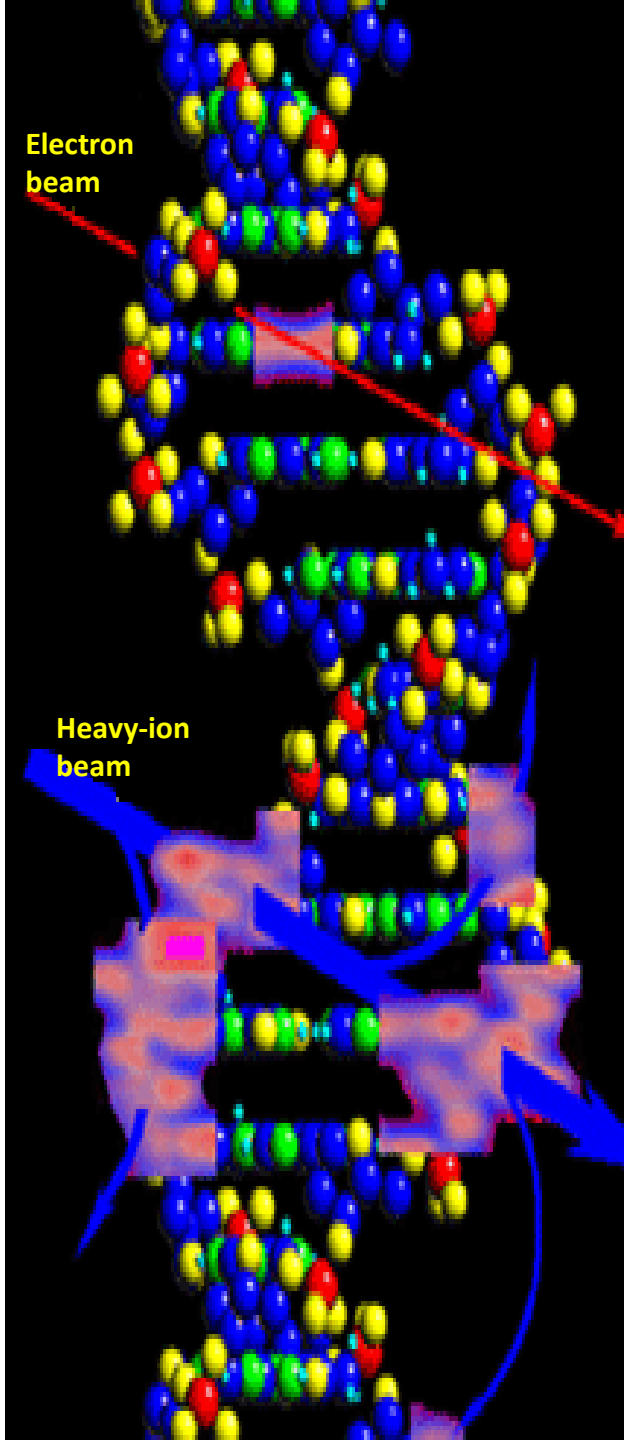
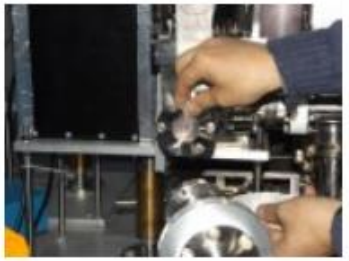
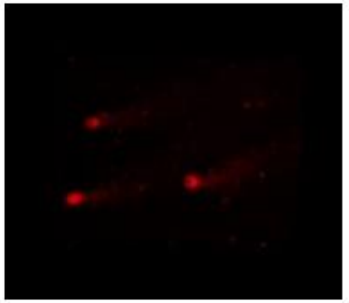
Genetic Effects of Ultrashort Pulsed Electron Beam Irradiation – 7 years of joint experience

Rouben Aroutiounian, Tigran Harutyunyan, Andrean Osipov, Natalya Vorobyeva, Galina Hovhannisyan, Anzhela Sargsyan, Bagrat Grigoryan, Samvel Harutyunyan, Thomas Liehr and Nelly Babayan

1.Yerevan State University
2.Institute of Molecular Biology NAS of RA
3.CANDLE Synchrotron Research Institute
4.Semenov Institute of Chemical Physics, RAS
5. Human Genetics Institute, Jena







February 18, 2015

The first Experimental results on AREAL facility

The first experimental results of DNA comets – human cultured cells with damaged DNA obtained on the AREAL facility. The experimental study is performed within the project “Study of Molecular-genetic Effects of Ultrafast Radiation” headed by Prof. Rouben Aroutiounian (Yerevan State University).

March 5, 2015

Presentation of the first Experimental Results on AREAL facility

The research groups from the Department of Genetics (Biology Faculty, Head Prof. Rouben Aroutiounian) and the Department of Molecular Physics (Physics Faculty, Head Prof. Eva Dalyan) of Yerevan State University have presented the first experimental results on the AREAL facility conducted in the framework of the project " Study of Molecular-genetic Effects of Ultra-fast Radiation". The main experimental set-ups, first results, nearest plans and new experimental highlights on AREAL facility have been discussed. The presented topics were the following:

- Study of Molecular-genetic Effects on AREAL

Prof. R. Aroutiounian (Dept. of Genetics, YSU)

Dr. N. Babayan (Dept. of Genetics, YSU)

- The AREAL electron beam irradiation of DNA / Porphyrin solutions

Dr. L. Aloyan (Dept. of Molecular Physics, YSU)

CANDLE team congratulates the Research Teams with the successful start-up and accomplishment of the first stage of the experiment on AREAL. It is a solid step forward to the creation of robust AREAL User community and the Facility further development.

AREAL

laser driven, photocathode RF gun based linear accelerators providing ultrashort electron pulses (sub-pico or femtosecond) with the electron energy in MeV domain

- pulse duration - **0.04×10^{-12} s**
- UHPDR - **1.6×10^{10} Gy/sec**

JETI and ELBE
~ $1\text{-}5 \times 10^{-12}$ s
~10^{8}-10^{9} Gy/sec

AREAL **produce electron pulses generated by UV laser and accelerated using high gradient RF resonator**

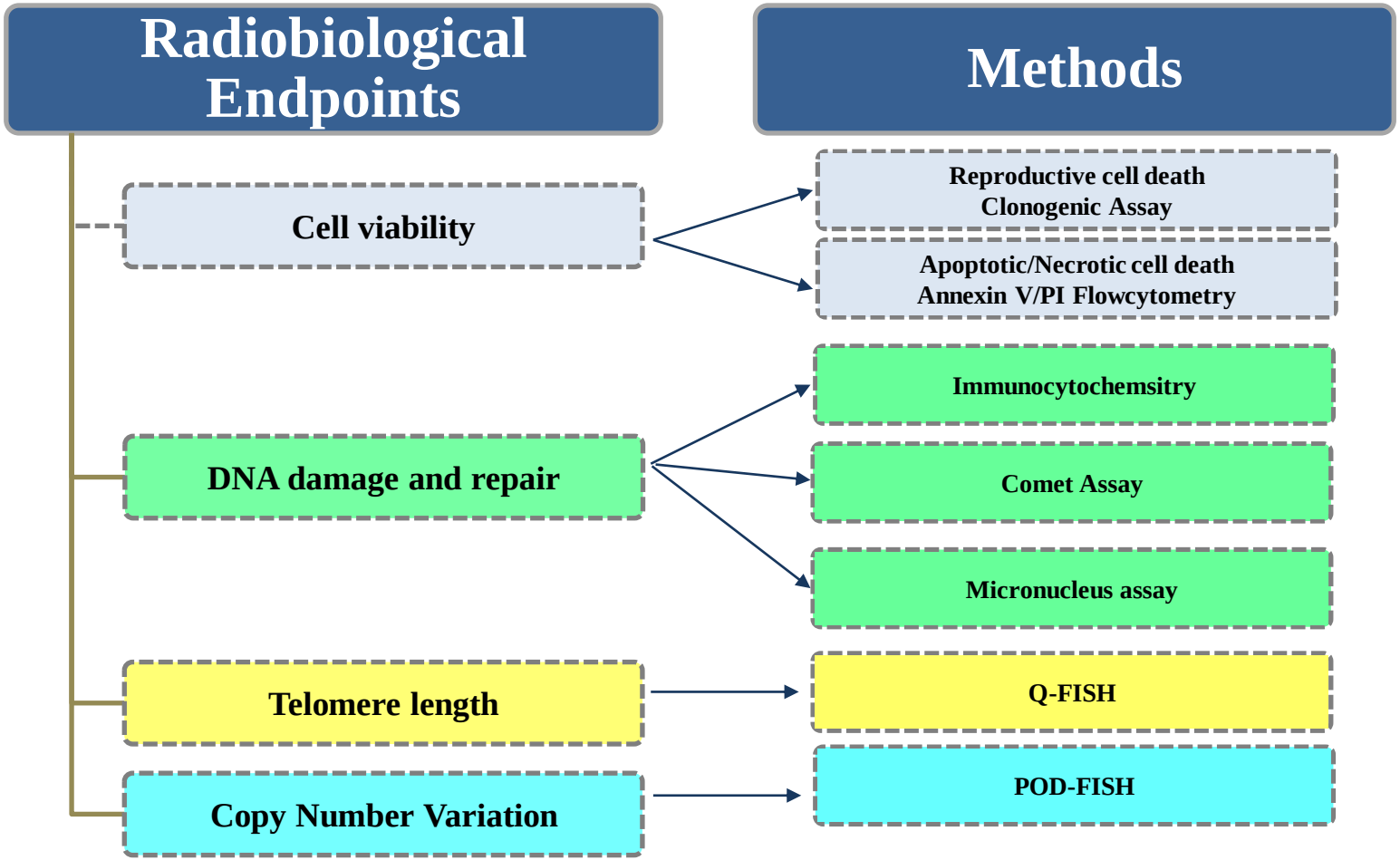
The advantage of laser-generated ultrafast electron beams for biological and clinical application:

-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 (sub-pc vs. pc), than many chemical reactions pulses of enormous capacity (10^{10} Gy/sec vs. 10^{8}-10^{9} Gy/sec).

-This allows the precise dose-control induction of local effects on cells and solid tumors with minimal exposure of normal tissues.

Radiobiological experiments



One of most important directions in radiobiology is the estimate of DNA damage, induced by different types of radiation.
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 lead to cancer.

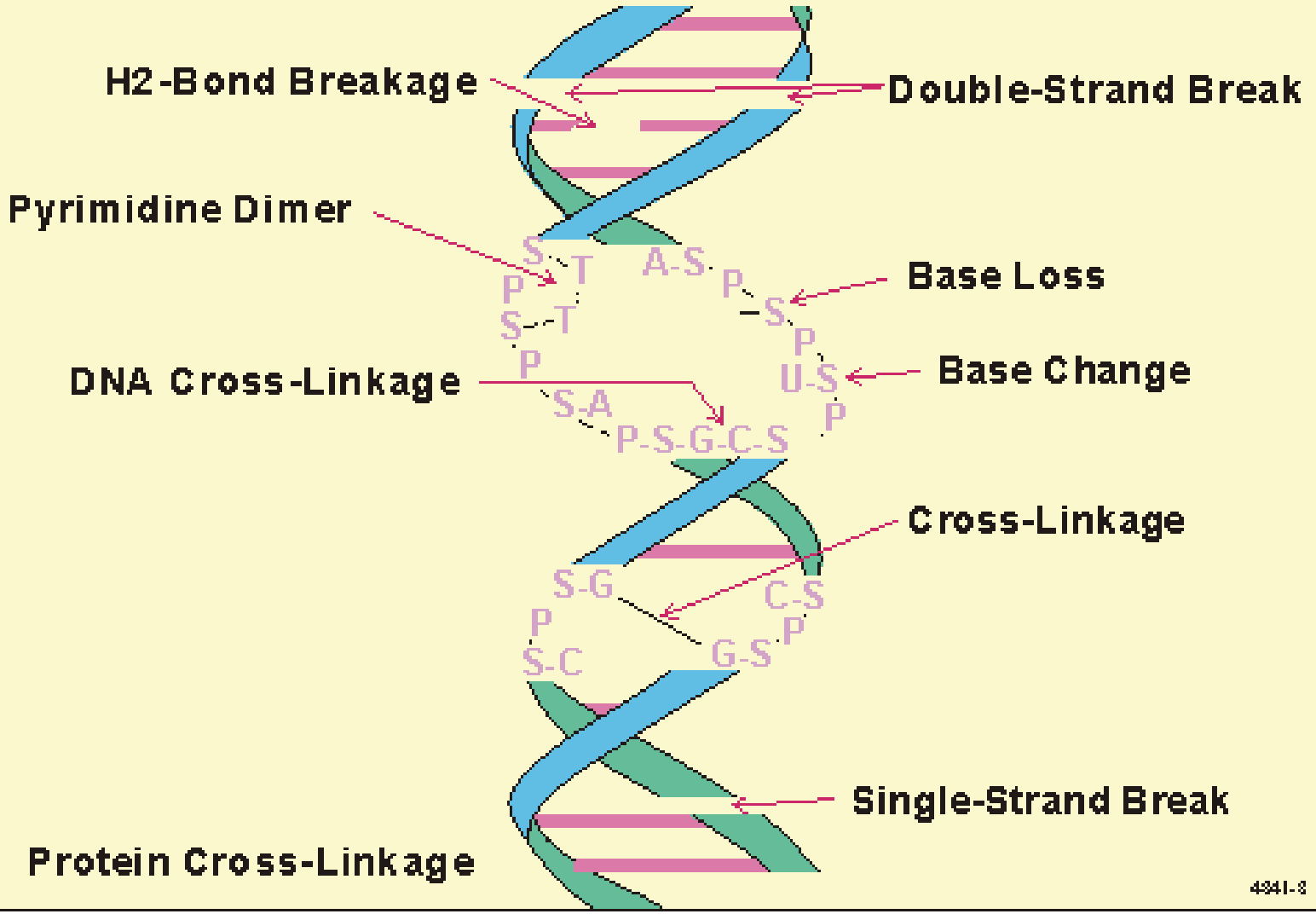
- **About 50 % of cancer cases are treated with radiation therapies (possibly in combination with surgery and/or chemotherapy).**

- **Among these treatments, more than 50 % 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.

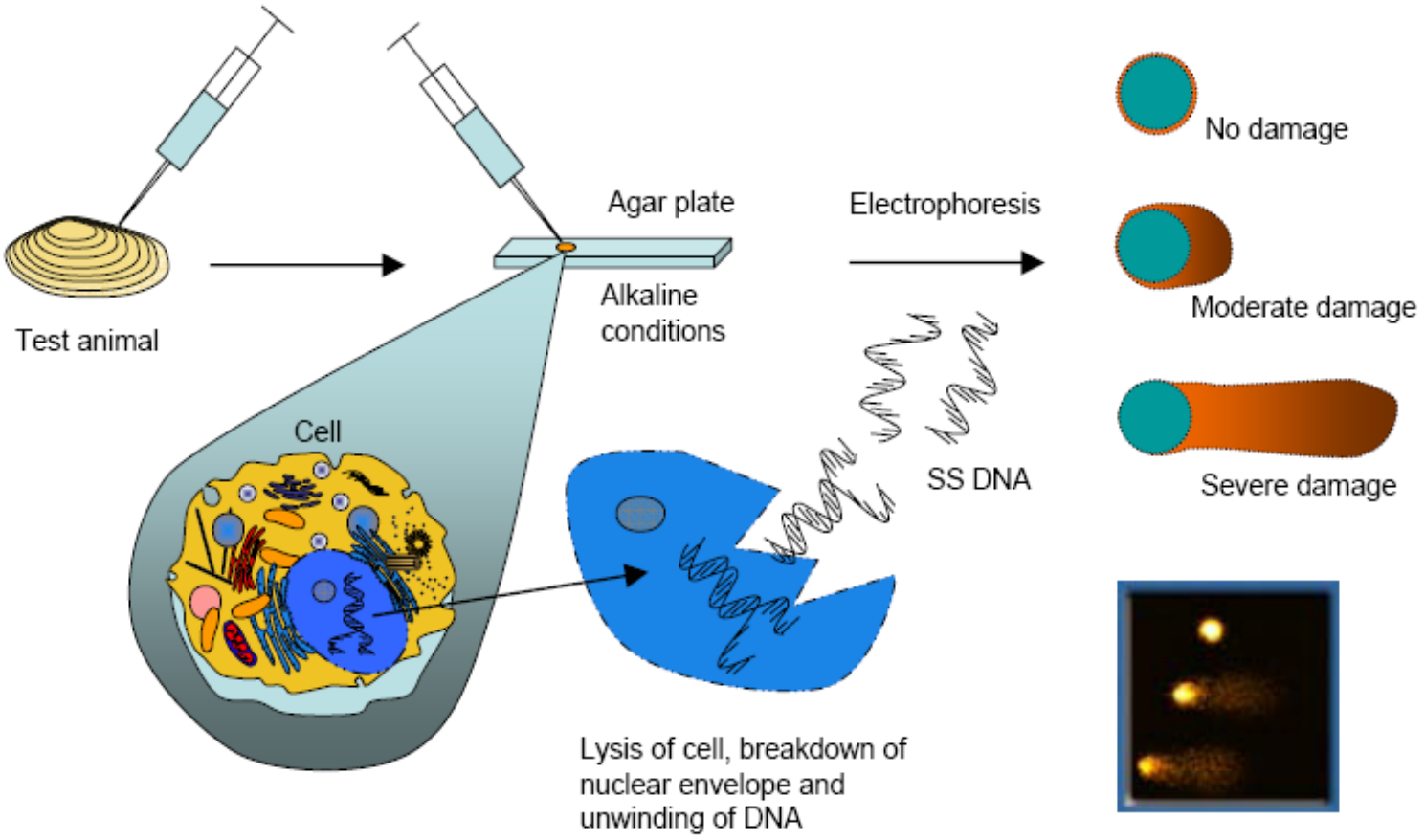
- **Electrons are used directly, either to cure superficial tumors or in the Intra-Operative Radiation Therapy which can be applied during operations of tumor.**

RADIATION DAMAGE TO DNA

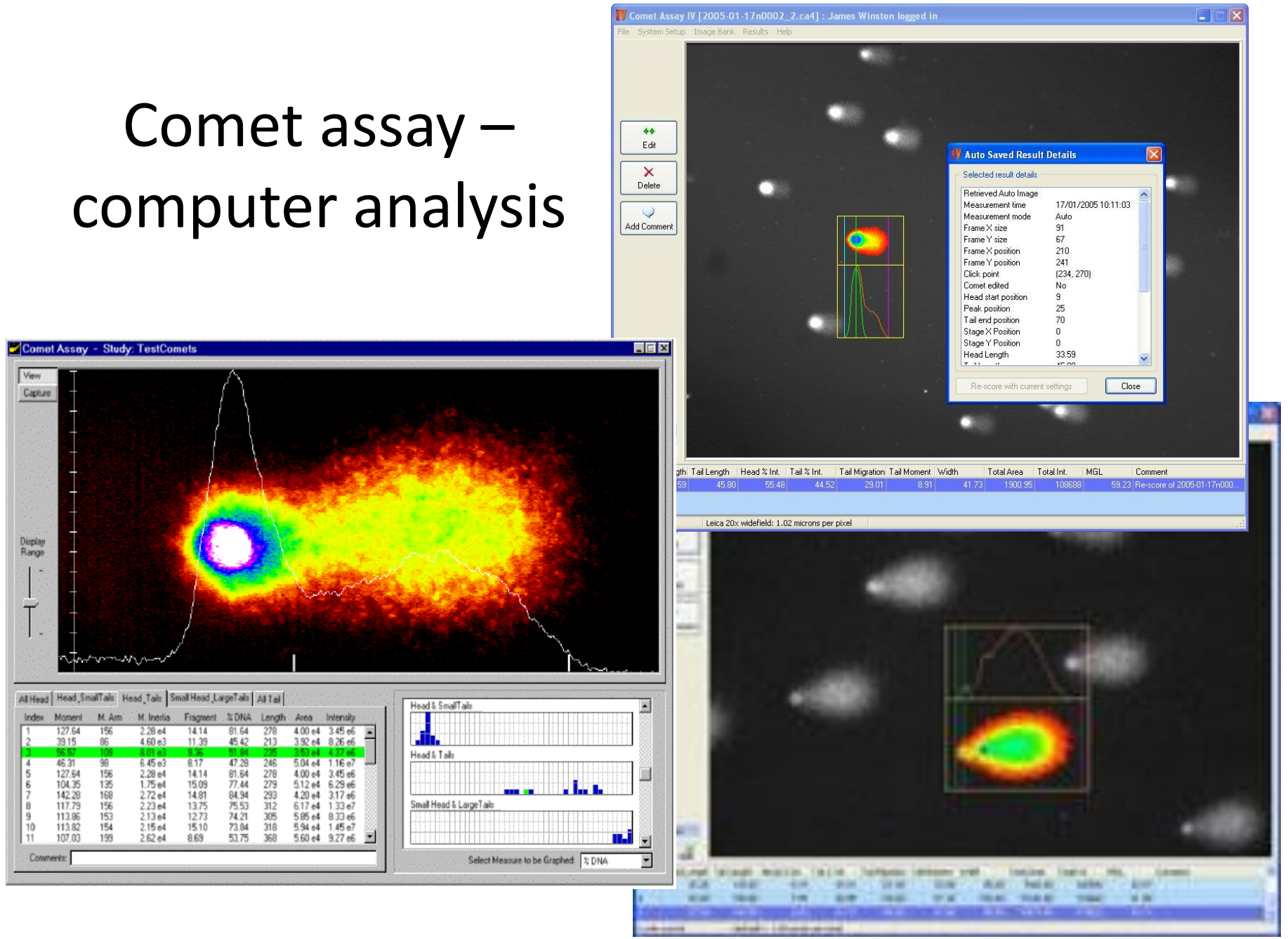


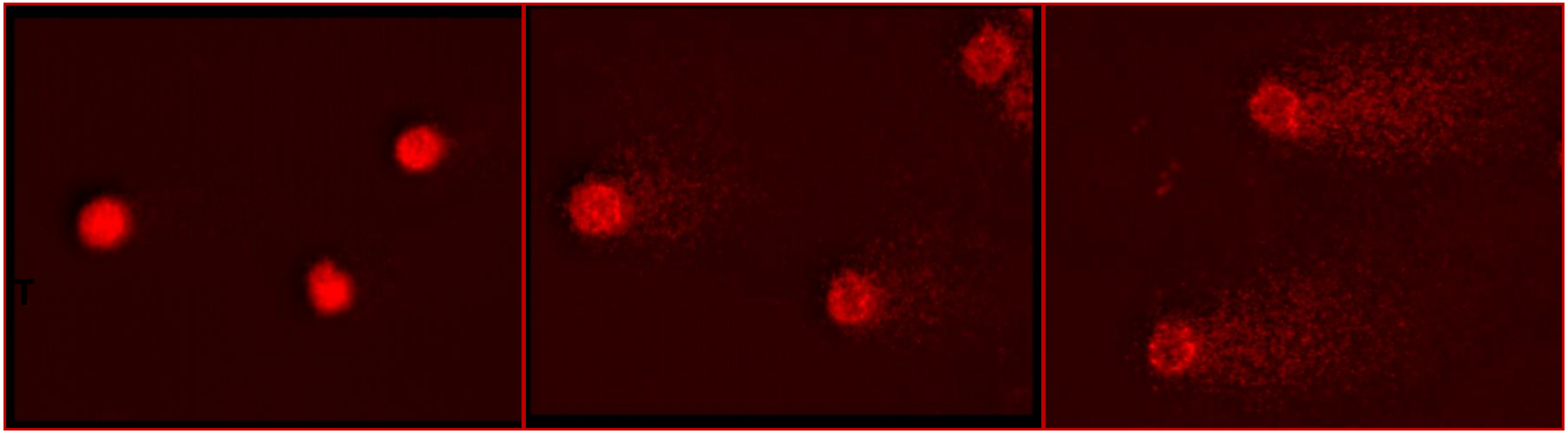
4341-3

Comet assay, was used during our experiments for evaluation of DNA damage

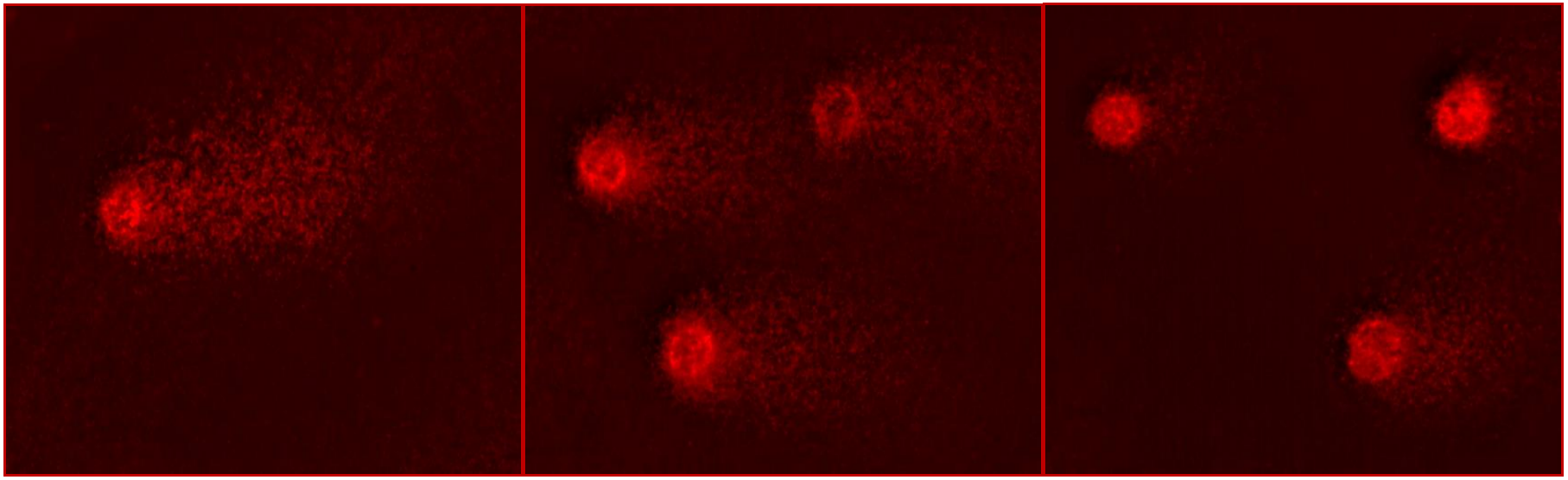


Comet assay – computer analysis





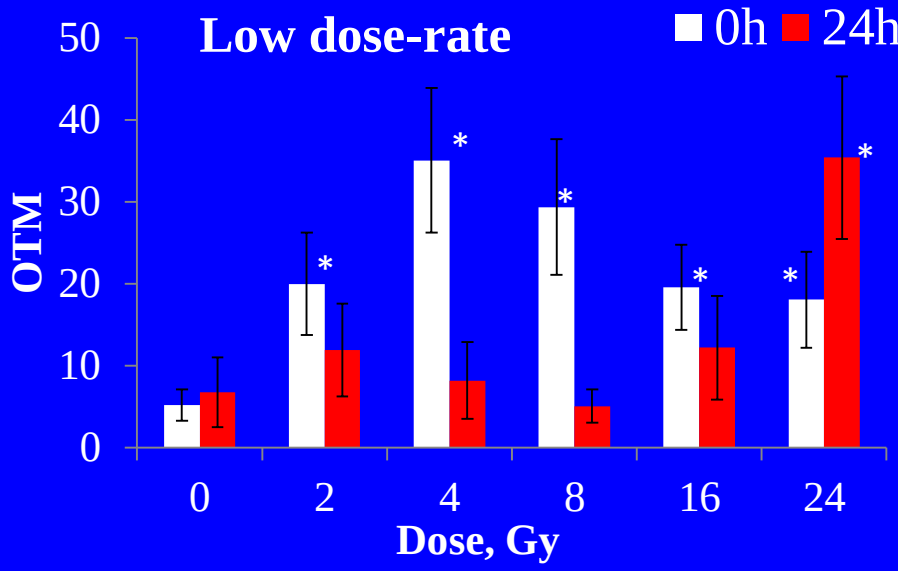
Non-irradiated control | Irradiation, dose 2 Gy, | Irradiation, dose 4 Gy,

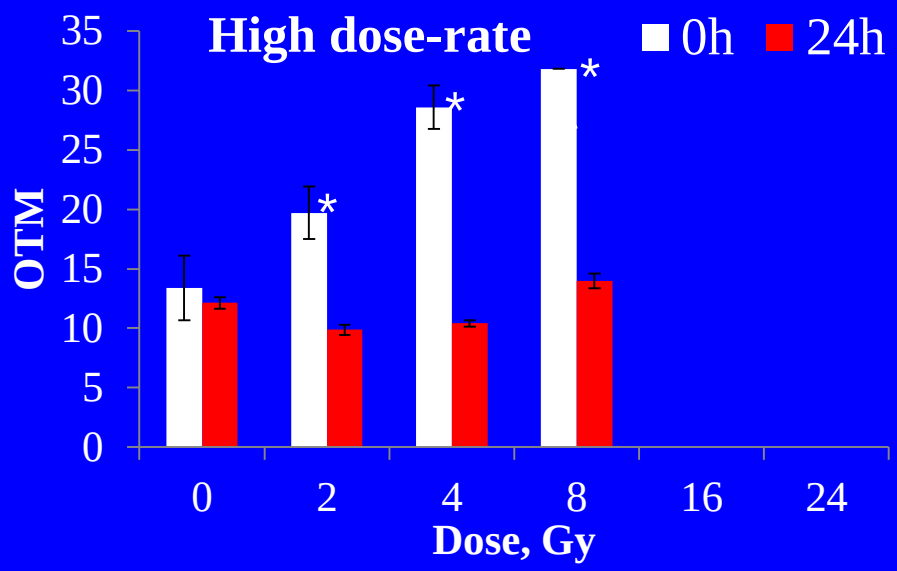


Irradiation, dose 8 Gy, | Irradiation, dose 16 Gy, | Irradiation, dose 24 Gy,

Comet assay images of K-562 (human chronic myelogenic leukaemia) cells after irradiation by AREAL

DNA damage (Olive Tail Moment) in K-562 cells 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 exposure of K562 to AREAL irradiation at different doses revealed the dose-dependent increase of the primary DNA damage. At the high doses the DNA damage decreases. The reason - the level of dead cells can be increased. Electrons, as a source of low LET radiation, lead to DNA lesions, including single-strand and double-strand breaks of DNA, which were generally repaired efficiently., except 24 Gy.

Distribution of cells on the base of DNA damage in K-562 cells after irradiation by low and high dose rate, by Olive Tail Moment

DNA damage

NT 3,6 Gy/min 36 Gy/min

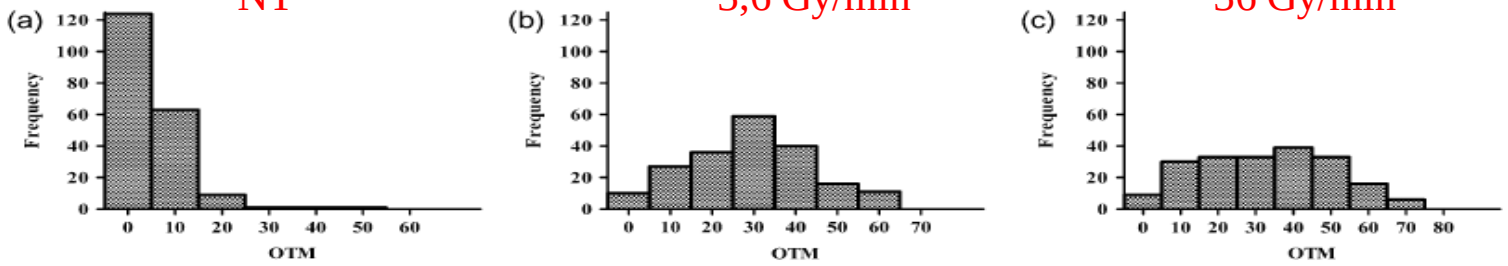


Fig. 1. Probability distribution of the OTM value for unirradiated K-562 cells (a) and those exposed to 8 Gy with the ultrashort electron beam at low (3.6 Gy/min) (b) and high (36 Gy/min) (c) dose rates at the 0 h time point after irradiation.

DNA repair

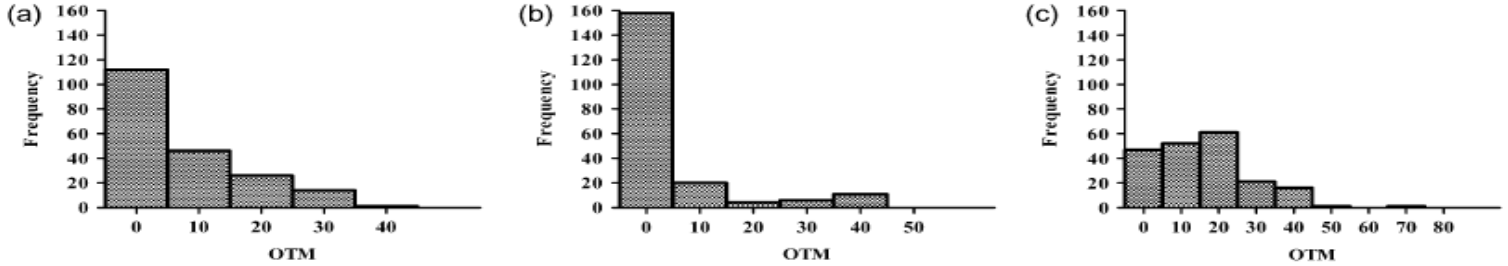
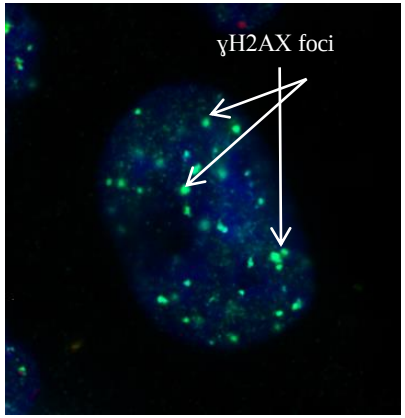


Fig. 2. Probability distribution of the OTM value for unirradiated K-562 cells (a) and those exposed to 8 Gy with the ultrashort electron beam at low (3.6 Gy/min) (b) and high (36 Gy/min) (c) dose rates at the 24 h time point after irradiation.

An analysis of the distribution of initial DNA damage after high power irradiation by increasing the dose-rate of ultrafast electrons revealed a shift towards an increase in the proportion of damage and expansion of the distribution. with a subsequent decrease in reparability due to more complex DNA damage.

Dynamic microstructures, called FOCI, are formed during recognition and repair of DSBs. DNA damage FOCI are distinct spots after DNA damage consisting of repair proteins of double-strand DNA ruptures.

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

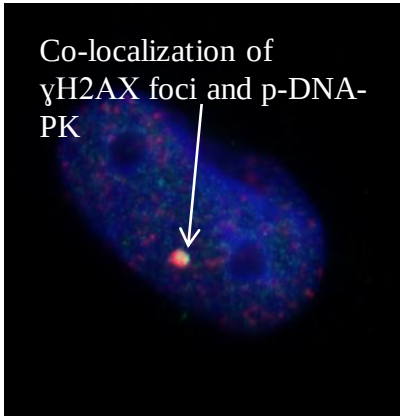


Visualization of phosphorylated γH2AX foci in irradiated cell

Fluorophores

γH2AX foci – Fluorescein (FITC) – green

p-DNA-PK - Texas Red - red



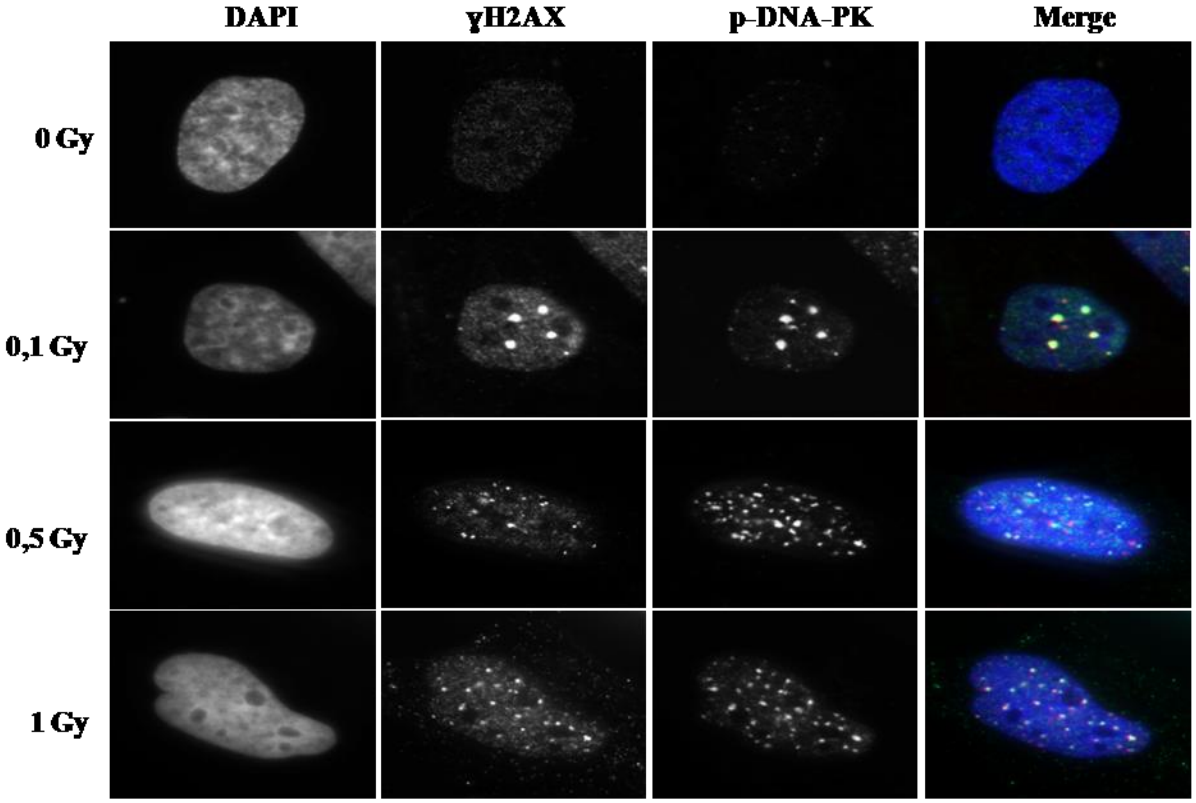
Co-localization of phosphorylated γH2AX and DNA-PK protein can be visualized

One of the earliest (several minutes) DNA repair events is the phosphorylation of a protein called histone H2AX, that involves many other proteins in the repair process. which are sensors of double-strand breaks of DNA (For low-LET we measured DNA-PK)

The quantitative analysis of the foci of repair proteins and their localization in the postradiation period **allows to determine not only the number of DSBs and their spatial distribution in the cell nucleus, but also the mechanisms of their repair.**

Co-localization of γH2AX foci and p-DNA-PK at different doses of AREAL irradiation

The ultrashort electron beam radiation effect on DNA DSBs formation



The γH2AX and p-DNA-PK foci and their co-localization at 1h post-irradiation in human fibroblasts

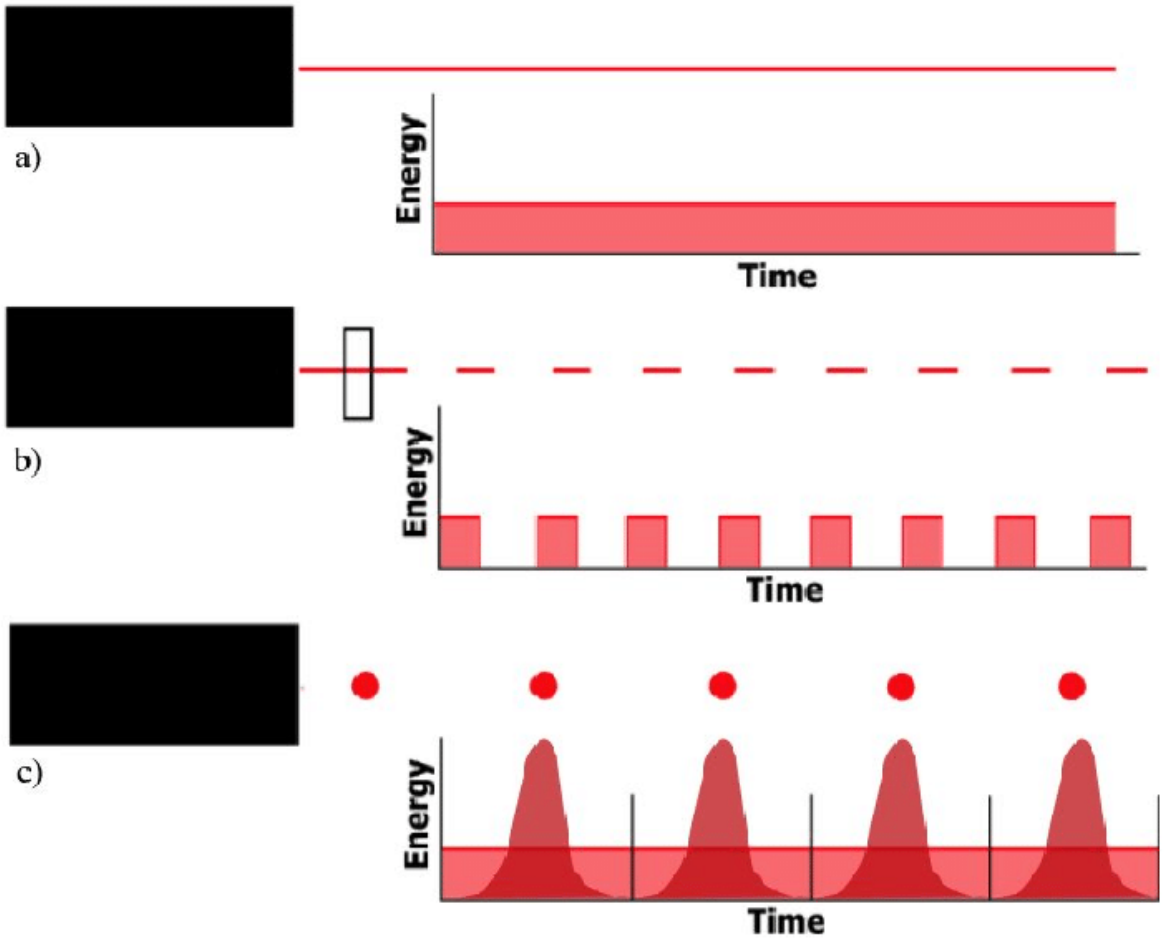
Comparative studies of radiobiological effects for AREAL and Varian Accelerators

Varian Trilogy linear electron accelerator (Varian Medical Systems, USA)

- For quasi-continuous irradiation, a Varian Trilogy linear electron accelerator (Varian Medical Systems, USA) was used.
- Characteristics: electron energy - 4 MeV, dose-rate 5.6 Gy/min, electron beam spot 250×250 mm.
- Dosimetry was carried out by the ionization chamber method of absorbed dose measurement in the water phantom according to the international protocol IAEA TRS-398.

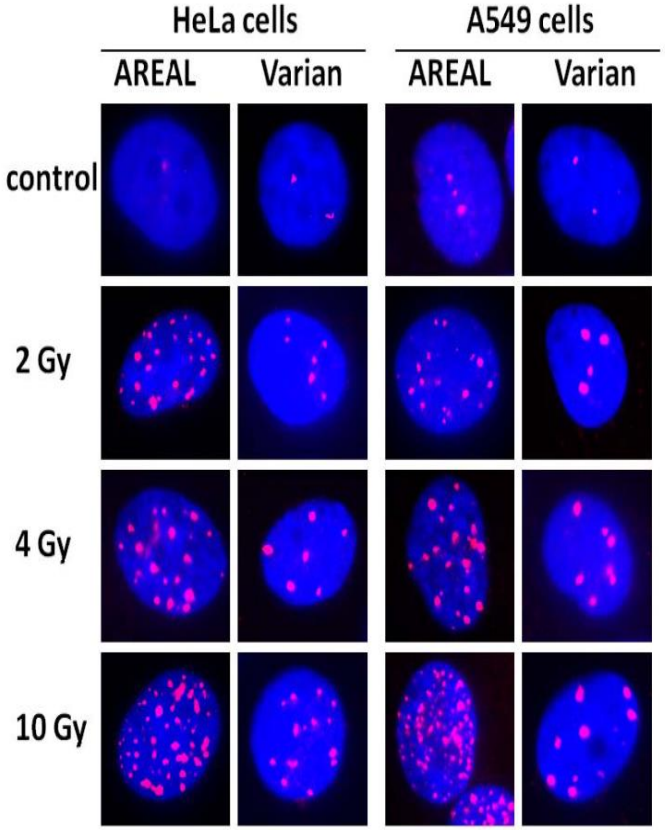
Energy vs. time emitted from a laser.

a) typical continuous wave (CW) laser; constant energy over time. b) CW laser 'chopped' with a shutter mechanism; average power is reduced but no gain in peak power. c) Ultrashort pulse laser; average power remains the same but peak power is greatly enhanced. Peak power is limited by how narrow each pulse is.



AREAL *vs* Varian

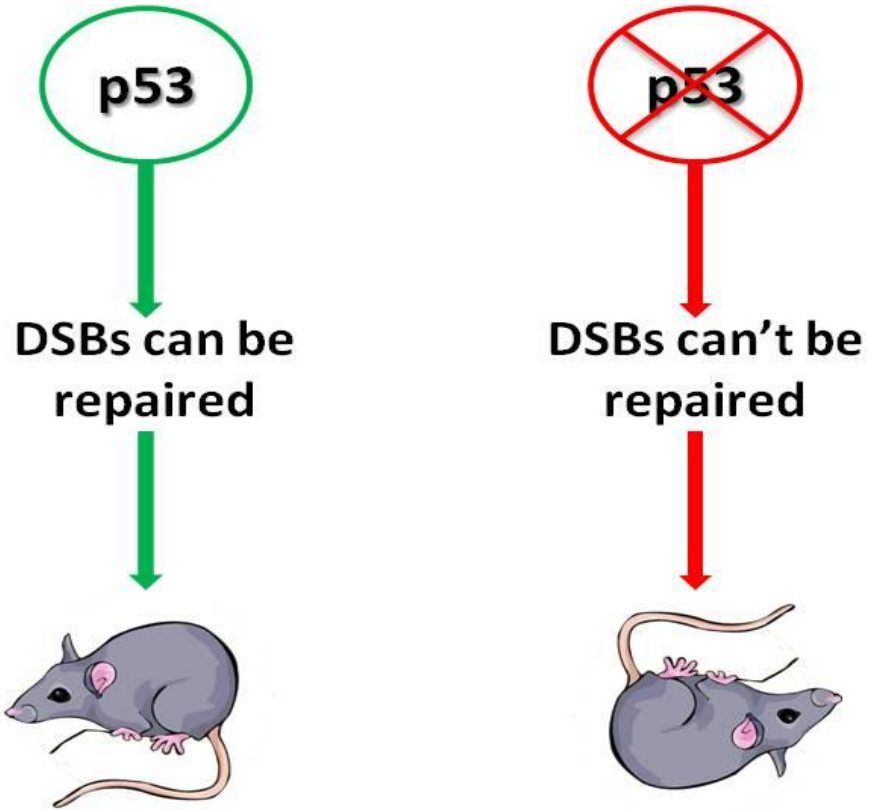
Dose-dependent changes in the γH2AX residual foci numbers in HeLa and A549 **lung carcinoma cells** **at 24 h post-irradiation**



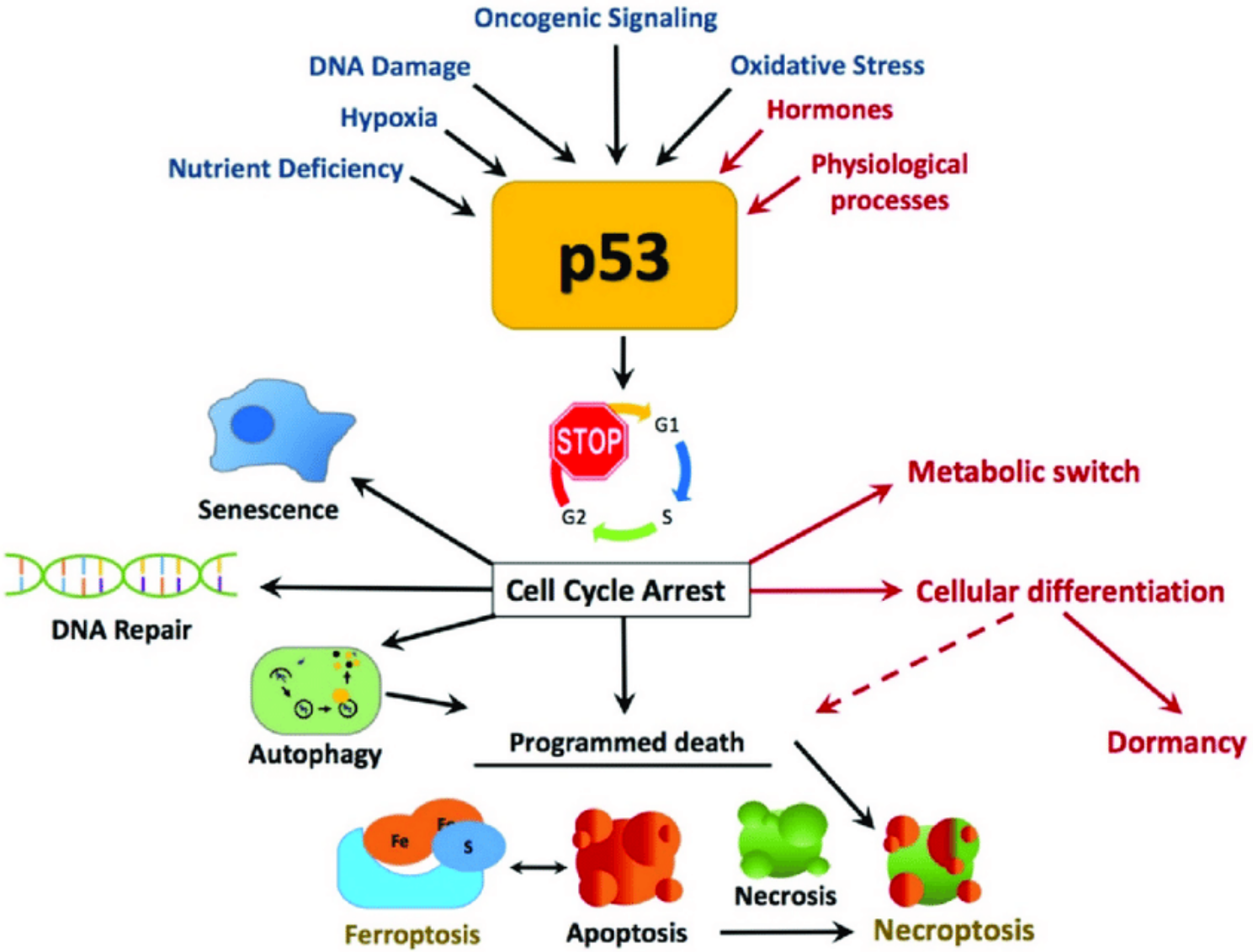
- **For AREAL irradiation is demonstrated slower DSB repair rate induced by ultrashort pulsed irradiation for both cell types , compared to Varian.**
- **Why? Pulse duration increases the possibility of DSB-complex with more difficulty repairable DSBs formation**.

Tumor protein P53, is cellular tumor antigen the “Guardian of the Genome”

*p53-binding protein 1 (**53BP1**) is a crucial component of*
***DNA double-strand break signalling** and **repair activator** in mammalian cells*



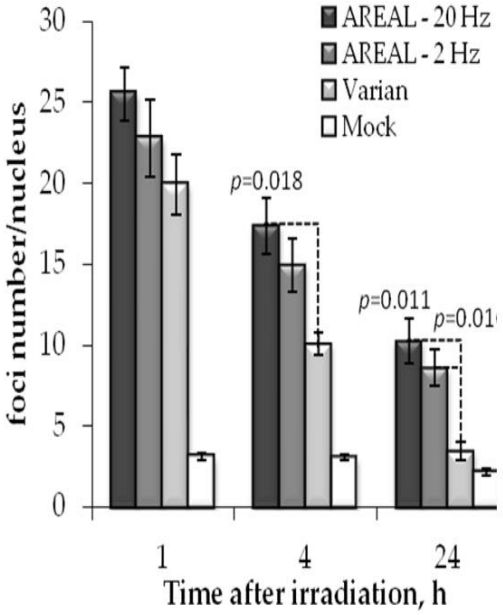
The biological role of p53 –
that arrests cell cycle until repairing DSBs



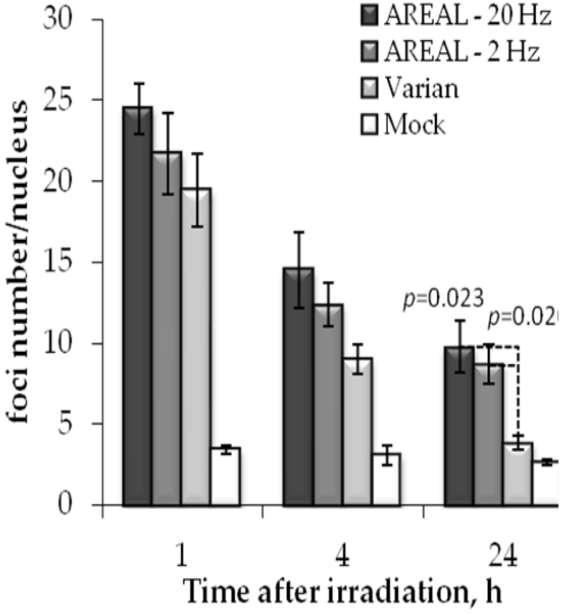
AREAL *vs* Varian

Post-irradiation changes of the γH2AX and 53BP1 FOCI (markers for DNA DSBs) in HeLa cells, irradiated on AREAL and Varian accelerators at a 1 Gy dose

γH2AX FOCI



53BP1 FOCI

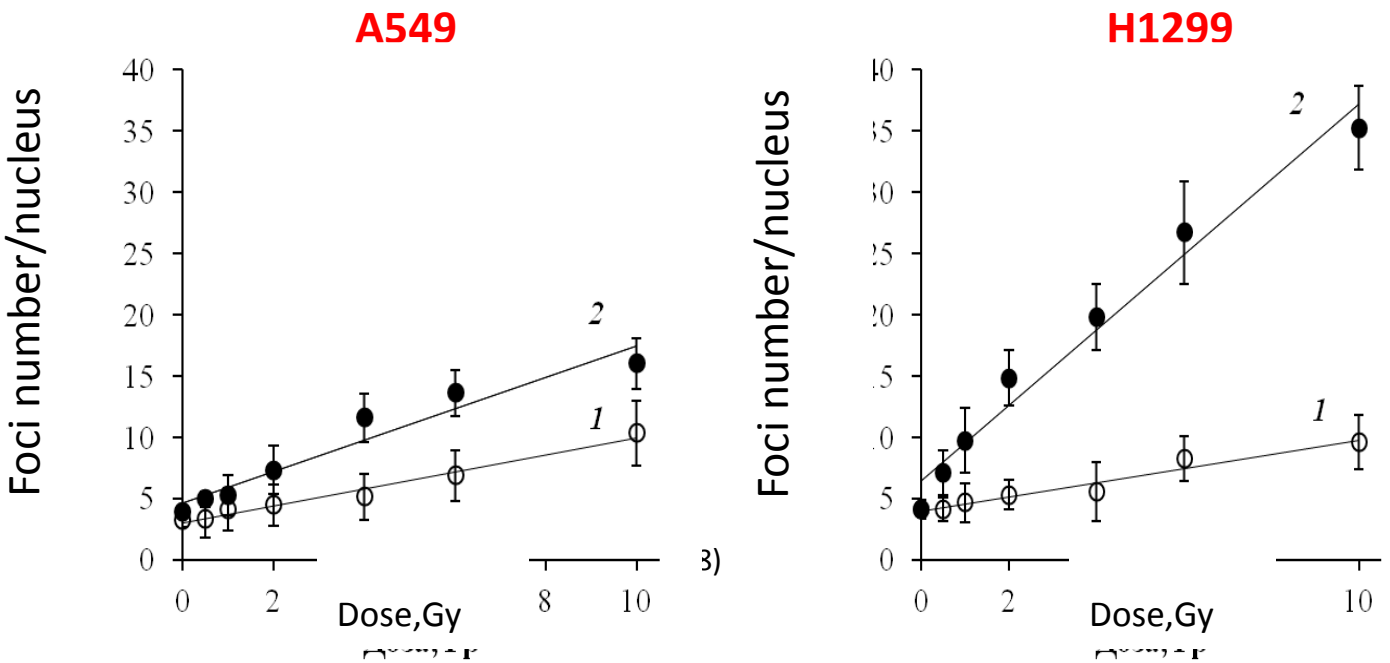


1. **No significant difference between the effects of pulsed irradiation at 2 and 20 Hz**
2. **24 h post-irradiation, the significant difference between the effects of pulsed and quasi-continuous irradiations was observed at both pulse rates (2 and 20 Hz)**
3. **24 h after exposure to pulsed irradiation, the foci number for AREAL was 2.5–2.9 times higher as compared with Varian. Similar trends were observed also for 53BPI foci.**

Pulsed irradiation induces more difficultly repairable complex DSBs

AREAL vs Varian

Residual γH2AX Foci in H1299 (p53-deficient Human Lung Carcinoma Cells) Exposed to Subpicosecond Beams of Accelerated Electrons



Comparison of the slope coefficients showed that yield of γH2AX foci for AREAL exposure was 1.8 times higher, than for Varian in A 549 (p53 wild type cells), but ~5.3-fold higher in H1299 (p53 deficient cells).

Conclusion: the yield of FOCI in human cells is p53-dependent for AREAL irradiation, but not for Varian!

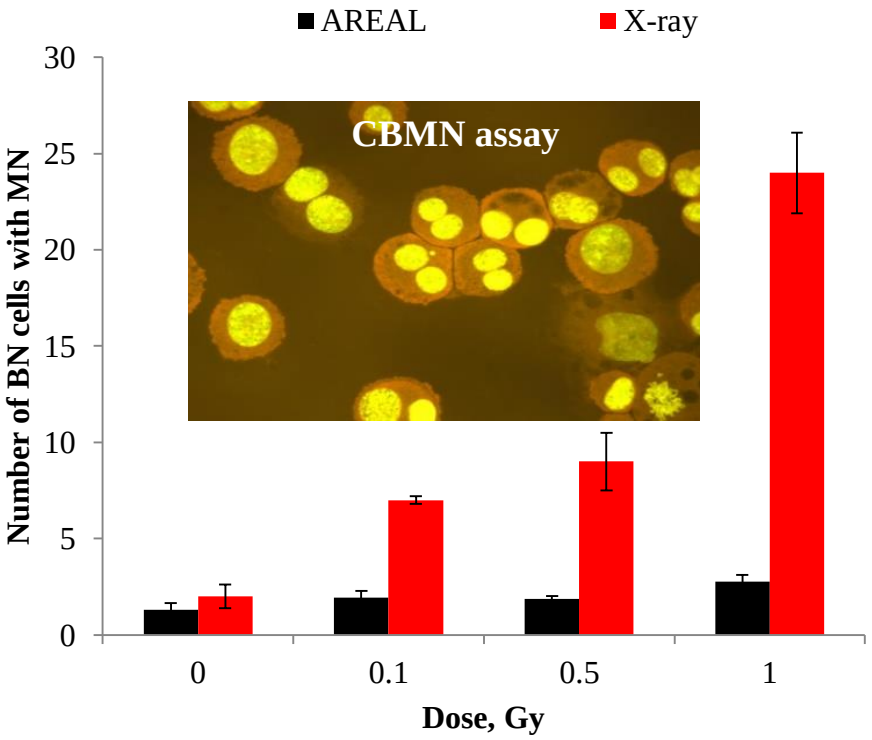
AREAL>VARIAN due to complex damages,

H1299>A549 (more FOCI) because not-complex damages of p53- can be repaired without repair activator.

Babayan, N.; Vorobyeva, N.; Grigoryan, B.; Grekhova, A.; Pustovalova, M.; Rodneva, S.; Fedotov, Y.; Tsakanova, G.; Aroutiounian, R.; Osipov, A.
Low Repair Capacity of DNA Double-Strand Breaks Induced by Laser-Driven Ultrashort Electron Beams in Cancer Cells. Int. J. Mol. Sci. 2020, 21, 9488

The aim of this work was to compare the formation and elimination of γH2AX and 53BP1 foci (well known markers for DNA double-strand breaks (DSBs)) in Hela cells exposed to ultrashort pulsed electron beams generated by Advanced Research Electron Accelerator Laboratory (AREAL) accelerator (electron energy 3.6 MeV, pulse duration 450 fs, pulse repetition rates 2 or 20 Hz) and quasi-continuous radiation generated by Varian accelerator (electron energy 4 MeV) at doses of 250–1000 mGy.

The induction of chromosomal aberrations, such as chromosomal fragments resulting from DNA breaks, or entire chromosome loss was evaluated in MRC5 cell line after 0.1 Gy, 0.5 Gy and 1 Gy doses of electrons and X-ray irradiation.



•The slight, but statistically significant increase was observed for AREAL only at the 1 Gy of electrons irradiation

•The dose-dependent increase of MN frequency was observed after X-ray irradiation, reaching the level of 24±2.1 of BN cells with MN at the irradiation dose of 1 Gy

To compare the mutagenic capacity of the UPEB with X-ray irradiation, the **RBE (relative biological effectiveness)** value was calculated as the ratio of the **slopes** for the two radiation types

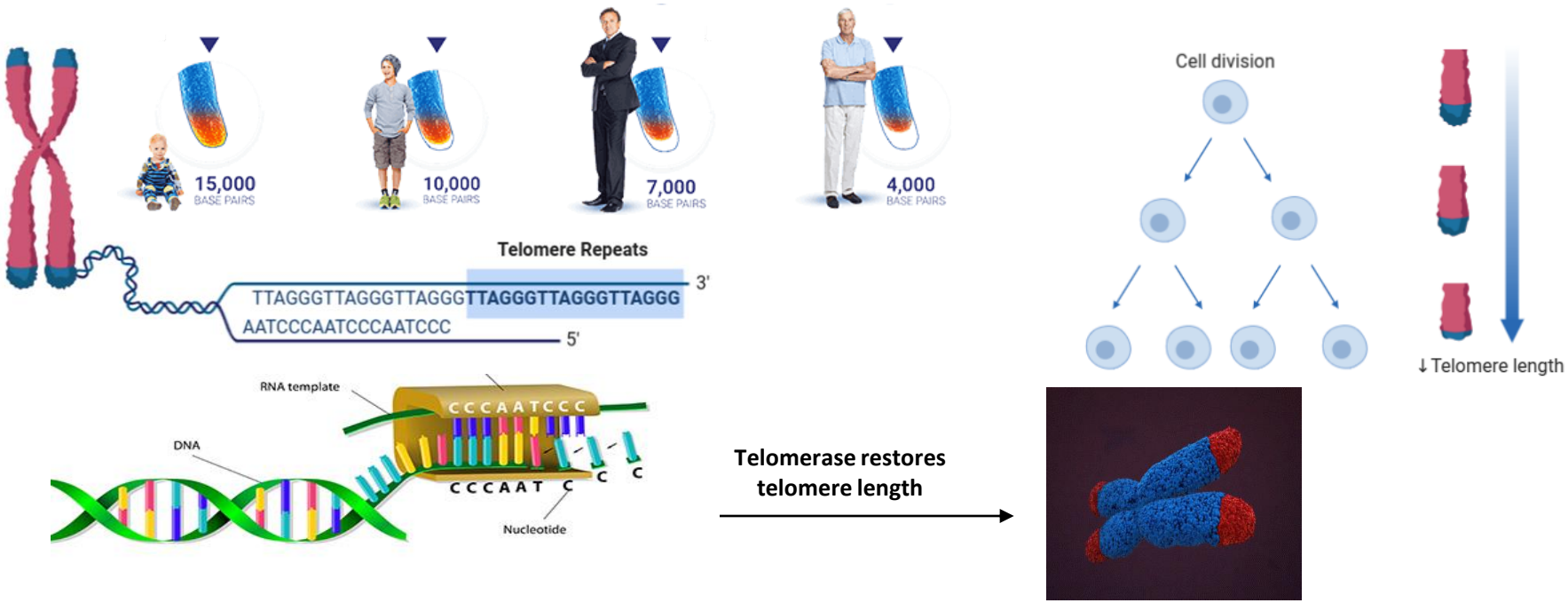
The dose-response functions were

•AREAL $y=\mathbf{1.1979}x+1.4864$ ($R^2=0.83$)

•X-ray $y=\mathbf{20}x+2.5$ ($R^2 = 0.92$)

The deduced RBE value of AREAL iradiation to X-rays to induce micronuclei in **MRC5 cells was about 0.06,** suggesting **lower level of mutagenic capacity in case of electrons iradiation due to death of cells, not their transmission in generations!**

Telomeres



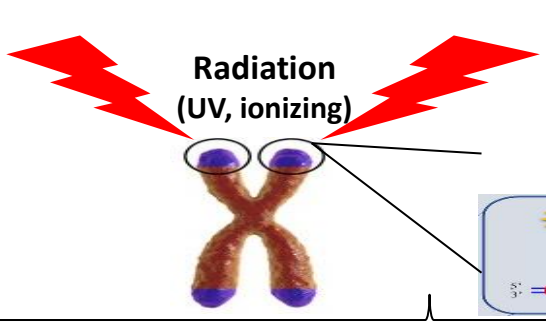
-Telomeres are nucleoprotein structures with repeated sequences of DNA (TTAGGG)n that cap the end of each chromosome arm and function to maintain genome stability.

-With each cell division the length of telomeres gets shorter which limits cell divisions. Also, telomere attrition is correlated with the process of aging.

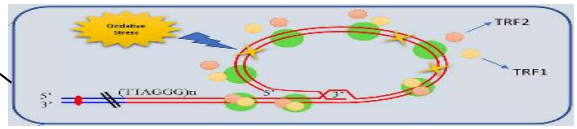
-However, telomere length can be restored by the enzyme telomerase which is constantly active in cancer cells.

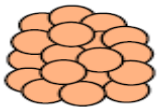
Therefore, a detailed understanding of the mechanisms of telomere loss and preservation is important for human health. 2009 - Nobel prize for their studies of telomeres and telomerase (Blackburn, Greider and Jack Szostak) .

Telomere attrition can be induced by environmental factors

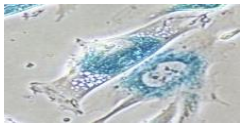


Environmental factors
(smoking, air pollution,
chemical mutagens)

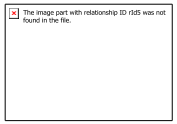




Cancer



Cellular senescence



Cardiovascular diseases

Neurodegenerative and
age-related diseases

-Alterations of telomere length are associated with various pathological conditions including cancer, cellular senescence, cardiovascular, autoimmune and neurodegenerative or aging-related diseases

-Moreover, environmental factors such as radiation and chemical compounds can increase telomere damage mainly via induction of DNA oxidative damage due to high concentration of G-rich parts in telomeres.

-Telomere shortening suppresses cell division by triggering senescence in normal cells but can promote cancer by triggering genomic instability in pre-cancerous or malignant cells.

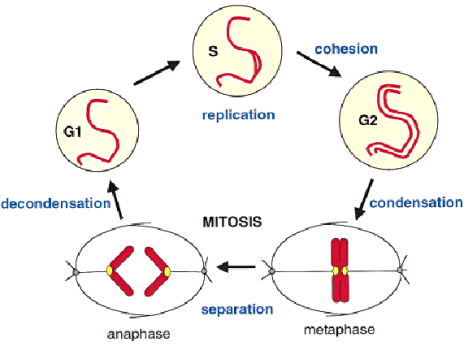
-In recent years telomere length alterations have been used as molecular markers for the evaluation of biological effects of radiation. However, the impact of accelerated electrons on telomere length was not studied yet.

Coluzzi et al. Cells. 2019 Jan 3;8(1):19.

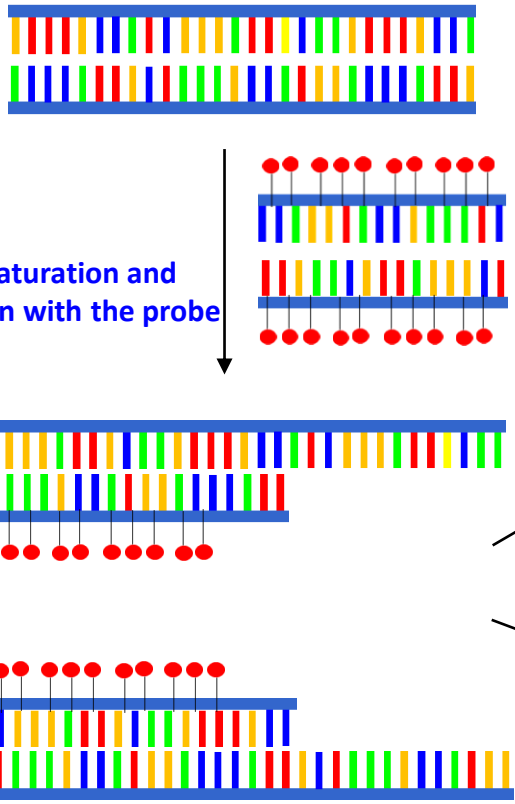
Quantitative Fluorescence in situ hybridization or Q-FISH

Telomeres

Telomeres



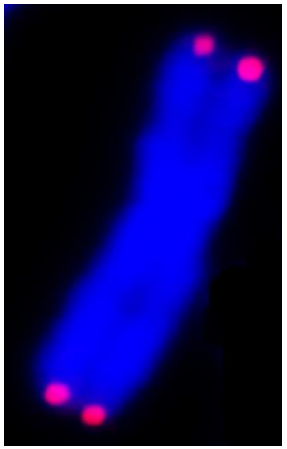
Telomeric DNA



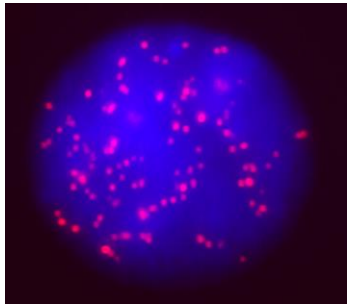
PNA probe

DNA denaturation and
hybridization with the probe

Q-FISH analysis



Metaphase chromosome

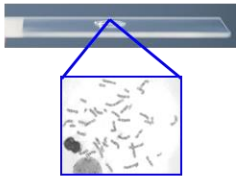


Interphase nucleus

Irradiation of human blood with AREAL electron accelerator and Q-FISH analysis

Irradiation
0.5, 1.5 and **3. 0 Gy**

Blood cultivation



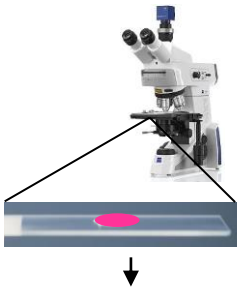
Slide with chromosomes

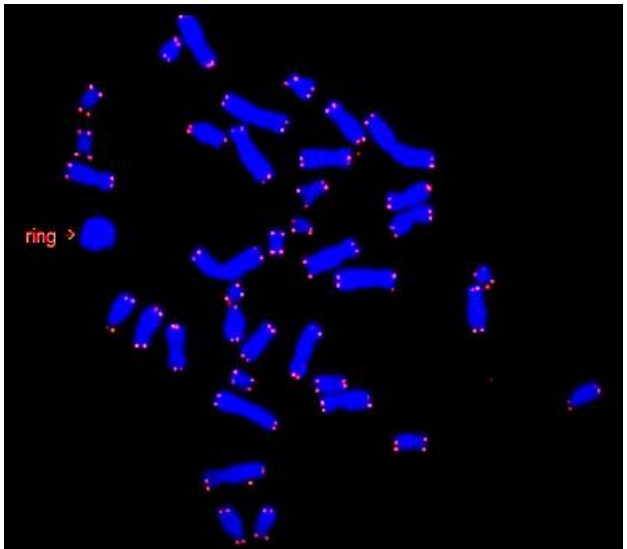
In the Institute of Human Genetics Jena (Germany)…

Hybridization with telomeric FISH probe

DNA denaturation (85-87˚C)

Overnight hybridization (37˚C) and analysis

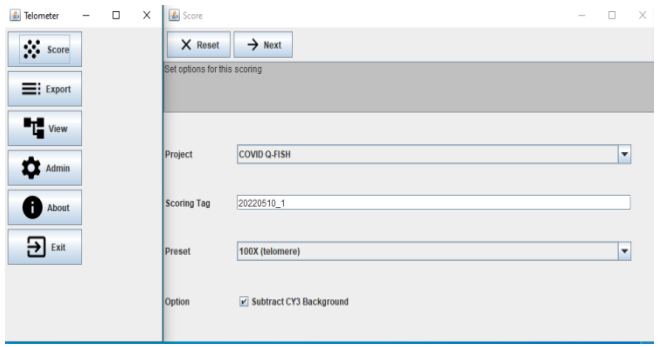




Telomeric Q-FISH image acquisition

Q-FISH was performed using Telometer plugin for ImageJ program

https://demarzolab.pathology.jhmi.edu/telometer/

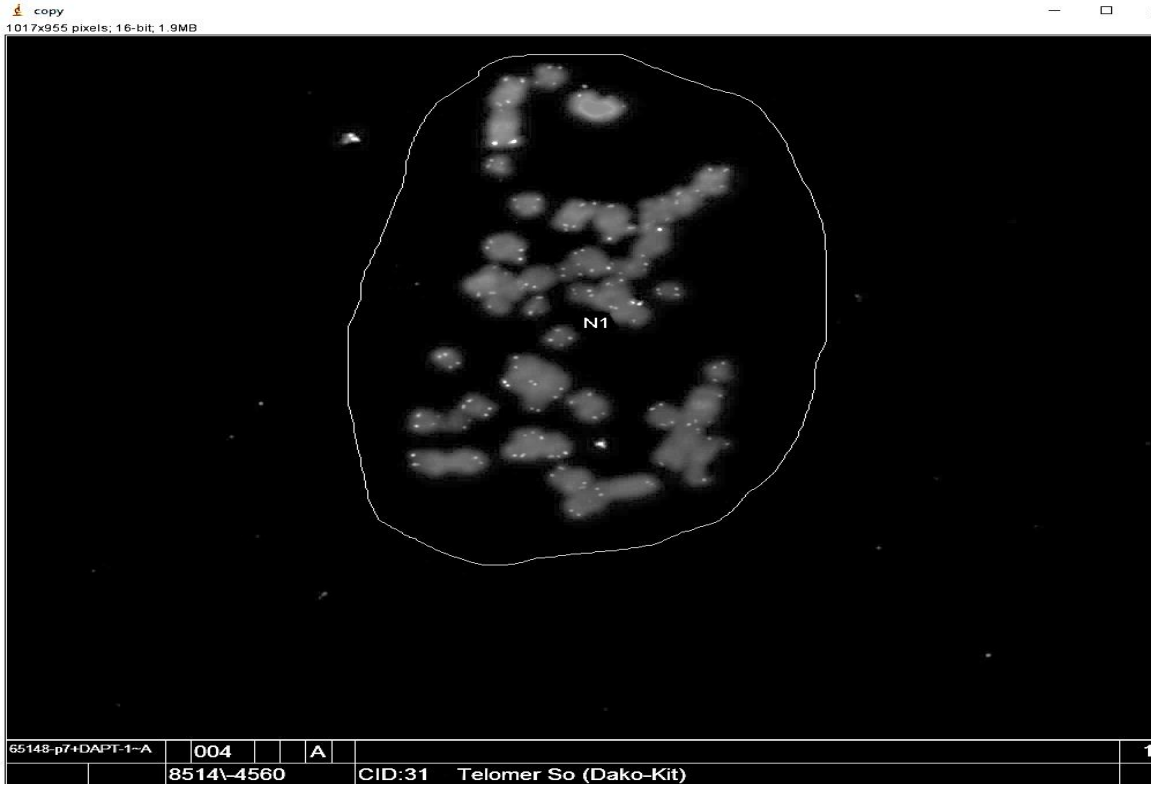


Projects | Scorings | Cells | Telomeres

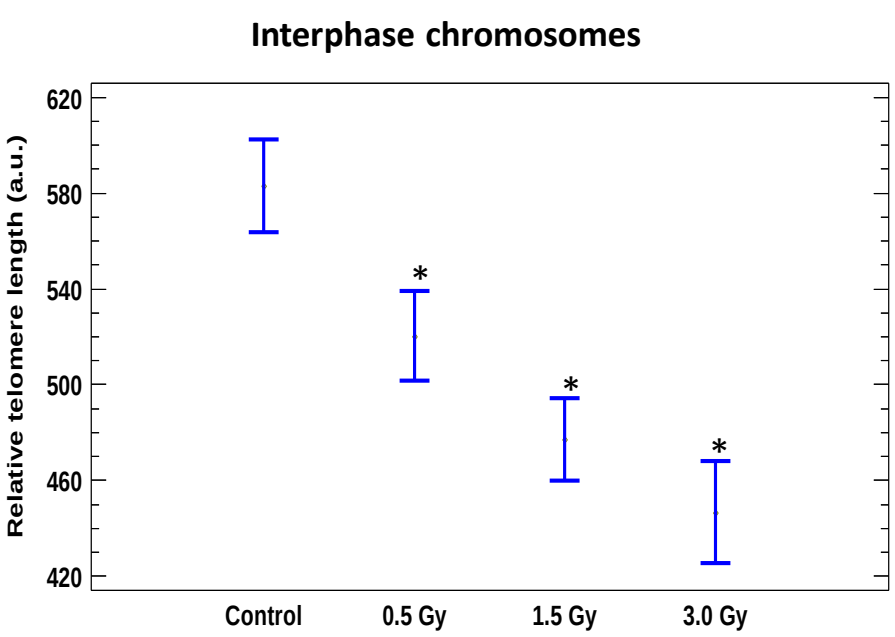
Cells | Export

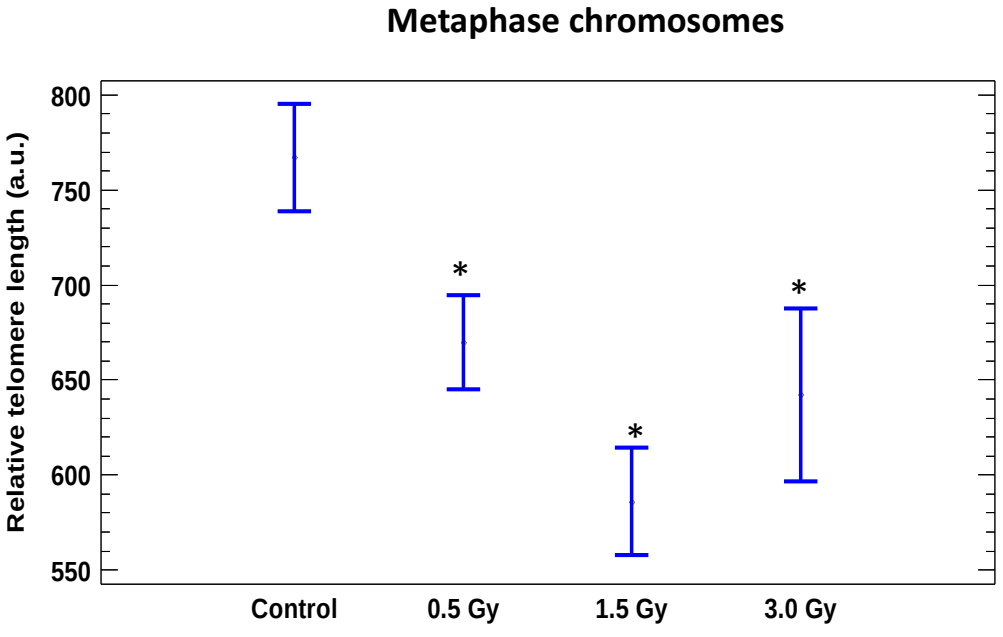
Telomeres:

TelomereId	TelemereNumber	CellId	CellName	X	Y	Sum	Area	Min	Max	StdDev	Mean
57975	1	1220	N1	495	609	184	10	12	27	5.641	18.4
57976	2	1220	N1	521	315	229	10	16	32	5.763	22.9
57977	3	1220	N1	609	170	349	16	13	37	8.432	21.813
57978	4	1220	N1	521	295	472	15	15	49	10.809	31.467
57979	5	1220	N1	420	158	227	10	13	34	7.009	22.7
57980	6	1220	N1	417	165	221	13	10	25	4.491	17.0
57981	7	1220	N1	453	352	392	16	13	39	8.132	24.5
57982	8	1220	N1	559	474	371	15	14	41	8.302	24.733
57983	9	1220	N1	488	384	227	10	13	32	6.584	22.7
57984	10	1220	N1	498	215	426	19	13	37	7.19	22.421
57985	11	1220	N1	484	243	676	29	13	40	7.217	23.31
57986	12	1220	N1	432	442	1409	38	11	71	16.323	37.079
57987	13	1220	N1	480	549	212	12	14	21	2.774	17.667
57988	14	1220	N1	354	545	506	20	12	42	8.779	25.3
57989	15	1220	N1	471	534	300	14	15	29	4.686	21.429
57990	16	1220	N1	355	496	433	14	14	50	11.125	30.929
57991	17	1220	N1	506	298	241	11	13	33	6.107	21.909
57992	18	1220	N1	471	387	312	12	15	40	7.886	26.0
57993	19	1220	N1	500	64	432	17	12	44	10.0	25.412
57994	20	1220	N1	509	485	263	13	12	30	6.379	20.231
57995	21	1220	N1	375	408	232	10	14	33	6.052	23.2
57996	22	1220	N1	434	83	376	15	15	37	6.912	25.067
57997	23	1220	N1	395	471	429	15	17	42	7.89	28.6
57998	24	1220	N1	496	75	306	13	13	40	8.866	23.538
57999	25	1220	N1	432	55	364	14	15	38	7.211	26.0
58000	26	1220	N1	406	536	265	12	13	37	8.522	22.083
58001	27	1220	N1	440	533	383	14	15	44	9.27	27.357
58002	28	1220	N1	541	244	467	21	14	35	6.008	22.238



Relative telomere lengths of human interphase chromosomes in control and after irradiation with AREAL accelerator





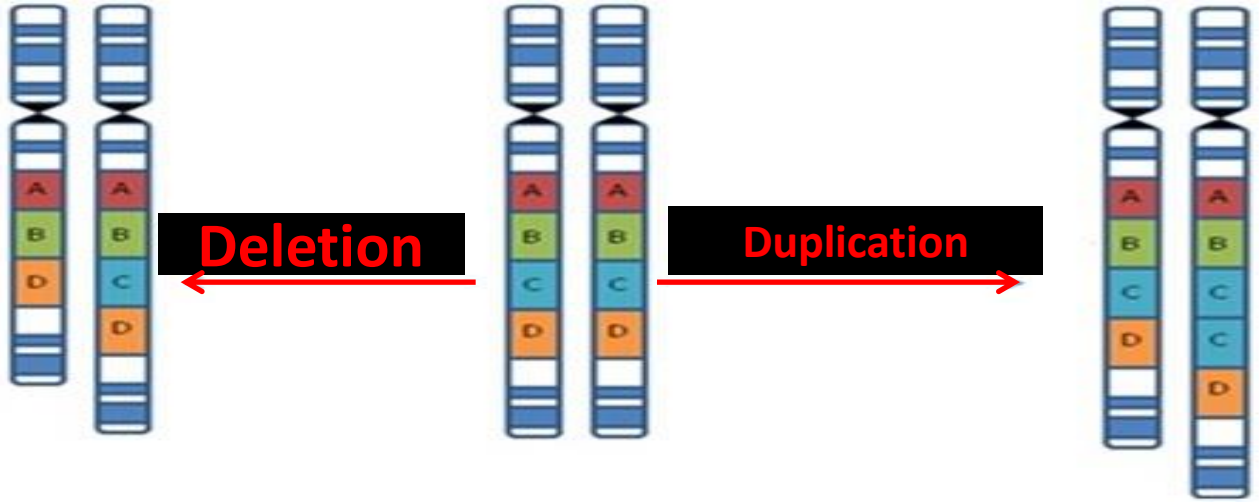
-The relative telomere lengths were quantified on a per-cell basis using the open-source plugin Telometer program after converting images to grayscale.

-It is revealed significant shortening of telomeres in all irradiation variants compared to control.

Interphase chromosomes are more suitable for the analysis of the dose-effect on telomere length and can be used as markers of genotoxicity of radiation exposure.

-Nevertheless, the **resolution of the relative telomere length is higher in metaphase chromosomes** which can be informative in combination with molecular-genetic methods for the mechanistic analysis of telomere length dynamics.

Copy number variation - CNV



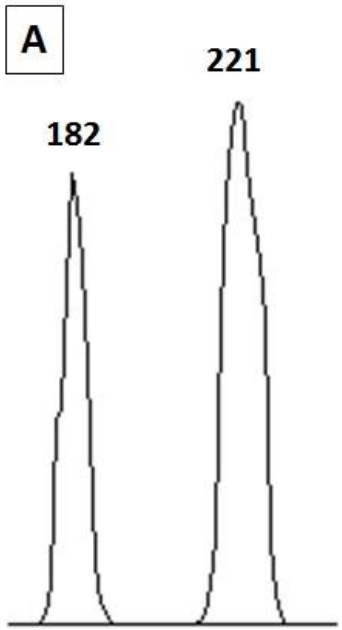
CNVs are sequences of DNA of 50 bp or larger compared with its reference genome, 9.5% of the genome contributes to spontaneous CNVs.

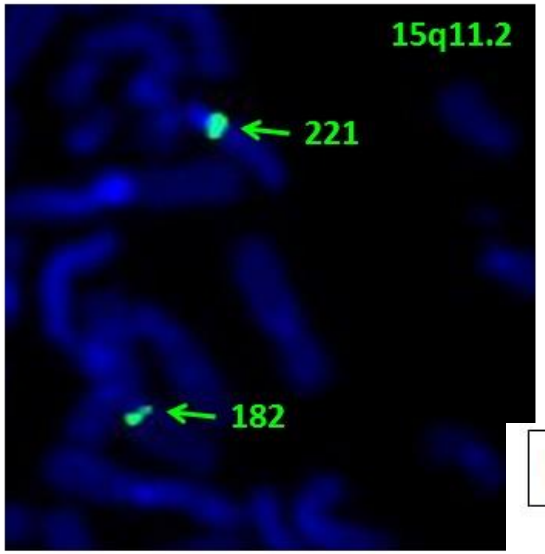
Copy number variation is now recognized as one of the major sources of genetic variation. Despite their importance little is known about environmental risk factors affecting CNVs.

CNVs are mutagenic! (duplicated sequences can predispose to new mutation events, like inversions, deletions and duplications)

De novo CNVs can arise after influence of replication inhibitors (aphidicolin, hydroxyurea, ionizing radiation), these findings have been confirmed by our group. We studied induced CNVs with application of chemical and physical mutagens.

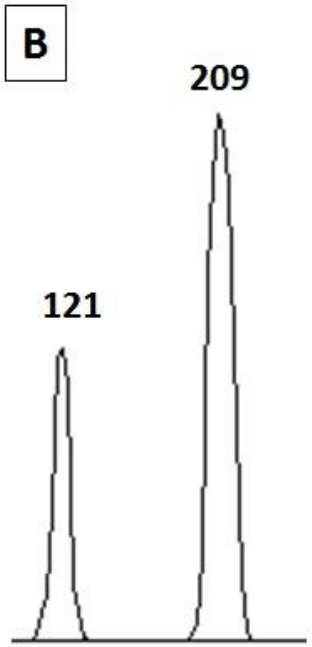
Analysis of fluorescence intensities using Scion Image - different sizes of probes signals

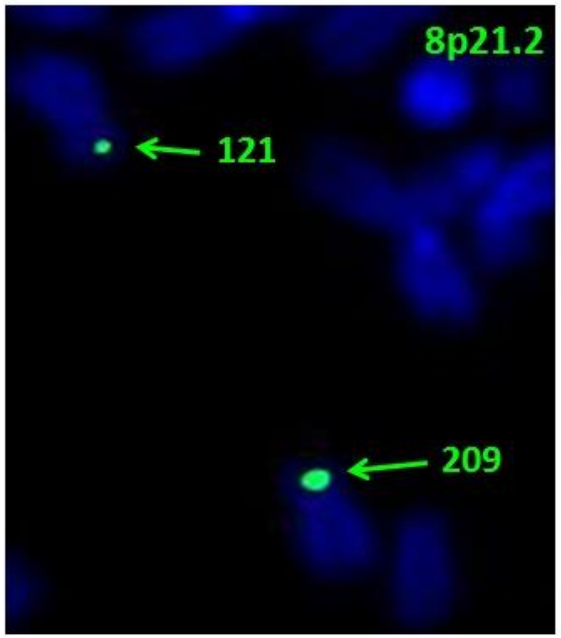




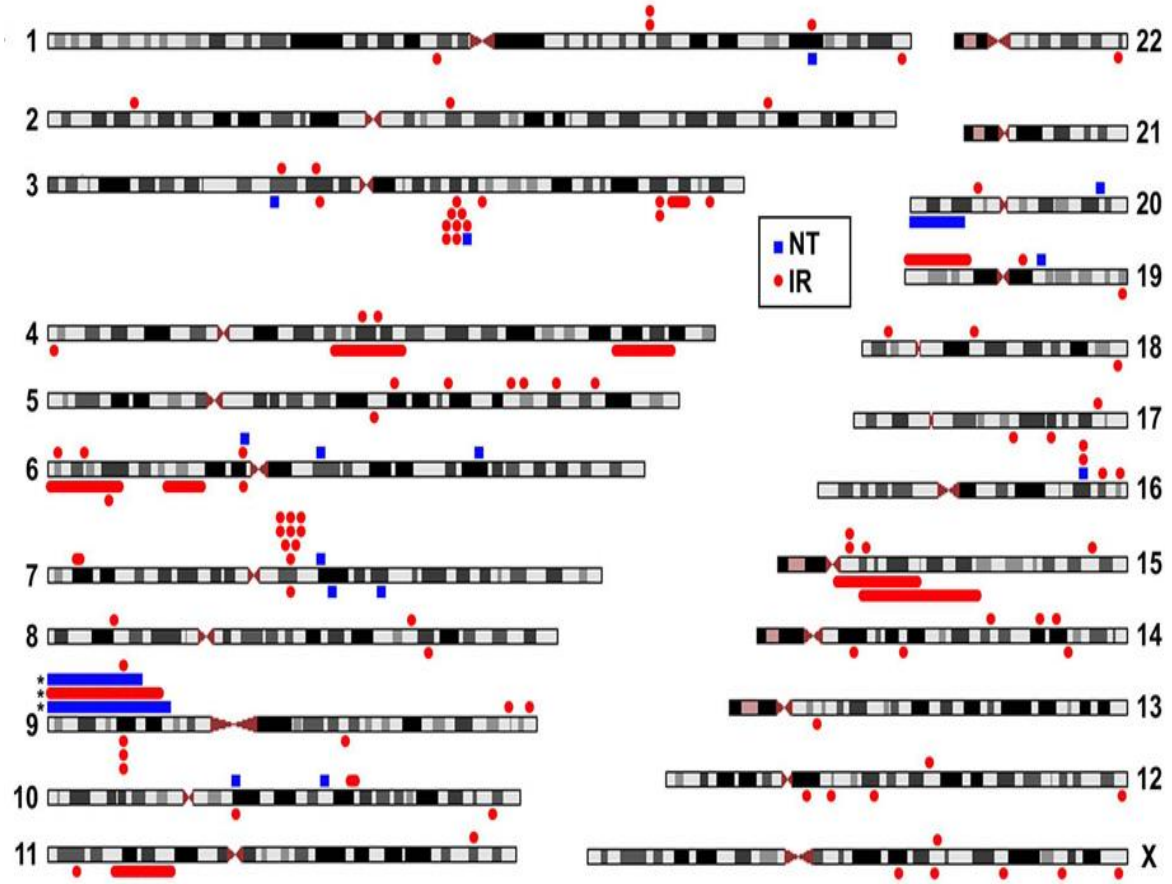
Fluorescence intensities of CNVs on homologous chromosomes 15q11.2

Fluorescence intensities of CNVs on homologous chromosomes 8p21.2

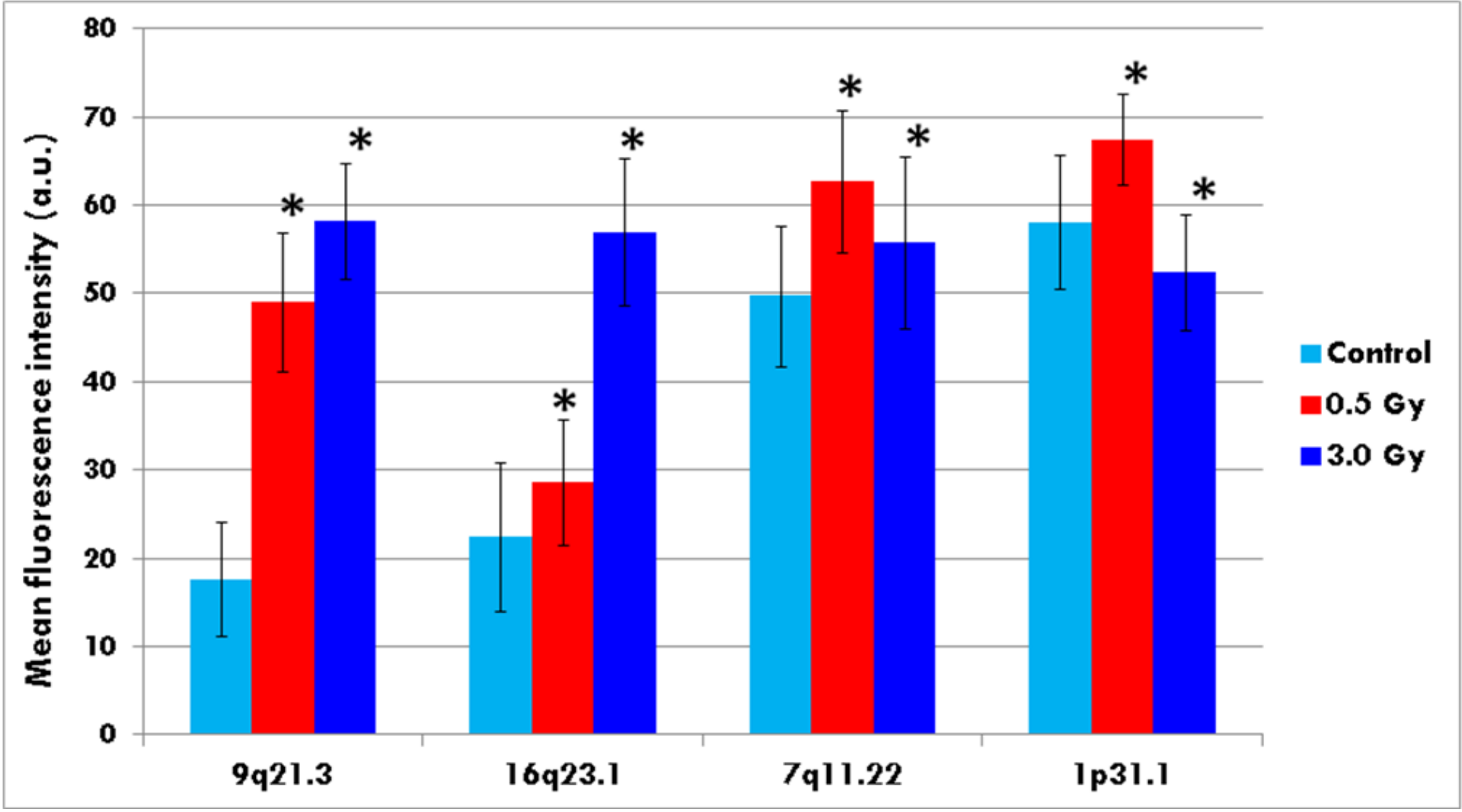




Ionizing radiation (1.5-3 Gy) induces copy number variations (CNVs) of DNA sites.



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. Irradiation by Philips RT250 (Kimtron Medical)



- **Mean fluorescence intensities of BAC signals at CNV loci** measured by ImageJ program.
- **Irradiation with 3 MeV, 2 Gy/min, 2 Hz**
- **Ultrashort electron pulses at a dose of 0.5 Gy induced duplications at 9q21.3, 16q23.1, 7q11.22 and 1p31.1.**
- **At the dose of 3 Gy irradiation induced duplications at 9q21.3, 16q23.1 and 7q11.22, and deletion at 1p31.1.**
- ***-p<0.05 compared to control.**

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), http://dx.doi.org/10.1016/j. nima.2016.02.028

Harutyunyan, T., Hovhannisyan, G., Sargsyan, A. et al. Analysis of copy number variations induced by ultrashort electron beam radiation in human leukocytes in vitro. Mol Cytogenet 12, 18 (2019).

Babayan, N.; Vorobyeva, N.; Grigoryan, B.; Grekhova, A.; Pustovalova, M.; Rodneva, S.; Fedotov, Y.; Tsakanova, G.; Aroutiounian, R.; Osipov, A. Low Repair Capacity of DNA Double-Strand Breaks Induced by Laser-Driven Ultrashort Electron Beams in Cancer Cells. International Journal of Molecular Sciences, 2020, 21, N 24 (9488), 1-10.

Babayan NS, Guryev DV, Vorobyeva NY, Grigoryan BA, Tadevosyan GL, Apresyan LS, Chigasova AK, Yashkina EI, Rodneva SM, Tsishnatti AA, Fedotov YA, Sarkisyan NK, Manukyan AT, Aroutiounian RM, Osipov AN. Colony-Forming Ability and Residual Foci of DNA Repair Proteins in Human Lung Fibroblasts Irradiated with Subpicosecond Beams of Accelerated Electrons. Bull Exp Biol Med. 172, 2021, pp. 22–25.

Conclusions

1. AREAL (Ultrashort Pulsed Electron Beam) **irradiation-induced DNA damages are characterized by slow DSB repair rate**

The pulse duration of ultrashort irradiation is only 0.4×10^{-12} s. So a huge peak dose-rate of 1.6×10^{10} Gy/s per pulse is achieved during the pulse. Apparently, it increases the possibility of complex difficulty repairable DSBs formation.

2. The radiosensitivity of cells towards AREAL irradiation is p53-dependent.

P53-deficient cells are more sensitive to AREAL irradiation compared to cells with wild type p53.

p53 is the tumor suppression protein whose function is most frequently lost in human cancer cells. So, this advantage of AREAl radiation can be used in the future to provide cancer cell specificity during irradiation, which will lower side effects associated with the damages occurred in normal cells.

3. AREAL irradiation induces apoptotic cell death

Apoptosis is a regulated cell death mediated by several signaling pathways triggered by multiple factors, such as cellular stress, DNA damage etc. Unlike necrosis, apoptosis is less immunogenic or inflammatory process. For over three decades targeting the apoptosis was a mainstay and goal of clinical oncology.

4. AREAL irradiation has low genotoxic capacity

Radiation-induced cytogenetic abnormalities, such as micronucleus formation, represent an early marker of possible delayed effects. These delayed effects during irradiation of the organism may lead to secondary cancers. A much lower level of micronuclei frequency in case of UPEB irradiation was shown in our study, compared to X-ray radiation. So, it can be assumed, that AREAL UPEB radiation has low potential to induce secondary cancers during irradiation of the whole organism.

5. According to in vitro studies AREAL irradiation is more effective compared to X-ray and quasi-continuous electron beam (Varian) irradiations used in clinical practice

6. Irradiation of human normal blood cells by AREAL can induce telomere shortening in a dose-dependent manner.

7. AREAL irradiation can induce molecular-cytogenetically visible CNVs in human blood leukocytes in vitro. These CNVs tend to inversely correlate with chromosome size and gene density. CNVs can last in cell population as stable chromosomal changes for several days. and can be used for characterization of genetic effects of accelerated electrons.

Acknowledgements

The work was supported through grants from the MES-BMBF, RSF (N 19-14-00151) and Science Committee of the RA (#21AG-1F068).

Best Wishes!