JOINT INSTITUTE FOR NUCLEAR RESEARCH
Veksler and Baldin laboratory of High Energy Physics

FINAL REPORT ON THE SUMMER STUDENT PROGRAM

Possibility of Study of Cellular Response to Light-Ion Beams of LHEP Nuclotron

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Abstract

Currently a standard way to investigate an individual response of a single cell to light ion irradiation is usually linked to the use of micrometric size beams i.e. the microbeams. This work proposes an alternative approach suggesting to use a wide light or medium size ion primary or secondary beams from an accelerator with silicon Double-Sided Strip Detector (DSSD) acting a role of position sensitive electronic analogue of the Petri dish housing the cells under investigation. The final goal of the research with proposed setup could be a study of kinetics of repair of double-strand breaks by measuring the SOID parameter. The advantages of this approach are the possibility of full automation of the experiment, low costs and large range of Linear Energy Transfer (LET) available.

Brief report below is twofold. Its first part is devoted to the biological aspects of the project describing a method for cell samples preparation while the second, instrumental, part presents results of the modeling experiment with realistic DSSD and a thin Mylar film with micrometric holes simulating the cells to demonstrate the feasibility of the proposed approach.

Introduction

At the end of the 20th century, radiobiologists began to carry out experiments on the effects of ionizing radiation (IR) to the individual cells, – cell monolayers attached to a surface of a dish or cell suspensions depending on the type of cells.

A study of an individual cellular response to an interaction with ionizing particle is still an actual task of today. Usually such experiments are performed with the help of micrometric size beams obtained by magnetic focusing or collimators to focus ions on a cell – the “microbeams” [1, 2]. Unfortunately, this approach is too complicated and costly for the most of biological laboratories since it requires an accelerator with special equipment and considerable expenditures on its use.

It is proposed hereby to use a different approach i.e. instead of focused beams to use primary or secondary wide beams of light ions together with silicon Double-Sided Strip Detector (DSSD) for accurate on-line measurement of an ion hit coordinates at the surface of the DSSD containing the cells. Positions of the randomly spread over the surface of the dish cells are optically measured prior to irradiation. During irradiation cells are randomly hit by the ions. The fact of the impact is identified by the event-by-event comparison of the optically and electronically gained coordinate data. Once the coordinates coincide the cell is marked as the hit one and the corresponding cell coordinates are stored in the look-up table used for the biological analysis to follow. The advantages of this approach are accessibility, opportunity for full automation and a large range of LET available.

Since the determining factor for further life of a cell is the occurrence of the double-strand breaks (DSbs) in DNA it is suggested to observe just this type of damage. Method for visualization of the DSb associated with the fluorescence of the DSb by the y-H2AX biomarker is to be used to investigate the DSb repair kinetics. Number of the DSbs and/or the SOID (Sum Of Integrated Density) parameter for the nuclei will be exploited for quantitative estimation of the LET dependence of the biological destiny of a cell nuclei after an ion hit. Hopefully, it would be possible to collect also experimental data on such puzzling phenomena as a bystander effect [3].

DNA damage induced by ionizing radiation

Damage Classification

Radiation which can cause ionization of atoms and molecules in an irradiated substance is called ionizing (IR). Because of the interaction between IR and a substance, atoms and molecules of the substance get enough energy to lose their electrons. 33 eV of energy is released in each act of ionization considerably exceeds the energy of any bond between atoms in a molecule therefore chemical links between molecules can be sometimes broken. When IR penetrates a cell (here and below – mammalian cell) all structures of the cell can be affected. The probability of interaction is determined by the size of a structure, – the smaller the structure the lower the probability of the damage. Due to this fact such a large molecule as DNA is often happens to be the main target for IR in a cell. It is DNA damage which is the main threat to the life of a cell.

DNA damage induced by IR can be divided into 2 groups. The first one accumulates all possible single damages caused by a single act of energy transfer from an ion to a molecule of a cell, such as single strand breaks (SSb), depurinations, depyrimidinations, double strand breaks (DSb), etc. The second group of damages contains all local multiple damages caused by several acts of ionization – two or more SSb and/or DSb which are located close to each other. The main types of damage are schematically shown in the Fig. 1.

It should be noted also that when primary structure of DNA is damaged the secondary one is also damaged since its hydrogen bonds are broken as well.

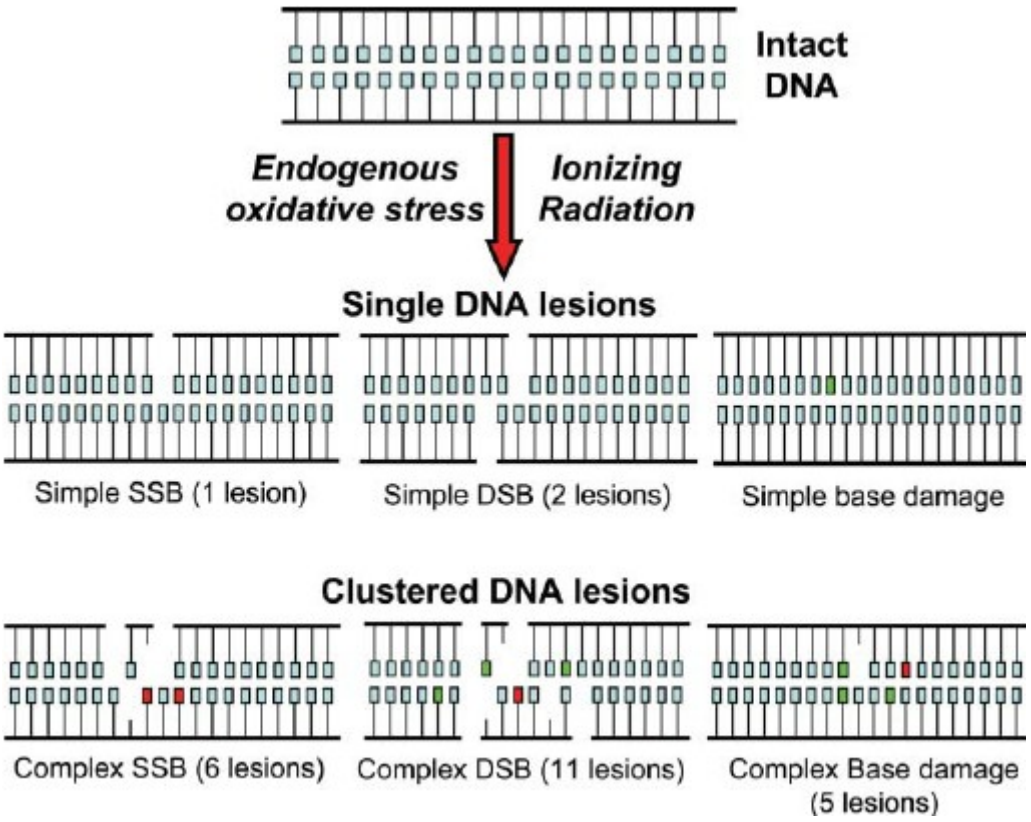

Fig.1. Examples of the DNA lesions induced by ionizing radiation [4]

The role of DSb in response of cells to IR

Absorbed 1 Gy dose induces about 1000 SSb and about 25-40 DSb in a diploid cell [5]. Although DSbs occur in DNA rather seldom namely they are to determine the fate of the cell. Improperly repaired or unrepaired DSb can lead to cell death, genome instability, carcinogenesis [6]. Cluster DNA damage, induced by heavy charged particles, makes it even more difficult to repair DSb of DNA. Cellular response to DSb of the DNA in a cell can result in cell cycle arrest, activation of repair pathways and initiation of apoptosis.

On the opposite, it turns out that a cell effectively repairs SSbs that only because of the endogenous processes (such as overload of free radicals, ions, etc.) arise at a daily rate of about 50000 breaks per cell [7].

Visualization of DNA DSb in a cell

The occurrence of DSb is always accompanied by the phosphorylation of the histone protein H2AX. H2AX is one of the variants of the H2A-family of proteins involved in the structure of chromatin. When a DSb occurs, this protein is phosphorylated by ATM kinase in serine-139, resulting in a modification of H2AX to y-H2AX. This modification attaches to the site of the DSb and starts the cascade of repair processes. Phosphorylation occurs quickly, right after the irradiation, with a constant number or a percentage of y-H2AX being formed at single DSb [9]. Thus, this protein can be used as a biomarker of a serious DNA damage. A possible way to visualize the DSb exploits yH2AX-specific antibodies as markers with fluorescent proteins attached to them.

The method essentials

The general scheme of the experiment

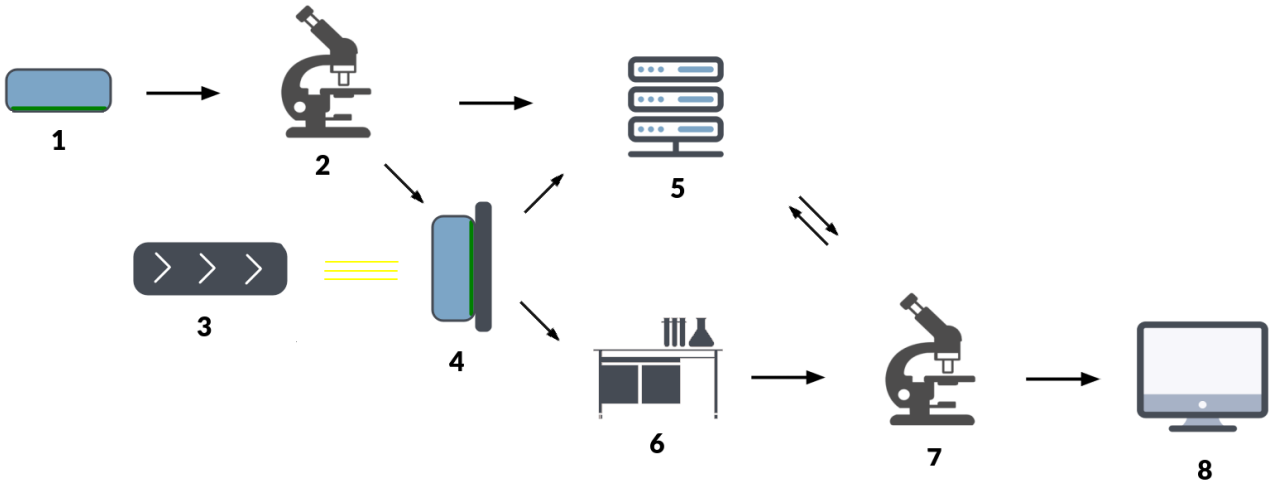

Figure 2. The bird-eye view of the layout of the experiment. Notation: 1 – a dish with a cell monolayer; 2 – a microscope for initial measurements of cell nuclei coordinates; 3 – accelerator beam line; 4 – cell sample fixed to the DSSD; 5 – server for the DSSD data taking and storing of coordinates of hit cells; 6 – biological laboratory; 7 – fluorescent microscope for analysis of the impact results on the cell nuclei, 8 – a computer for control of the position guide and analysis of the images from the fluorescent microscope

A monolayer of cells is located onto cell-friendly thin plastic slide with four fiducials defining the “dish” coordinate system. Method of obtaining cells with required concentration in a dish with the dish slide on the bottom is described below. On the day of the experiment, coordinates of the centers and radii of the nuclei of all cells in the monolayer are measured relative to the “dish” fiducials with the help of the microscope. The results are stored in a “dish” look-up table at a server which will later use them for the on-line triggering on the occurrence of the ion hitting a cell. The slide covered with thin protective layer to keep the cells alive is attached in a fixed position to a DSSD having the “detector” fiducials of its own. The “dish” fiducials positions are measured relative to the DSSD fiducials to establish translation between the two coordinate systems.

The sample and DSSD assembly is placed in a primary or secondary wide beam from an accelerator, for example, – products of peripheral fragmentation of accelerated gold nuclei on a thin target deflected in magnetic field differently due to quite different values of Z/A of primary gold ion beam, gold, and its secondary beam of light fragments predominantly produced with Z/A=0,5. A mixture of these light ions bombards a stack of a sample with a cell monolayer and the DSSD. The readout system of the DSSD analyzes the amplitude of the signal from the DSSD which in case considered is proportional to $\sim Z^2$ and registers a cell nuclei hit in event-by-event mode by comparison of the DSSD measured coordinates of the ion crossing the assembly with that recorded for the cells prior to the irradiation. The detector hit coordinates are transferred to the coordinates in the sample system according to

$$x' = \frac{x - x0 + y - y0}{2\cos(\theta)}$$
$$y' = \frac{-x + x0 + y - y0}{2\sin(\theta)} \qquad (1)$$

where x, y are coordinates of the point in the “dish” coordinate system; x`, y` are coordinates of the point in a “detector” coordinate system, θ is the angle between coordinate vectors, x_0, y_0 is a position of a center of the “detector” coordinate system relative to the center of the “dish” coordinate system.

If an angle between coordinate vectors $\theta = 0^0$ (a new coordinate system is formed by the parallel translation of the old one) then the conversion rules simplify as follows

$$x' = x - x_0$$
$$y' = y - y_0 \qquad (2)$$

By translating coordinates of the hits into the coordinate system associated with the dish, a program determines whether the particle has hit a nucleus or not. If a hit occurs, then coordinates of the affected nuclei are transferred to a control program of a fluorescent microscope together with the LET released in the cell by the penetrating ion.

The irradiated sample is sent to biology laboratory where DSbs are visualized by adding to the cell culture antibodies to the phosphorylated histone yH2AX with fluorescent proteins attached to them. After that the fluorescent microscope connected to the server at regular intervals makes photos of IR induced foci of those nuclei that suffered interaction with the ions.

As a result received photos are processed by the program for each nuclei which interacted with a charge particle the amount of DSb / SOID is calculated. Eventually, the amount of the transferred energy to the cell needed to produce the measured DSb / SOID response is evaluated

Cell culture preparation

Human breast carcinoma cells – Cal51 were chosen as a material for research. The cell culture was stored in a freezer at temperature of -80^{0}C. To prepare the cells for the experiment, they were placed in a culture flask with a «Gibco DMEM/F-12» medium supplemented with 10% fetal bovine serum ("DIAM") and 1% antibiotics ("Sigma"). Cells were cultured in a thermostat at 37°C with a 5% CO_2 content. Medium in a flask was updated every 24 hours . A flask was changed twice a week. To do this a monolayer of cells was processed with 0.05% trypsin solution of resuspended cells in fresh medium and then transferred to a new culture flask. A cell concentration in the suspension was determined using a TC20 ("Bio-Rad"). A monolayer of Cal51 cell line is shown in Fig.3. The size of cell nucleus of this culture depends on a cell cycle phase and averages from 11 (stage G1) to 20 (stage G2) μm.

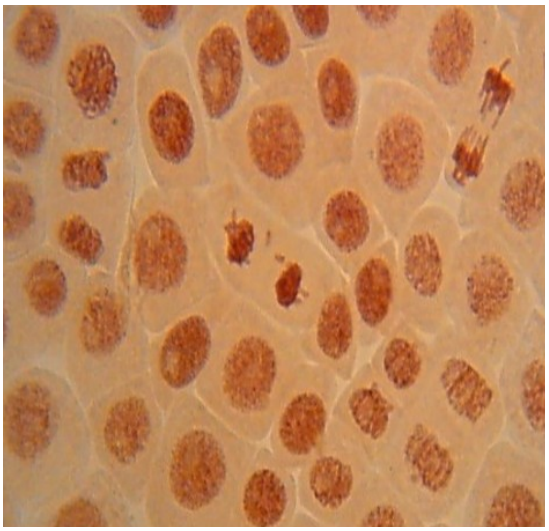

Fig. 3. Monolayer of Cal51 cells

Optimal cell concentration

To select a suitable cell concentration, several samples were prepared in Petri dishes with a diameter of 30 mm: 16, 32 and 80 thousand in 3 ml of medium. After 24 hours a monolayer was fixed with a methanol: acetic acid (3:1) on the bottom of the Petri dish for 20 minutes, dried and stained with 4% Giemsa solution in Sorensen buffer (1 / 15M KH2PO4 + 1 / 15MNa2HPO4) for 13 minutes. After that the solution was drained, the cells were dried and the dyed cells attached to the surface of Petri dish were examined under a microscope. It was found that concentration of 16*10^3 cells per Petri dish with a diameter of 3 cm is the most optimal one from the set of the above values. Obtained monolayers of different concentrations are shown in Fig. 5.

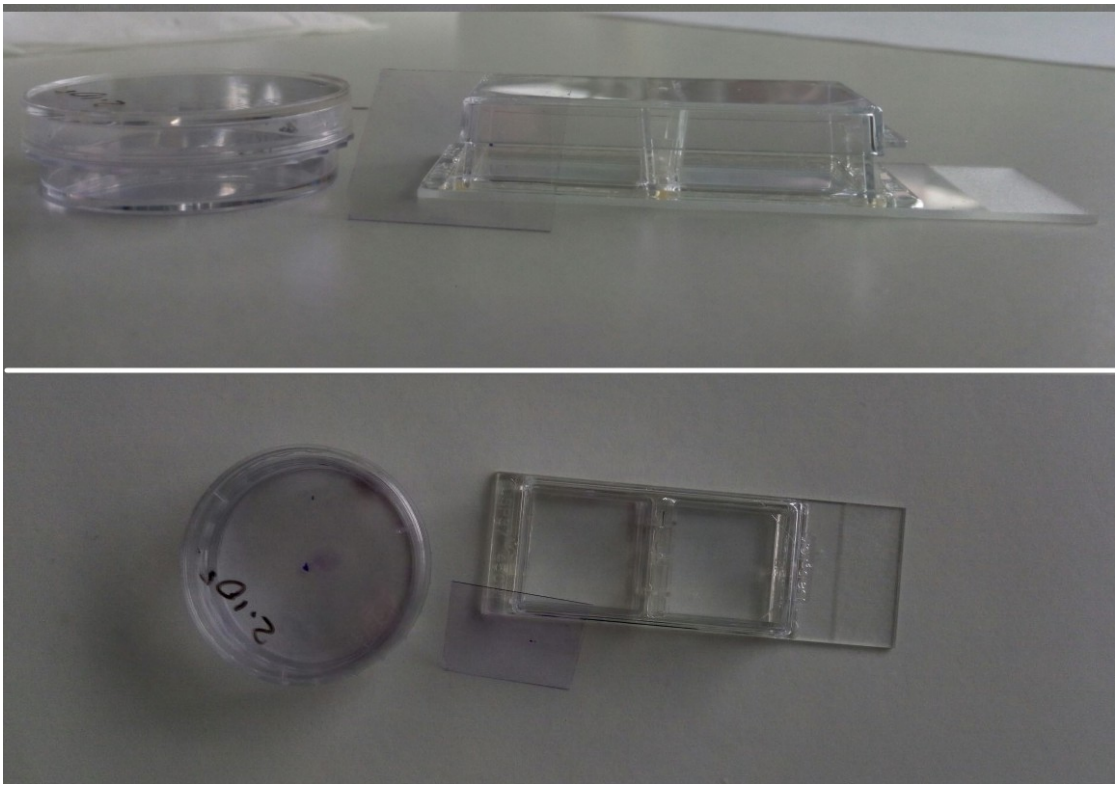

Fig. 4. Examples of containers that can be used to grow a monolayer of cells in them

One of the steps in preparations for the experiment is to determine coordinates of individual cells of a monolayer culture growing on the surface of a slide put on the bottom of the Petri dish. To increase accuracy of the measurements it is necessary to choose suitable concentration of cells in the monolayer. They should be uniformly distributed over the surface and have to be separated from each other by a sufficient distance for clear identification. For the experiment, for example, Petri dishes, slide glasses with chambers and Petri dishes with an inner cover glass can be used (Figure 4).

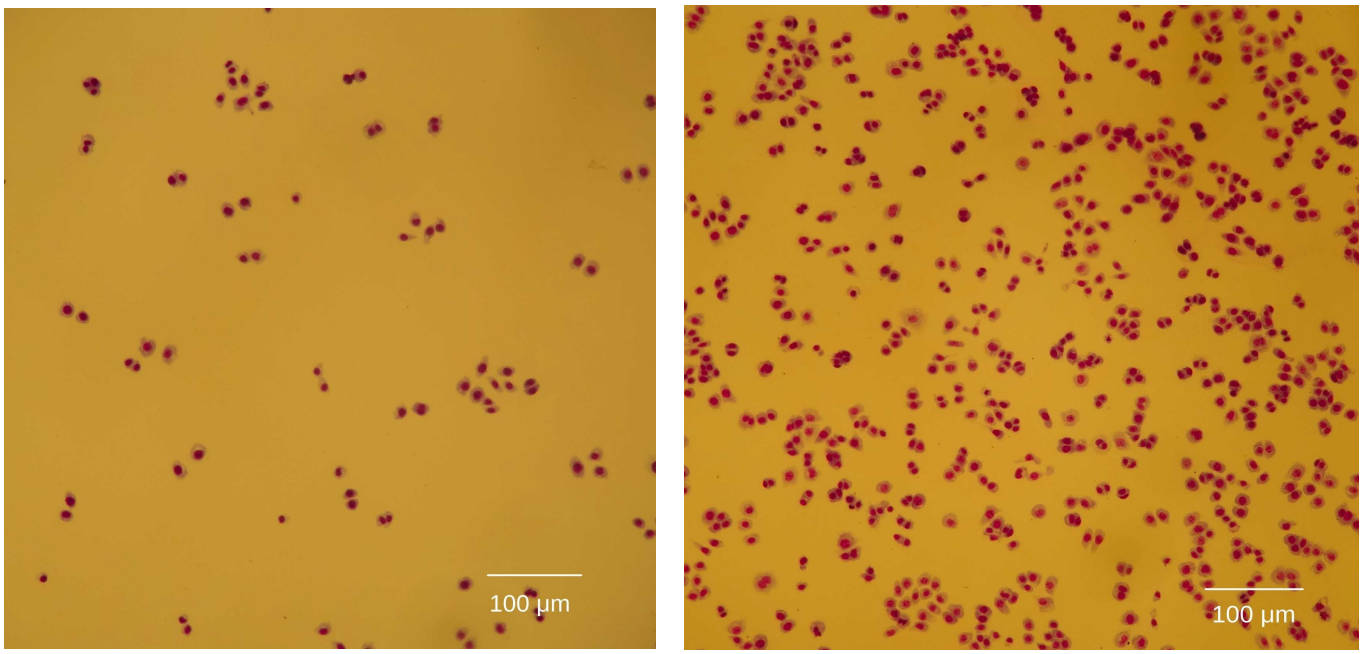

Fig. 5. Monolayers of the stained Cal51 cell population in Petri dishes with a diameter of 3 cm

The SOID parameter

As mentioned above, induction and repair of DSb phosphorylated y-H2AX histones can be visualized with fluorescently labeled antibodies attached to the DSb sites and analyzed with the help a fluorescence microscope. Fluorescent clusters are called y-H2AX foci. An example of visualization of y-H2AX foci is shown in Fig. 6.

In the case of IR with a low LET it can be assumed that one DSb corresponds to one foci [13]. However, in the case of IR with high LET the probability of formation of two closely spaced foci increases making it difficult to apply such traditional approach to the analysis of IR induced DSb as calculating the number of foci. To more accurate assess to DSb repair kinetics, it has been proposed to use the SOID parameter i.e. the sum of area of the total pixel numbers at each focus multiplied by mean fluorescence intensity per pixel of each focus. There are studies that prove that this parameter is more accurate for type of ion induced IR of our interest [15].

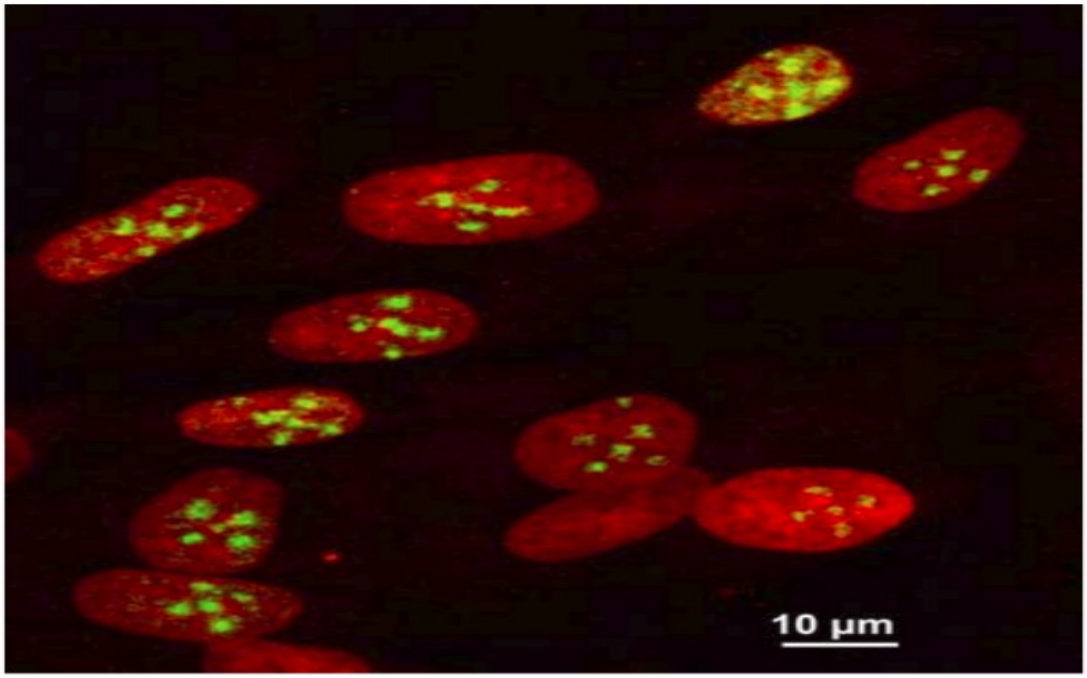

Fig. 6. Visualization of foci in human fibroblast nuclei in 30 minutes after irradiation (carbon ions, 4.8 MeV per nucleon) [12]

Secondary beams

Fragmentation of heavy projectile-nuclei in inelastic peripheral nucleus-nucleus collisions can serve as an efficient source of light and medium atomic number secondary beams which could be used for the biology studies [10, 11]. A secondary beam, consisting of products of the projectile fragmentation, from He to Fe nuclei, can be easily separated by magnetic optics from the primary beam. The event-by-event measurements of the amplitude of signal from DSSD can tell exactly the charge of the ion that penetrated the cell. Thus, a mixture of ions with different values of charge number and, accordingly, with different LET values makes it possible to study the dependence of the biological impact of the LET values on the cells in experiment with a mixture of light ions which could be organized as a “parasitic” experiment during the beam session of the Nuclotron.

Modeling of the experiment

Modeling of the experiment was carried out with a DSSD covered by an aluminized Mylar film in which the round holes of 100 μm radius were perforated to simulate a cell culture while Am^{241} 5.6 MeV alpha particle source was acting as an "ion accelerator". The film thickness was (50 ± 2) μm which was considerably more than the range of alpha particles in Mylar of about 35 μm (SRIM by James F. Ziegler) thus making the particles pass through the holes to mimick the cell hit.

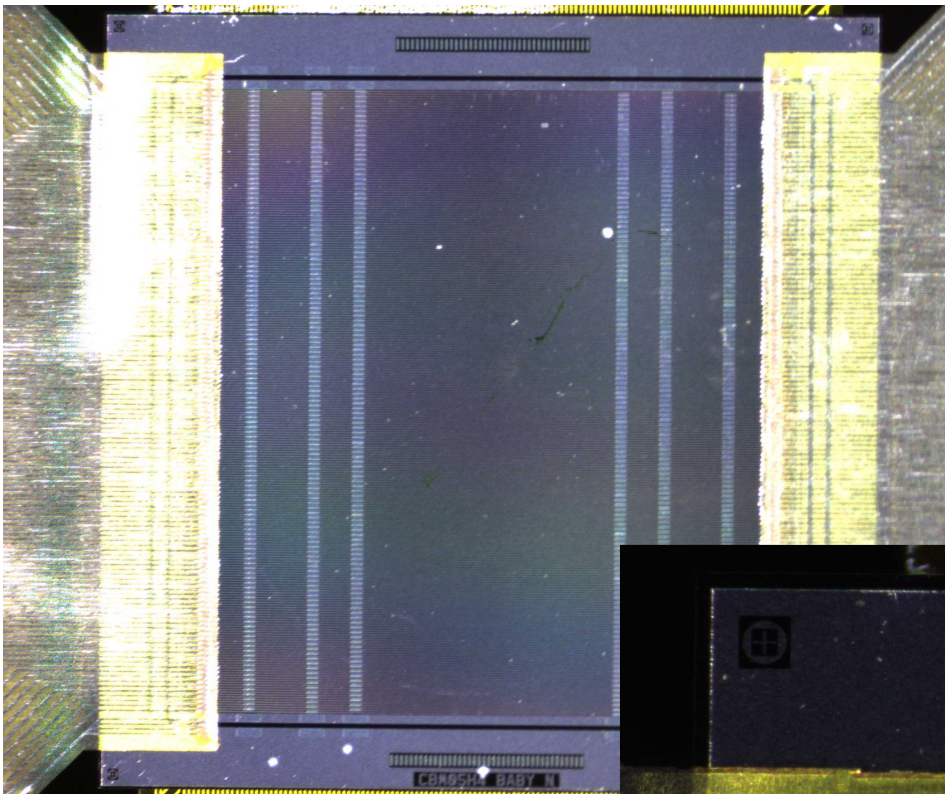

Fig. 7. Photo of the Double-Sided Strip Detector used in the modeling experiment and with its fiducials used for coordinate measurements (in the lower right corner)

A microstrip detector used was 1.6 x 1.6 cm^2 square, with four fiducials located in its corners (see Fig.7). 256 strips on each side of the DSSD had stereo angle of 90° and strip pitch of 50 μm resulting in X-Y spatial resolution of the DSSD of about 14 μm.

For simplicity the corners of the modeling foil were cut out making the detector fiducials and the holes visible to measure their position relative to the detector fiducials. At first, the coordinates of the centers of the holes were measured optically with the help of the microscope and after that with the help of the DSSD detecting the pass of the alpha particle through the holes. The results are compared below.

The film was fixed onto the DSSD with a polyimide tape (Fig.7). The alpha source plate was placed on top of the assembly, and data on alphas pass through the holes was accumulated within an hour. The collected data was analyzed using the ROOT software [https://root.cern.ch/]. A large re-scattering of alpha particles was observed because of an air gap between the film and the DSSD. As a result particles entering a hole at large angles passed some way through the gap and were detected at greater distance from a center of the hole measured optically (Fig.8a). If the air gap is reduced by pressing the film against DSSD, noticeable decrease of the above mentioned discrepancies can be observed (Fig. 8b).

It should be noted that energy release of the alpha particles was greater than dynamic range of the electronics used, so energy values of detected particles were not considered.

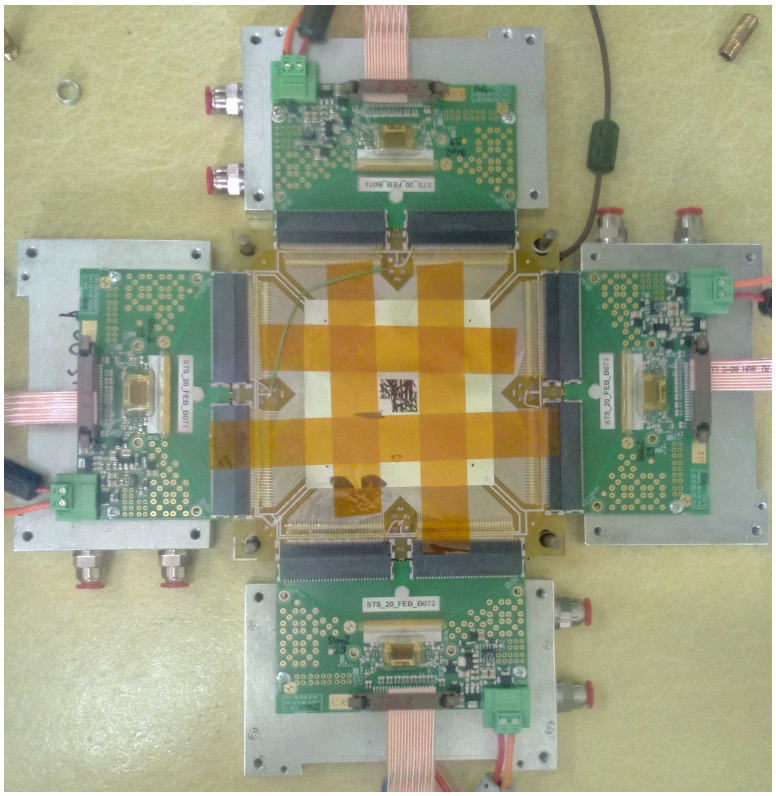

Fig. 8. Electronic setup used in the modeling experiment with a DSSD mounted in the center

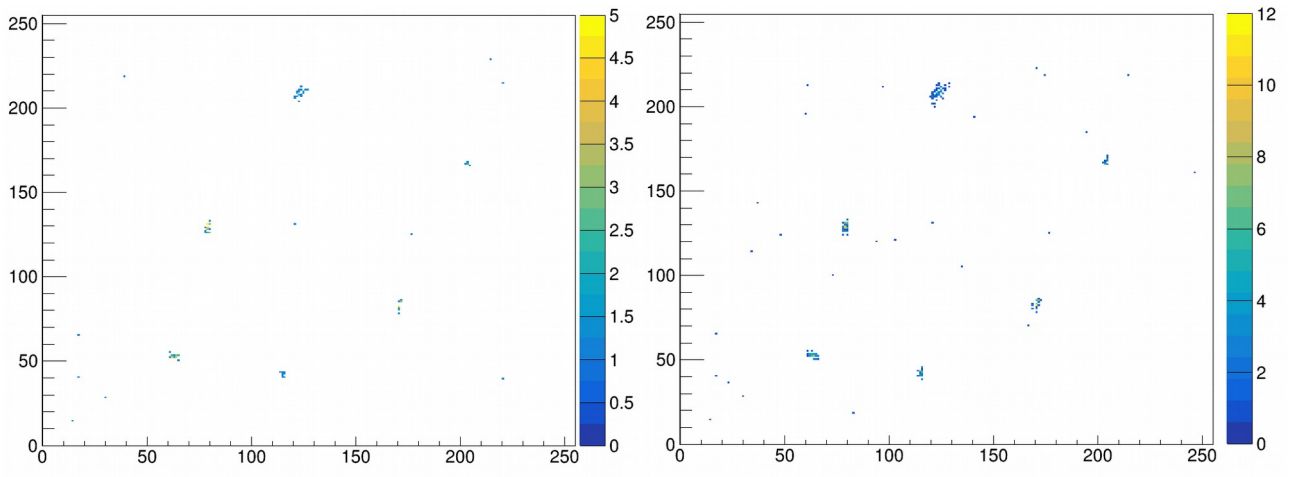

Fig. 9. Hits recorded by the DSSD: a - configuration with non-pressed Mylar film, b - the film was pressed to reduce air gap between the film and DSSD (see the text for details)

The number of hits per strip were stored in histograms separately for X and Y projections and then each fitted by a normal distribution to estimate the resolution with which the method could tell the coordinates of the hits, – the mean and standard deviation σ for each perforated hole in the film (Fig. 10).

Transform of the data from “detector” coordinate system of the DSSD to the “dish” coordinate system plays important role in the experiment. If the angle between the coordinate systems is zero degrees then only parallel translation of an origin of coordinates

takes place defined by Eq.2 above. The error resulting from such transformation is equal to the error in measuring positions of centers of the coordinate systems which are relative to each other. In this experiment, due to technical limitations of the equipment used, the error in optical measuring of the coordinates was around 3 μm. The strip topology of the DSSD produced by photolithography is known with much greater accuracy and it is possible to recalculate the obtained coordinates of the electronically measured coordinates relative to the DSSD fiducials in a straightforward manner. The accuracy, however, is defined by finite strip pitch i.e. 50/SQRT(12) microns. Fig.11 demonstrates the hit map of alpha particles scaled to the absolute measures of the DSSD coordinate system. Table 1 summarizes the results of the measurements with the estimates of mistakes the data was taken with.

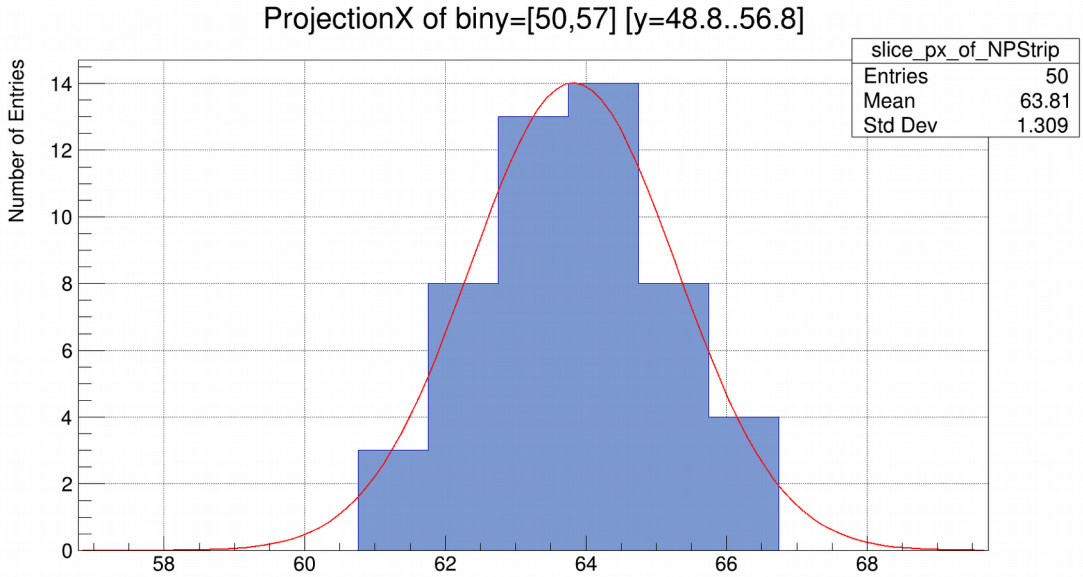

Fig. 10. Example of data processing. The resulting histogram shows the dependence of the number of the alpha particle hits on the strip number of the DSSD identifying a hole in the Mylar foil (X axis).

The maximum difference of expectated and measured centers of the holes was 72 μm, the minimum difference was 3 μm. Accuracy could be slightly improved by setting hit threshold and thus leaving only points at which the particles hit most often. A relatively large error in hit coordinates measurements is mostly due to a large size of the holes.

Coordinates of hole centers									
Visually measured		Mean of hit distribution		Mean error		σ of hit distribution		σ error	
X, ± 3 μm	y, ± 3 μm	x, μm	y, μm	x, μm	y, μm	x, μm	y, μm	x, μm	y, μm
7502	11732	7583	11668	6	9	100	124	6	9
5297	7760	5323	7757	1	5	40	100	1	5
4515	3963	4521	3990	4	1	71	17	4	4
7105	3451	7126	3404	1	7	29	80	3	8
9854	5486	9884	5428	6	8	42	98	4	7
11536	9676	11575	9658	3	12	33	74	6	11

Table 1. The results of the modeling experiment: The measured coordinates of the hole centers by the microscope are on the left . The rest are the parameters obtained after processing the distribution of the alpha particle hits from the DSSD data (see details in the text).

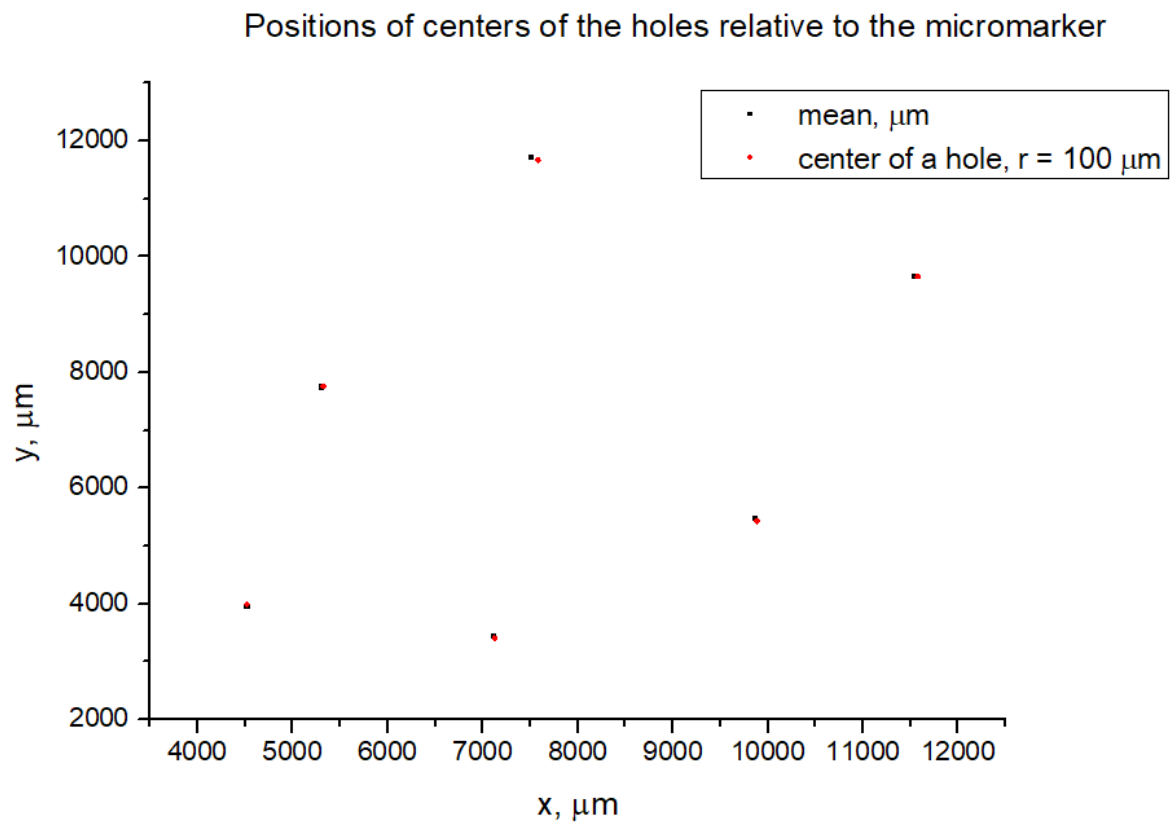

Fig. 11. Map of hits of alpha particles in a film with holes with coordinates in micrometers in DSSD coordinate system

Conclusions

Conclusions for *the biological part* of the project are as follows

1. There is a need to develop the method of visualizing IR foci as described in detail in [16].
2. For proper investigation of the kinetics of the DSb repair, only those cells that are in a desired phase of the cell cycle should be considered and those cells that are in other phases should be excluded.
3. The time between irradiation of the cells and examination of foci in a microscope should be reduced including the delivery time of the irradiated dish to the biological laboratory, antibody injection and installation of a sample at the fluorescence microscope. The best would be to examine foci in 15-30 minutes after the irradiation [16].

Instrumental R&D has to be undertaken deeper since the performed modeling experiment only pointed to those factors that need to be taken into account during the experiment.

First of all, it is necessary to minimize the errors that arise from the technical limitations of the existing equipment and measure the position of DSSD fiducials relative to the dish fiducials more accurately. This requires fiducial pattern recognition system. It is necessary to computerize the measurement of the position of a center and a radius of nucleus of each cell in a monolayer. For this it will be necessary to stain cells and use a cell pattern recognition software which needs to be custom developed for this particular approach.

Secondly, one needs to take care of the ion beam to penetrate the “dish-DSSD” stack perpendicular. In the model experiment, the Am^{241} plate was used which was located on a

film with holes. Thus, an alpha particle flux consisted of particles flying in a variety of directions leading to their large rescattering and, as a result, a large standard deviation of values of the holes centers measured optically and electronically.

Thirdly, due to the same reasons thin slides with cell culture have to be still developed with accurately made fiducials in order to minimize scattering of particles and, consequently, deviation from the original direction, as well as the energy loss. At the same time, it must be thick enough not to break during further transportation to a fluorescent microscope.

The DSSD used in the model experiment does not have the necessary resolution (about 14 μm) to determine a hit in a cell nuclei (10 μm in the G1 phase, 20 μm in the G2 phase). The best microstrip detector currently available in the house is a DSSD with 512 strips on each side and pitch 25 μm resulting in a spatial resolution of 7 μm. This would be a satisfactory value for the planned experiments to follow.

Finally, the energy transferred from charged particle to a cell nucleus has to be determined as accurately as possible. According to studies [12], linear energy transfer for C and Ar with an energy of 200-400 MeV/nucleon is between 16-100 KeV/μm. Energy is determined from the known particle velocity and charge number of the ion with mass of the most stable isotope used. Therefore, for a more accurate determination it is necessary to purify a secondary beam from admixture of other isotopes hitting the cells.

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