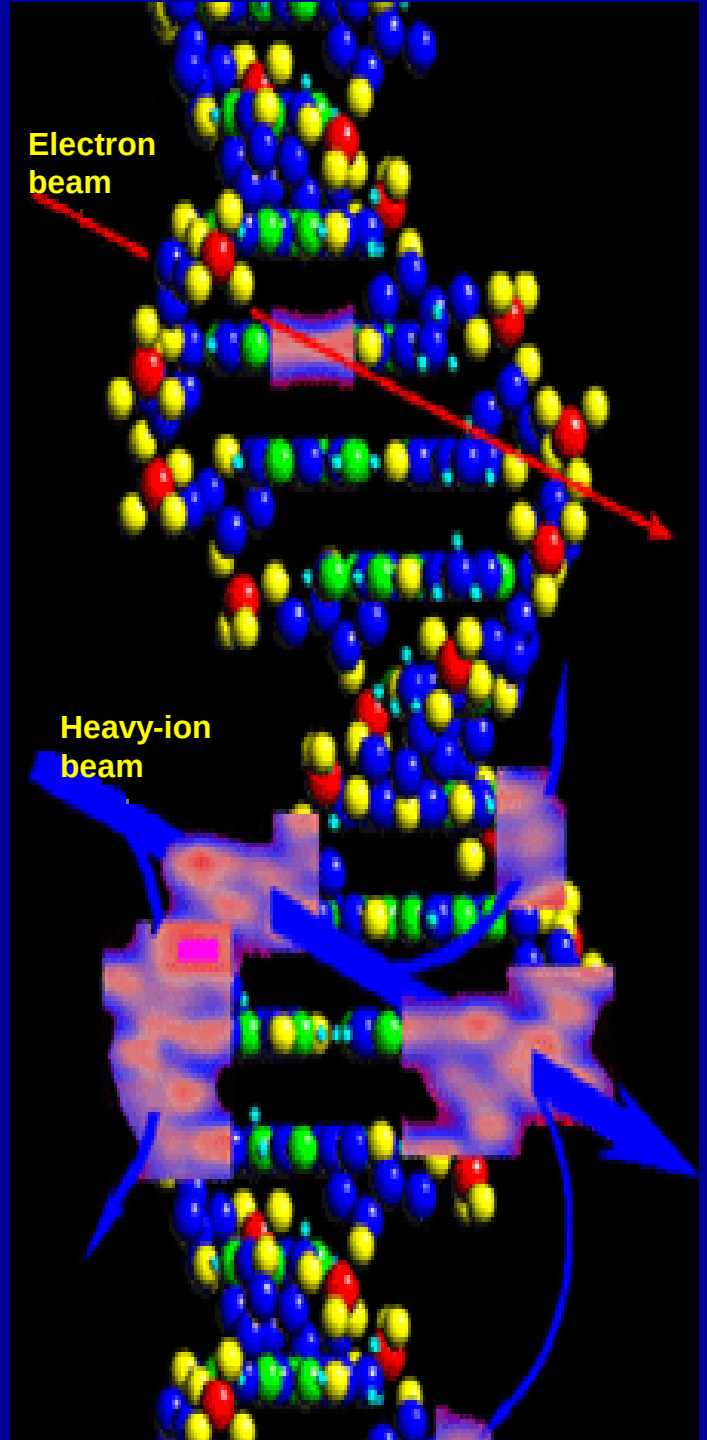
Ultrafast electron beam irradiation effects on DNA damage and repair in normal and cancer cells

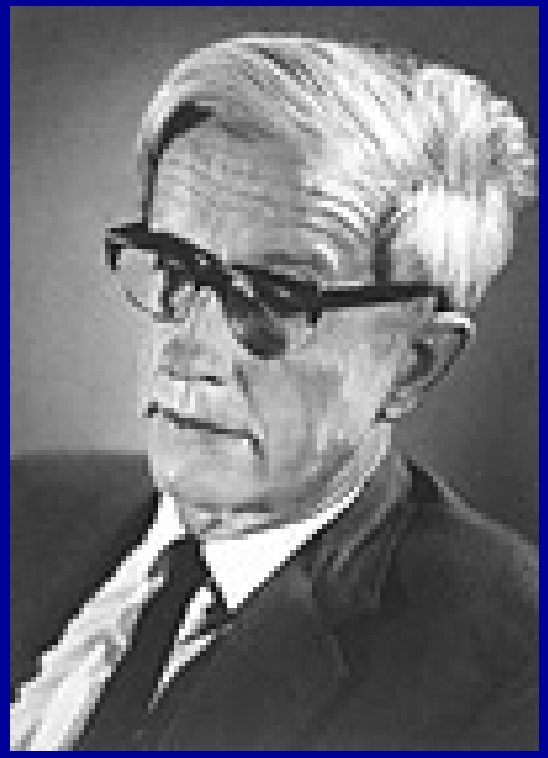
Rouben Aroutiounian
Galina Hovhannisyan,
Nelly Babayan

Faculty of Biology, Yerevan State University;

Institute of Mol.Biology
of National Academy of Sciences RA



Nikolay Timofeev-Ressovsky 1900-1981



Max Delbruck

Niels Bor

Max Born

Timofeeff's principal discovery was his observation of a linear relation between the total radiation dose and the number of mutations. Whether the dose was administered in a single shot, in several fractions or continuously at a low level over an extended period appeared irrelevant.
The intensity of the dose did not affect the number of mutations produced.
He also found no minimum dose below which mutations were not generated.

These properties suggested that **X rays produce mutations much like bombs hitting targets**. Timofeeff, along with his German co-workers Karl G.Zimmer and Max Delbruck, set out the target-or hit-theory based on this analogy. **The classic "three-man paper"** "On the Nature of Gene Mutation and Gene Structure" (1935) **describing their work inspired Erwin Schrodinger to deliver his 1943 course of lectures, later published as the book What Is Life?,** which helped draw many physicists to molecular biology.

In the target model, an **X-ray photon expels electrons from atoms**. These unbound electrons hit other atoms, dislocating more electrons, and so on. The free electrons eventually settle in the electron shells of other atoms**. In this way, an X ray creates positively charged ions (atoms missing electrons) and negatively charged ones (atoms having a surplus of electrons).** One ionization in a gene causes a mutation.

Timofeeff and his collaborators set out to estimate the size of a single gene by calculating the number of ionizations produced in a certain volume of tissue and by recording the increased number of mutations of a particular gene in that tissue. Timofeeff and his co-workers found the gene to be a sphere one to 10 microns across

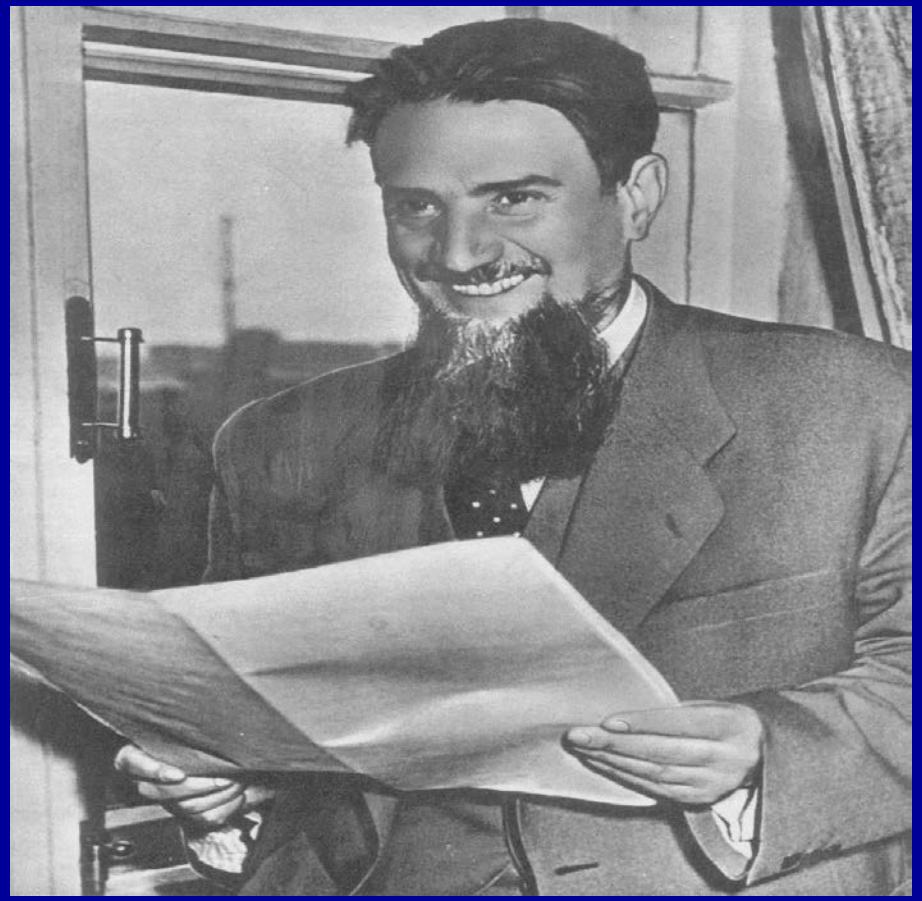
Uncertainty principle of Heisenberg-

What Werner Heisenberg formulated for quantum physics (non commuting operators: coordinate and impulse, electric and magnetic fields) to a certain extent has its analogy in biology: In the living state one can observe the collective movement of molecules in cells, which makes it however difficult to determine their exact positions

Formal description of a cell's genetic information should provide the number of DNA molecules in that cell and their complete nucleotide sequences. The genome sequence of any actual living cell cannot physically be known with absolute certainty, independently of the method used. There is an associated uncertainty, in terms of base pairs, equal to or greater than μs (where μ is the mutation rate of the cell type and s is the cell's genome size). The genetic information that makes living cells work is thus better represented by a probabilistic model rather than as a completely defined object.

Erwin Schrodinger (1887-1961)
What is Life? The Physical Aspect of the Living Cell. 1945

Карикатура на генетика



Igor Kurchatov 1903-1960

National Research Centre "Kurchatov Institute"

Medical Radiological Research Center of the Russian Academy of Medical Sciences (Obninsk)

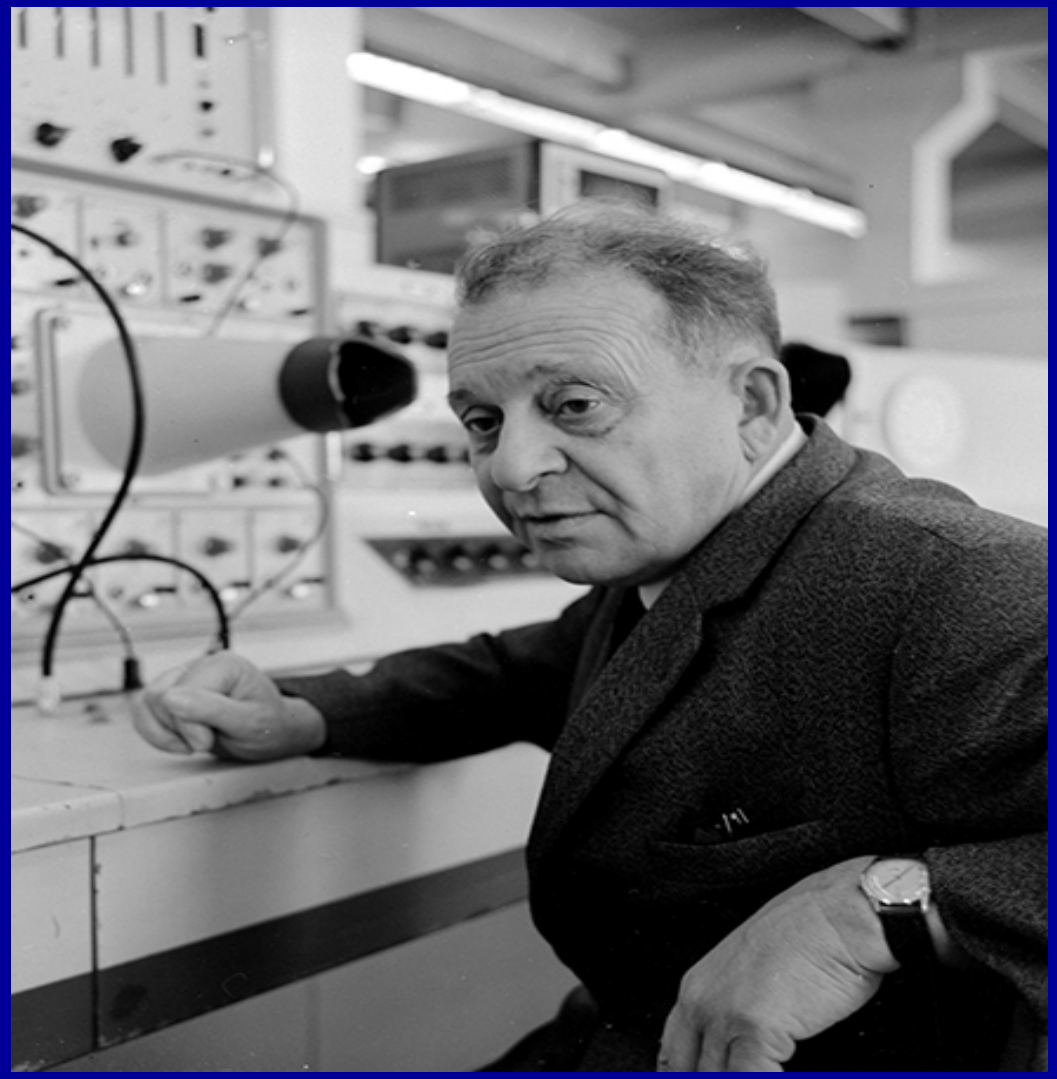
DUBNA

LRB

JINR

Joint Institute for Nuclear Research in Dubna

Laboratory
of Radiation Biology



Artem Alikhanian 1908-1978

A. ALIKHANYAN
National Laboratory

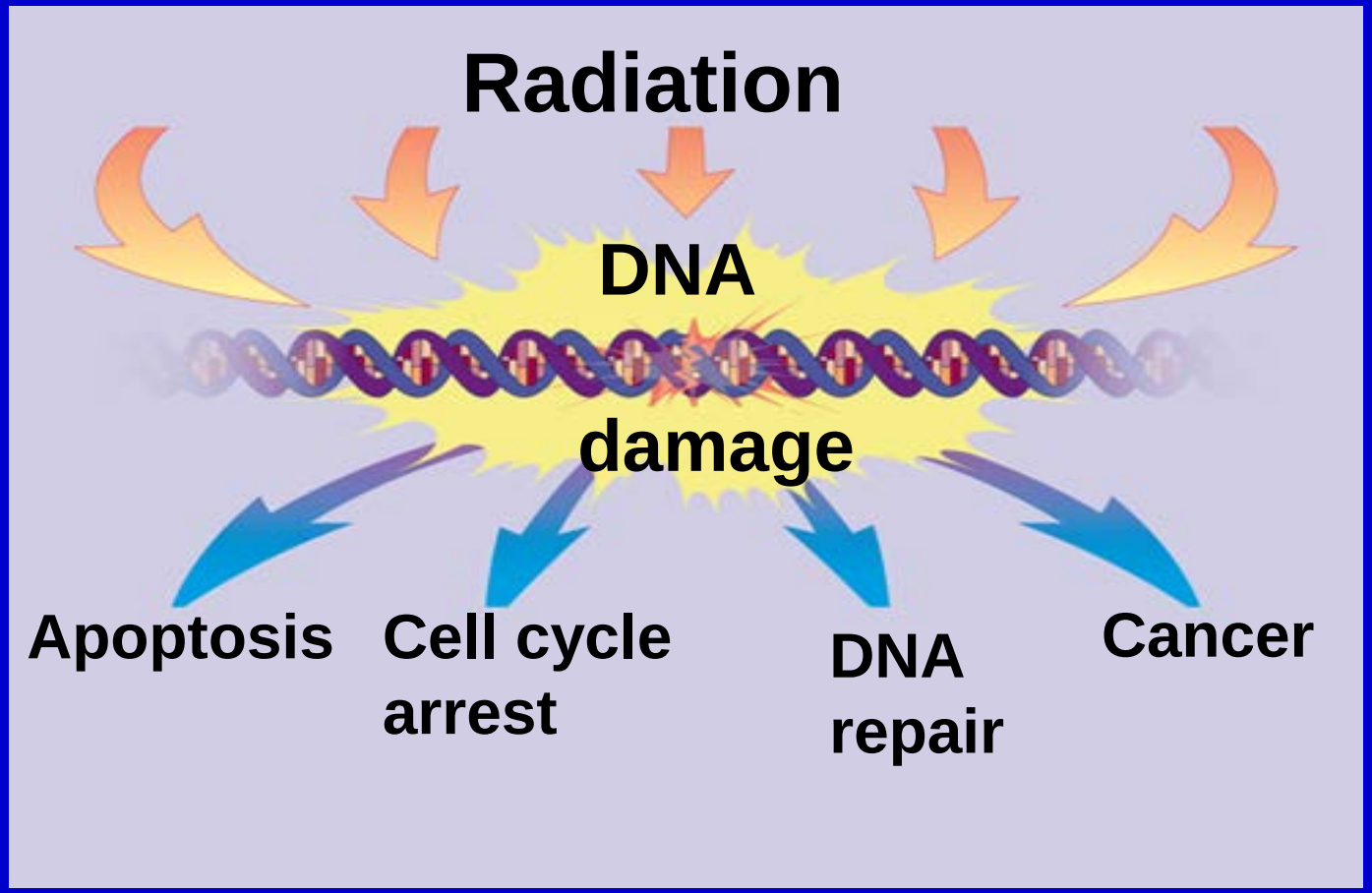
For geneticists, the most important direction in radiobiology is the DNA damage, induced by different types of radiation, including ultrafast electron beams.

_The damaged DNA can be repaired, and if repair is not successful, then:

-cells can die by apoptosis.

-arrest in cell cycle can be induced.

-DNA damage can also lead to cancer.

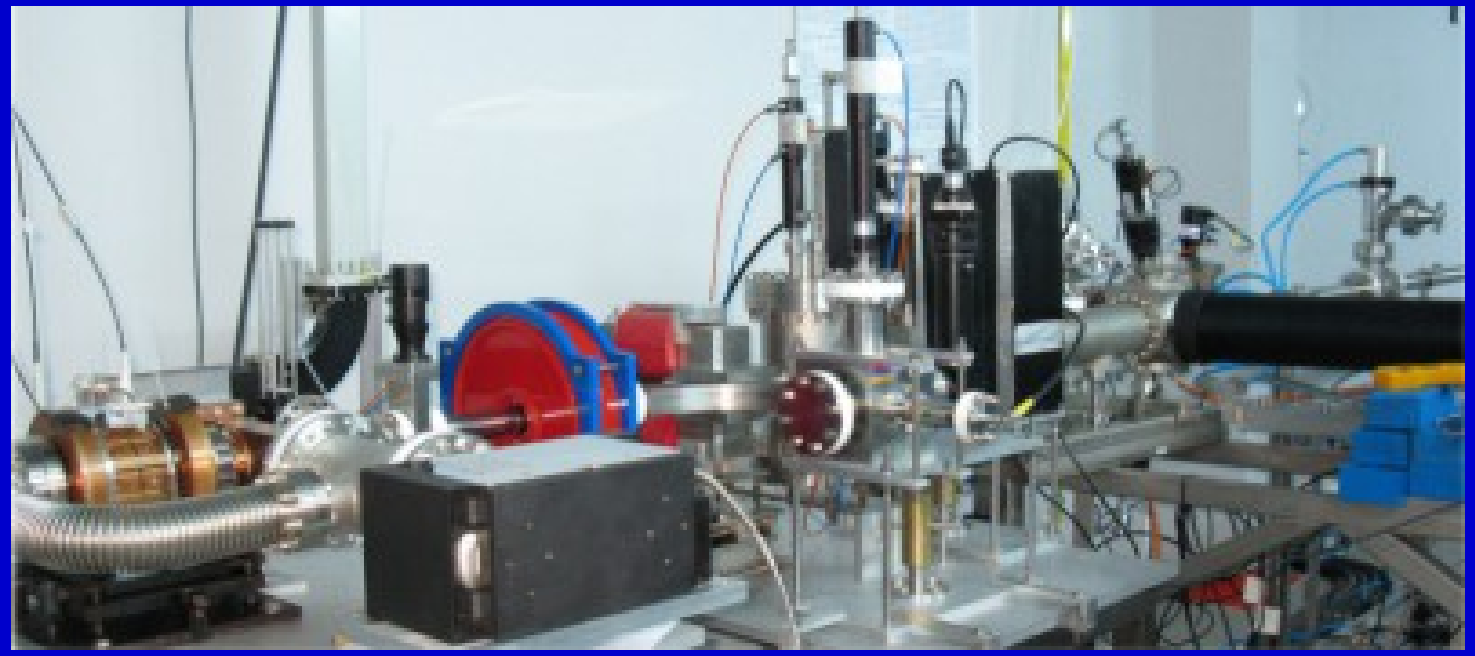


The advantage of laser-generated ultrafast electron beams for biological and clinical application is obvious, since they...

- typically feature a monoenergetic spectral profile and are better directed (less lateral spread) than other laser-driven ions.
- They have very high instantaneous dose delivery within a time interval shorter than many chemical reactions pulses of enormous capacity (up to kGy /s), but with small dose of 1 pulse. This allows the precise dose-control induction of local effects on solid tumors with minimal exposure of normal tissues.
- Engineering development activities in this field are far ahead of radiobiological research. There are only a few publications on the biological effects of pulsed ionizing radiation.
- It is assumed that accelerators of this type can improve radiation therapy of tumors.

The role of renovations in radiation therapy is important, because about 50 % of cancer cases are treated with radiation therapies (possibly in combination with surgery and/or chemotherapy). Among these treatments, more than 90 % use RF-driven linear accelerators of electrons (RF-Linac).

- Other techniques include internal radiation (brachytherapy) and proton-ion beams (hadrotherapy). In most cases electrons delivered by a RF-linac are not used directly on the tumor but converted into photons (hard X-rays) by bremsstrahlung through a suitable target. In some case electrons are used directly, either to cure superficial tumors or in the Intra-Operative Radiation Therapy (IORT) which can be applied during surgical operation of a tumor.



AREAL (Advanced Research Electron Accelerator Laboratory) is the laser driven RF gun based electron linear accelerator producing small emittance ultra-short electron beam pulses for advanced experimental study

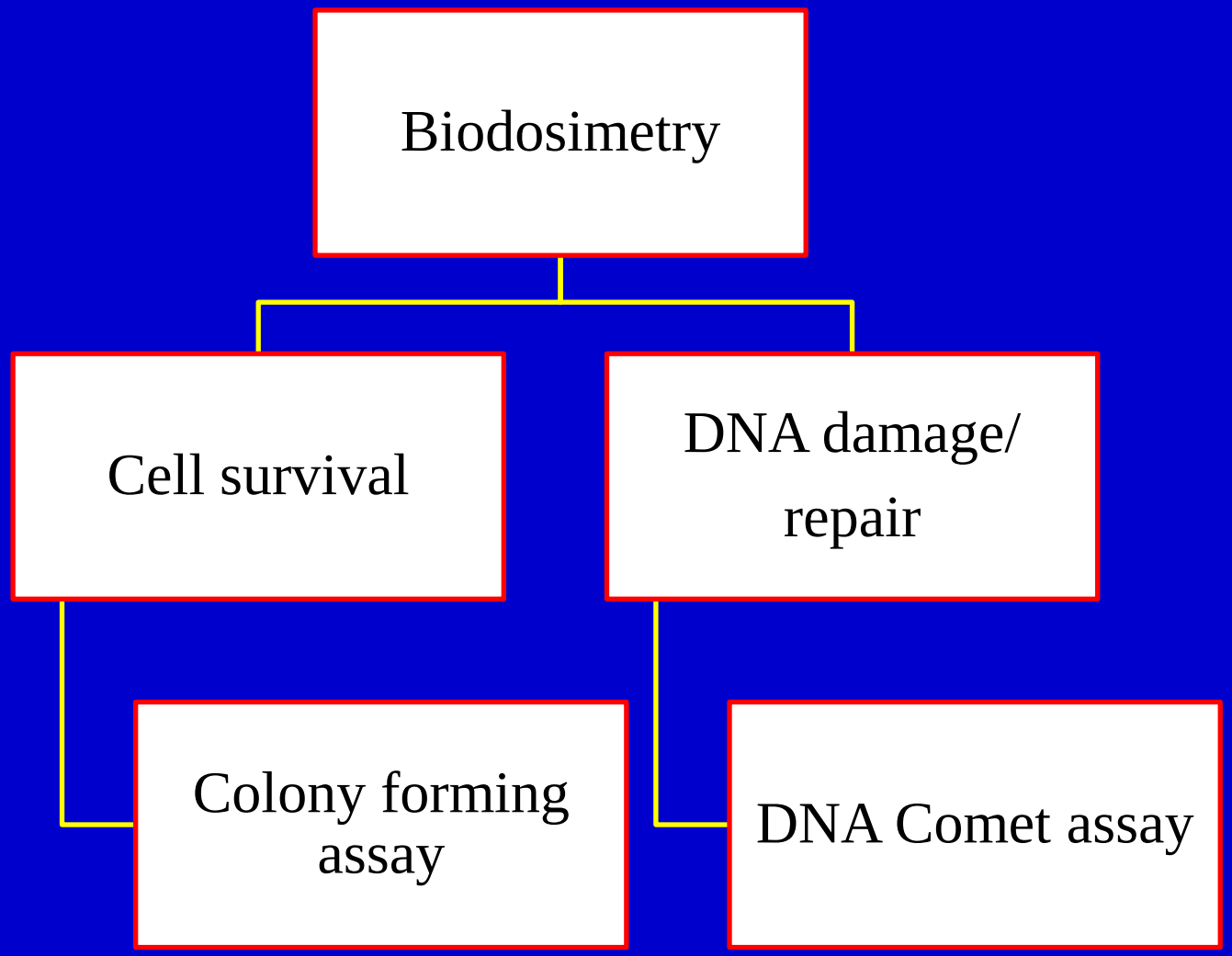
The aim of our study:

- to analyze ultrafast electron beam irradiation-induced DNA damage and repair in normal and cancer cells

Parameters used in our experiments

AREAL beam parameters		UV laser parameters	
Beam charge (pC)	30	**Wavelength (nm)**	258
Electron energy (MeV)	4	**Pulse energy**	200
Pulse duration (fs)	400	**Repetition rate (Hz)**	2 /20
Pulse repetition rate (Hz)	2/20	**Energy stability**	<2%
Beam spot (mm)	15	**Beam divergence (mrad)**	<0,3
Norm. emittance (mm-mrad)	<0,5	**Beam diameter (mm)**	4,0
RMS energy spread	<1,5%		
Online dose information	Faraday cup		

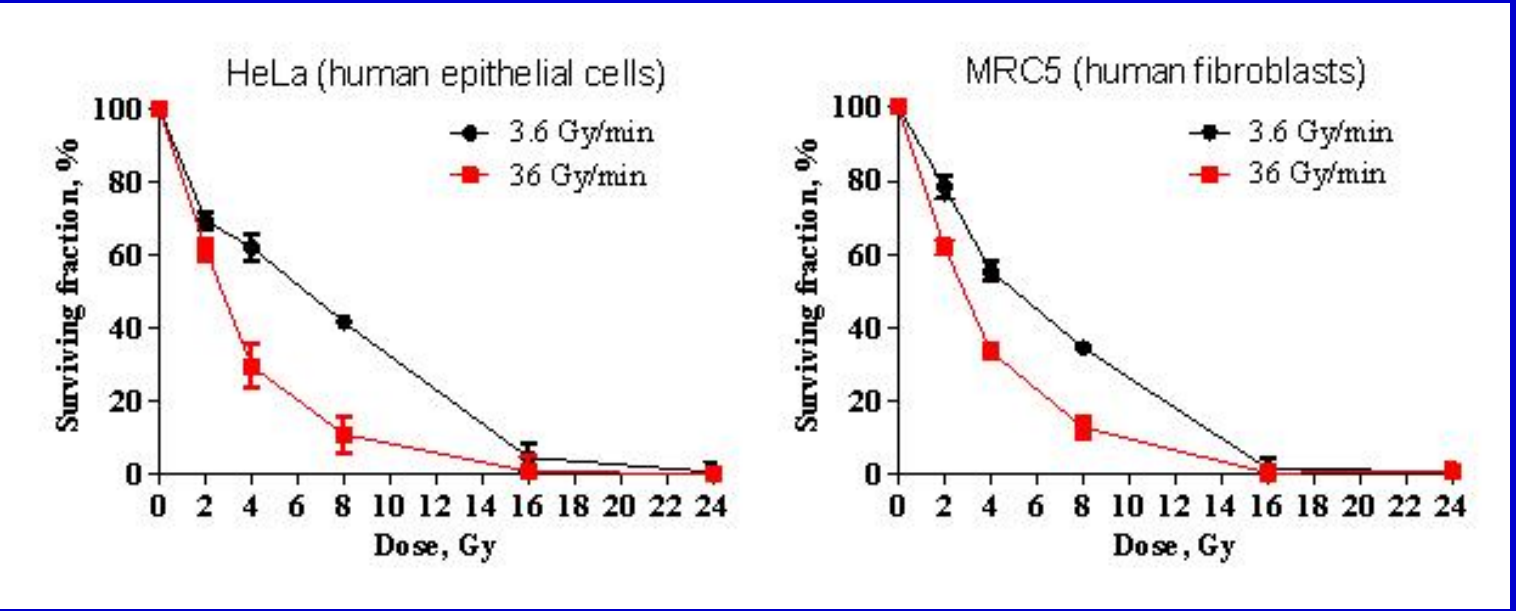
Schematics of our primary radiobiological research

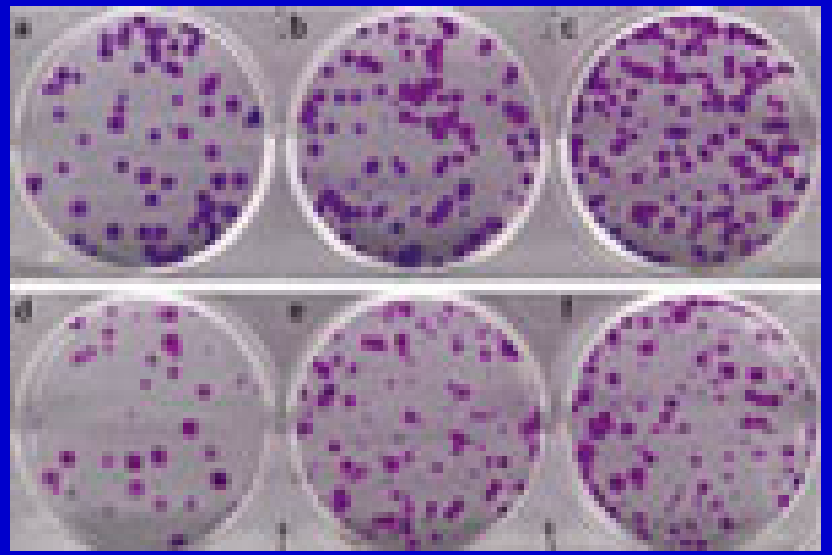


Cell survival

Colony forming assay

The gold standard in radiobiology, which shows the sensitivity of the cells to ionizing radiation





The clonogenic survival curve obtained after irradiation of HeLa (a) and MRC-5 (b) cells with the ultrafast electron beam at the low (3.6 Gy/min) and high (36 Gy/min) dose-rates

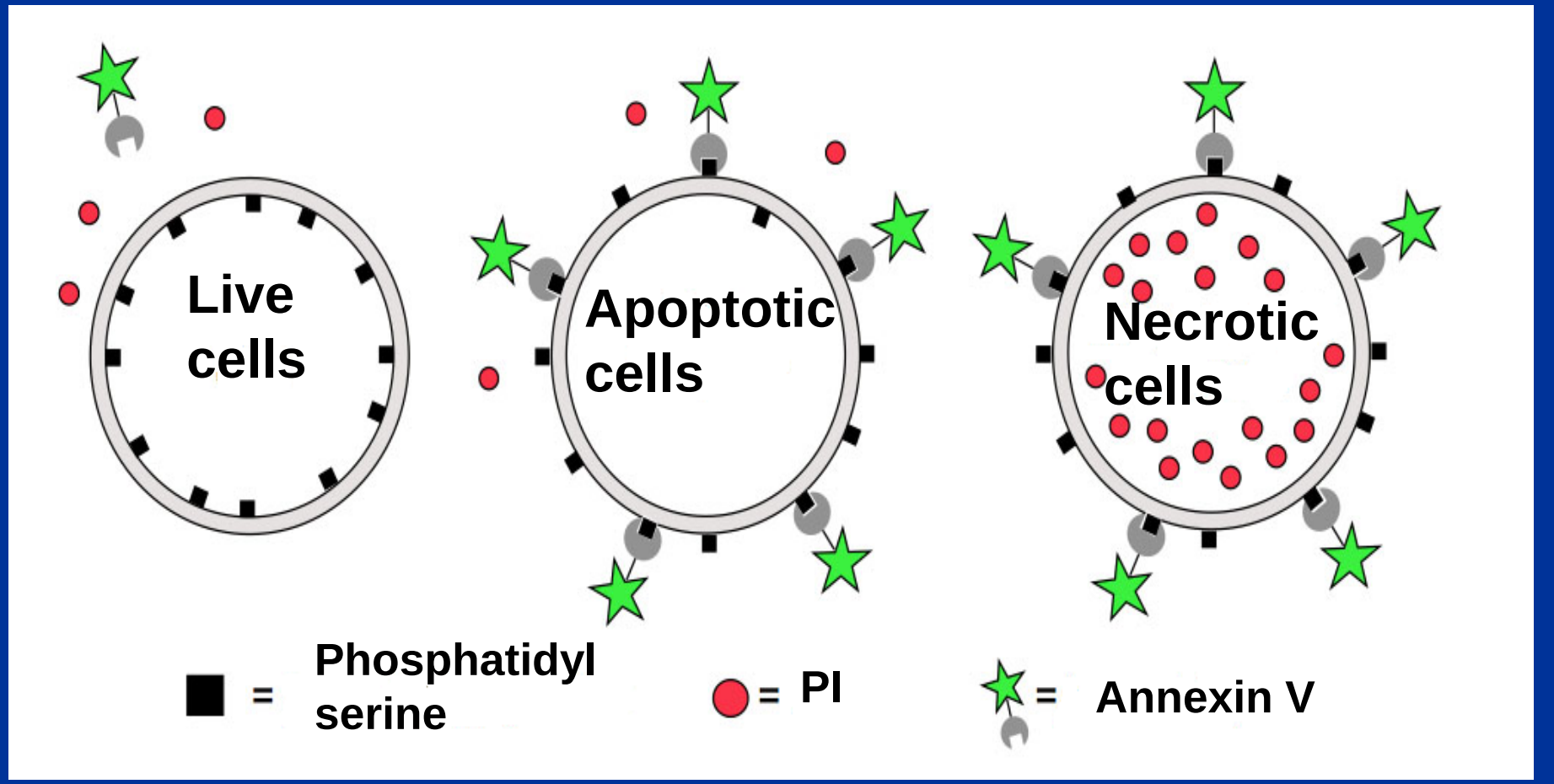
Apoptosis and Necrosis

Apoptosis the cell death without inflamation has been considered **a major mechanism of radiotherapy**-induced cell death, and the regulation of apoptosis is very important point of therapy.

Necrosis is a form of cell death that is uncontrolled and pathological. The fundamental features of necrosis include cellular energy depletion, damage to membrane lipids, and loss of function of homeostatic ion pumps/channels. Symptoms during necrosis: inflammation, decreasing blood flow at affected site, and even tissue death.

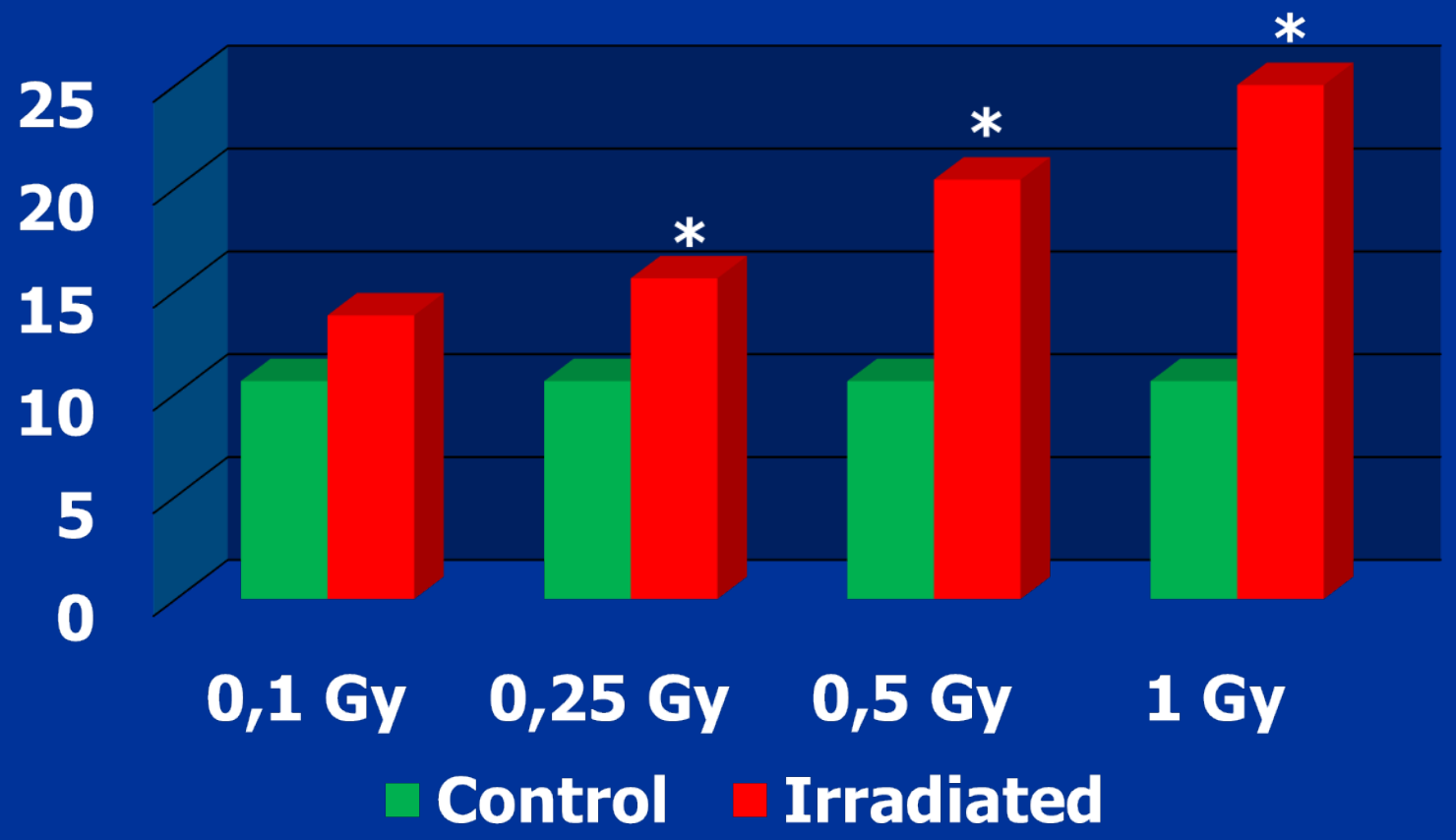
So, apoptosis, which can also occur as a defense mechanism during healing processes, is almost beneficial to organism, while necrosis is always abnormal and harmful.

The effect of ultrafast electron beam irradiation on the level of cell viability, apoptosis and necrosis was investigated using Annexin V/propidium iodide assay



Cell survival

Annexin V/PI assay



The increase of apoptotic cells as a result of irradiation is dose-dependent and means that cancer cells can be safely destroyed by apoptosis when irradiated with ultrafast electron beams.

DNA damage

DNA-Comet assay

Comet classification

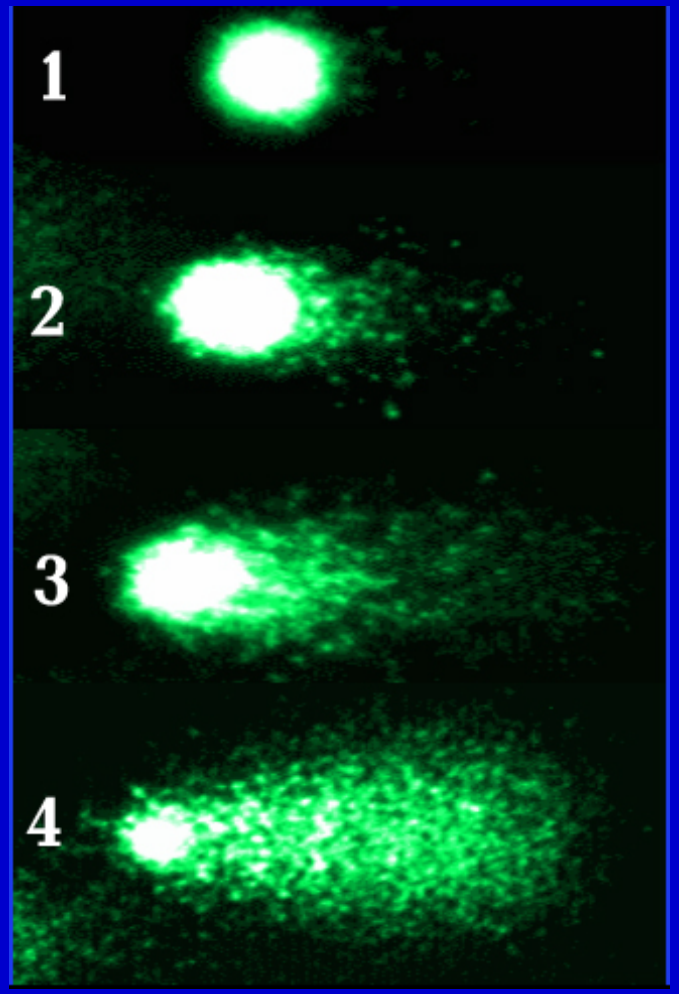
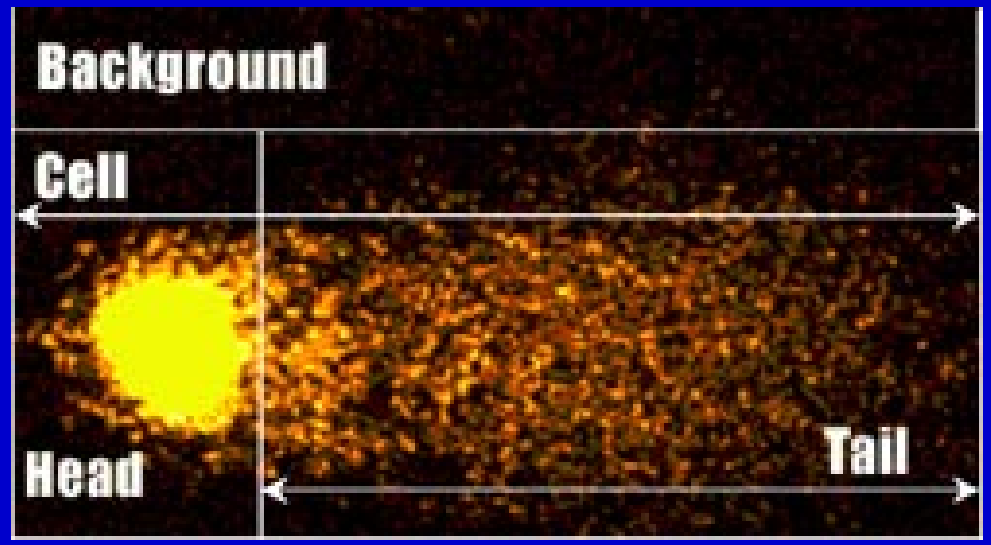
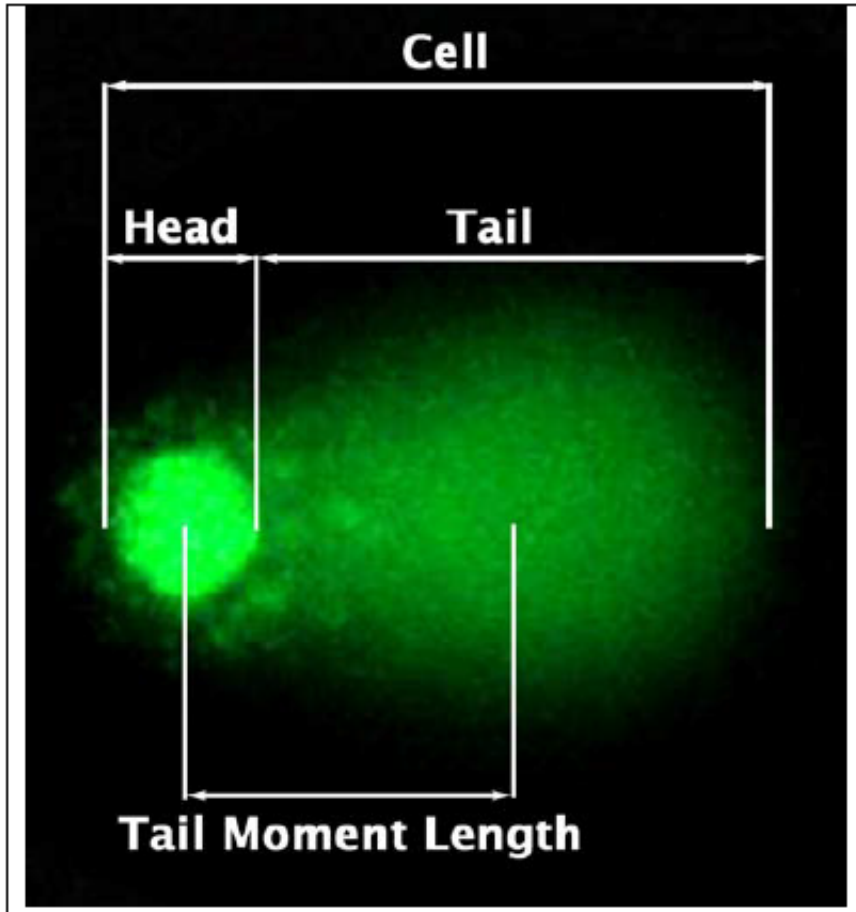


Image of comet in Komet 4 software



Olive Tail Moment definition



Tail DNA% = 100 x Tail DNA Intensity/Cell DNA Intensity

Tail Moment can be measured using one of the following methods:

(a) Olive Tail Moment = Tail DNA% x Tail Moment Length*

(b) Extent Tail Moment = Tail DNA% x Length of Tail (see diagram on left)

A number of Comet analysis software programs are commercially available, such as LACAAS from Loats Associates, Inc.) and Comet Assay IV from Perceptive Instruments.

*Tail Moment Length is measured from the center of the head to the center of the tail (see diagram)

CELL BIOLABS, INC.
Creating Solutions for Life Science Research

The exposure of K562 human chronic myelogenic leukaemia cells to AREAL ultrafast electron irradiation at different doses revealed the dose-dependent increase of the primary DNA damage.

The comet assay was performed immediately after the irradiation to avoid DNA repair. Reparable and non-reparable DNA damages were assessed after 24h-incubation of irradiated cell culture.

The level of DNA damage was defined by OTM– the amount of DNA in the tail of the comet multiplied by median migration distance.

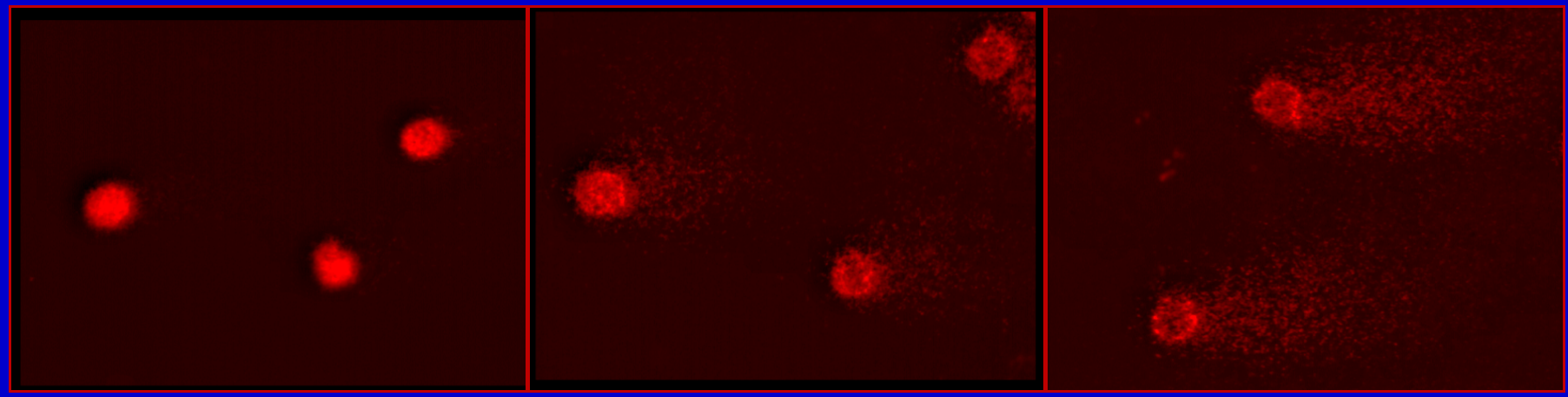
At the doses higher than 4 Gy the DNA damage decreased. The reason - the level of dead cells can be increased in relation to viable cells. After 24 hour of cell incubation, the damaged DNAs have repaired until 24 Gy.

It was shown that electrons, as a source of low LET radiation, led to isolated DNA lesions, including single-strand and double-strand breaks of DNA, which were generally repaired efficiently.

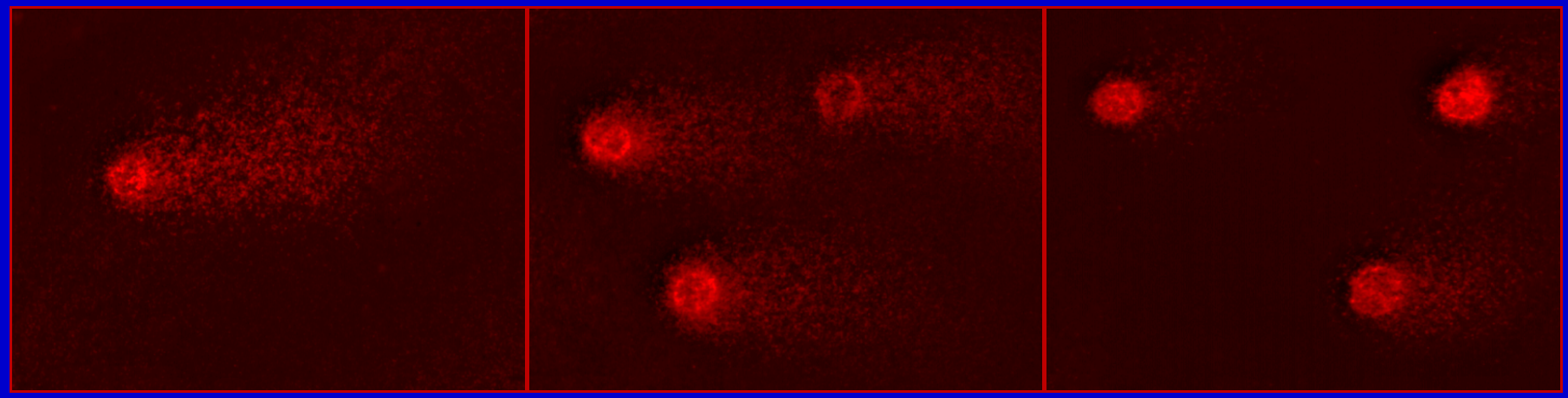
DNA damage

DNA-Comet assay
K-562 cell line

Comet images in K-562 cells after irradiation by AREAL accelerator

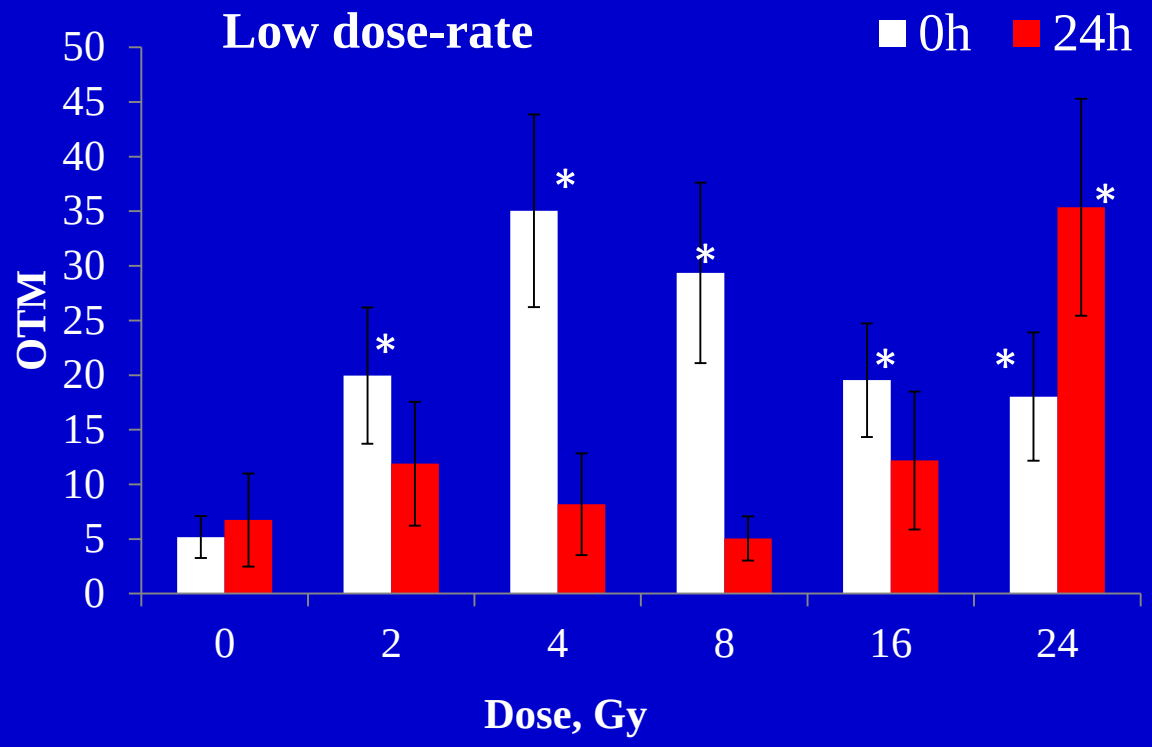


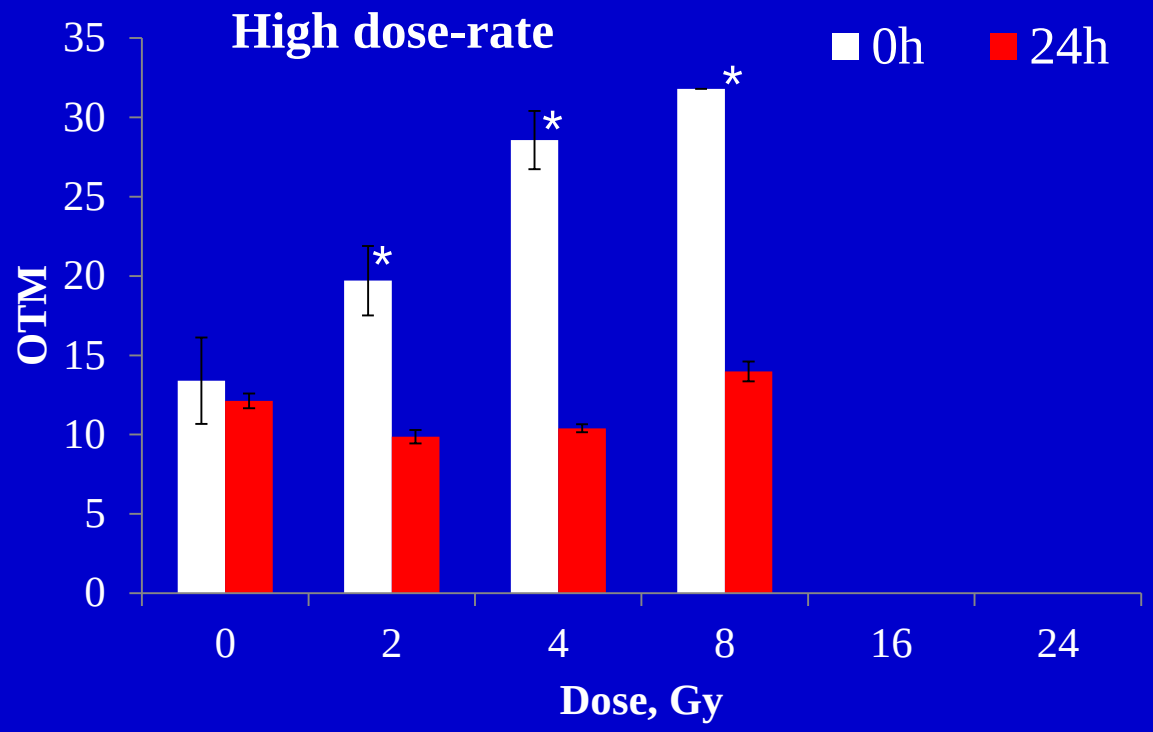
Non-irradiated control | Irradiation dose 2 Gy | Irradiation dose 4 Gy



Irradiation dose 8 Gy | Irradiation dose 16 Gy | Irradiation dose 24 Gy

DNA damage (Olive Tail Moment) in K-562 cells after 0 and 24 hours after irradiation (pulse frequency **2 Hz and 20 Hz**)





Low dose-rate – 3,6 Gy/min (**pulse frequency 2 Hz**), High dose-rate – 36 Gy/min (**pulse frequency 20 Hz**)

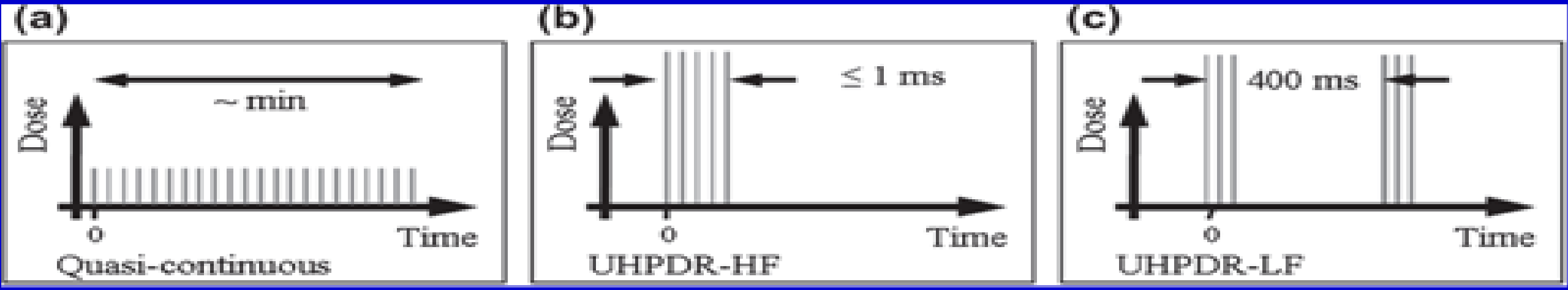
- The radiation-induced levels of DNA damage depend on dose and dose-rate

The publications mainly present biological data obtained on following laser electron accelerators

JETI	• Techinsche Instrumente GmbH • Jena, Germany
LINAC	• Oncor Impression, Siemens AG, Healthcare • Erlangen, Germany
ELBE	• Forschungszentrum Rossendorf • Dresden, Germany

Beyreuther et al., **2015**, I*nternational Journal of Radiation Biology, 91(8): 643–652*

Modern laser electron accelerators



Beam Parameter	20 MeV ELBE electron beam			JETI laser acc[a]	Clinical LINAC[a]
	Quasi-continuous	Ultra-high pulse dose rate			
		High frequency	Low frequency		
Pulse frequency	13 MHz	13 MHz	2.5 Hz	2.5 Hz	50 Hz[b]
Pulse length	~ 5 ps	~ 5 ps	~ 5 ps	~ 1 ps	~ 4 μs[b]
Dose per pulse	~ nGy	~ mGy	~ mGy	~ mGy	~ mGy
Irradiation time	~ min	≤ 1 ms	≤ 30 min	≤ 33 min	~ min
Mean dose rate/Gy min^{-1}	4.1 ± 0.2	(0.54 ± 0.05) × 10^6	0.38 ± 0.03	0.36 ± 0.01	3.0 ± 0.3
Pulse dose rate/Gy min^{-1}	~ 10^4	~ 10^{10}	~ 10^{10}	~ 10^{11}	~ 10^4

Beyreuther et al., 2015, *International Journal of Radiation Biology*, 91(8): 643–652

- The radiation source ELBE

- **Effect of quasi-continuous electron beam of clinical linear accelerator (LINAC) was realized** for comparison with:
- electron pulses at the ultra-high pulse dose rate of 10^{10} Gy min^{-1}
- **-either at the low frequency of a laser accelerator 2.5 Hz**
- **-or at 13 MHz avoiding effects of prolonged dose delivery.**
- The impact of pulse structure was analyzed by clonogenic survival assay and by the number of residual DNA double-strand breaks remaining 24 h after irradiation of two human squamous cell carcinoma.
- **Response was independent from electron pulse frequency**

RBE measurements - Praveen electrons vs. Areal electrons

OTM	**Praveen *et al.*** Microtron accelerator **8 MeV**	Areal accelerator **3,6 MeV**	RBE	Mean
	Electron dose, Gy			
10	1.5	3	2	**2.1**
20	2.62	5,4	2.1	
25	3.08	7	2.3	

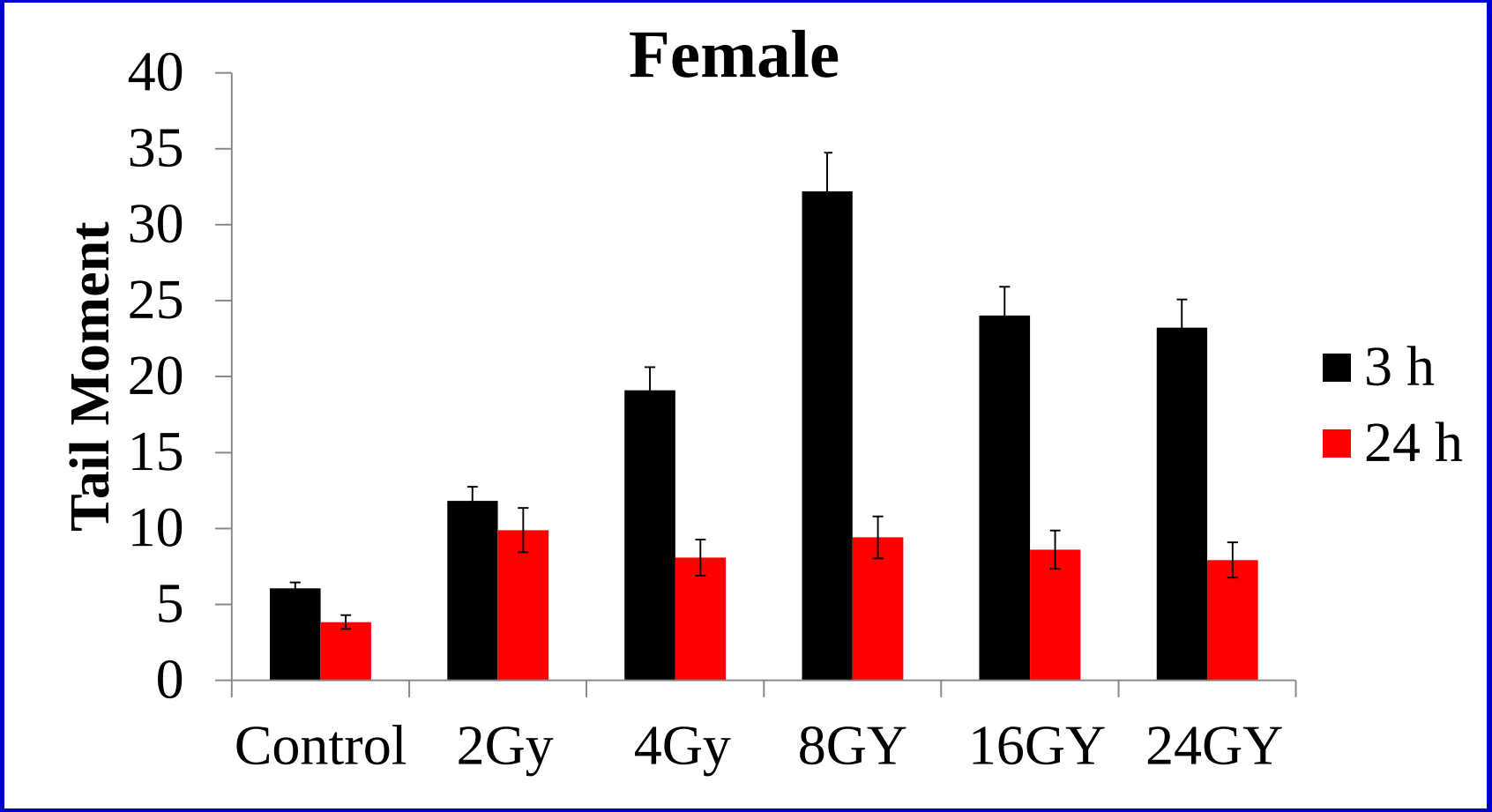
Praveen et al.2014, experiment - Radiation treatment has been carried out using electron beam from microtron accelerator at Mangalore University. It is a pulsed mode circular accelerator (*we – pulsed mode linear accelerator*) offering an electron beam with a maximum pulse current of 50 mA and pulse duration of 2.5 μs (*we-400 fs*), electron energy of 8 MeV (*we – 3.6 MeV*).

- **The SSB and DSB yields linearly depends on the electron beam energy** !

DNA damage

DNA-Comet assay

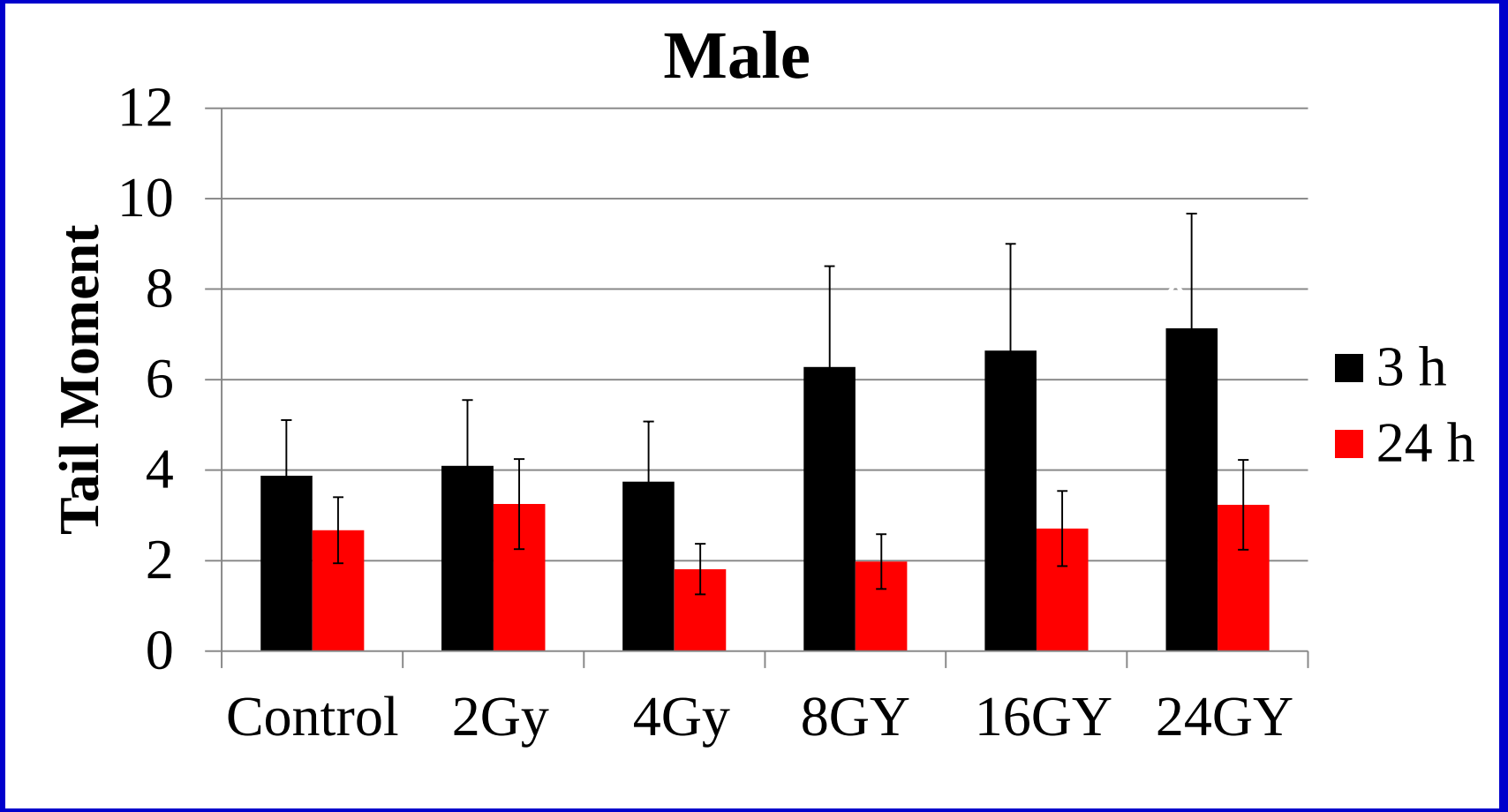
PBMC



DNA damage level in female peripheral blood mononuclear cells 0 and 24 hours after irradiation with femtosecond electron beams

DNA damage

DNA-Comet assay
PBMC



DNA damage level in male peripheral blood mononuclear cells 0 and 24 hours after irradiation with femtosecond electron beams

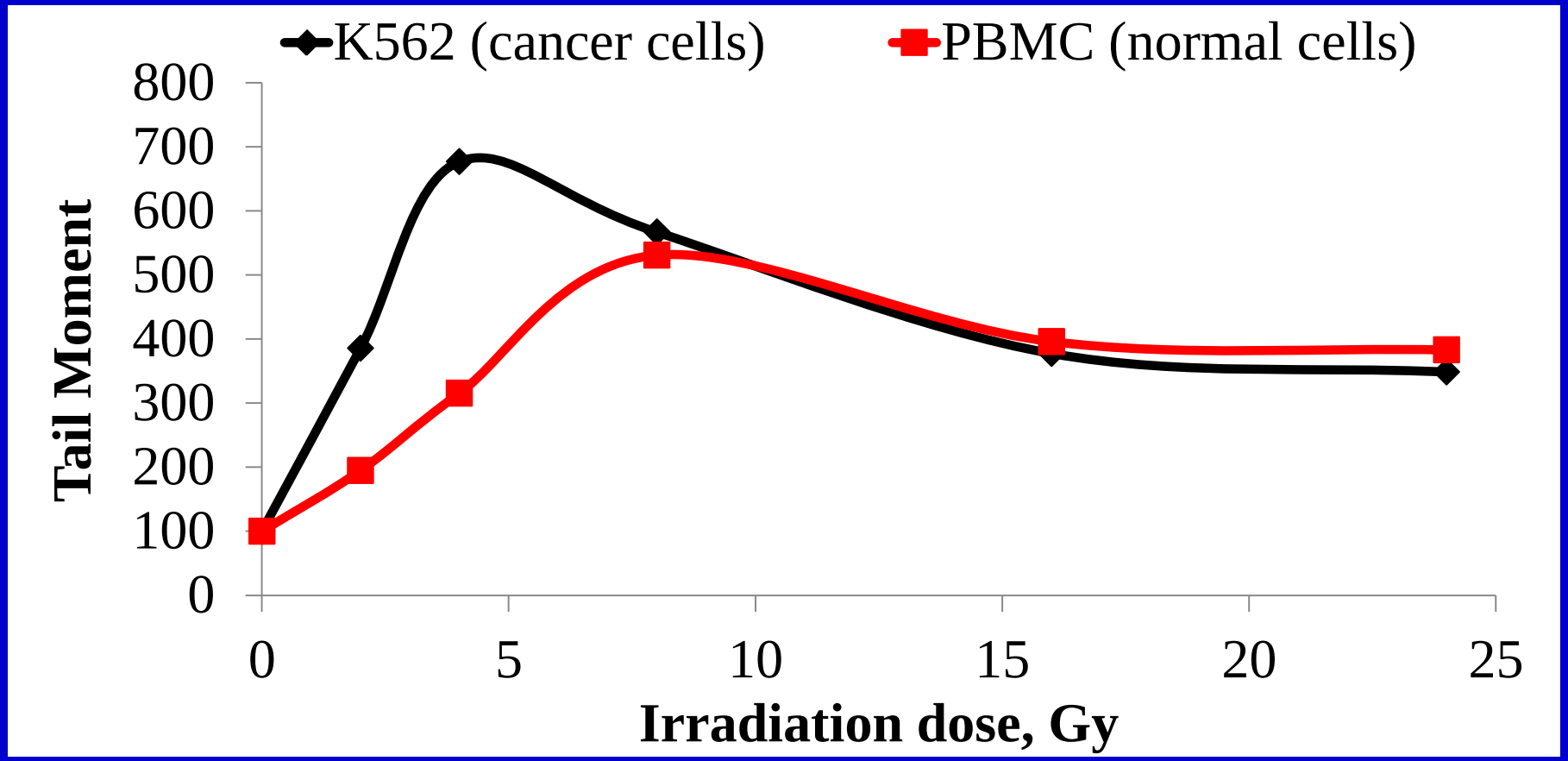
The comparison between male and female PBMCs response to the irradiation was demonstrated, that female PBMCs are more sensitive, as the DNA-damage level in female PBMCs increased more than 5 times (8Gy) in comparison with negative control, which leads to the formation of unrepairable DSBs and SSBs.

In male's cells even at the highest dose of irradiation (24Gy) the level of DNA-damage increased less than 2 times in comparison with negative control, and those damages was totally repairable.

DNA damage

DNA-Comet assay

Cancer/Normal cells



DNA damage level in peripheral blood mononuclear cells of a female (normal cells) and in cell line K562 (cancer cells, female) 0 hours after irradiation with electron beams

For comparison of cancer and normal cells the female data were used, as the cancer cell line K562 was also obtained from female.

Neoplastic cells show higher effects at the doses of 2-4Gy of irradiation, but starting from 8Gy the similar behavior between normal and cancer cells response was evident. The slopes of the two dependences, calculated by linear interpolation, are 0.31 Gy^{-1} for normal cells and 2.59 Gy^{-1} for neoplastic HeLa cells. These values suggest that normal cells are less damaged by radiations than neoplastic ones, which is a good aspect in case of cancer treatment using electron beams.

By Borcia et al.,2014 “The slopes of dose-effect dependences for Olive Tail moment ,calculated by linear interpolation, are 0.31 Gy^{-1} for normal cells and 2.59 Gy^{-1} for neoplastic HeLa cells. These values suggest that normal cells are less damaged by radiation than neoplastic ones, which is a good aspect in case of cancer treatment using electron beams.”

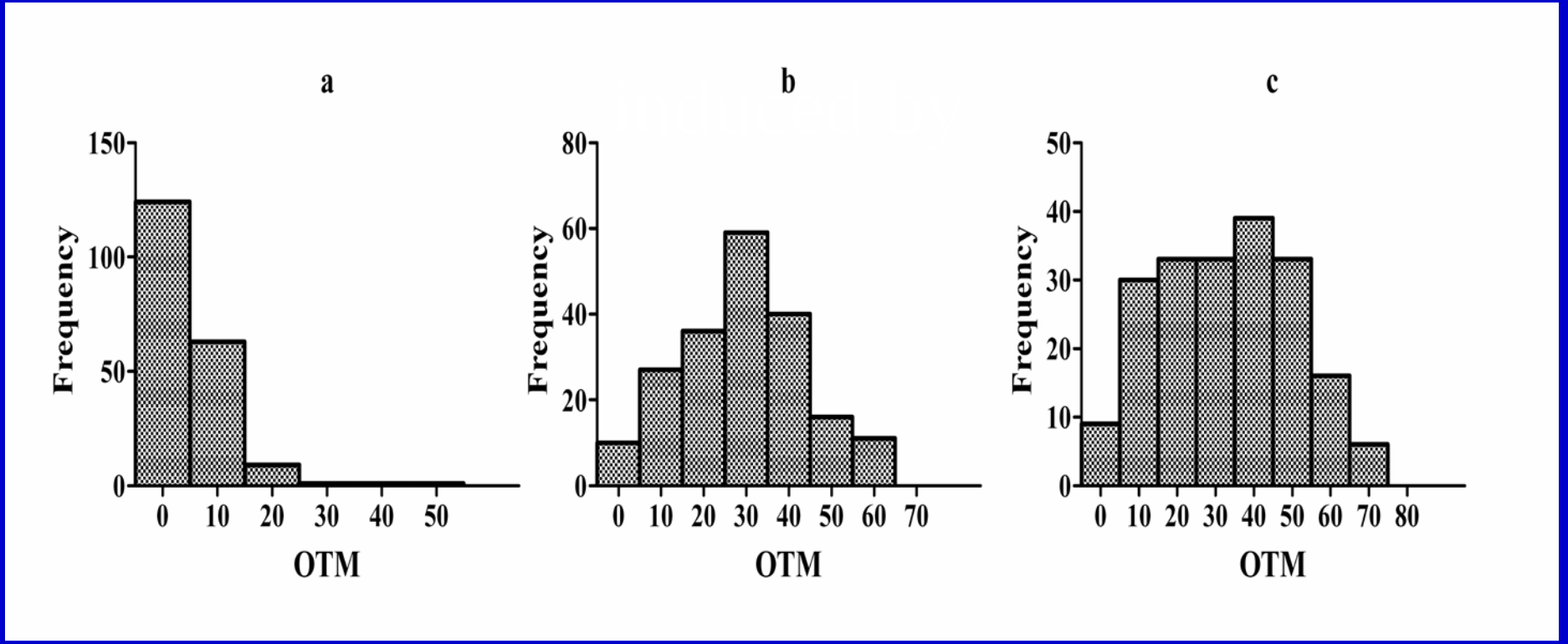
Comet assay permits to analyse DNA damage in single cells

Data distribution of comet assay is usually asymmetric.

We can assume the distribution of comet assay data is a mixture of two distributions - normal, and exponential. However, that mixture is difficult to characterize.

Di Giorgio et.al. (2004). Evaluation through comet assay of DNA damage induced in human lymphocytes by alpha particles. Comparison with protons and Co-60 gamma rays.

Examples of distribution of DNA damage in cells



(a) unirradiated K-562 cells and exposed to 8 Gy with the ultrashort electron beam at low (3,6 Gy/min) (b) and high (36 Gy/min) (c) dose-rates.

After irradiation at HDR (high dose-rate) the shift to higher OTM and broadening in comet distribution is observed, in comparison to LDR (low dose-rate). The shift to the higher OTM values in this case suggests the formation of highly damaged DNA leading to the accumulation of un-repairable DNA damages.

We applied F O C I (DNA DSB repair proteins assay)

- It is believed that DNA DSBs are the main triggers of cellular processes, which are responsible for responding to the impact of ionizing radiation.
- DNA DSB repair is quite slow and if left unrepaired, it will lead to serious cytogenetic abnormalities, cell death, inactivation of gene suppressors or the activation of oncogenes.

- DNA DSB present key mechanism through which radiotherapy and some chemotherapeutic agents kill cancer cells.

- An immunocytochemical analysis of proteins participating in the processes of DNA DSB (FOCI assay) was used

Ionizing radiation induced foci are subnuclear areas at or near the DNA damage sites and consisting of repair proteins of double-strand DNA ruptures.

One of the earliest (several minutes) of DNA repair events is the phosphorylation of a protein called histone H2AX.

This is a variant of histone H2A, which is a core-core component, nucleosome structures around which DNA is wrapped.

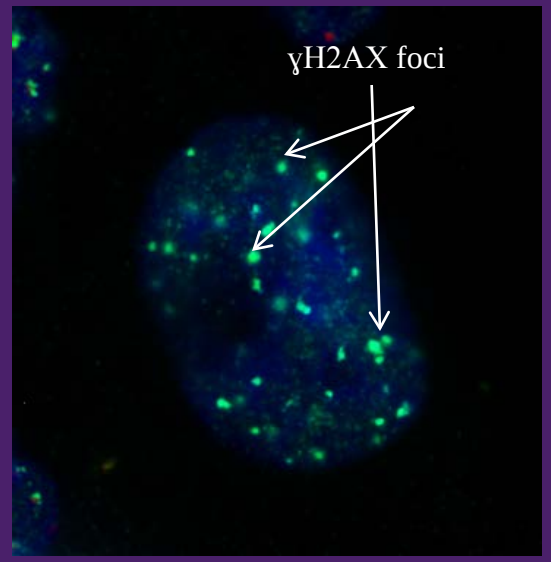
Phosphorylated protein, which is designated as γH2AX, is required to involve many other proteins in the repair process, followed by the formation of IRIF.

Phosphorylation of H2AX occurs with the participation of kinases, which are sensors of double-strand breaks of DNA (ATM-MRN complexes, DNA-PKcs-KU and ATR-ATRIP).

Recently γH2AX and other FOCI have been used as biomarkers in radiotherapy, diagnostics and biodosimetry.

Detection of DNA DSBs formation and repair after ultrashort pulses of electron beam irradiation in human fibroblasts using immunohistochemistry.

FOCI contain hundreds to thousands of copies of various proteins involved in DNA DSB repair

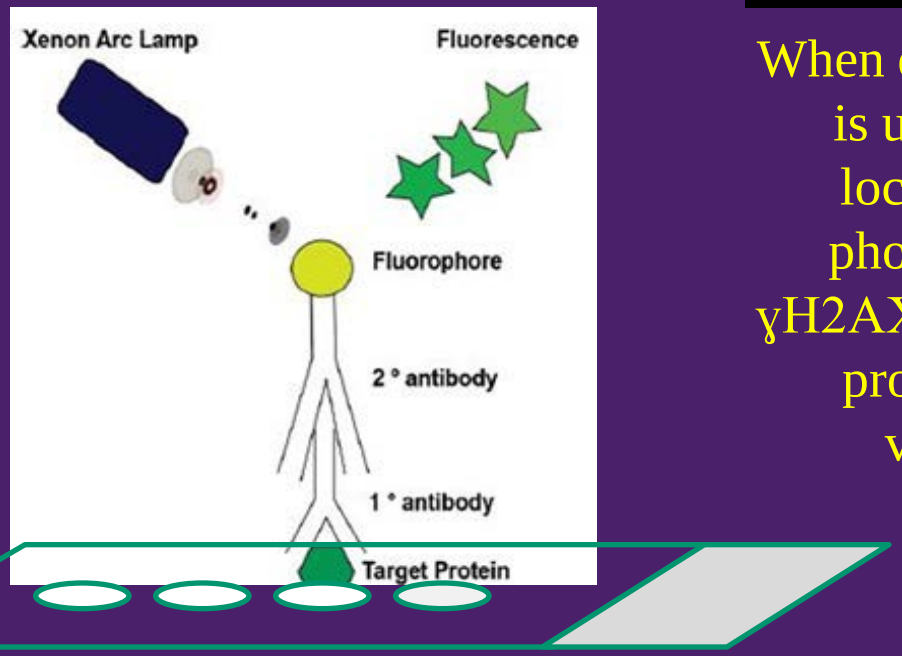


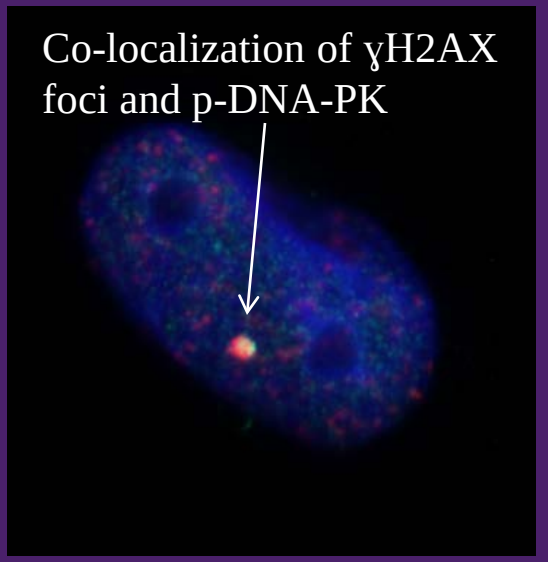
Visualization of phosphorylated γH2AX foci in irradiated cell

Fluorophores

γH2AX foci – Fluorescein (FITC) – green

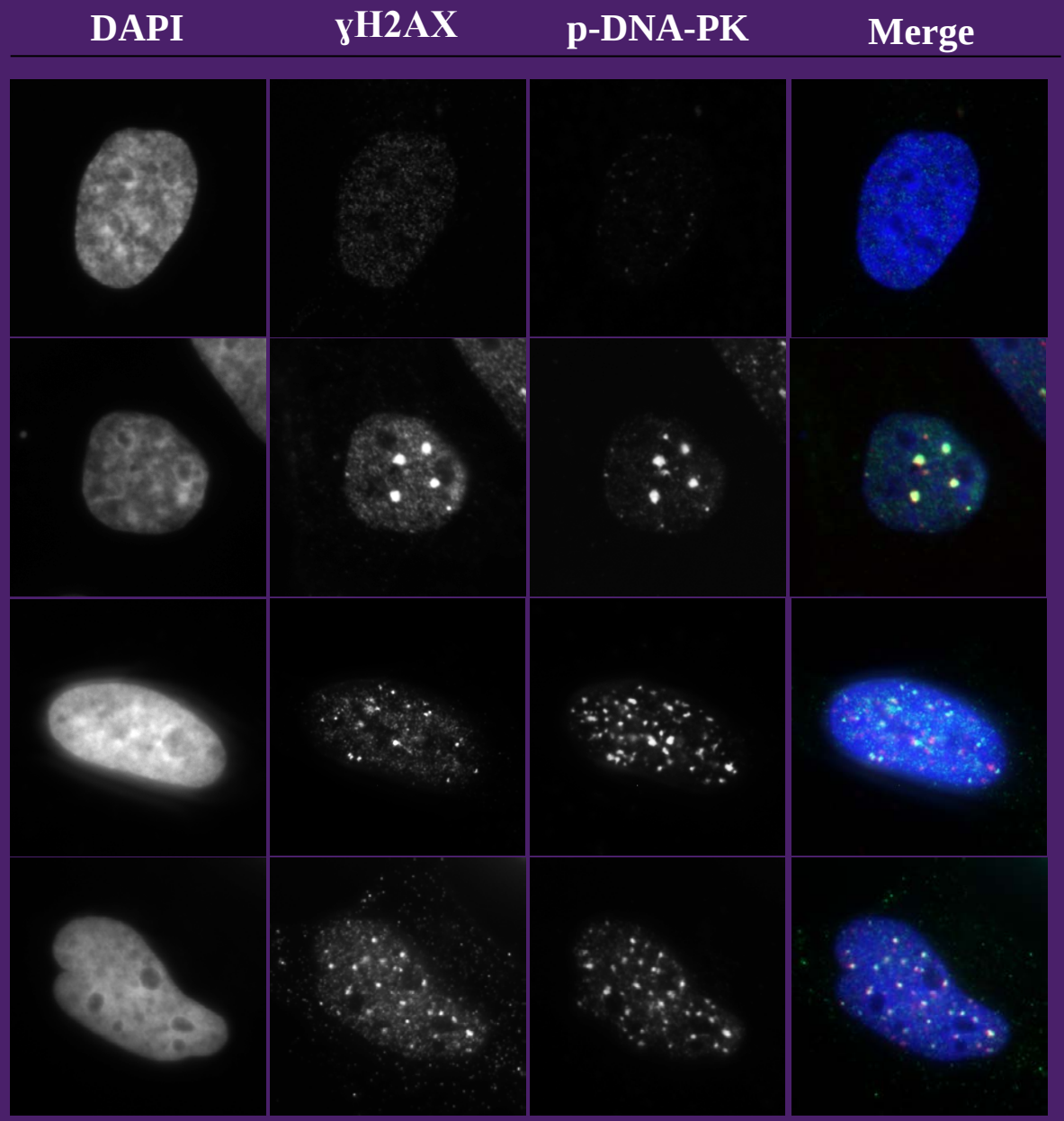
p-DNA-PK - Texas Red - red



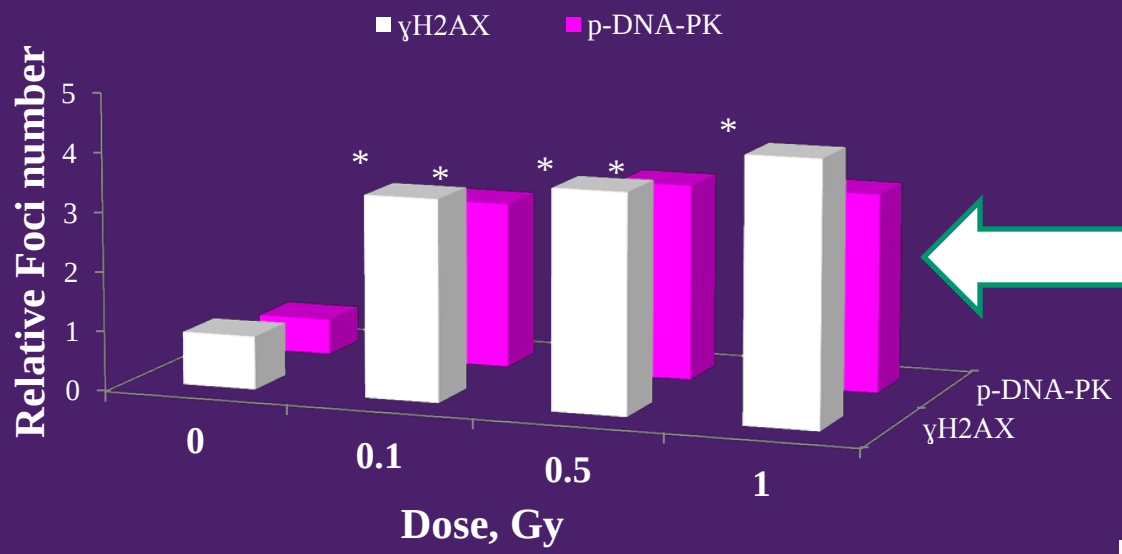


When double-staining is used, the co-localization of phosphorylated γH2AX and DNA-PK protein can be visualized

DNA double-strand breaks induced by ultrashort pulses of electron beam in human fibroblasts

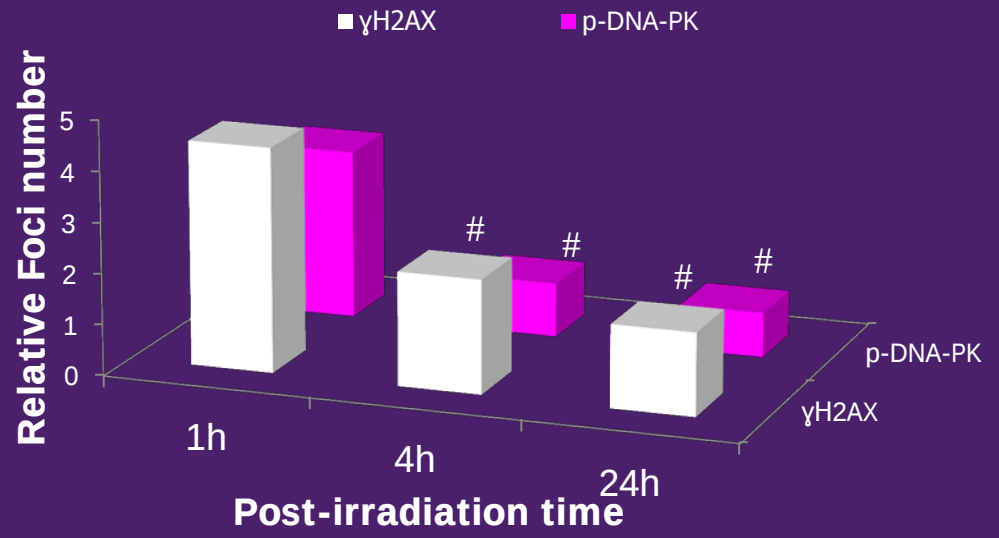


Dose- and Time-dependent DSBs induction and repair after ultrashort pulses of electron beam irradiation in human fibroblasts



Dose-dependent increase of γH2AX and p-DNA-PK

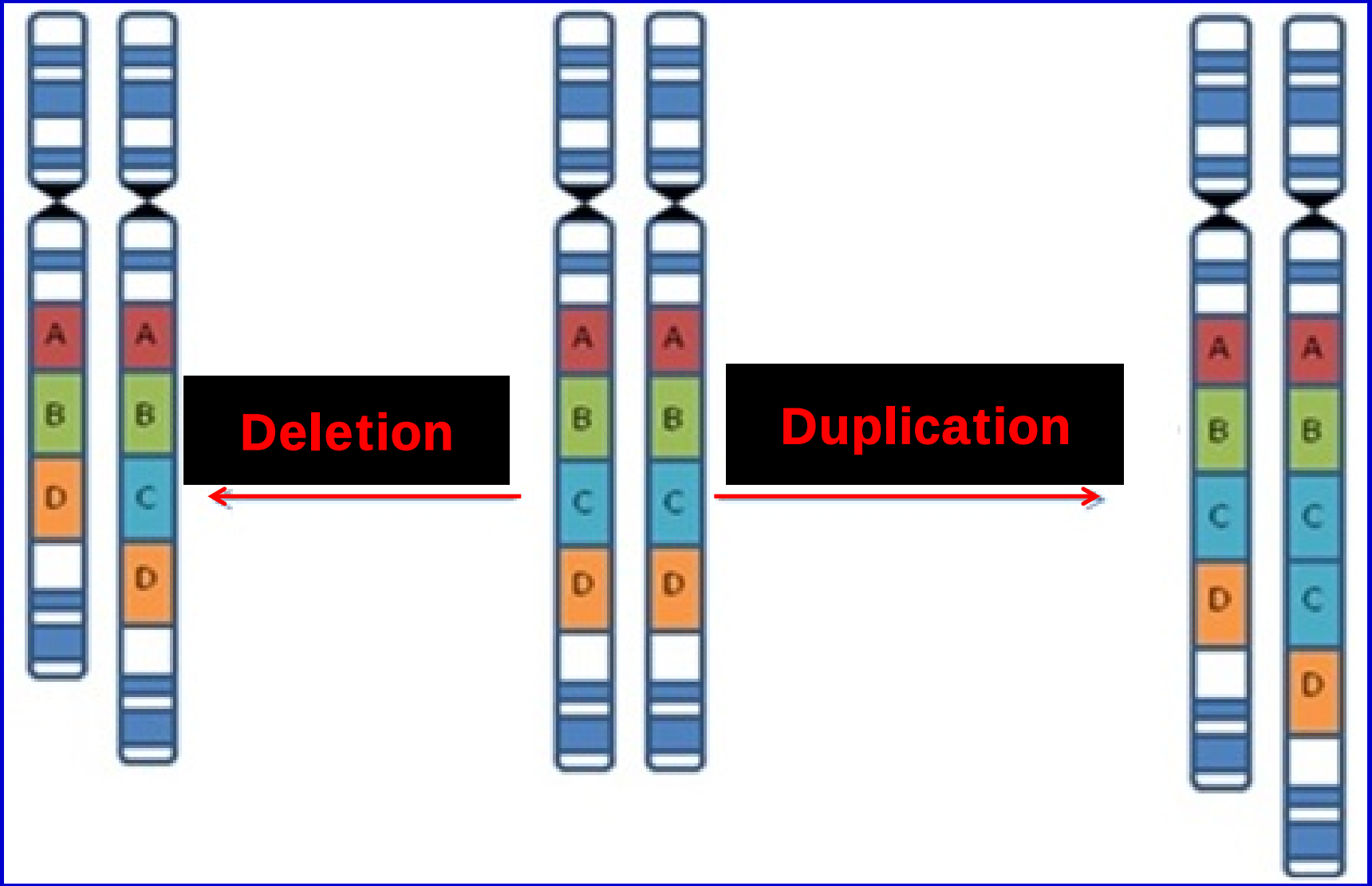
Time-dependent decrease of γH2AX and p-DNA-PK (Irradiation dose 1 Gy)



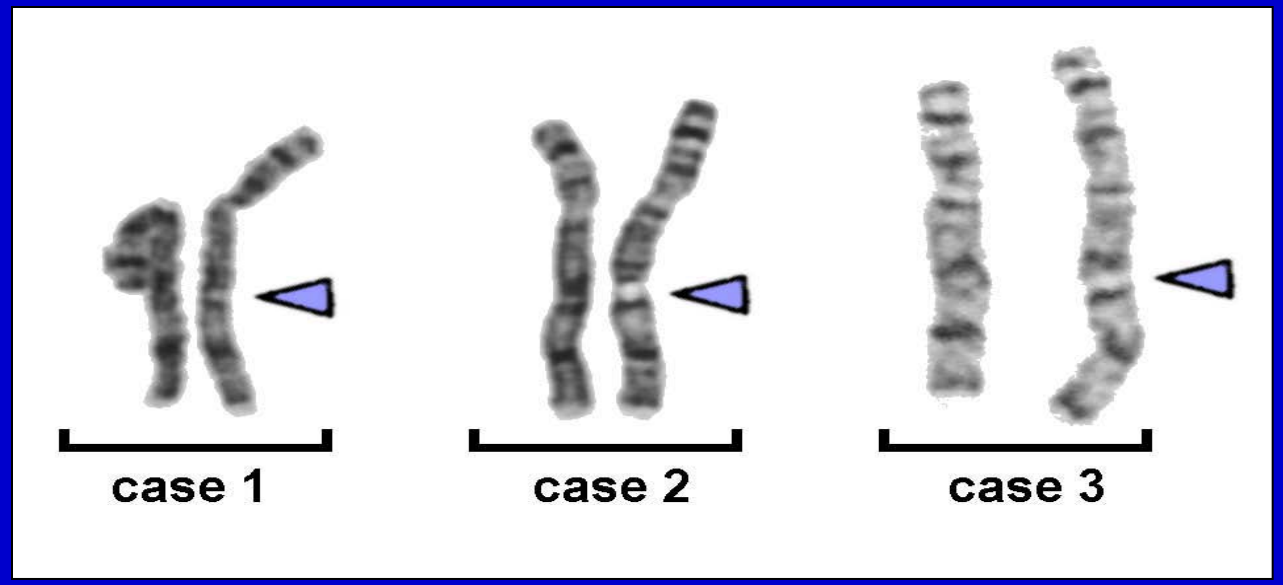
* - p<0,05 in comparison to control

\# - p<0,05 in comparison to pprevious time-point

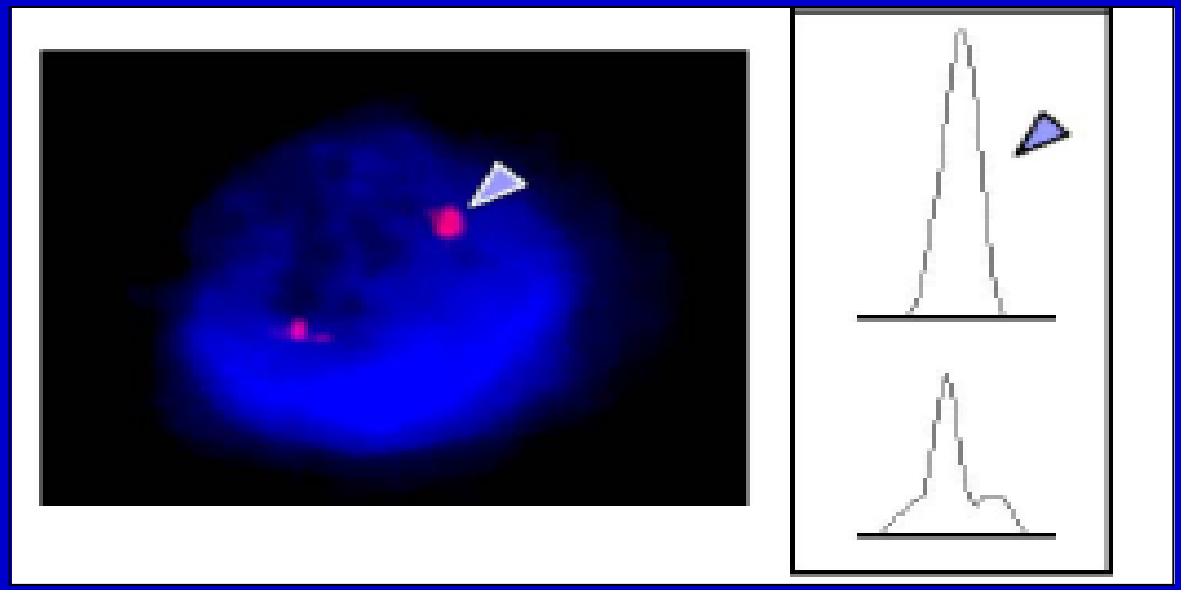
Copy number variation - CNV



Variation of CNVs



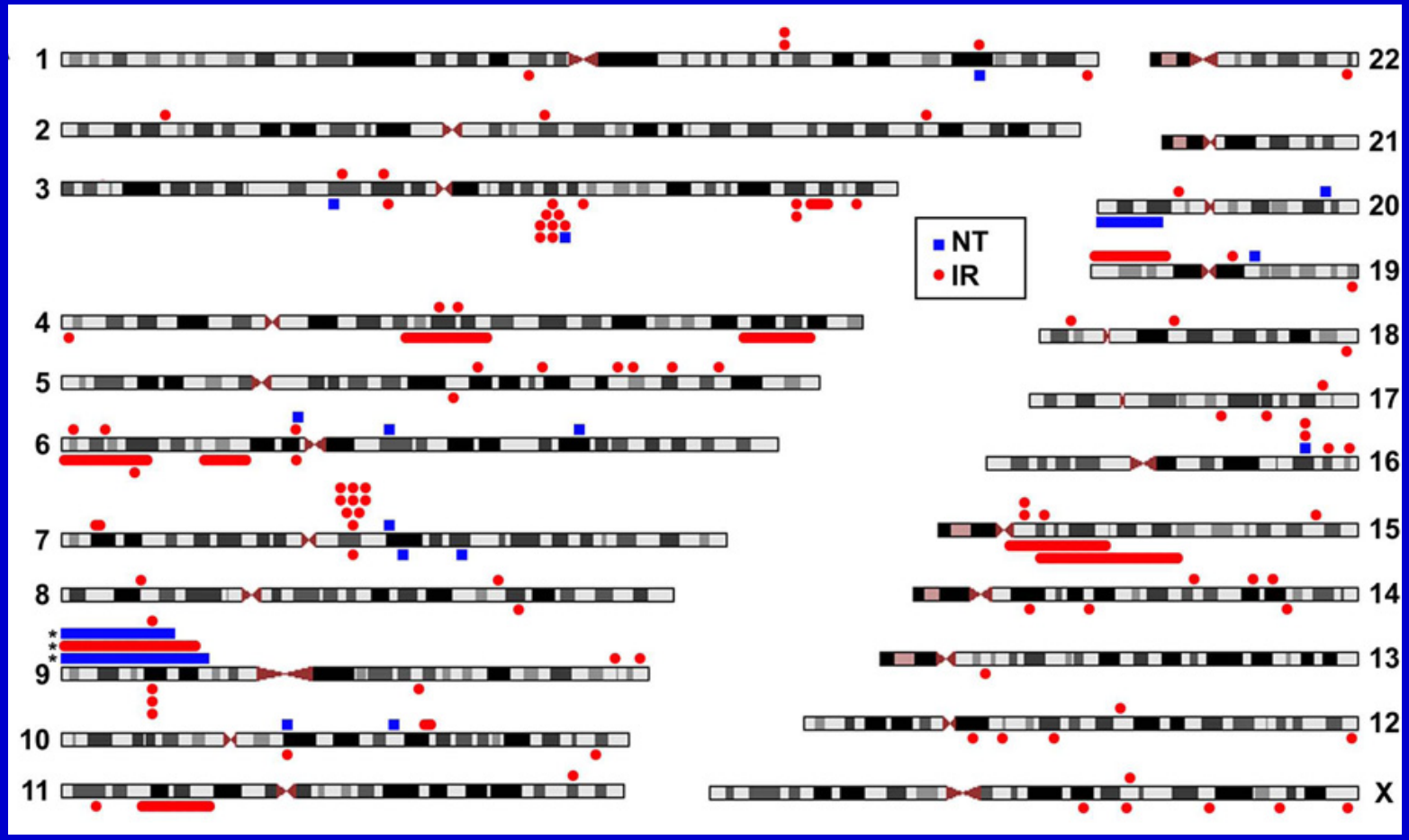
Extra CNV band in 3 persons in **8q21.2** (показан стрелкой).



CNV by FISH in **8q21.2**

Manvelyan M. et al. New cytogenetically visible copy number variant in region 8q21.2. Molecular Cytogenetics 2011, 4:1

Ionizing radiation (1.5-3 Gy) induces copy number variations (CNVs) of DNA sites.

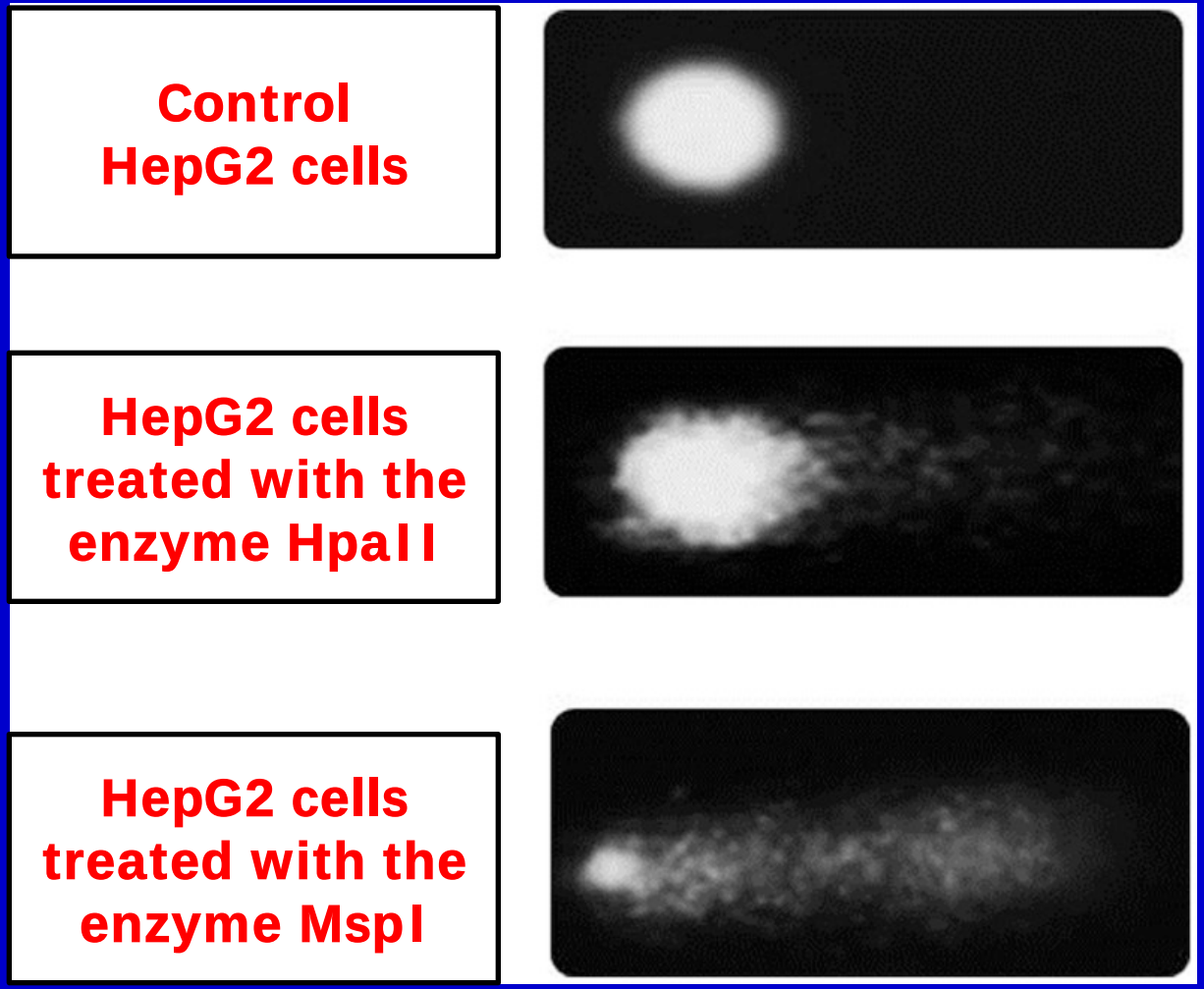


Arlt et al. Copy number variants are produced in response to low-dose ionizing radiation in cultured cells. Environ Mol Mutagen. 2014; 55(2):103-13.

Now we plan to obtain new results on electron beam induced **epigenetic effects (DNA methylation, the influence of microRNA inhibitors on genetic effects)** important for cancer radiation therapy.

EPIGENETICS include any process that alters gene activity without changing the DNA sequence

Analysis of global DNA methylation by DNA-Comet modified assay



Lewies et al. Using a medium-throughput comet assay to evaluate the global DNA methylation status of single cells. Front Genet. 2014; 5:215.

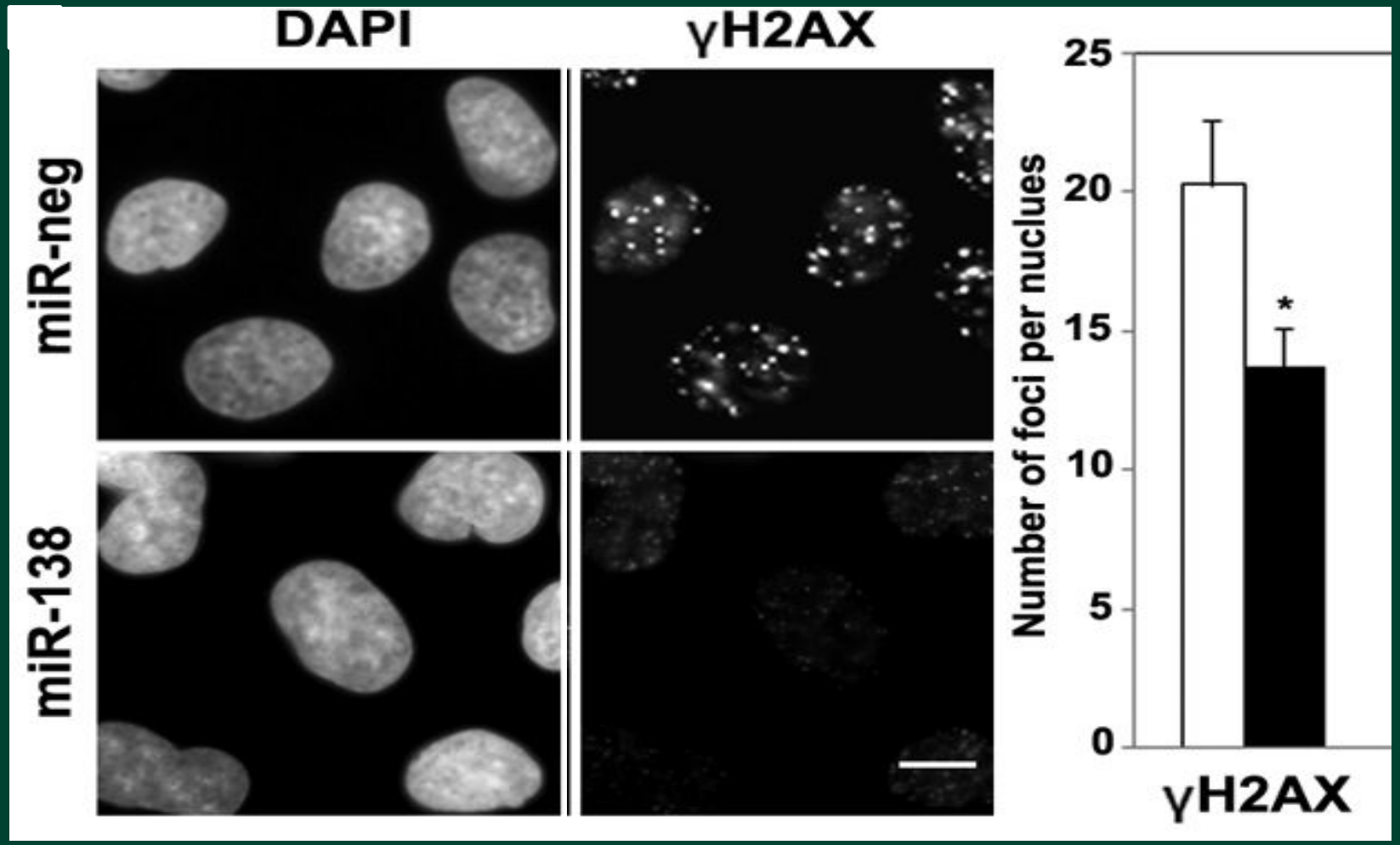
To get comprerhensive picture of electrons beems molecular effects we plan to apply microRNA inhibitors.

MicroRNAs are small non-coding RNA molecules 18-25 nucleotides in length (22 on average) that can inhibit gene expression.

MicroRNA controls the expression of many genes, that participate in the functioning of the DNA repair complex and apoptosis, reproduction and migration of cells.

The expression of microRNA is important for regulation of radiation effects.

Micro-RNA regulate DNA DSB repair



Wang et al. MicroRNA-138 modulates DNA damage response by repressing histone H2AX expression. Mol Cancer Res. 2011; 9(8):1100-11.

So, using modern methods of molecular and cell biology we have realized the presented work and are planning to investigate the radiobiological effects of modernized ultrafast pulsed electron beam obtained on upgraded AREAL accelerator, including:

-The main ways of DNA damage and repair in tumor and normal human cells

-The cell cycle and cell death pathways (apoptosis, autophagy, reproductive cell death) in human normal and tumor cells.

-The tumor specific epigenetic alterations, based on methylation status, CNVs and microRNA.

As the result, the optimal mode of pulsed ultrafast electron beams, which causes the pronounced radiobiological effect will be estimated.

V.M. Tsakanov, et al.

AREAL low energy electron beam applications in life and materials sciences.

Nuclear Instruments and Methods in Physics. Research Section A: Accelerators, Spectrometers, Detectors and Associated Equipment. 2016

Nelly Babayan, Galina Hovhannisyan, Bagrat Grigoryan, Ruzanna Grigoryan, Natalia Sarkisyan, Gohar Tsakanova, Samvel Haroutiunian, Rouben Aroutiounian

Dose-rate effect of ultrashort electron beam radiation on DNA damage and repair in vitro

Journal of Radiation Research (2017 July, in press)

Our research was implemented by grant of State Commitee of Science RA

We express our gratitude to our colleagues from AREAL, who created the opportunity to realize our work

We thank Professor Andreyan Osipov from Federal Medical Biophysical Center named after A. I. Burnazyan of FMBA of Russia for active help.


Cal

Thank you!