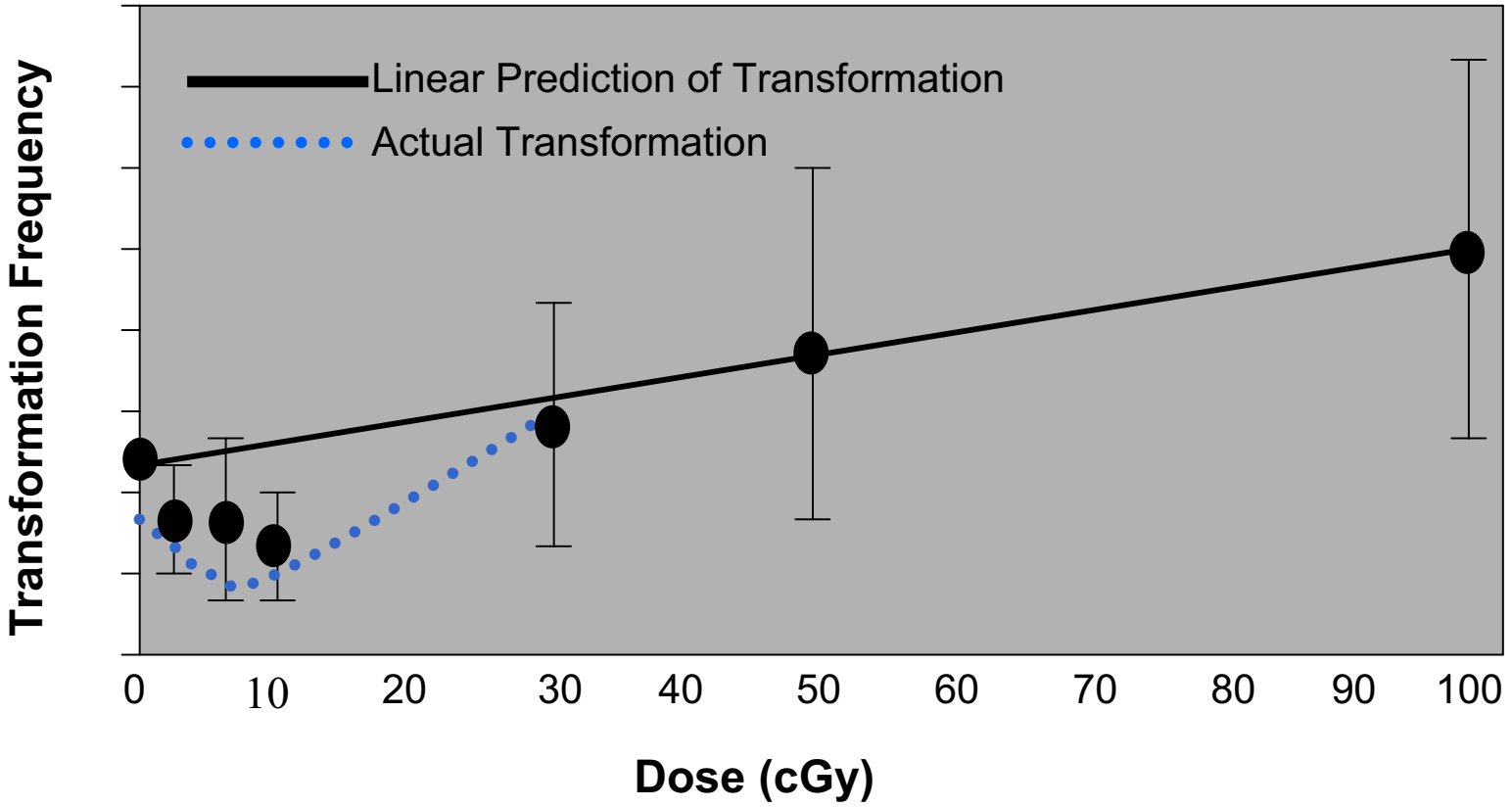
Cells transforming to cancerous cells

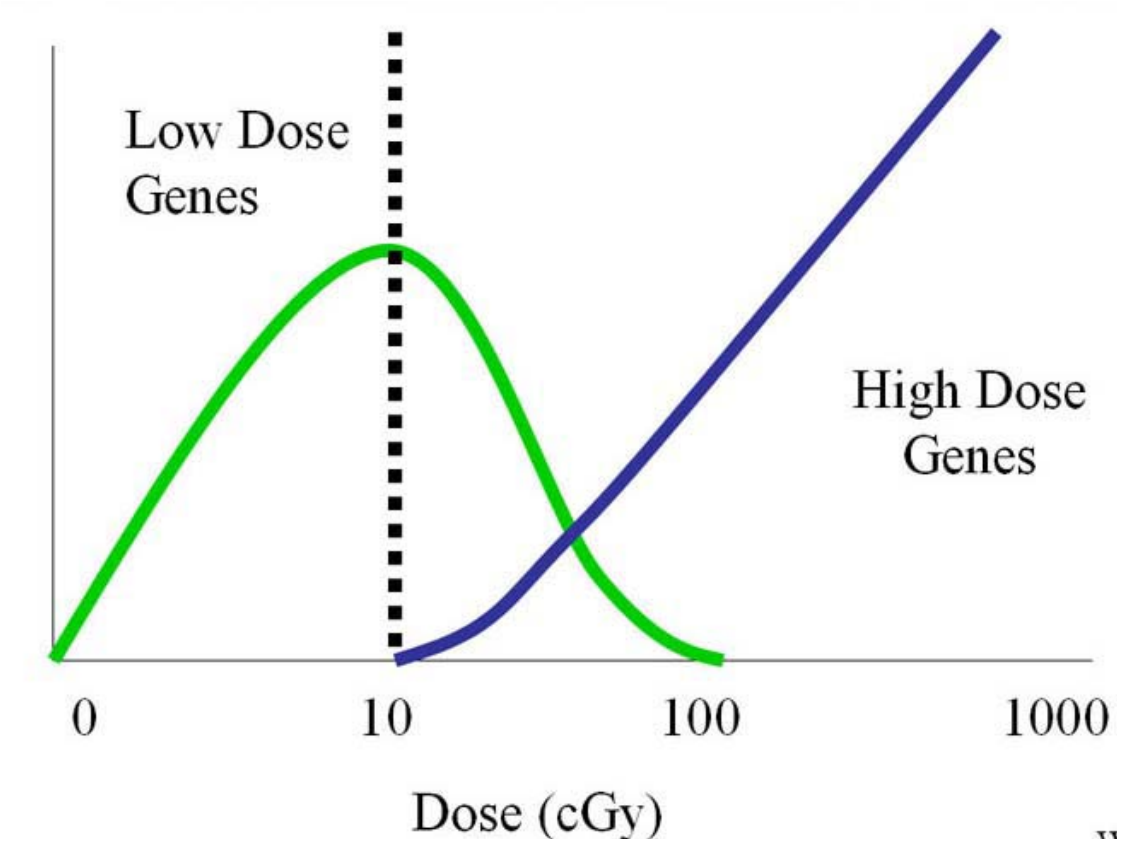


At very low doses, the transformation frequency is below that predicted by linear extrapolation

Redpath *et al.* 2001

Gene induction

- Gene array technology allows many thousands of genes to be analysed after irradiation exposure
- Both the dose effect relationship and the types of genes expressed is dose-dependent

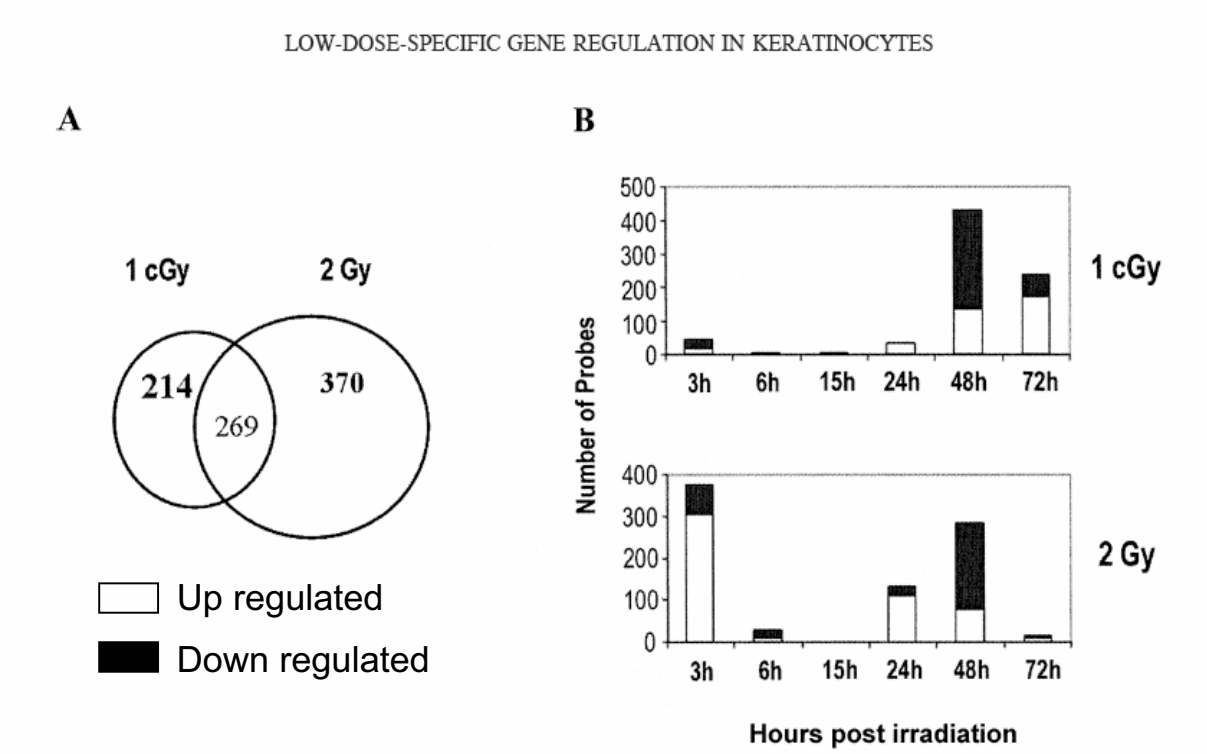


Yin *et al., Int J Radiat Biol,* **79**, 759-75.

Gene expression at low and high dose

- Specific genes are induced at low dose

  Franco N *et al.* Radiat. Res. 163, 2005

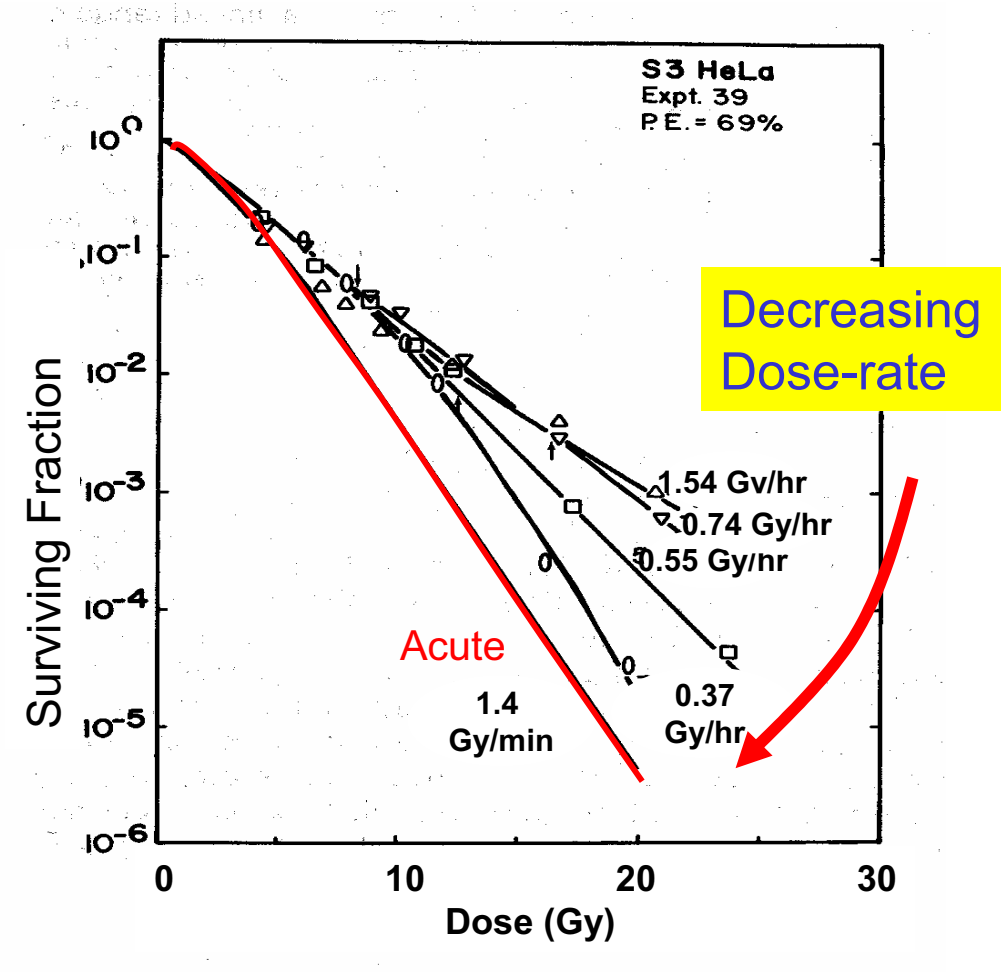


1- Genes induced at 1 cGy include genes involved in homeostasis, stress, cellular signaling, cytoskeleton, RNA synthesis, membrane function and transport

2- Low dose-responsive genes rarely include DNA repair genes

Inverse dose-rate effect

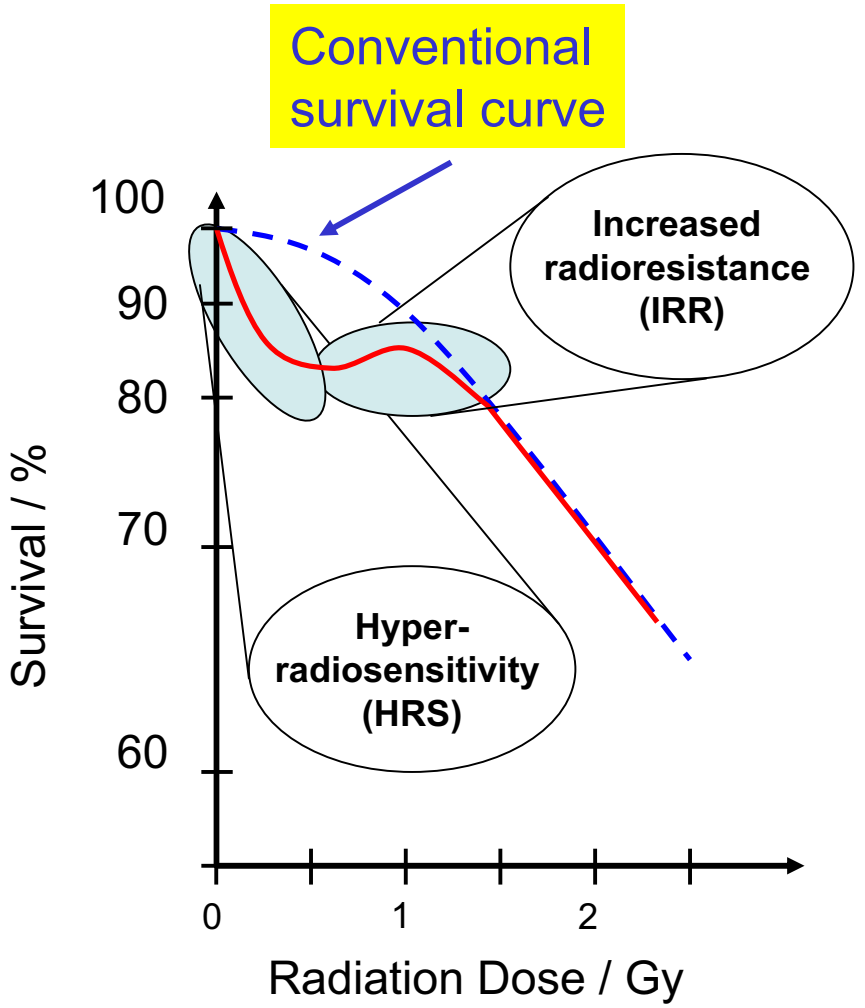
- Normally reducing the dose-rate leads to increased survival due to the repair of sub-lethal damage
- At very low dose-rates, increased radiosensitivity is observed due to increased proliferation
- Increase sensitivity is due to cells becoming blocked in the radiosensitive G2 phase of the cell cycle



Mitchell *et al.*, 1979, *Radiation Res.* **79,** 520-536

Low dose hypersensitivity

- Survival at low dose (< 0.2Gy) shows a hypersensitive response (HRS)
- Survival at high dose shows induced radioresistance (IRR)
- Cell cycle dependent
- Radioresistant tumour cells show biggest HRS/IRR ratio
- Observed for ultrafractionation of tumours *in vivo*



Joiner *et al.*, 2001, IJRBO, 49, 379

Key points – Non-targeted responses

- Non-targeted responses are changes in cells to radiation exposure not related to direct energy deposition in the DNA
- A range of non-targeted responses have been found
- Seen for many type of radiation-including ions
- They have been measured in model systems and *in vivo*
- A common feature is that they are important at low dose and saturate at high dose
- Many non-targeted responses may be interrelated

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Useful References

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- Morgan WF. (2003). Non-targeted and delayed effects of exposure to ionizing radiation: II. Radiation-induced genomic instability and bystander effects in vivo, clastogenic factors and transgenerational effects. *Radiat Res*, **159,** 581-96.

- Prise KM, Schettino G, Folkard M and Held KD. (2005). New insights on cell death from radiation exposure. *Lancet Oncol*, **6,** 520-8.

- Tapio S and Jacob V. (2006). Radioadaptive response revisited. *Radiat Environ Biophys*. EPUB DOI10.1007/s00411-006-0078-8

- Joiner MC, Marples B, Lambin P, Short SC and Turesson I. (2001). Low-dose hypersensitivity: current status and possible mechanisms. *Int J Radiat Oncol Biol Phys*, **49,** 379-89.

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