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IRRADIATION OF CELLS WITH TARGETED IONS USING OPTICAL AUTOMATIC RECOGNITION

Dissertation for the Doctor of Philosophy Degree in Experimental Physics

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CONCLUSIONS
ABBREVIATIONS

CMB Cracow Microbeam
DIC Differential Interference Contrast
DMEM Dubelco Minimum Essential Medium
DNA Deoxyribonucleic Acid
DSB Double Strand Break
FBS Fetal Bovine Serum
FLED Fuzzy Logic Edge Detection
GHT Generalized Hough Transform
GUI Graphical User Interface
HMC Hoffman Modulation Contrast
HT Hough Transform
LED Light Emitting Diode
LET Linear Energy Transfer
PBS Phosphate Buffered Saline
PIXE Proton Induced X-ray Emission
QPI Quantitative Phase Imaging
QPm Quantitative Phase microscopy
RAM Random Access Memory
RBS Rutherford Back Scattering
RCD Random Circle Detection
RHT Random Hough Transform
SIHF Single Ion Hit Facility
STIM Scanning Transmitted Ion Microscopy
BASIS OF THE THESIS

The thesis has been based on the original material that has been presented in the following publications:


Some results of the thesis have been also published as the Reports of Henryk Niewodniczański Institute of Nuclear Physics PAN:


The results of the thesis have been also presented at the international scientific conferences and consequently in the abstract books of the conferences:


INTRODUCTION

Topicality of the work. The collaboration of nuclear physicists with biologists and medicines has led to the development of such fields of science as radiobiology and radiomedicine. Each year brings a new knowledge related to current questions how the ionising radiation influence the living matter, how to protect the living organisms from the harmful effects of radiation and even how to use different types of ionising radiation in disease treatment and healthcare. However, to answer these questions the assistance of nuclear physics is necessary now as before.

Microbeams of ionizing radiation are one of the powerful tools proposed recently by nuclear physicists to radiobiology. They enable a delivery of small dose of radiation to the biological targets. Moreover, on the base of microbeam setup, the so-called Single Ion Hit Facilities (SIHF) can be developed, providing very precise dose delivery and improved targeting accuracy (up to exactly one ion to the defined cell or even to subcellular structures). Thus, SIHF provides the opportunity to carry out the particular radiobiological investigations on the cellular and subcellular level which is not possible to realize using other techniques.

There are only a few working SIHF over the world (see Section 1.1). However, in order to provide the extensive studies of the radiobiological effects caused by different types of ionizing radiation, there is a need for the development and construction of even more SIHFs basing on different types of ions with different parameters.

The present work is dedicated to the development of the SIHF on the base of existing Cracow microbeam. A particular attention is paid to the development of the system for the automatized (computer controlled) cell irradiation process, which is very important for high performance SIHF functioning. It should be taken into account that for further application of SIHF for cell irradiation experiments, it would be beneficial to make a conversion from the number of ions to the units of dose adsorbed by the target. However, the concept of absorbed dose, originally invented for X-ray and gamma ray broad beam irradiations, is less adequate for targeted irradiation. Especially, for ion beam bombardment the deposited energy is well
localized in a small volume along an ion track in the biological object. In such case, taking the mass of the whole object (e.g. cell or nucleus) as input for dose calculation is doubtful. Therefore, in order to better characterize the process of irradiation, in the present work the proposition is made to apply Geant4 object-oriented toolkit for this aim (see Section 3.7). The work includes both the results of the preliminary tests carried out to verify functional performance of the SIHF and the application of the developed facility for the irradiation of living cells.

**Connection of the work with scientific programs, plans and topics.** The present work was performed in the confines of

i) the statutory activity of the Henryk Niewodniczański Institute of Nuclear Physics Polish Academy of Sciences (theme 5, task 7: “Investigations of biological, environmental and complex systems with the use of spectroscopic methods”);

ii) the 6th FP EU project No MRTN-CT-2003-503923 “Studies on cellular response to targeted single ions using nanotechnology” (CELLION, cellion.ifj.edu.pl)

iii) the 5th FP EU project No QLK4-CT-2002-02678 “Quality of Skin as a Barrier to ultra-fine Particles” (NANODERM, http://www.uni-leipzig.de/~nanoderm/)

**Objectives and main tasks of the investigation.**

The main objective of the present work is to develop the Single Ion Hit Facility for automatic cell irradiation on the base of the existing Cracow microbeam.

The first task is the development of the hardware. It is worth to notice, that the whole hardware construction has been developed due to large involvement of several scientists, while the subtasks assigned for the present PhD work are mainly the development of cell visualisation system, cell positioning system, blanking system, and modifications of data acquisition system.

The most important task of the work is the development of the automatic SIHF controlling system, which automatizes cell recognition, cell positioning, blanking process, and data acquisition. The system links together all parts of the hardware and thus makes SIHF a powerful tool for precise cell irradiation.
A determination of the main functional characteristics of the developed facility, such as the targeting precision and the precision of the dose control, is a next essential task.

The main prove and justification of the work is demonstration of the application of the developed SIHF for automatic irradiation of living cell.

Last, but not least task is to perform a conversion from the number of irradiating protons to the units of the dose adsorbed by the target, in order to express how the radiobiological effects observed in irradiated cells and subcellular structures depend on the dose.

Practical significance and scientific novelty of the obtained results.

The developed SIHF is the only facility of such type in Poland. It has been based on the existing Cracow microbeam (CMB) and enlarges the field of the CMB applications by allowing novel, unique measurements. Since the CMB has the shortest focusing system, existing up to now in the world, the presented SIHF is the shortest focused one. Thus, its development may be considered as the essential step on the pathway of the vertical focused SIHF development, where cells can be irradiated in more preferable, horizontal position.

The automatisation of the SIHF, which has been provided by the specially designed controlling system, allows carrying out different types of advanced radiobiological experiments, where individual cells (in cell population) can be targeted with precisely defined number of single ions. The very automatisation enables providing several experiments with the same parameters and, in that way, obtaining the statistically significant results. An important part of the control system – the software particularly designed for automatic cell recognition – is modifiable for different types of cell images and, moreover, can be used as independent software for various image processing tasks.

Designed protocols (algorithms) of the optical system and moving stage calibrations, beam position determination, beam profiling, cell irradiation, etc. may be applied separately or may constitute the compound protocol used for different types of radiobiological experiments in future.
For the first time in SIHF, the on-line visualization of the unstained living cells has been realized via introducing the illumination source inside the irradiation chamber of the SIHF which allows the cell observation in the transmitted light.

For the first time in SIHF, the constructive solution has been found to use the Quantitative Phase microscope for on-line visualization of cells, which improves significantly the quality of on-line cell visualization.

The simulation of the proton pathway through a biological cell, developed using the Geant4 toolkit, may be extended for further simulation of more complicated proton-cell interactions.

_The approbation of the results._ The results included in the thesis were presented at 9 scientific conferences and meetings:

- The 9th International Conference on Nuclear Microprobe Technology and Application, September 13-17, 2004, Cavtat, Dubrovnik, Croatia
- The 1st CELLION Meeting, February 24-30, 2005, Cracow, Poland
- The 14th IBC Annual Workshop and Training Course for Young Researchers, April 4-8, 2005, Guildford, United Kingdom
- The 17th International Conference on Ion Beam Analysis, June 26 - July 1, 2005
- Sevilla, Spain
- The 2nd CELLION Meeting, October 5-7, 2005, Leipzig, Germany
- The 14th Symposium on Microdosimetry, November 13-18, 2005, Venice, Italy
- The Mid-Term CELLION Meeting, February 3-5, 2006, Bordeaux, France
- The 7th International Workshop “Microbeam Probes of Cellular Radiation Response”, March 15-17, 2006, New York, USA
- The 10th International Conference on Nuclear Microprobe Technology and Application, July 9-15, 2006, Singapore
Publications. The results included in the thesis were published in 8 scientific papers, including 5 publications in scientific profile journals [1-5] and 3 scientific reports of IFJ PAN [9-11]. The materials of the thesis are also presented in 6 abstracts of scientific conferences [12-17], 2 papers accepted [6,7] and one paper sent [8] to be published in scientific profile journals.

Individual contribution. It is worth to notice, that the hardware modifications and improvements were made during a long period of time by a group of scientists of the VdG Accelerator and Nuclear Microprobe Laboratory of the Department of Applied Spectroscopy of the IFJ PAN. Therefore, a part of the hardware described in the chapter is a contribution of other people. In the case when the individual contribution due to the author of the thesis is insignificant in a certain hardware modification, only the brief description of this modification is presented and the reference is given to distinguish the main performer.

The author of the thesis tried to pay more attention to his main contributions in the Cracow SIHF development, such as visualisation, recognition and positioning of cells, certain details of data acquisition system modifications and blanking system performance, as well as automatisation of the whole irradiation experiment.

Publications with co-authors contain the following contribution of the author: in the papers [1, 3-7, 10-16] – design of the software, participation in hardware development, participation in the experimental work, data processing, interpretation and discussion of the results; in the papers [8, 17] – design of the software, participation in hardware development, participation in the experimental work and discussion of the results; in the paper [2] – work concerning cell visualisation, recognition and positioning; in the paper [9] – participation in the project discussion.

Structure of the thesis. The thesis is composed of the Introduction, five Chapters, Conclusions, and References. The total size of the thesis is 121 pages (including the main part of 99 pages). The thesis includes 71 figures and 3 tables. References (65 issues) take 6 pages.
1.1 Single Ion Hit Facility (SIHF)

The Single Ion Hit Facility (SIHF) enables bombarding (irradiation) of a studied target at an exactly chosen location by a defined number of single-ions. Term “single-ion” implies that the control of targeting ions is assured with a precision down to one particle. The SIHF is a powerful tool to investigate individual effects induced by single-ion bombardment in specific area of the sample which has micron-scale structures, for instance, biological cells or semiconductor devices.

One of the serious problems in reliability of space electronics is the ion induced charge current which causes serious malfunctioning of semiconductor chips because the injection of an ion to materials produces various radiation effects along the track, such as ionization, excitation, or damage. This effect is called a Single Event Effect. Moreover, it depends not only upon a kind of incident particles or their energy, but also upon the hit position on the semiconductor devices.

SIHF provides also a unique opportunity to control precisely the dose delivered to individual cells in vitro and the localization of the dose within the cell. This makes possible to study a number of important radiobiological processes in a way that cannot be achieved by other methods. For example, at the dose levels relevant to environmental exposure to naturally occurring radioactive radon gas, virtually no cell receives more than one charged-particle traversal in its lifetime. As the SIHF enables exact delivering of one particle (single-ion) to a cell and is therefore ideally suited to developing an in vitro experimental model for reproducing the levels of exposure that occur in vivo.

The first SIHF design and application has been reported in 1991 by the scientific group of Columbia University (NewYork, USA; Geard C.R. et al., 1991; Randers-Pehrson G. et al., 2001). This Radiological Research Accelerator Facility (RARAF) has been dedicated to providing radiation source for research in biology, radiation biology, and radiation physics. Later a number of scientific laboratories and centres engaged in the construction of SIHFs as JAERI (Takasaki, Japan; Kamiya T. et al.,
2004), Gray Cancer Institute (Northwood, England; Folkard M. et al., 2002; Folkard M. et al., 2003), Texas A&M (USA; Braby L.A., 1992), GSI (Darmstadt, Germany; Cholewa M. et al., 2003), CENBG (Bordeaux, France; Moretto Ph. et al., 2001), LIPSION (Leipzig, Germany; Reinert T. et al., 2004), SNAKE (Munich, Germany; Dollinger G. et al., 2005), PTB (Braunschweig, Germany; Greif K.D. et al., 2004), and MIT (Boston, USA; Wang R. and Coderre J.A., 2005) etc.

Generally, all these facilities can be classified by two main features: the beam orientation (horizontal, vertical) and the beam formation, i.e. the way to reduce beam spot size (collimation, focusing). Existing SIHFs present a wide spectrum of the fine technical solutions to accomplish the bombarding of the defined location by the exact number of ions. Schematic depiction of vertical and horizontal SIHFs are presented in Fig. 1.1 a and b, respectively.

Independently on the SIHF type (vertical or horizontal), the basic elements of the facility are an ion source (IS), an accelerator (A), an analyzing magnet (AM), a beam current reducing element (BRE), a beam shutter (BS), a beam formation element (BFE), a scanning system (SS), and an ion detector (ID). Various types of ion sources (radio frequency source, duo-plasmatron, ECR, PIG, Penning, Cs-ion sputter, etc.) of
different ions (p, d, α, and heavy ions) are used. In order to impart the certain energy (up to 20 MeV) to the ions, various types of accelerators (Van de Graaff, Singletron, Cyclotron, Tandem, and single-ended Pelletron) are applied. Since the particle beam produced by the ion sources is not homogeneous, the analysing magnets are applied next to obtain the monoenergetic beam of the same species. To decrease the beam spot size up to the size of a few micrometers or even less, the beam formation system is used – either focusing or collimating one. Focusing systems can be based on either electromagnetic quadrupole lenses set as doublets, triplets (Oxford), quadruplets (Russian) or superconductive multiple lenses. The collimator is usually based on a glass (silica, stainless steel) capillary with internal diameter about 1 µm. Ion detection together with beam shutter gives a possibility to stop beam (so called beam blanking) after detection of the defined number of ions passed to the detector, thus providing the mode of single ions (i.e. control the number of bombarding ions). To facilitate the beam blanking, the reduction of the beam current is essential and it is realised using the micrometer sized slit. Scanning system is applied to precisely define the place where the ions are delivered. The scanning system can be based on electrostatic (magnetostatic) beam positioning or mechanical sample positioning.

Most of above-mentioned horizontal SIHF's have been based on existing microbeam (MB) systems. Microbeam implies the ion beam with a micron or even submicron spot size. In such a way, the SIHF represents the next generation of MB family that makes its application even wider than before via such additional research directions as non-targeted effects in biology, single event upset phenomena in microelectronics, etc.

Nowadays, there is only one microprobe in Poland – the Cracow microbeam (CMB) in the Henryk Niewodniczanski Institute of Nuclear Physics in Cracow (Polish Academy of Science). The CMB has been extending the frontier of its application from solid state physics through geology (Lekki J. et al., 2003) to biology (Nowak J. et al., 2005). Developing the SIHF based on the microbeam is a great opportunity to enlarge the field of the CMB applications and to carry out novel, unique measurements. The special particularity of the CMB, which is the shortest
focusing system (FS) of about 2.3 m (Lebed S., 1999; Lebed S. et al., 2001), has additional attraction for the further SIHF development. Since the conventional focusing systems are about 12 m long, there are certain difficulties arise to build a vertical focusing SIHF. The up-to-date vertical SIHFs have been realized basing on collimators only. If the Cracow SIHF based on the shortest horizontal focusing system provides satisfactory results, such short FS will have a perspective to be used as a base for the vertical focusing SIHF.

Cracow microbeam group has been involved in the CELLION project, dedicated to the investigation of the radiation-induced damage in biological samples. The participation in CELLION defined the main direction of the SIHF development oriented on the biological investigation of the single ion single cell irradiation.

For precise single ion irradiation living cells, some additional elements should be introduced to the basic design of the SIHF (Fig. 1.2).

![Fig. 1.2. The schema of the SIHF modifications required for the living cell irradiation](image)

For the irradiation of the living targets, such as biological cells, experiments must occur in the air conditions (not in vacuum). Therefore, external ion beams are used in such experiments. The system of cell visualization (see Section 1.2) and cell recognition (see Section 1.3) are essential to determine the precise target (cell) location. The cell recognition can be performed manually or automatically. Both cell visualization and recognition, closely connected with the positioning system, are responsible for the precision of the ion delivering to the defined cell location.
1.2 Cell visualisation

An important issue of the construction of the SIHF dedicated to cell bombardment is the visualisation of cells during irradiation experiments (a so called on-line cell visualisation). There are two main problems of the on-line cell visualization in SIHF. First, living cells, being almost transparent objects, are invisible in the reflected light. Second, although they can be (hardly) visualised in transmitted light illumination, there have been no constructive solutions for the on-line introduction of illumination source.

Some SIHF groups do not use the on-line cell visualisation and apply pattern irradiation (Reinert T. et al., 2004; Dollinger G. et al., 2005). In most of existing SIHFs, the on-line cell visualisation is made by the staining of cells using fluorescent dyes (Folkard M. et al., 2002; Greif K.D. et al., 2004; Randers-Pehrson G. et al., 2001). However, it is evidently more advisable to study unstained cells and cells unaffected by UV irradiation. In the case of living cells, staining may change the nature of the analyzed cells. Furthermore, the UV radiation used to observe the fluorescence of stained cells may be highly damaging to the living cells.

The alternative to staining can be i) use of brightfield (darkfield) microscopy, if the problem of illumination is solved; ii) use of the so called phase sensitive techniques, which are able to “see” differences in phase of light passing through different parts of the object. Some of phase sensitive techniques (Trigg et al., 1995) such as 'Standard Phase Contrast' (e.g. Zernike Phase Contrast), 'Nomarski Differential Interference Contrast' (DIC), 'Hoffman Modulation Contrast' (HMC) or 'Interference-contast', are difficult to implement at the SIHF end-station because of non-standard construction requirements: they must be tailored to work in a horizontal position and, usually, space for placing them at the beam line is rather limited. Therefore, the most appropriate phase sensitive technique of cell imaging without staining seems to be the Quantitative Phase microscopy (QPm).
1.2.1 Brightfield microscopy

The conventional brightfield microscope (Trigg et al., 1995) is most widely used for investigation of samples which partially absorb illuminating light. The technique is best suited for stained specimens, samples naturally absorbing light, or specimens thick enough to absorb a significant amount of light despite being colourless. When illuminating light passes through such specimen, its amplitude or intensity is reduced; therefore, specimen imaged with brightfield illumination is termed an \textit{amplitude objects}, while the image of such objects is considered to contain so-called \textit{intensity (amplitude) information}. In a brightfield microscope, illuminating light is aimed through a specimen and through an objective lens to a camera or an eyepiece. We see objects in the light path because the investigated specimen, being an \textit{amplitude object}, absorbs light differentially. Images produced with brightfield illumination appear dark and/or highly coloured against a bright, often light grey or white, background.

1.2.2 Quantitative Phase microscopy (QPm)

QPm is an image-capturing and analysis tool that has been developed in the IATIA company (Australia, www.iatia.com.au). The method uses an optical microscope utilizing conventional brightfield optics without the need for specialized optical systems to measure transparent samples. Next, a patented digital Quantitative Phase Imaging (QPI) algorithm (Barone-Nugent E.D. \textit{et al.}, 2002; Curl C.L. \textit{et al.}, 2004) provides quantitative phase and intensity data. QPm provides independent digital acquisition of both quantitative phase and amplitude (intensity) information. It should be stressed that no other phase visualization technique delivers the pure phase information only – in other phase sensitive techniques the phase image contains always some intensity information, too.

The QPI algorithm is based on the paraxial approximation of the propagation of intensity distribution as described by the Transport of Intensity Equation (Teague M.R. 1983):

\[
k\partial_z I(\vec{r}) = \nabla_{\perp} \cdot [I(\vec{r})\nabla_{\perp} \varphi(\vec{r})],
\]  

(1.1)
where the light at wavelength $\lambda$ travels along the z direction, $k = \frac{2\pi}{\lambda}$, $\vec{r} = (x, y)$, $I(\vec{r})$ is the irradiance, $\phi(\vec{r})$ is the phase and $\nabla_\perp = \left(\frac{\partial}{\partial x}, \frac{\partial}{\partial y}\right)$.

The solution for phase in the Transport of Intensity equation can be presented by the following formula:

$$\phi = -k\nabla_\perp^{-2} \left\{ \nabla_\perp \cdot \left[ \frac{1}{I} \nabla_\perp \nabla_\perp^{-2} \partial_z I \right] \right\}. \quad (1.2)$$

The powerful ability of the QPI algorithm is to regain the phase information from only two ordinary brightfield images which are taken at two planes, slightly different from the focal plane (Fig. 1.3). In addition, a third image, generally used for normalisation, is taken at the focus plane. The phase data is rendered as a grey scale image (the phase map), and represents the phase or optical density across a sample that is entirely free of intensity based data.

Fig. 1.3. Principle of the Quantitative Phase microscopy.

QPm has a number of key advantages, including: a) operating with phase and intensity information independently; b) working in both transmitted and reflected illumination; c) providing quantitative, absolute phase; e) working with non-uniform and partially coherent illumination.
1.3 Cell recognition

The cell recognition can be realized either manually or automatically. In both cases, it is used to localize the place of the targets (cells) where the ions must be delivered. To obtain statistically significant results, many cell irradiation experiments with a large number of cells have to be carried out. Obviously, such task can be realized only using an automatic cell recognition.

The cell recognition is a particular case of pattern recognition. Thus, the cell recognition can be based on such techniques as Neural Networks (Egmont-Petersen M. et al., 2001; Han M. and Xi J., 2004), Fuzzy Logic (Chi Z., 1996; Nachtegael M.D. et al., 2006), direct Image Processing (Gonzalez C. and Woods E., 2001), etc.

Some of SIHF groups use the direct Image Processing for recognition of cell colonies (Barber P.R. et al., 2001). The others use commercial software (mainly Image Pro, Media Cybernetics) also based on the direct Image Processing. The main disadvantage of the commercial software is that it cannot be linked directly with the SIHF controlling software or it cannot include such software in itself. Additionally, the commercial software can be limited by the number of image processing techniques already realized by a company. Thus, while developing the SIHF for cell irradiation, in the present work, the tailored software for the automatic cell recognition has been chosen to be developed.

1.4 Recent experiments of cell irradiation

Development of SIHF and focused microbeam systems for single cell irradiation opens new possibilities for radiobiological researchers. Targeted irradiation is extremely useful in such studies, since it delivers a well-defined number of charged particles directly to cellular and sub-cellular structures. Therefore, the main contribution of SIHF in radiobiology is seen in so-called cellular micro-irradiation investigations (Folkard M. et al., 2001). The main aims of cellular micro-irradiation are to study and understand the following issues:
- mechanisms of radiation action on cells (energy deposition in cell, subsequent damage of cellular and sub-cellular structures, etc.);
- response of cells to irradiation (radiation-induced cellular effects, like death, apoptosis, damage repairing, intracellular and cell-to-cell signalling, mutation induction, etc.).

Such knowledge is crucial in the area of radiomedicine and radiodosimetry to improve the efficiency of radiotherapy strategies and radiation risk estimates.

Examples described below show some mechanisms of radiation interaction with cells and cellular responses to this action studied using the SIHF technique.

1.4.1 Mechanisms of radiation action on cells

The effectiveness of an ionizing radiation (i.e. the number and the quality of the effects induced by radiation) critically depends both on a radiation type (i.e. photon, particle) and on its energy. Therefore, there is a need to introduce a certain classification. Radiation is often classified in terms of its ‘ionization density’. The quantity used to measure ionization density is called Linear Energy Transfer (LET). LET measures the amount of energy lost by a particle or a photon traversing a unit distance. This quantity is often expressed in units of keV/µm.

The ionizing radiation is divided into two types (see Table 1.1):
- low LET (sparsely ionizing) – γ-rays, X-rays and neutrons;
- high LET (densely ionizing) – α-particles, protons and heavy ions.

<table>
<thead>
<tr>
<th>Radiation</th>
<th>LET (keV/µm)</th>
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<tr>
<td>250kV X-rays</td>
<td>&lt;1</td>
</tr>
<tr>
<td>γ-rays</td>
<td></td>
</tr>
<tr>
<td>protons</td>
<td>1 – 50</td>
</tr>
<tr>
<td>neutrons</td>
<td></td>
</tr>
<tr>
<td>α-particles</td>
<td>100 – 200</td>
</tr>
<tr>
<td>heavy ions</td>
<td>≥ 1000</td>
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Table 1.1. LET values for different types of ionizing radiation.
The main reason of such distinguishing is the fact that radiation of different types and LET values produce different patterns of ionization passing through a cell. Fig. 1.4 presents two examples of ionizing patterns induced in a cell by low LET X-ray or \( \gamma \)-ray radiation (left image) and high LET \( \alpha \)-particle radiation (right image).

In the first case (Fig. 1.4 a), a photon passing through a cell transfers its energy to electrons via either photoelectron or Compton effects or producing electron-positron pair. In dependence on the occurring effect, these electrons have different energies and, next, transfer energy to subsequent electrons. All secondary electrons produce a pattern in the form of a cascade. In the second case (Fig. 1.4 b), an \( \alpha \)-particle induces the ionization in the well-determined volume along its flight path.

![Fig. 1.4. Ionization patterns induced in cells by low LET X-ray radiation (a) and high LET \( \alpha \)-particle radiation (b).](image)

Both types of ionization patterns lead to subsequent damages of cellular and subcellular structures. The most serious consequences for a cell appear in the case of DNA helix damage.

Radiation might induce DNA damage either directly or indirectly. Direct damage results from direct targeting a DNA strand by the irradiating particle. Indirect damage is induced by free radicals originating from the reactions between radiation-induced secondary electrons and intracellular water (see Fig. 1.4 a).
DNA damages are classified into several types in dependence on the number of breaks and the distance between them (see Fig. 1.5).

SSB – single strand break

DSB – double strand break

Fig. 1.5. Classification of the DNA strand breaks by complexity (Charlton D.E. and Humm J.L., 1988; Nikjoo H. et al., 1997).

Some additional types of the radiation-induced DNA damage are i) base damage (knocking out basic nitrogens – building blocks of DNA) and ii) formation of anomalous DNA-protein bonds.

1.4.2 Cellular response to irradiation

The described above radiation-induced DNA damage may lead to different consequences for a living cell.

In the case, when radiation doses exceeds the cell possibility for self-repairing two types of cell death may occur: apoptosis or necrosis. Apoptosis (suicidal death) is a natural physiological death of a cell, being a part of the normal growing process. It prevents the subsequent transferring of induced mutations. Oppositely, necrosis is an uncontrolled sudden death of a cell. Both cell death types significantly differ in consequences.

One more type of the cell death may occur as a result of radiation-induced DNA damage – a mitotic death. The mitotic death appears if radiation breaks a DNA strand into several pieces and this damage is not repaired till cell division (mitosis). Then, the cut-off pieces form the structures, called micronuclei. This process leads to the death of cells of the next generation, which has lost a part of the genetic information included in micronuclei.
Certainly, a lot of other biological effects may occur in living cells in response to radiation-induced damages. However, the effects presented above are presently the most studied using targeted irradiation facilities.

It is worth to notice, that most of effects appearing in biological objects in response to radiation are supposed to follow the so-called ‘linear no-threshold model’ (Fig. 1.6; Trott K.R. and Rosemann M., 2000). According to this model, the probability of the given effect (radiation risk) is directly proportional to the radiation dose.

The model was mainly developed from the data obtained studying the large groups of people who received doses much higher than background (survivors of atomic bomb explosions, power plant disasters, uranium miners, etc.).

Effects induced by doses in the range of usual background (low-dose effects) are not known at the same extent. During the last century, they were usually extrapolated from higher doses data using the ‘linear no-threshold model’. However, some serious apprehensions for applicability of this model to low-dose effects appear. This was caused by recent discovery of several low-dose and non-targeted effects where cells are found to give an indirect response to ionizing radiation. These are:

a) *adaptive response* – cells subjected to a low priming dose show a lesser response when subsequently challenged with a higher dose (Wolff S., 1998);

b) *low-dose hypersensitivity* – cells exhibit increased radiation sensitivity at low doses (Joiner M.C. *et al*., 2001);
c) inverse dose–rate effect – cells manifest the increased levels of mutations or transformations at very low dose rates (Vilenchik M.M. and Knudson A.G., 2000);

d) bystander effect – the unirradiated cells die in response to the signalling processes arising from irradiated cells (Prise K.M. et al., 2003);

e) gene expression – which is the up, or down regulation of genes at doses below levels significant for direct DNA damage (Amundson S.A. et al., 1999);

f) genomic instability – cell lines manifest chromosome changes and mutations in the surviving progeny of irradiated cells (Wright E.G., 1999).

Although the investigations of these effects are far from complete, by analyzing them one can conclude that the ‘linear no-threshold model’ requires modifications in the low-dose range. And there is still a question which of possible models (see Fig. 1.7) will properly describe the low-dose effects.

![Fig. 1.7. Several possible models of the radiation risk for low-dose range.](image)

Such uncertainty shows the necessity for careful studies of the effects described above, in order to construct the model for the low-dose radiation risk. Taking into account the capabilities of SIHF, this technique gives a great opportunity for such studies, which is confirmed by the increasing number of recently reported scientific papers about SIHF applications in low-dose effects’ investigations.
1.4.3 Recent radiobiological investigations using SIHF

Last years have brought a number of papers reporting the cell irradiation experiments with defined number of charged particle (Zhou H. et al., 2000; Prise K.M. et al., 1998; Belyakov O.V. et al., 1999; Folkard M. et al., 2001; Sawant S.G. et al., 2002; Prise K.M. et al., 2000; Kobayashi Y. et al., 2003) or with X-ray radiation (Prise K.M. et al., 1998; Belyakov O.V. et al., 1999; Schettino G. et al. 2005). The studied radiation induced effects such as bystander effect (Prise K.M. et al., 1998; Zhou H. et al., 2000; Sawant S.G. et al., 2002), micro nucleus formation (Belyakov O.V. et al., 1999; Prise et al. 2000), cell lethality (Belyakov O.V. et al., 1999; Kobayashi Y. et al., 2003) has been performed for different cell lines, e.g. human fibroblasts (Prise K.M. et al., 1998; Belyakov O.V. et al., 1999) and chinese hamster cells Prise K.M. et al., 2000; Sawant S.G. et al., 2002; Kobayashi Y. et al., 2003).

The above mentioned studies employ different types of ionizing radiation (α-particles, heavy ions, and X-rays) with a broad range of LET values. After the analysis of these works, one may conclude that among the most studied cellular effects induced with application of SIHF there are: micronuclei formation, cell lethality and bystander effect.

In the present work, the micronucleus formation and cell lethality effects were chosen as the principal effects to be studied using the developed SIHF. In addition, the study of radiation-induced double strand breaks of DNA of irradiated cells was carried out as well.

Most of SIHF groups use α-particles, heavy ions or X-ray setups. Taking into account the importance to provide the experiments with different types of ionization, the Cracow SIHF would extend the present knowledge about radiation-induced biological effects providing the possibility to study the effects induced by protons in living cells.
2.1 Introduction

In the literature review, it has been shown an expediency of SIHF development on a base of working microbeam systems. Such approach has been applied in the present work: the presented SIHF has been developed on a base of fully operational microbeam setup. For this aim, the Cracow microbeam (CMB) has been used.

The present chapter gives a brief description of the CMB and explain the main modifications that have to be performed in order to construct a SIHF on the base of it. The main hardware modifications are explained in the present chapter, while the software specially developed for the SIHF is described in Chapter 3.

2.1.1 Cracow microbeam (CMB) – a base for SIHF development

The nuclear microbeam in Cracow (Fig.2.1) is based on a HVEC KN-3000 Van de Graaff accelerator (A) with a proton (alpha particle) beam energy $\leq 3$ MeV and an energy stability of $\Delta E/E \approx 10^{-3}$. Brief depiction of the CMB is presented in Fig.2.1, while the detailed information about the system construction can be found elsewhere (Lebed S. et al., 2001; Lekki J. et al., 2001).

The CMB currently provides a minimum beam spot size of about 7-8 microns at beam current of 100 pA (for beam current of 0.5 – 1 nA, which is more suitable for PIXE measurements, the beam spot size is of 10 microns). The scanning system provides the scanning area up to 0.25 x 0.25 mm (can be extended twice, if required). The CMB enables measurements using such micro analytical techniques (Cambell J.L., 1993; Maydell E.A., 1993) as Proton Induced X-ray Emission (PIXE), Rutherford Back Scattering (RBS), and Scanning Transmitted Ion Microscopy (STIM).
Fig. 2.2. Scheme of the Cracow MB. The ion beam (B) from the accelerator (VdG) is delivered to the circular slit (CS) via a 90° double-focusing analyzing magnet (AM) and three doublets of magnetic quadrupole lenses (QL). Only central part of the beam can pass through the circular slit to the object slit (OS). Passing an angular collimator (AC), the beam is next focused by means of two doublets of magnetic quadrupoles, forming the so-called divided Russian quadruplet lenses (RQL). The beam is focused on a target (T) inside the vacuum chamber (VCH). The detectors placed inside the chamber enable the PIXE, STIM and RBS measurements. A ferrite-cored scanning system (SS) is used to carry out the micro-analytical measurements in two dimensions. The detectors and the scanning system are connected to a controlling computer (CC) via data acquisition system (DAS) based on PC data acquisition cards and CAMAC.

2.1.2 From CMB to SIHF

The CMB facility is a fully operational system for micro analytical analysis. In the present work, however, it is used only as a base for the SIHF development. The main point is that the CMB provides the solution of well focused microbeam formation in vacuum. Whereas, for single ion irradiation of living cells it is required to introduce additional solutions. Fortunately, the CMB is an open system to which a new hardware (detectors, CAMAC units, new end-station, etc.) can be easily added.
For irradiation of the living targets (cells), the ion beam should be led out from the vacuum and focused in air. For this purpose, a special chamber had to be designed, since the end-station of the CMB includes a vacuum chamber designed in such a way that the focus point of the ion beam is inside it.

For the precise ion number control, two requirements has to be realised: i) the beam current should be decreased to about 1000 protons/s and ii) the possibility should be provided to switch off the beam after detection of every single ion (the so-called beam blanking). To provide the accurate beam blanking, the effective ion detection should be carried out. It can be realised via introducing the particle detector either inside, or outside the chamber.

The scanning system of the CMB could be used for delivering the ions to the defined target point. However, it covers too small region of interest (0.25 x 0.25 mm area). Therefore, it is reasonable to introduce a special system for positioning the targets (cells) in the SIHF. Another important requirement is the construction of special holders for living cells, which would be suitable for both culturing and irradiation of cells.

In order to observe and localise living cells for their subsequent irradiation, an optical system should be introduced at the end-station of the SIHF. The task of cell visualisation is complicated, since living cells are usually semi-transparent or even transparent objects almost not visible in the conventional optical system.

Taking into account the hardware modifications, certain data acquisition modifications should be performed as well.

All above-mentioned requirements for the SIHF development are summarized in Fig. 2.3, which presents the schematic illustration of the SIHF.

In general, for single ion irradiation of living cells it is required to solve the following issues:

- beam focusing in air;
- precise ion detection and ion number control, down to one ion;
- cell positioning and cell visualization;
The implementation of the first three issues, dealing mainly with the hardware modifications, is described below. It involves both the development of a new end-station, and modifications of the beam line and data acquisition system.

For automatisation of cell recognition, cell positioning and cell irradiation, a special software has to be developed. It is entirely described in the next chapter (see Chapter 3 “Developing SIHF Software”).

2.2 The beam line modifications

The beam line modifications have been aimed for better beam control. First of all, they have included the replacement of the object slit (Fig. 2.2, item OS) with the high precision beam slits (Fig. 2.3, item PS) (construction Technisches Büro S.Fisher,
Ober-Ramstadt, Germany). These slits have allowed the adjustment of the beam size from 50 \( \mu \text{m} \) downwards and thus reduction of the beam current to the required flux of about 1000 protons/s (0.16 fA). In that way, the time between two protons hitting the target one after another has been extended (to about 1 ms in average) in order to separate those protons in time for the more effective electronic reduction and computer control.

The precision slits may be also fully opened and therefore their placement in the ion guide is not harmful for the normal microprobe operation (i.e. for the beam intensity of 100 pA and more, not for the single protons regime).

The deflection plates (Technisches Büro S.Fisher) have been located next to the beam reduction slits. Their function is to turn off the beam, when required. Deflection plates are electrostatically driven by a fast high voltage amplifier (HV-amplifier, Technisches Büro S.Fisher), which allows rising the deflection voltage up to 1000 V/\( \mu \text{s} \). The voltage value, sufficient to deflect the beam of protons towards the ion guide and therefore to stop it completely, has been estimated as follows.

![Fig. 2.4. Proton moving in electrostatic field of the deflection plates with an initial velocity \( \vec{v}_0 \) perpendicular to the vector of electric field intensity \( \vec{E} \).](image)

The proton moving in the electromagnetic field with the velocity \( \vec{v} \) is affected by the Lorentz force

\[
\frac{d}{dt}(m_p \vec{v}) = p\vec{E} + p(\vec{v} \times \vec{B})
\]

(2.1)

where \( m_p \) is the proton mass, \( p \) – proton charge, \( \vec{E} \) is electric field intensity, and \( \vec{B} \) – magnetic induction. The protons moving between the deflection plates are considered as the charged particles moving in the electrostatic field (\( \vec{B} = 0 \)). Thus, for the proton
moving in such field with an initial velocity $\vec{v}_0$ ($t = 0$) perpendicular to the vector of intensity $\vec{E}$ (Fig. 2.4), Eq. 3.1 is equivalent to the equations

$$m_p \frac{d^2x}{dt^2} = 0, \quad m_p \frac{d^2y}{dt^2} = pE. \quad (2.2)$$

Taking into account the initial parameters, the solutions of the Eqs. 2.2 are

$$v_x(t) = v_0, \quad v_y(t) = \frac{p}{m_p} Et, \quad x(t) = v_0 t, \quad y(t) = \frac{p}{2m_p} Et^2. \quad (2.3)$$

The proton moves along the parabolic trajectory until the end of the deflection plates. The transversal proton velocity component $v'_y$ and the proton deflection $h'$ from the initial direction of its movement in the moment when the proton reaches the end of the deflection plates, can be estimated using Eqs. 2.3

$$v'_y = \frac{p}{m_p} E \frac{l}{v_0}, \quad h' = \frac{p}{2m_p} E \frac{l^2}{v_0^2} \quad (2.3)$$

The further movement of the proton is described as a uniform motion with a constant initial velocity $\vec{v}_{xy} = \vec{v}_0 + \vec{v}'_y$. Thus the total proton deflection $h$, from the initial direction of its movement in the moment when the proton reaches the angular collimator, is

$$h = h' + v'_y \frac{L}{v_0} = \frac{p}{m_p} E \frac{l}{v_0} \left( \frac{l}{2} + L \right) \quad (2.4)$$

Taking the velocity $v_0$ of the 2 MeV protons of about $1.96 \cdot 10^7$ m/s, the plates length $l$ of 0.1 m, the distance $L$ between the plates and the angular collimator of 0.5 m, the distance $D$ between two deflection plates of 0.002 m, the deflection $h$ has been estimated for the values of the voltage $U = E \cdot D$ applied to the deflection plates. Finally, the voltage value of 440 Volts has been applied to the plates. It has resulted in a proton beam deflection $h$ of 3.06 mm, which is sufficient to omit the aperture of the angular collimator (the aperture size $d$ is of 100 µm).

2.3 The end-station

The new end-station for the SIHF has been developed (Fig. 2.5). It is based on the special designed target chamber (Лебедь С.О. et al., 2000).
It includes the outlet window enabling leading the ions out of vacuum, the moving stage, the particle detector and the microscope objective of the on-line optical system (described in Section 2.4).

Fig. 2.5. The end-station of the SIHF.

Chamber. Fig. 2.6 presents the design of the irradiation chamber. In the SIHF, the focus of the beam has to be located in air, outside the chamber. The focusing distance of the CMB is 15 cm. Therefore, during the construction of the new irradiation
chamber for the SIHF, the existing limitation of the depth of the chamber has been taken into account.

![Fig. 2.6. The schematic view of the irradiation chamber of the SIHF end-station (A – top view, B – side view): 1 – part of the divided Russian quadruplet; 2 – scanning system; 3 – target chamber; 4 – connection of the chamber with the ion guide; 5 – outlet window; 6 – target holder as a part of the positioning system; 7 – secondary electrons detector (channeltron); 8 – illuminating diode. Figure were taken from (Лебедь С.О. et al., 2000)]](image)

The chamber includes both the outlet window (see below) and the quartz window. The latter one is used for preliminary beam spot visualization under the conditions of a high current beam (see Section 3.4.2). The details of the chamber construction can be found elsewhere (Лебедь С.О. et al., 2000).

Outlet window. The outlet window used for leading the ions out of the vacuum have been purchased from Silson, UK. The window is a thin (100-200 nm) Si$_3$N$_4$ membrane in the frame made of Si (Fig. 2.7). Different sizes and thicknesses of both the windows and the membranes are commercially available.
Fig. 2.7. The Si₃N₄ windows. Two differently sized windows are shown: (a) 3 x 3 mm (7 x 7 mm with Si frame) and (b) 1.5 x 1.5 mm (5 x 5 mm with Si frame).

As the outlet window in the SIHF, the 5 x 5 mm windows with the 200 nm thick, 1.5 x 1.5 mm membranes have been used. The membrane of 200 nm thickness has assured tolerable beam scattering and satisfactory mechanical endurance, withstanding the atmospheric pressure.

_Petri dishes_: Three different modifications of the standard Petri dishes (made off polystyrene, 3.5 cm in diameter) have been used for the cell irradiation experiments. For two of them (Fig. 2.8 a and b), a small hole (1 cm in diameter) has been drilled in the centre of the dish and either Si₃N₄ window (500 nm thick, either 1.5 x 1.5 mm, or 3 x 3 mm sized) or Mylar foil (2.5 µm thick) has been glued by wax or by epoxy adhesive glue specially formulated for medical applications (EP30MED, Master Bond Inc.), correspondingly.

Fig. 2.8. Three modifications of the Petri dishes: (a) window based on the Si₃N₄ window and (b) Mylar foil glued over the hole in the dish bottom, (c) Mylar foil stretched on the place of removed dish bottom.
For the third modification (Fig. 2.8 c), a whole bottom of the Petri dish has been removed and it has been replaced with the Mylar foil (2.5 µm thick) in such a way that the foil has been stretched using plastic rings. During irradiation experiments, all types of cell dishes have been covered with a Mylar foil to prevent contamination of cell culture.

**Moving stage.** A precise two dimensional V-106 voice-coil moving stage (Physics Instruments, Fig. 2.9) mounted in a vertical position has been used as a base of the positioning system. As the beam line is horizontal, Petri dish with the cells must be placed (and irradiated) vertically. The stage motion in the third direction has been provided via introduction of the extra micrometer screw.

![Image of V-106 Voice-Coil Scanning System](www.physikinstrumente.com)

The V-106 voice-coil scanning and positioning system is designed for applications where small samples have to be positioned or scanned with high velocity and high resolution. It provides superior responsiveness compared to micropositioners with conventional leadscrew drives. The mechanics is driven by an integrated, non-contact, zero-friction, voice-coil actuator. Scan velocities up to 50 mm/sec over a range of 5 mm are feasible with typical loads of less than 100 grams.
Integrated linear encoders provide position resolution of 0.1 µm and repeatability of about 0.2 µm.

**Ion detection.** The first attempt of ion detection has been provided using a couple of Si₃N₄ window covered with CsI as a source of the secondary electrons and a channeltron (Sjuts Electronics, Fig. 2.10 a) installed in the chamber (Fig. 2.6 item 7).

![Fig. 2.10. a) Sjuts Electronics channeltron detector (www.sjuts.com) and b) ORTEC silicon surface barrier detector.](image)

However, this way of the ion detection appeared to be not enough reliable – the measured effectiveness was not better than 61 % (Polak W. et al., 2004). Therefore, another way of ion detection has been developed using a particle silicon surface barrier detector (ORTEC, Fig. 2.10 b). This detector has been installed outside the chamber, behind the Petri dish with cells (Fig. 2.3 item SiD, Fig. 2.5). It has provided the proton detection with effectiveness about 100 % (Polak W. et al., 2004).

### 2.4 Optical system for cell visualization

Observation and evaluation of cell samples must be assured before, during and after the irradiation. Moreover, to make the subsequent automatic cell recognition more efficient, the high quality of cell images must be provided. Two main solutions have been developed at IFJ PAN for cell visualization purposes. These are brightfield (conventional) cell visualisation and cell visualisation based on Quantitative Phase microscopy (QPm). It has been shown, that both techniques can be used either for off-line cell inspecting (in preparatory room before and after irradiation) or for on-line cell observation (during cell irradiation).
2.4.1 Brightfield (conventional) cell visualisation

Generally, the brightfield microscopy is applied to visualize samples that absorb a significant amount of visible light (see Section 1.2). The main equipment required for brightfield cell visualisation is a light source for sample illumination, a lens (objective) for image magnification and a camera for image registration. These basic elements are present in both off-line and on-line visualisation systems, which have been proposed for the SIHF.

Off-line cell visualisation system. The proposed brightfield off-line cell visualisation system is based on research Olympus BX-51 research microscope (Fig. 2.11 a). The Olympus BX-51 allows using not only a high quality light source (built-in Koehler illumination for transmitted light 12V 100W halogen bulb) and a wide range of objectives (4x, 10x, 20x, 50x), but also other microscope modules (condenser, filters etc.) serving to enhance the quality of the obtained image.

Fig. 2.11. Olympus BX-51 microscope (a) and QICAM 12-bit digital camera (b).

For image registration, a QICAM 12-bit digital camera has been used (Fig. 2.11 b). This camera provides 1392 x 1040 pixel (1.4 million) image with 1/2” optical format.

In our SIHF, the realization of brightfield on-line cell visualisation has become possible mainly due to the proposed method of sample illumination. The illuminating emitter has been installed directly inside the chamber of the SIHF and thus the ion exit window serves as an illumination source. The chosen Philips Lumiled LUXEON emitter (Fig. 2.12 a) is characterized by the brightness of 45 lumens, the power consumption of 1 Watt, and the lifetime of 50000 hours. Its representative spatial radiation pattern is presented in Fig. 2.12 b. The emitter size is 9.6 x 9.6 x 5.8 mm. The light beam of the emitter contains no infrared or ultra violet components.

The used Zoom 125C setup (Fig. 2.13 a) delivers a magnification in the range of 1x – 12.5x zoom ratios. The Zoom 125C is characterized by a 89 mm working distance and a field of view ranging from 3.4 x 4.5 mm$^2$ to 0.27 x 0.37 mm$^2$. Its performance is enhanced with a remote motorized zoom and internal 5 mm and 15 mm fine focuses controls.

![Fig. 2.12. Philips Lumiled LUXEON emitter (a) and representative spatial radiation pattern for the emitter (b) (www.lumileds.com).](image)
The images have been registered using KOBI B/W CCTV Camera (SG32M-1CE AI/DC, Fig. 2.13 b) attached to the Zoom 125C. The camera is characterized by the high sensitivity of 0.005 lx (important in low illumination conditions), signal to noise ratio of 48 dB, 1/3" optical format, and resolution of 537 x 505 pixels.

2.4.2 QPm visualisation

The advantage of the Quantitative Phase microscopy is its ability to obtain only the phase information about imaged object, enabling thus the observation of the transparent and semi-transparent samples, even those not visible for conventional visualization methods (see Section 1.2).

For both off-line and on-line optical systems proposed in the work, the QPm construction requires the only mechanical detail – a P-721 PIFOC® High-Speed Piezo Nano-Focussing Device with direct metrology feedback (Physics Instruments, Fig. 2.14). This device allows fast moving of the objective from the focal plane to two planes located on the both sides from the focal plane. The images are taken in each of these three planes.
P-721 PIFOC® provides a positioning and scanning range of 100 μm with sub-nanometer resolution (± 0.7 nm) and a very high linearity (0.03 %). The distance between the focal plane and the plane out of the focus is in a range of 0.5 – 20 μm and depends on a magnification of used objective (see Table 2.1).

**Table 2.1. Dependence of the defocusing distance on the objective magnification.**

<table>
<thead>
<tr>
<th>Objective magnification</th>
<th>2.5x</th>
<th>10x</th>
<th>20x</th>
<th>40x</th>
<th>100x</th>
</tr>
</thead>
<tbody>
<tr>
<td>Defocusing distance</td>
<td>20.0 μm</td>
<td>5.0 μm</td>
<td>2.0 μm</td>
<td>1.0 μm</td>
<td>0.5 μm</td>
</tr>
</tbody>
</table>

**Off-line system.** Just like the brightfield off-line system, the QPm off-line cell visualisation has been based on the research Olympus BX-51 microscope (Fig. 2.11 a). In the QPm system, additionally, the P-721 PIFOC® device has been mounted between the microscope objective and revolver. For image registration, the QICAM 12-bit digital camera described above (Fig. 2.11 b) has been used.

**On-line system.** The QPm on-line visualisation has been developed using the P-721 PIFOC® device, Olympus objectives (4x, 10x, 20x, 50x), and the QICAM digital camera (Fig. 2.15). All elements of the system have been directly mounted as the part of the end-station as shown in Fig. 2.15.
2.5 Data acquisition modifications

The aim of data acquisition system (DAS) is to provide an interface between electronic parts of the SIHF and a computer. The data acquisition system of the CMB (Lekki J. et. al., 2000) has been used as base of the DAS for the SIHF. However, the added electronic modules have caused certain data acquisition modifications.
First of all, the beam blanking has required the addition of PCI-DDA 08/16 analog output and digital I/O board (Measurement Computing Company). The PCI-DDA 08/16 (Fig. 2.16 a) provides eight analog output channels, which are characterized by 16 bit resolution, ±0.63 mV accuracy, 12 µs settling time and 2.5 V/µs slew rate. The board receives signal related to the ion passage and generates the signal to switch off the beam.

![a) PCI-DDA 08/16 computer board; b) GA-VD201G High Quality Video Creator (www.cooldrives.com).](image)

Secondly, the V-820.20 Voice-Coil Scanning Controller (see Fig. 2.9) has been added for direct stage control. This digital controller comes on an ISA-bus-card for industrial PCs and features the on-board linear amplifiers for two individual axes, a 32-bit PID-V-ff servo system, as well as I/O lines and hardware interrupt capability.

Thirdly, the GA-VD201G High Quality Video Creator (Fig. 2.16 b) has been added to digitize the video signal from KOBI B/W CCTV Camera. It supports NTSC, PAL video inputs and high resolution still image capture at 720 x 576 pixels.

### 2.6 Results and discussion

*Beam line.* Influence of the equipment newly introduced in the beam line, such as the precise slits and the deflection plates, has been checked in the following way. First, under the conditions of the slits being open and zero voltage on the deflection plates, the beam flux of about 10000 protons/s was set. Next, the flux was measured while operating with the precise slits. The slits allowed, for smooth control, reducing the beam down to the chosen optimal current value of about 1000 protons/s. Then,
the voltage of 440 V was applied to the deflection plates. It reduced the measured current to zero value, which indicated the successful beam deflection (beam blanking). After the setting, the system has been put back to the initial conditions with open slits and zero voltage on the deflection plates, and the previous beam flux was restored.

**End-station.** The next important part of the SIHF is the end-station. Its main task is getting the beam out of vacuum. To verify the beam behaviour in air, the following test has been carried out. The ion beam has been guided through the outlet window outside the chamber. Directly behind the window, a copper grid has been mounted on the holders designed for the Petri dishes. Using the CMB scanning system and the externally mounted particle silicon surface barrier detector, the scanned image of the grid has been obtained Fig. 2.17. The image has been constructed as the map of number of counts corresponding to the energy value of the beam coming through the grid in each pixel of the scanned area. On the map, the lighter colour corresponds to the higher number of counts.

![Fig. 2.17. Copper Kα image (128 x 128 pixels) of the Cu grid (mesh size 63 µm, Cu bar diameter 9 µm). Estimated beam spot size was of about 15 µm (FWHM).](image)

The first detecting system based on the channeltron has shown the efficiency of 60 % (Polak W. *et al.*, 2004). It is definitely not enough for SIHF that requires efficiency up to 100% and thus control of every single ion. Efficient ion detection is
vital, since it must precisely indicate when a proton has been delivered to the target, which information is next used for the beam blanking. Moreover, knowledge about a precise number of ions delivered to a cell is used for dose estimation. Higher efficiency has been achieved using the silicon detector placed behind the cell dish. This method is complicated by the fact that protons lose their energy passing through the medium in the cell dish. According to the SRIM2003 simulations, 2 MeV proton loses the energy of 16.42 keV per 1 µm of water (H\textsubscript{2}O, 1 g/cm\textsuperscript{3}); thus, medium layer of 122 µm completely absorbs the energy of 2 MeV proton. Therefore, there is a necessity to remove liquid (medium) from the cell dish in order to assure that all ions pass to the detector.

Other inconvenience is that this detector must be situated in front of the optical system. Thus, during image registration it must be moved aside (this task is realized via a specially designed moving arm, see Fig. 2.5). Despite the mentioned inconveniences, the high efficiency of the second method makes it more satisfactory one for the SIHF performance.

**Off-line optical systems.** The cultures of the living cells (from metastatic prostate carcinoma) have been visualized using both brightfield and the QPm based off-line optical systems. The obtained images are presented in Fig. 2.18. Due to the transparency of the sample, the intensity information of the background is similar to the cytoplasm area in the brightfield image and thus the cells are nearly invisible. The QPm phase map (QPm image) displays larger contrast by calculating the phase shift through each point in the image. The cells are covered with a medium of refractive index higher than the background and the light travels more slowly through the cell than through its external environment. This phase shift or retarding of the light is the method by which QPm can differentiate objects using the algorithm described above (see Section 1.2.2). In the phase image one can even see some structural details of the cell, e.g. nuclei.
Fig. 2.18. Comparison between brightfield and the QPm based off-line optical systems. Images taken with 20X magnification of living unstained cells (from metastatic prostate carcinoma) plated on the bottom of the Petri dish. The QPm processing of the 1280 x 1024 pixels (480 x 385 µm$^2$) image took about 5 seconds using Athlon XP 2800+ PC.

Hence, due to the evident difficulty in using the brightfield visualisation the QPm based system is the preferred solution used for off-line visualisation of living cells in our experiments.

The main feature of the QPm system to obtain separately phase and intensity information allows also the simulation of other optical techniques which use both types of information, e.g. 'Standard Phase Contrast' (i.e. Zernike Phase Contrast),
'Differential Interference Contrast' (DIC), and 'Hoffman Modulation Contrast' (HMC).

Fig. 2.19. Example of DIC and HMC images simulated from pure phase and intensity information obtained by QPm.
Fig. 2.19 presents the example of DIC and HMC simulations derived from the pure phase and intensity information obtained by QPm. This ability may be used, for instance, for preliminary evaluation of the particular optical method applicability for the visualisation of a given type of sample.

*On-line optical systems.* Although some scientific groups have shown the way of using the system of upright microscope (where cell dish is placed horizontally) for horizontal SIHF (Gerardi S. *et al.*, 2006; Gerardi S. *et al.*, 2005), this solution is rather complicated. Whereas, the on-line cells observation is very important for their effective irradiation in the case when cell dish is placed vertically. The on-line cell visualization allows reducing both the time of the whole experiment and the time between cell recognition and cell irradiation, which reduce an influence of cell movement and, thus, enhance the experiment purity (effectiveness).

The main obstruction in the on-line cell visualization is a lack of the high-quality transmitted illumination. Thus, it is needed to use a technique than can work in a reflective light (e.g. QPm) or to invent the method of placing a light source behind the target. Therefore, we have applied the Philips Lumiled LUXEON emitter, placed inside the chamber, for the both QPM and the brightfield imaging.

The chosen Philips Lumiled LUXEON diode is an energy efficient (45 lumens per 1 Watt) and ultra compact light source. It combines the advantages of light emitting diodes (long lifetime and high reliability) with the high brightness of the conventional lighting. Additional benefit is that there are no infrared or ultraviolet components in the light beam of the emitter, while conventional light sources (including some LEDs) contain not only the visible component but also the invisible tails both below and above the visible band (ultraviolet and infrared). The ultraviolet and infrared light components can also damage materials, cause colour changes and breakdown of some materials. They are especially inappropriate for illuminating living cells, since they may cause additional effects in the living objects (thermal shock and even cell death).
Such technical solution can be easily applied with the systems based on beam focusing. Collimated beam systems would require special construction, because of difficulties to place a light source on the other side of the cell sample.

The advantage of QPM over all other phase sensitive techniques is the small size of the setup that has been used to mount it as a part of the end-station (see Fig. 2.15).

The cultures of the living cells (fibroblasts) have been visualized on-line using both the brightfield and the QPM based optical systems. The obtained images are presented in Fig. 2.20.

Fig. 2.20. Comparison between brightfield (a) and the QPM based (b) on-line optical systems. Images of living unstained cells (fibroblasts) plated on the bottom of Petri dish.

Comparing two on-line systems, it has been concluded that QPM based system still shows better results than the brightfield one. In some cases, however, the images taken with the brightfield microscope are good enough to recognise the cells. In these cases, there is no necessity to improve the images using so advanced method as QPM. There is also another advantage of the on-line system based on the brightfield microscope: the Zoom 125C optical setup used by this system provides more convenient working distance (about 8 cm) than Olympus objectives used by QPM (about 1 cm only). Therefore, the Zoom 125C setup is much easier in operation as it provides much space for the cell dish placement.

It is worth to notice, that introducing the possibility of a quick switching between two optical systems (brightfield and QPM) in the on-line mode could be convenient. It would allow quick performing of some routine observation tasks (such as beam
spot visualisation during beam adjustment) by the conventional brightfield method as well as carrying out the advanced tasks (such as visualisation of transparent samples) using QPm.

Summarising, the results obtained using off-line and on-line optical systems, both QPm and brightfield methods have proved to be satisfactory for observation of living cells in SIHF experiments, without necessity to stain the samples or to use harmful UV illumination.

**Petri-dishes.** All three above-described types of Petri-dishes have been used for cell growing for cell irradiation experiments in the SIHF. Each type has its advantages and disadvantages. The images obtained while visualizing the cells deposited on the Si$_3$N$_4$ windows (Fig. 2.21 a) are better than those for the cells deposited on the Mylar foil (Fig. 2.21 b). The reason for this effect is probably the attraction of talcum powder from biological gloves to the Mylar foil.

![Fig. 2.21. Comparison between images with fibroblasts seeded on Si$_3$N$_4$ window (a) Mylar foil (b). The images have been taken using the on-line brightfield system.](image)

However, construction of the Petri-dishes on the base of the Si$_3$N$_4$ window is too expensive, while the quality of images obtained from the dishes with the Mylar foil is still possible to be improved due to extremely accurate sample preparation. At the same time, the dishes of the Mylar foil have the advantage of providing larger region of interest. It is also worth to notice that the dishes with the Mylar foil stretched in
place of the removed bottom have a large foil surface, which is readily attracted to other surfaces via electrostatic forces. Therefore, using this type of Petri dishes increases the risk to break the outlet-window, since the dish with cells is placed in the close vicinity of the outlet window. Concluding, we have chosen the usage of the Petri-dishes with the Mylar foil glued over a small hole. However, the procedure of sample preparation should be improved to avoid the talcum powder contamination on the foil.

Summarizing, the successful implementation of all above-described hardware innovations and modifications introduced to the existing CMB with the purpose of the SIHF development have been shown. However, to use the full experimental power of the SIHF, the automatisation of the whole system is necessary.
CHAPTER 3. DEVELOPING THE SIHF SOFTWARE

3.1 Introduction

The previous chapter has shown the main hardware modifications introduced in order to develop the SIHF on the base of the CMB. The existing CMB facility has the software providing the data acquisition and interaction between the separate parts of the microbeam system. However, taking into account the installation of the new hardware and logical interactions to be realised between the hardware and computer during the targeted irradiation experiments (Fig. 3.1), it has become necessary to develop new software system dedicated to this task.

Fig. 3.1. Schematic illustration of the SIHF with the defined new lines of interaction between the computer (CC) and hardware. The proton beam (B) enters the SIHF beam line via circular slit (CS). The abbreviations in figure denote precise slits (PS), electrostatic deflecting plates (DP), angular collimator (AC), beam blanking (BB), Russian quadruplet of quadrupole lenses (RQL), outlet window (OW), chamber (CH), 3D moving stage (MS) with a Petri-dish, channeltron detector (CHD), silicon surface barrier detector (SiD), optical system (OS), illuminating diode (D), beam off signal (BOS), high voltage amplifier (HVA) and data acquisition system (DAS).
For the full functionality of the SIHF, the newly developed software should provide and maintain: i) automatic cell recognition, ii) automatic localisation of the beam position, iii) automatic cell positioning iv) correspondence between the cell recognition and the cell positioning, v) correspondence between the ion detection and beam blanking, vi) data acquisition, vii) automatisation of the whole irradiation process.

The present chapter is dedicated to the development of algorithms and protocols and the full design of corresponding software to realize the tasks listed above.

3.2 Algorithm of automatic cell recognition

The aim of the cell recognition is to find the coordinates of the cells in an image obtained with either on-line or off-line visualization systems. Subsequently, these coordinates are used in the SIHF control program responsible for the cell irradiation process.

The cell recognition is a task similar to the fingerprint recognition, optical character recognition, pattern recognition etc., which have a great number of different techniques (neural networks, fuzzy logic etc.) at their disposal. However, the cell recognition has its own features, which do not allow the direct use of the techniques from other fields of recognition. Therefore, the analysis of the existing recognition techniques was performed and the most appropriate of them were chosen, and adapted to the cell recognition task, taking into account our specificities.

For convenient perception, the present section contains not only main results and discisions of the executed work but also short introductions into each of the studied techniques.

The existing recognition techniques usually solve the task reduced to a certain form. Thus, in our case, cell images should be preliminary analyzed and transformed into the form required for the chosen technique. For this aim, the image processing is used.

Generally, the image processing has a large number of image transformation procedures (filters, logical operations, morphological transformations etc.) at its
disposal. Nevertheless, there is no universal way to get directly the required result. Therefore, the image processing is usually performed as a sequence of consecutive procedures (methods). An analysis of some methods used in such sequences has been carried out in view of application to our task, and is presented below.

3.2.1 Thresholding (Binarization)

Image thresholding converts a greyscale image to a black and white image in such a way that one colour corresponds to the background, while another one to the sought objects (cells). The thresholding provides an easy and convenient way to perform a separation between the objects and the background regions of the image. In simple implementations, the separation is determined by a single parameter known as the intensity threshold. In a single pass, each pixel in the image is compared with this threshold. If the pixel's intensity is higher than the threshold, the pixel is set to the one colour. If it is less than the threshold, it is set to another colour.

In more sophisticated implementations, multiple thresholds can be specified, so that a band of intensity values can be set to white while everything else is set to black (or vice versa).

Such multiple thresholds can be chosen after analysis of the image intensity distribution as low and high intensity values. Fig. 3.2 presents an image of a cell after thresholding applied with varying low and high thresholds. As it can be seen, the colour of both the object (cell) and the background may be either black or white, depending on the thresholds chosen (compare the images with the bands of 30-130 and 130-230).
Fig. 3.2. Original image of a living cell (taken with QPm) with corresponding intensity distribution and this image after thresholding carried out for different intensity bands (100-200, 100-130, 30-130, 130-230, and 160-190).

The choice of the band determines also how many details remain in the image. The band 30-130 is applied when only the cell location must be found in the image. The band 100-130 can be useful in the case, when the task of recognition is to find also the internal structures of the cell.

Another example of thresholding applied to the image of a cell is given in Fig. 3.3. Here, one can see that none of the proposed thresholds has given the satisfactory result (the object does not stand out clearly against the background). Such effect appears because the object, due to nonuniform illumination, contains both dark and light regions.
Fig. 3.3 Original image of a living cell taken with the brightfield microscope (a) with corresponding intensity distribution and results of the image thresholding carried out for different intensity bands; (b) two examples of addition of the results of thresholding with two different intensity bands.

Such problem can be solved via separate use of two different intensity bands for thresholding of the image, and next summing two resulting images (Fig. 3.3 b). Another solution of such problem can be based on the fuzzy logic methods, described below (Section 3.2.5).
3.2.2 Median filter

The obtained image often contains undesired information (noise). It can appear not only during image registration, but also as a side-effect after applying various image transformations.

In order to reduce impulse noise effects in the image, the median filter is widely used. The median filter processes each pixel in the image in turn and analyses its nearby neighbours to decide whether it is representative to its surrounding. Instead of simply replacing the pixel value with the mean of neighbouring pixel values (like mean filter does), it replaces it with the median of those values. The median is calculated by sorting all pixel values from the surrounding neighbourhood into numerical order and then replacing the pixel being considered with the middle pixel value (Fig. 3.4).

![Fig. 3.4. An example of the median and the mean values estimation.](image)

<table>
<thead>
<tr>
<th>123</th>
<th>125</th>
<th>126</th>
<th>130</th>
<th>140</th>
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<tr>
<td>111</td>
<td>116</td>
<td>110</td>
<td>120</td>
<td>130</td>
</tr>
</tbody>
</table>

**Neighborhood values:**
115, 119, 120, 123, 124, 125, 126, 127, 200

**Median value:** 124
**Mean value:** 131

Actually, using the median filter, not only the nearest neighbours (3 x 3 analyzed field), but also further ones (5 x 5, 7 x 7 fields, etc.) can be chosen, as it can be seen in Fig. 3.4.

The figure presents the median applied to the cell image (after preliminary thresholding with different intensity bands) using different analyzed fields. The median applied to the original (grey scale) image has not given the significant result (only the median 7 x 7 has resulted in slight blurring of the image details). The application of the median to the binary images has efficiently removed the debris both inside and outside of the cell.
From the results obtained by the median and mean filters (Fig. 3.4), one may conclude that the median filter has two main advantages over the mean value filter. First, analyzing small (one pixel) debris, a single very unrepresentative pixel in a neighbourhood has not affected the median value significantly, since the median is a more robust average than the mean. Second, analyzing the pixels at the edges, the median filter has not created new unrealistic pixel values, since the median value must actually be the value of one of the pixels in the neighbourhood. For this reason the median filter is much better at preserving sharp edges than the mean filter.

Fig. 3.5. Large binary image (610 x 640 pixels) of cells before (a) and after (b) application of median filter (with 3 x 3, analyzed field).
Finally, the demonstration of the result of median application to the large binary image (610 x 640 pixels) of cells is shown (Fig. 3.5).

3.2.3 Opening transformation

The opening is an important operator from mathematical morphology. It is derived from the fundamental operations of erosion and dilation. The erosion of image I by a structuring element B can be expressed as the union of all centres of B, if the element has all pixels lying inside the I (see Fig. 3.6). Thus, those translates of B that have at least one pixel outside of I are removed from the image. In other words, the structuring element is being moved along the internal side of the image boundary and the subsequent positions of the element centres determine a new boundary of the image.

![Fig. 3.6. Erosion of the image I by the structural element B.](attachment:image)

The dilation is the inverse of the erosion operation. The structuring element is being moved along the external side of the image boundary and the subsequent positions of the element centres determine a new boundary of the image. The opening can be defined as erosion followed by dilation using the same structuring element for both operations. Thus, opening by a structural element is provided in such a way that moving the element inside an object, along the internal side of its boundary, all parts of the object where the element cannot reach are removed. All three operators (opening, erosion, and dilation) are normally applied to binary images.
Analysis of the opening transformation by the circular structuring element with different radius value has been performed for the binary images of cells before (Fig. 3.7 a) and after (Fig. 3.7 b) the median filter application. It has been shown that, in both cases, opening has effectively removed small objects (smaller than the structuring element) and has separated narrowly connected objects (if the width of the junction between the objects is smaller than the size of structuring element).

Fig. 3.7. Opening transformation by circular structuring element (with R = 3 and R = 8 pixels) applied to two different input images: binary image of cells (a), the same image after 3 x 3 median filter (b).

However, in the first case (Fig. 3.7 a), the opening transformation has removed too many true objects. Such effect happens due to the presence of small size debris inside the objects after original image thresholding. This debris prevents the structuring element to fit into the object, resulting in removing of the whole object. In the case when median has been applied before opening transformation, such debris has been preliminary removed (by median), thus, the above-described effect has not occurred.
Therefore, in the case of cell recognition, opening by circular structuring element has been chosen as ancillary to the median filter method. It is used, if required, to separate narrowly connected objects (cells) and to remove small objects, which are smaller than cells but bigger than debris removed by the median filter.

The exact operation of opening is determined by the structuring element. It may lead to an interesting effect of the opening transformation by a circle structuring element. If the circle size is close (not bigger) to the cell size, the opening transformation may preserve the objects regions that have a similar shape to the structuring element (circle), or that can completely contain the structuring element, while eliminating all other regions of object pixels. Such effect can be observed in Fig. 3.7 (transformation using a circle with R = 8).

3.2.4 Edge detection

Some methods require not all information about recognizing objects, but only information about the objects edges. The edges of the objects contain information about objects location, their shape and size. An edge is a place, where the intensity of an image changes from a low value to a high one (or vice versa).

There is an infinite number of edge orientations, widths and shapes (Fig. 3.8). Some edges are straight while others are curved with varying radii.

![Image of different types of edges](image)

Fig. 3.8. Some types of edges: roof edge, line edge, step edge, and ramp edge (from left to right).

Many edge detection techniques are based on so-called convolution method. The idea of the convolution is that a sliding window (called the convolution window) centres on each pixel in an input image and generates a new output pixel. The new pixel value is computed by multiplying each pixel value in its neighbourhood with the corresponding weight in the convolution mask (kernel) and summing these products. If \( K(x, y) \) is an \( (m \times n) \) kernel and \( I(x, y) \) is an image, then the convolution of \( K \) with \( I \) is written as
\[ C(x, y) = K(x, y) * I(x, y) = \sum_{i=0}^{m-1} \sum_{j=0}^{n-1} K(i, j) I(x + i, y + j). \] (3.1)

Often the kernel is not allowed to shift off the boundary of the image, so the resulting image is normally smaller than the first one. For example:

\[
\begin{pmatrix}
  1 & 0 \\
  0 & 1 \\
\end{pmatrix} \times
\begin{pmatrix}
  1 & 1 & 3 & 3 & 4 \\
  1 & 1 & 4 & 4 & 3 \\
  2 & 1 & 3 & 3 & 3 \\
  1 & 1 & 1 & 4 & 4 \\
\end{pmatrix}
= 
\begin{pmatrix}
  2 & 5 & 7 & 6 & x \\
  2 & 4 & 7 & 7 & x \\
  3 & 2 & 7 & 7 & x \\
  x & x & x & x & x \\
\end{pmatrix}
\]

Fig. 3.9. An example of applying kernel \( k \) to image \( I \) with result \( R \). Where \( x \) is no value. Here the \( 2 \times 2 \) kernel is applying to a \( 4 \times 5 \) image, giving \( 3 \times 4 \) results. For example, the value 5 in the result \( R \) is obtained from \( 5 = (1 \times 1) + (0 \times 3) + (0 \times 1) + (1 \times 4) \).

For edge detection, the following kernels are usually used:

- **Prewitt**
  \[
  \begin{pmatrix}
    1 & 1 & 1 \\
    0 & 0 & 0 \\
    -1 & -1 & -1 \\
\end{pmatrix}
  \quad \text{and} \quad 
  \begin{pmatrix}
    -1 & 0 & 1 \\
    -1 & 0 & 1 \\
    -1 & 0 & 1 \\
\end{pmatrix}
\]

- **Sobel**
  \[
  \begin{pmatrix}
    1 & 2 & 1 \\
    0 & 0 & 0 \\
    -1 & -2 & -1 \\
\end{pmatrix}
  \quad \text{and} \quad 
  \begin{pmatrix}
    -1 & 0 & 1 \\
    -2 & 0 & 2 \\
    -1 & 0 & 1 \\
\end{pmatrix}
\]

- **Lapassian**
  \[
  \begin{pmatrix}
    1 & 1 & 1 \\
    1 & -8 & 1 \\
    1 & 1 & 1 \\
\end{pmatrix}
\]

Each of the edge detection kernels can be applied both before and after any other image processing procedure. The analysis of the resulting images obtained after application of the edge detection to different input images (original, binary, and median processed binary images) has been performed (Fig. 3.10 a, b-c, and d respectively). It has been noticed that the edge detection has achieved the best result if it has been applied after binarization and the median filtering (Fig. 3.10 d).
In order to make a comparison between the effects produced by the three above-mentioned kernels (Prewitt, Laplasian, Sobel), the same median filtered binary image (Fig. 3.11 d) has been processed using these kernels. The resulting images are presented in Fig. 3.11.

The sharp thin edge of the object has been detected using Prewitt kernel; though the obtained edge is not solid (it has some gaps – missing pixels). Laplasian has resulted in a solid edge, but it has added some noise (grey pixels around the edge line) to the image. Sobel edge detection has no such disadvantages. The edge line obtained by applying the Sobel is a little bit thicker than those obtained using two other kernels.

There are many edge detection techniques to go with different types of edges, each having its own strengths. Some edge detectors may work well in one application
and perform poorly in others. Thus, it is human operator’s responsibility to determine what is the best edge detection technique for the given application.

### 3.2.5 Fuzzy Logic edge detection

The thresholding→edge detection sequence has not shown sufficient result for some kind of images often obtained in our experiments (Fig. 3.12 a). This case can be explained in a following way. The presented images have been obtained using the illuminating light falling not perpendicularly to the sample plane. Therefore, cells are observed as the couples of light and dark borders. Thus, the one band thresholding can make visible either dark or light parts. Evidently, such images could be processed using the two-band thresholding in such a way, that the colour of the analyzing pixel is changed to the object colour if its original colour belongs to one of the bands; otherwise, it is changed to the background colour (Fig. 3.12 b). Unfortunately, such solution has the disadvantages mainly caused by the need to predefined four parameters, which determine the two-band thresholding. First, it is difficult (almost impossible) to use the same parameters for images that slightly differ by the intensity distribution value (e.g. such difference can be observed if one image is taken closer to illumination source than another one); then, the parameters must be found for each type of images separately. Second, the two-band thresholding is ineffective for the images with the irregular background (e.g. in the case, when there is a light field in the middle-right part of the image, see Fig. 3.12 a).

The most appropriate solution for such problem is the Fuzzy Logic edge detection (FLED). FLED is based on the analysis of the neighbourhood pixels. The developed FLED model that differentiates two classes (edge and background) is realized in a following way.

1) The $p_0$-$p_8$ pixels are analyzed:

\[
\begin{array}{ccc}
  p_1 & p_2 & p_3 \\
  p_4 & p_0 & p_5 \\
  p_6 & p_7 & p_8
\end{array}
\]
The grey-level difference magnitudes between the analyzing pixel \( p_0 \) and its neighbors (\( p_1-p_8 \)) designated by feature vector \( \bar{X} = [X_1, X_2, ..., X_8] \) are calculated as 
\[
X_i = p_i - p_0.
\]

2) Using the user defined low (\( L \)) and high (\( H \)) constants to substitute 0 and 255 grey values correspondently, the feature vectors for these classes are

- class background : \( \bar{B} = [L, L, L, L, L, L, L, L] \),
- class edge : \( \bar{E} = [H, H, H, H, H, H, H, H] \).

3) The fuzzy membership functions by radius \( \beta \) for these classes with Epanechnikov functions (Looney C.G., 2001; Liang L.R. and Looney C.G., 2003) are defined as

- class background : \( b = \text{Max} \left( 0, 1 - \frac{\parallel \bar{X} - \bar{B} \parallel}{\beta} \right) \),
- class edge : \( e = \text{Max} \left( 0, 1 - \frac{\parallel \bar{X} - \bar{E} \parallel}{\beta} \right) \).

4) The maximum fuzzy truth-value of the fuzzy set of two membership functions (\( b \) and \( e \)) is the so-called winner, which determines the class of the pixel.

A smaller \( H \) value yields more sensitivity to edges (and displays more noise), whereas a larger \( L \) value maps results in greater number of the weak edges to the background. Any \( \beta \) value greater than 200 appears to yield similar results.

The performed comparison between Fuzzy edge detection and thresholding→edge detection sequence is shown in Fig. 3.12.
Fig. 3.12. A comparison between the results of Fuzzy logic edge detection and thresholding→edge detection sequence. a) Original image and the same image after application of b) thresholding, c) thresholding→edge detection sequence, d) Fuzzy edge detection.

It is clearly seen that the fuzzy technique is more effective for this particular type of images than its opponent with comparable calculation time (about 1 s). The big thickness of the obtained edges (Fig. 3.12 d) in comparison with those in Fig. 3.12 a could be a disadvantage of FLED, but it can be improved due to varying the $H, L, \beta$ parameters.

3.2.6 Hough transform

The Hough transform (Hough P.V.C., 1962) is a technique which can be used to recognize features of a particular shape within an image. The classical Hough
transform is most commonly used for the detection of regular curves such as lines, circles, ellipses, etc.

**Hough transform for line detection.** The simplest example of the Hough transform is used to detect straight lines. The main idea of line detection is to determine all the possible pixels of line \( l \) in the image, to transform all lines that can go through these pixels into corresponding points in the parameter space, and to detect the point \( p(\theta, r) \) in the parameter space that most frequently resulted from the Hough transform of lines in the image.

\[ r y \cos(\theta) + r y \sin(\theta) = r, \]  \hspace{1cm} (3.2)

where \( r \) is the length of the normal from the origin to the detected line \( l \) and \( \theta \) is the angle between the normal and the positive \( x \)-axis, the line is transformed to a single point \( p(\theta, r) \) in the parameter space Fig. 3.13.

Lines of any direction may go through one pixel (Fig. 3.14, left plot). In theory, the number of these lines is infinite. However, in practice this number is limited by the resolution of the analyzed image. Thus, the parameter space (Fig. 3.14, right plot) is not continuous, but it is rather represented by a rectangular array of cells. This array of cells is called the *accumulator array* \( A \), whose elements are *accumulator cells* \( A(\theta, r) \). For each pixel of a line, parameters \( \theta, r \) are determined, which represent lines of allowed directions going through this pixel.
Fig. 3.14. Set of lines passing through the same point in the image space \((x, y)\) and the corresponding set of points in the parameter space \((\theta, r)\). The set of points forms a sinusoidal curve in the parameter space.

Next, if a line represented by the equation [Eq. 3.2] is present in the image, the value of the accumulator cell \(A(\theta, r)\) will be increased as many times as the line is detected as a line possibly going through any of the line pixels. Thus, a line present in the image is detected as the high-valued accumulator cell in the accumulator array, and the parameters of the detected line are specified by the accumulator array coordinates. As a result, detection of the line in the image is transformed to detection of local maxima in the accumulator space.

In other words, through one pixel in image space \((x, y)\) we can draw a lot of lines which make a sinusoidal curve in the parameter space \((\theta, r)\), like it is shown in Fig. 3.14. Analyzing all pixels from the same line we obtain several sinusoidal curves (number of the curves is equal to the number of pixels in the line) which intersect in one common point, like it is shown in Fig. 3.15. Exactly this point contains the sought information about the line.
Fig. 3.15. Set of pixels forming a straight line \( l \) in the image space \((x, y)\) and corresponding set of the sinusoidal curves in the parameter space \((\theta, r)\). If all pixels belong to the same line then the sinusoidal curves intersect in the same point \( p \) corresponding to the line \( l \).

**Hough transform for circle detection.** The same procedure can be used to detect other features with analytical descriptions. For instance, in the case of circles, the parametric equation is

\[
(x-a)^2 + (y-b)^2 = r^2, \tag{3.3}
\]

where \( a \) and \( b \) are the coordinates of the centre of the circle and \( r \) is its radius.

Fig. 3.16. Circle with radius \( r \) and with the centre in the point \((a, b)\) in the image space \((x, y)\) and corresponding point \( p(a, b, r) \) in the three dimensional parameter space \((a, b, r)\).

In this task, the computational complexity of the algorithm starts to increase, since the parameter space has three dimensions (coordinates). The task can be simplified if the radius \( r \) of the sought circle is known. In that way, analogously to the line detection the set of the points corresponding to all possible circles passed through the same pixel \( p_1(a_1, b_1) \) in the image space forms a circle with radius \( r \) and with the centre in the point \((a_1, b_1)\). Thus, the intersection of such circles in the parameter space contains the sought information about the circle in the image space (Fig. 3.17).
Unfortunately, the radius \( r \) is often unknown. So, it is needed to provide the analysis of the three dimensional parameter space. If some pixel \( p_1(a_1, b_1) \) belongs to a circle, the locus of the parameters of this circle will be a right circular cone in the three dimensional parameter space (Fig. 3.18). It can be seen from the equation of a circle by treating \( x \) and \( y \) as fixed and letting \( a, b \) and \( r \) vary.

In this way, if a set of pixels in the image are arranged as a circle with parameters \( a, b \) and \( r \), the resultant loci of parameters for each of such pixels will pass through the same point \( (a, b, r) \) in the parameter space. Thus, a lot of such right circular cones
will intersect at a common point containing the sought information about the circle in the image space.

Additional improvement of the algorithm is based on the use of the directional information associated with the circle, so-called generalized Hough transform (Ballard D.H., 1981). For the point \( p(x, y) \), the centre of the circle \( c(a, b) \) must lie \( r \) units along the direction of the gradient. Formally, the circle involves three parameters, but using the Eq. 3.3 together with its derivative significantly reduces the computations as follows.

Let \( X = x - a \) and \( Y = y - b \), then Eq. 3.3 transforms into

\[
X^2 + Y^2 = r^2, \tag{3.4}
\]

Differentiating Eq. 3.4 with respect to \( X \), one obtains

\[
X + Y \frac{dY}{dX} = 0, \tag{3.5}
\]

hence

\[
X^2 = Y^2 \left( \frac{dY}{dX} \right)^2. \tag{3.6}
\]

Substituting Eq. 3.6 in Eq. 3.4, the \( X \) and \( Y \) values are obtained

\[
Y = \pm \frac{r}{\sqrt{1 + \left( \frac{dY}{dX} \right)^2}} \quad \text{and} \quad X = \pm \frac{r \frac{dY}{dX}}{\sqrt{1 + \left( \frac{dY}{dX} \right)^2}}. \tag{3.7}
\]

Finally, the circle centre can be found as

\[
a = x \pm \frac{r \frac{dy}{dx}}{\sqrt{1 + \left( \frac{dy}{dx} \right)^2}} \quad \text{and} \quad b = y \pm \frac{r}{\sqrt{1 + \left( \frac{dy}{dx} \right)^2}}. \tag{3.8}
\]

Thus, only one value of the circle centre position \( c(a, b) \) will correspond to the specific radius \( r \) if the value \( \frac{dy}{dx} \) is known. This solution reduces the parameter locus to a line, as shown in Fig. 3.18 (C).

Derivative \( \frac{dy}{dx} \) can be found directly from the analysed image. If this image may be described by a two dimensional function \( I(x, y) \) then, from the definition of the
derivative, in point \( p(x_i,y_i) \), \( \frac{dy}{dx} = \frac{I(x_i,y_i) - I(x_{i+1},y_i)}{I(x_{i+1},y_i) - I(x_i,y_i)} \). Further, the line in Fig. 3.18 (C) can be made shorter via the restriction of the radius of the sought objects, which also reduces the computation time.

Fig. 3.19. Hough transform illustration: (a, c) recognized circles (marked as grey crosses) found by the algorithm and (b, d) the corresponding parameter space.

The great advantage of the Hough transform is its powerful object detection (Fig. 3.19), even when working with poor quality images (e.g. images with a noise, images with the destructed or partially overlapped objects, etc.). On the other hand, the direct realization of this method is very time-consuming and the calculation time grows significantly with the increasing object’s degree of freedom. Therefore, in spite of the fact that searching for the elliptical shapes is more adequate for cell localization, it is reasonable to apply the Hough transform for circle detection.

For further improvement the performance of the code, several modifications of the Hough transform were coded in and tested experimentally, i.e. the direct Hough transform (HT, Hough P.V.C., 1962), the random Hough transform (RHT, McLaughlin R. A., 1962), the generalized Hough transform (GHT, Ballard D.H., 1981), and the random circle detection (RCD, Chen T.-C. and Chung K.-L., 2001). According to these tests, the fastest results were achieved by GHT. Unfortunately,
even this modification is time consuming for large images (about 35 seconds for 1.3 megapixels image using a Mobile AMD Sempron(tm) 3000+ processor, 1.8 GHz). The time performance of some of these algorithms is shown in Fig. 3.20.

![Graph showing time performance of full recognition processing based on GHT, RHT, and clustering.](image)

As an alternative fast method, a clustering has been developed (see Section 3.2.7).

### 3.2.7 Clustering

This algorithm is based on the erosion method with the defined threshold value and the circle with radius of \( r = 1 \) pixel as a structuring element. In the clustering algorithm, erosion is iterated until the whole object is eroded. All eroded neighbouring pixels are collected in a cluster. This cluster defines the area of the sought object.

The clustering has some advantages over the Hough transform: i) it is several times faster (only about 8 s vs. 35 s, correspondently for 1.3 megapixel image using the same PC), ii) the time of calculation is not affected by the object shape. However, its disadvantages are also serious. Particularly, the clustering may interpret the noise as an object and treat the overlapped objects as a single object (see the results in Fig. 3.21). This method requires additional processing of the image, such as filtering of recognized objects by the given size, shape, and other parameters.
Fig. 3.21. The example of the clustering applied to the different originals (a and b). White crosses correspond to the centres of the recognized clusters (black objects).

3.2.8 Final algorithm

As it has been mentioned above, the idea of cell recognition is based on the applying a sequence of consecutive image transformations to obtain specific information (e.g. the coordinate of the centre, size, orientation etc.) about the sought objects (cells). It is worth to notice that a universal way (sequence) to get directly the required result does not exist. The methods to be used and their order in such sequence are often determined by the key method.

Since biological cells have a shape close to circular (ellipsoidal) one, the sequence can be based on Hough transform for circle (ellipse) detection. In this way, the whole algorithm (Fig. 3.22) for original image (a) consists of thresholding (b), median filter
(c), opening transformation (d), Sobel edge detection (e), and, finally, the Hough transform (f).

Fig. 3.22. An example of cell recognition sequence (a→f) based on Hough transform. An output image of each step is an input for the next step. For demonstration purposes, only a small portion of the full image is shown.

The image analysis for this sequence has been provided basing on the above-described methods in the following way. The best performance of the Hough transform has been achieved using an image with objects edges as an input image. So, the method used before the Hough transform must be one of edge detection techniques (in our case it is the Sobel edge detection). Most of the techniques have shown certain restrictions:

– if an input image is a greyscale one then the result image does not contain clearly (sharply) defined edge information (Fig. 3.10 b);

– noise in an input image significantly reduces the effectiveness of the edge detection (Fig. 3.10 c).
Thus, before the edge detection two steps must be applied, \textit{i.e.} the thresholding (binarization) and the noise reduction. Since the noise can appear both during image registration and after the thresholding, the noise reducing method should follow the thresholding. For the noise reduction, two techniques have been chosen. These are the median filter used to remove impulse noise from the image and the opening, which is ancillary to the median filter method. The opening has been used to separate narrowly connected objects and to remove small objects that are smaller than cells, but bigger than debris removed by the median filter.

In this way, the highest effectiveness of the cell recognition has been achieved using the presented methods applied in the following order: thresholding $\rightarrow$ median filter $\rightarrow$ opening transformation $\rightarrow$ Sobel edge detection $\rightarrow$ Hough transform.

Additionally, analyzed above sequence can be based on the Hough transform either for the circle or for the ellipse detection depending on the shape of the cell to be recognized. However, the time consuming of calculations is significantly higher for the ellipse detection than for the circle detection. Thus, it has been proposed to use the Hough transform for the circle detection even when cells have ellipsoidal shapes. It can be sufficient if, before proposed Hough transform, one applies the opening by the circular structuring element with the radius slightly less than the minor ellipse diameter. This solution uses the effect of opening to preserve objects’ regions that have a similar shape to the structuring element. Thus, it certainly changes ellipsoidal object shape to circular one, but does not change the centre of these shapes.

Similar analysis has been carried out for sequence based on the clustering method. Since the clustering requires all information about objects (not only the edge information), the Sobel edge detection has been omitted. All other argumentation is the same as described above. Thus, the final order is as follows: thresholding $\rightarrow$ median filter $\rightarrow$ opening transformation $\rightarrow$ clustering.

Alternatively, the sequence with the clustering method can be realized using the fuzzy logic edge detection. Sometimes FLED does not give the images of the whole cells. Therefore, in order to connect separate parts of the same cell, it must be
supplemented by the closing transformation, which is reverse to the opening operation. Thus, the final sequence is as follows: fuzzy logic edge detection $\rightarrow$ median filter $\rightarrow$ closing transformation $\rightarrow$ clustering (Fig. 3.23).

Fig. 3.23. An example of cell recognition sequence (a→e) based on the clustering and fuzzy logic edge detection techniques. An output image of each step is an input for the next step.

This sequence can be calculated much faster than the sequence based on the Hough transform, because the complexity of the clustering calculations does not depend on the object shape. However, its disadvantages, such as treating of the overlapped objects as a single object, are strengthened via splitting the closely spaced
objects by the closing transformation. Thus, this sequence is recommended to be applied in a case of the images with a low cell density only.

3.3 Software for automatic cell recognition

The previous section has shown the techniques (and certain sequences of their application) for the automatic cell recognition. Nevertheless, there is no single universal algorithm for such task, since any small changes in the optical environments can introduce significant changes in the obtained image of cells. Such crucial effects can be induced due to changing the type of the cell culture, medium, Petri dish design, parameters and location of the illumination source (even changes in voltage applied to the source), and many other conditions.

Though the image processing operates with a large number of image transformation procedures (filters, logical operations, morphological transformations etc.), it is difficult to predict which procedure will be useful or useless for future tasks or for future environments (conditions).

The main requirement for the developed software is to provide a flexible and easy-to-operate (convenient) way for different cell recognition tasks. This requirement must be realized at two planes – as a convenient graphical user interface (GUI) and as advanced mathematics.

The software has been developed for the Microsoft Windows XP platform. Windows hosted applications, due to uniform user-friendly GUI and functional standardisation, have many advantages in data visualisation, image manipulation, data storage and printing. Additionally, Windows XP has built-in the .Net Framework (www.microsoft.com/net) that includes C# (C Sharp) programming language compilator, which has been one of the reasons to choose this programming language for realisation of the program. It has reduced unnecessary costs of buying software for program development. Applications written in C# can be modernized using any Windows PC with .Net Framework (even Windows 98) or Linux PC with Mono Framework (www.mono-project.com). Moreover, C# allows using such advanced
techniques as *Reflection*, *Dynamic code compilation*, etc. (Robinson S. *et al*., 2004; Liberty J., 2005), which have not been realized in other programming languages.

The program GUI (Fig. 3.24) has been designed as window container (MDI) for three main types of windows: image visualisation windows (A), information windows (B) and code editor window (C). This design allows using large number (limited only by the computer RAM) of such windows easily switching between them via tabs.

*Image visualisation windows* (Fig. 3.24 A) have been used for the presentation of the registered image and all image changes due to application of transformation procedures, which helps to estimate the effectiveness of image processing. Such estimation may show the necessity of applying certain procedures to current tasks and may help to determine the most effective parameters for the procedure.

*The information windows* (Fig. 3.24 B) have been developed as an additional way to improve and to make easier the estimation of the effectiveness of the chosen procedure. They have been designed to present and visualize diverse information about current image (“Img Info” tab) as well as about current recognized objects (“Obj Info” tab). The information in these tabs is updated after any user action that changes it. Generally, this information is based on statistical data such as image intensity (object size) distribution, value and position of the highest peak, mean and median values *etc*.

The key possibility of the software, which uses the full power of the C# programming language, has been realized in the *code editor window* (Fig. 3.24 C). This is the *dynamic code compilation*. It means that user can write any programming code, compile it and run inside the cell recognition program. It is some kind of macros editor, but with full functionality of C# language. The very technique allows, in a flexible way, creation, modernization, edition and enahcing image processing sequences to perform either the simplified processing (for fast preliminary investigation) or compound sequences of image transformations (for precise cell recognition).
Fig. 3.24 The screenshot of the cell recognition program: A) image visualization window, B) information window, C) code editor window.
The possibility to store the successfully applied codes for later use facilitates the processing in the case of multiple experiments carried out under the same environments.

The code editor window has been designed as the code editor field (Fig. 3.24 C, left white field), the list of already realized image processing procedures (Fig. 3.24 C, white field in the middle) and the field with user instruction about current selected item in the list of procedures (Fig. 3.24 C, grey field). Existing procedures can be easily inserted with default parameters to the code editor field by mouse double-click on desired item in the list of procedures.

About 70 image processing procedures have been realized for the mathematical part of the software. The most commonly used procedures are presented in Table 3.1.

<table>
<thead>
<tr>
<th>Add</th>
<th>Dilatation</th>
<th>Intersect</th>
<th>Rotate</th>
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<tbody>
<tr>
<td>Blur</td>
<td>Edges</td>
<td>Invert</td>
<td>SaturationCorrection</td>
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<tr>
<td>BrightnessCorrection</td>
<td>Erosion</td>
<td>LevelsLinear</td>
<td>Sepia</td>
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<tr>
<td>Closing</td>
<td>GammaCorrection</td>
<td>MaskedFilter</td>
<td>Sharpen</td>
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<tr>
<td>Clustering</td>
<td>Gaussian</td>
<td>Mean</td>
<td>Shrink</td>
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<tr>
<td>ContrastCorrection</td>
<td>GHT</td>
<td>Median</td>
<td>SimpleSkeletonization</td>
</tr>
<tr>
<td>Convolution</td>
<td>Greyscale</td>
<td>Merge</td>
<td>SobelEdgeDetector</td>
</tr>
<tr>
<td>Correlation</td>
<td>HitAndMiss</td>
<td>Opening</td>
<td>Subtract</td>
</tr>
<tr>
<td>Crop</td>
<td>HSLFiltering</td>
<td>Pixellate</td>
<td>Thickening</td>
</tr>
<tr>
<td>Difference</td>
<td>HSLLinear</td>
<td>Resize</td>
<td>Thinning</td>
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<tr>
<td>DifferenceEdgeDetector</td>
<td>HueModifier</td>
<td>RHT</td>
<td>Threshold</td>
</tr>
</tbody>
</table>

The image processing procedures have been enhanced via additional procedures for filtration of already recognized objects by size, shape, etc. This possibility is often used in cell recognition sequences based on clustering technique.

3.4 **Algorithm of automatic cell irradiation with SIHF**

The cell irradiation process requires a precise interaction between hardware and software. The crucial aspect of this interaction is a spatial correspondence (consistency) of the positioning system, the optical system and the beam location.
3.4.1 Stage and Optical system calibrations

Stage calibration. In spite of the high precision of the positioning stage (0.1 µm), it has such inconvenient feature that after switching it on the current position is accepted as the origin. Thus, a special algorithm for the determination of the stage origin has been developed. The algorithm uses the possibility the stage to move relatively to the current position until it reaches the border (i.e. current position stops changing). In this way, all four stage borders that define the region of the stage motion are determined. After that, at any time the stage origin can be set in the same location, e.g. in the centre of the stage motion region, in the top-left corner of the stage motion region (like in computer coordinate system), etc.

Optical system calibrations. Cell coordinates, previously determined using the automatic cell recognition, define a map of targets. Just after the recognition process, the map is still in pixel units, not suitable for the positioning table control program. In order to transform these coordinates to micrometers, an image calibration (pixels to micrometers) has to be performed. For this purpose, two methods have been proposed. The first one is a conventional calibration using an optical standard placed in the plane of a sample. The second method is more sophisticated: it uses a diaphragm and benefits from the high precision of the positioning stage (0.1 µm). The procedure requires taking three photographs of the diaphragm aperture (Fig. 3.25).

![Fig. 3.25. The schema of the on-line optical calibration using the stage movement.](image)

The first recorded image is used as the origin. The second image, taken after moving the stage along X axis to a certain distance (e.g. 300 µm rightwards), is used for horizontal calibration. The third one, taken after moving the stage along Y axis (e.g. 200 µm downwards), is used for vertical calibration. The XY calibration is
based on the known distances (in pixels and µm) between the centres of these three diaphragm aperture images.

The Hough transform for circle detection has been proposed to be used for precise determination of these aperture centres in the images. The automatic method obtains, at any time, the same diaphragm centre in comparison to any manual method, e.g. there is a difficulty to select twice the same pixel in the image by a computer mouse. At first glance, for the precise determination of the beam position and the precise calibration, the best choice is the smallest diaphragm. However, the proposed method has achieved the best result with 20 µm diameter diaphragm, due to the fact that the more bright pixels correspond to the diaphragm aperture, the more precisely the Hough transform can detect it.

3.4.2 Beam position determination and Beam profiling

Beam position determination. In order to deliver an ion to the defined place (target), not only the target location, but also the beam position in the plane of a sample must be determined. The preliminary beam position is obtained as follows. The experimental chamber is rotated by 5° from its axis and the beam is focused on a quartz window located off-axis of the experimental chamber (2 cm left to the ion exit window, see Fig. 2.5). The region of quartz with a bright spot (corresponding to the beam spot) is imaged using the on-line optical system. The coordinate of the spot centre is determined, stored and used for the subsequent transformation of cell position relatively to the beam position. Finally, the chamber is rotated back to the central position to allow the ions passage through the exit window.

However, the use of the quartz window defines well the desired beam position, only if the beam falls strictly perpendicular to the sample plane. Any imperfections of the relative directions cause the shift of the beam position in the sample plane. Therefore, a more precise method for beam localization has been developed. This method implies the following steps: i) the region of the beam spot location is preliminary determined using the quartz, as described above; ii) this region is automatic scanned using a diaphragm (1.5 cm diameter) having an aperture (5 µm
diameter) placed in the plane of the sample; iii) the particle detector is located behind the diaphragm; iv) the position of the diaphragm corresponding to the strongest signal registered by the detector is determined (such position corresponds to the beam spot location); v) the image of the diaphragm aperture in the determined position is taken; vi) the coordinate of the aperture centre in the image is determined either manually or using the Hough transform; vii) this coordinate is stored and used for the subsequent transformation of the cell position (usually obtained relatively to the top-left corner of the image) relatively to the beam position. The difference between the beam spot positions determined optically using the quartz and measured using the scanning method can reach even 60 µm, which shows the importance of this method.

**Beam profiling.** In order to obtain the profile of the beam, the beam region is automatically scanned using a diaphragm (1.5 cm diameter) having an aperture (5 µm diameter) placed in the plane of the sample in the same way as described above. The scanned data are used to determine the beam spatial parameters, such as beam form, beam direction, etc.

### 3.4.3 Final algorithm

The whole algorithm of the several successive cell irradiation experiments consists of two phases (Fig. 3.26). The first one is the phase of preliminary beam settings that takes place only once before two other phases. The settings include beam adjustment and focusing on the quartz to obtain the beam in the required place (e.g. in the centre of the outlet window) and with required parameters (e.g. 1000 ions/s). Also in this phase, the moving stage and the on-line optical system are calibrated (see Section 3.4.1).

Steps (actions) of the second phase are applied as many times as many cell dishes must be irradiated. First of all, before placing the next cell dish, the current beam position is determined. After each irradiation experiment the beam position is checked to be the same as before the experiment. Next steps of the second phase are: i) turning beam off in order not to affect the cells by accident, ii) cell dish placement on the moving stage, iii) taking the image of cells, iv) cells recognition, v) setting the
required number of irradiating ions per cell, and vi) start of the single dish processing.

Fig. 3.26. Flowchart of the cell irradiation algorithm.

The single dish processing deals with every cell on the current cell dish and includes the following steps: i) moving the stage to the next cell position, ii) turning
the beam on, iii) waiting until the detector registers the chosen number of ions, and iv) blanking the beam.

The alternative, but far less accurate method to control the number of ions, applied in case of thick samples, i.e. when the ions are not able to reach the detector, uses turning the beam on for a certain period of time dependent on the beam current, when the average number of the bombarding ions is equal to the chosen value.

3.5 Software for automatic cell irradiation with SIHF

For the most efficient realisation of the above-described algorithm, most of the steps must be carried out or controlled by a computer. Therefore, taking into account the specificities and uniqueness of the developed SIHF and the assigned task, the specialized software has been designed.

A specificity of our SIHF is that it has been based on the Cracow MB, which is the fully operational setup at the present time. In order to prevent a possible conflict between these two systems and to provide an easy switching between systems, the special two-computer control system has been developed. The main idea of such system is to separate the hardware control from the image acquisition, analysis and processing (three tasks that require an exclusive use of a modern, powerful computer). In addition, one standard computer does not have enough slots and connectors to plugin all control cards required by the CMB and the SIHF. The schema of the developed two-computer control system for the automatic cell irradiation is presented in Fig. 3.27.

The schema presents the interactions between the hardware part of the SIHF (the top of the schema) and the two-computer control system consisting of the “manager” and “executor” computers. The executor has a direct access to all parts of the SIHF hardware (the blanking, positioning and detection systems) except the optical system, which is connected to the manager. On the other hand, the executor has a minimal graphical user interface with limited functionality available to the user (SIHF operator). Its task is executing the commands obtained from the manager after checking their appropriateness. In this way, it hides (protects) the direct access to the SIHF from a human factor.
The brain of the control system is the manager. This powerful computer is responsible for the image acquisition, cell recognition and analysis of the data received from the executor. It also provides a convenient graphical user interface for all these tasks.

The communication between computers has been realized by means of the standard network connection using the TCP protocol and the “client-server” model. For the SIHF control software, this model has been realized in such a way, that both computers run both the client and the server (see Fig. 3.27). The clients are created in the case when there is a need to transfer some data from one computer to another (see Fig. 3.28). Moreover, the manager’s client transfers the data that contain only a command or a command sequence for the executor, while the executor’s data contain either information resulting due to those commands’ execution or information about the current state of the SIHF.
Fig. 3.28. The schema of the communication between the manager and the executor including the client-server and the multithreading models.

The servers always exist and “listen” if the client from the other computer has anything “to say”. If any server receives some information, then it checks its integrity. In the case this information contains the executor’s command, the command
is checked for its appropriateness to be implemented in the current moment. After that, the server sends back a short receipt (report) about the successful (unsuccessful) data receiving.

To make the SIHF software more effective, all main functions are performed in the separate program threads (the so-called multithreading model). It allows almost simultaneous (parallel) execution of the functions and, in this way, it prevents the software from the hang-up.

The GUI for the SIHF control has been developed as an extension of the cell recognition software via its additional tab (it is situated in the field B, see Fig. 3.24). Fig. 3.29 is the screenshot presenting this tab. The realized GUI provides both manual and programmable SIHF controls and supports the following features:

1) optical system calibration via stage movement (see Section 3.4.1, Fig. 3.29, item 1);
2) measurement of the selected object (cell) on the current image (Fig. 3.29, item 2);
3) manual control of the stage movement with a possibility to define a motion step (Fig. 3.29, item 3);
4) generation of the targets’ map using the data of recognized cells or addition of some cells to the map, already generated (Fig. 3.29, item 4);
5) possibility to generate different patterns for a broad beam simulation, beam profiling, etc. (Fig. 3.29, item 5);
6) selection of the irradiation mode (Fig. 3.29 item 6); three modes (pulse/time/scan) are available: “pulse” is a standard technique for controlling the number of irradiating particles via counting the pulses from the detector; “time” is the above-mentioned method to control the number of ions irradiating thick samples; “scan” mode, in combination with a grid pattern, is used for a broad beam simulation or beam profiling (see Section 3.4.2);
Fig. 3.29. The screenshot of the SIHF control tab in the cell recognition program.

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<td>image calibration</td>
<td>2</td>
<td>measurement of selected object on current image</td>
<td>3</td>
<td>manual stage movement control</td>
</tr>
<tr>
<td>4</td>
<td>irradiate or add and irradiate recognized cells</td>
<td>5</td>
<td>pattern generator</td>
<td>6</td>
<td>irradiation mode selector</td>
</tr>
<tr>
<td>7</td>
<td>open/save current settings/patterns/results</td>
<td>8</td>
<td>communication log</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>stage position map</td>
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1. Image calibration
2. Measurement of selected object on current image
3. Manual stage movement control
4. Irradiate or add and irradiate recognized cells
5. Pattern generator
6. Irradiation mode selector
7. Open/save current settings/patterns/results
8. Communication log
9. Stage position map
7) possibility to save the current settings, patterns, scanned data, etc. and to use them later in the similar environments (Fig. 3.29, item 7);

8) communication log that reflects the interaction between the manager and the executor (Fig. 3.29, item 8);

9) mapping of the stage positions (Fig. 3.29, item 9) that roughly shows the current position (red marker) of the moving stage.

The designed software has a number of features that have no visual realization, but are, nonetheless, very important. These are, for instance, an automatic monitoring of critical parts of the code, execution of which may crash the program, a sequential saving of a log file, an automatic saving (storage) of the information about the experiment (including the experiment settings), original cell images and corresponding images with the recognized cells, the targets’ maps in units of the stage coordinate system, etc.

### 3.6 SIHF accuracy verification

Before irradiation of living cells, several tests and control experiments have been carried out to check each part of the system and its overall performance. The results shown in this section present the performance of the whole system, assuring the close cooperation of the hardware and the software. Since the task of the SIHF is to deliver the preset number of protons to the exact location, two main parameters, that fully characterize the assigned task, are the precision of the protons number control and the targeting accuracy.

**Protons number control.** For the precise proton number controlling, the main requirement is that the reaction time of the blanking system has to be less than the time between two consecutive protons reached the detector. This reaction time has been measured as a difference in time between two events: a signal on the particle detector and the appearance of the high voltage on the deflection plates. The reaction time has been determined to be of 20 µs, which corresponds to the beam current of about 50000 ions/s.
In order to measure the blanking system effectiveness, the following experiment was carried out at low beam currents ($10^3$–$10^4$ ions/s): i) in the single ion mode, the beam was switched off after each proton has reached the detector, ii) simultaneously, an additional subsystem was steadily registering the protons passing to the detector, iii) the ratio between the required number of protons and the number of protons actually passed to the detector determined the effectiveness of the blanking system. Such experiment has been repeated several times for different beam intensities (Fig.3.30).

![Graph showing the effectiveness of the blanking system for different beam intensities.](image)

Fig. 3.30. The effectiveness of the blanking system for different beam intensities.

At large ion intensities, there is a chance that some unwanted ions will pass to the detector due to insufficient performance of the blanking system. As it has been measured for beam intensities of up to 1000 protons/s and blanking triggered after the detection of every single ion, the effectiveness of the blanking system is not worse than the limit of 0.98. In such case, for every 100 protons only two unwanted ones may pass to the detector, which is an acceptable performance for the purposes of the planned experiments.
**Targeting accuracy.** The targeting accuracy of the SIHF is mainly determined by the beam resolution, which can be measured using the beam profiling technique (see Section 3.4.2). An example of the measured beam profile is presented in Fig.3.31.

![Beam profile](image)

*Fig. 3.31. An example of the beam profile (~200 µm from the exit window).*

The full width at half maximum (FWHM) of the beam has been obtained from the standard deviation $\sigma$ of the Gaussian fit of the beam profile: $FWHM = 2\sigma\sqrt{2\ln 2}$.

An example displayed in Fig.3.31 shows that the beam FWHM measured outside the vacuum chamber, 200 µm from the outlet window, is about 15 µm. It has been confirmed using a polymer CR-39 detector (Giacomelli G. *et al.*, 1997) in a following way: i) CR-39 has been placed in the sample holder on the moving stage, ii) grid pattern has been generated with a step of 100 µm, iii) the stage has been moved according the defined pattern, iii) in each point, the beam has been switched on for 3 ms, which corresponds to 3 protons per point for 1000 proton/s beam intensity, iv) CR-39 has been etched in NaOH (6.25 mol/dm$^3$) in 70°C, v) proton tracks have been observed on the CR-39 as the dark dots on the light background (Fig.3.32).
Taking into account the sizes of the irradiated targets, the obtained targeting accuracy of 15 µm is sufficient for irradiation of cells (cell size is about 20 x 60 µm, see Section 3.7). However, in a case of subcellular structures (e.g. the cell nucleus) chosen as irradiated targets, not all protons of such beam will target them precisely.

3.7 Modelling proton-cell interaction using the Geant4 tool-kit

The results of the studies of various radiation-induced effects are usually presented in dependence on the dose adsorbed in a cell (or nucleus). Therefore, in the case of targeted irradiation, the number of irradiating ions has to be recalculated into the units of dose (Gy) absorbed by the target.

It is worth to notice, that the cell nuclei become the main target in most radiobiological experiments (including those carried out with use of SIHF). It is due to the fact that radiation induces the most serious consequences for a cell in the very case of DNA helix damage. Thus, it is reasonable to study the radiation-induced effects in dependence on the dose adsorbed exactly in nuclei. However, the estimation of such dose is a difficult task if the targeting accuracy of SIHF does not allow the precise determination of the number of irradiating ions per nucleus. It is
difficult to find an analytical solution of such task, taking into account the stochastic nature of the occurring processes.

Therefore, in the present work, the estimation of the doses adsorbed in the cell and in cell nucleus has been proposed to be made via developing the theoretical simulation of the proton-cell interaction using the Geant4 code (Agostinelli S. et al., 2003). Geant4 is an object-oriented toolkit, written in C++, for the Monte Carlo simulation of the particles passage through the matter. It has been extensively used by nuclear physicists’ community for more than 20 years. In the toolkit, all physical processes, models and visualization modules are entirely accessible to the user. The last version of the Geant toolkit (G4) has included an additional low energy electromagnetic processes package (down to tens of electron-volts; Apostolakis J. et al., 1999), which has made it more applicable for the medical physics needs. Thus, Geant4 can be applied to simulate the particle-cell interaction assuming the conditions and parameters of our SIHF.

Description of the performed simulations. The Geant4 strictly determines the structure of the code being developed for the simulations of particle-matter interactions. The code must contain a run manager object, controlling the whole simulation process, and user designed special classes, describing the experimental setup (the geometries of elements and materials used) and the primary particle generator (primary beam characteristics). It must also contain a list of all particles that can be generated during the simulation process and all the physical processes describing the interaction of these particles with the matter (Wright D.H., 2006).

The construction of the simulated experimental setup closely repeats the construction of the end-station of our SIHF, starting from the outlet window and ending on a cell as the irradiated target. It includes the 200 nm thick outlet window (chemical formula Si$_3$N$_4$, density 3.44 g/cm$^3$), the 2.5 µm thick cell support made of polymer Mylar (formula C$_{10}$H$_{8}$O$_4$, density 1.397 g/cm$^3$) placed 200 µm from the window, and the cell (formula: H-60%, O-26%, C-11%, N-2.4%, density 1 g/cm$^3$,
Klyszejko-Stefanowicz L., 2002). The space around the elements of the simulated setup is filled by air.

Geant4 requires construction element shapes to be defined in an analytical form. The analytical description of the cell shape, however, is a difficult task. It becomes even more difficult if ones use the cells of intricate shapes (for instance, fibroblasts) as the irradiated targets. In order to simplify the analytical description of the biological cell, we have made the following assumptions. First, we have assumed that the simulated cell has an ellipsoidal shape. Next, taking into account, that in our experiments cells are deposited on a support, the assumed shape of the simulated cell has been defined as a half-ellipsoid. The cell nucleus has been assumed to be of ellipsoidal shape.

The dimensions of cells (fibroblasts used for the further radiobiological experiments) have been estimated from cell images obtained using the fluorescent microscope Olympus BX51 and atomic force microscope PSIA XE. The AFM images have been kindly granted by Dr. Małgorzata Lekka.

To obtain the width and the height of the cells and their nuclei, the fluorescent images of fibroblasts have been collected (two examples of the images are presented in Fig. 3.33). The cell width and height are of 17.6 ± 6.4 µm and 67.7 ± 17.3 µm, respectively, whereas the width and height of the cell nuclei are of 10.0 ± 1.4 µm and 18.0 ± 2.7 µm, correspondingly. To obtain these values, 20 cells were measured.

Fig. 3.33. Examples of (a) fluorescent and (b) AFM images of fibroblasts.
Average cell thickness has been measured from the AFM topographical images of fibroblasts (examples are presented in Fig. 3.33 and Fig. 3.34). The obtained average cell thickness is of $3.5 \pm 0.7 \, \mu m$ (estimated for 18 cells).

![Fig. 3.34. a) AFM topographical image of a fibroblast, b) two profiles corresponding to two different cell regions, marked in the AFM image.](image)

The cell nucleus thickness has been estimated as the difference between two thicknesses – the cell thickness and the thickness of the cell edge region ($0.4 \pm 0.1 \, \mu m$), where is certainly no nucleus there (see Fig. 3.34). The resulting average nucleus thickness is of $3.1 \pm 0.8 \, \mu m$ (estimated from a set of 18 cells).

The final construction of the simulated setup is presented in Fig. 3.35.

![Fig. 3.35. Final construction of the simulated setup.](image)
Figure 3.35 presents the outlet window, the cell deposited on the Mylar foil, and the cell nucleus shown inside the cell.

The primary particle generator is the next class defined by the user. It describes the primary characteristics of the simulated beam. The 2 MeV proton beam has been simulated. The position of the generated protons has been assumed to have a bivariate Gaussian distribution, which parameters have been estimated from the previous measurements of the beam profile (see Section 3.6).

Main physical processes describing the particle-matter interactions have been allowed in simulation, including the ionization, photo-electric effect, Compton scattering, Rayleigh scattering, gamma conversion, bremsstrahlung, and fluorescence of excited atoms (Wright D.H., 2006). The low energy electromagnetic package G4LOWEM and the electronic stopping power table ICRU R49p have been used (Allisy A. et al., 1993). The low energy data libraries cover the energies ranged from 100 GeV down either to 1 eV for Rayleigh and Compton effects, or to the lowest binding energy for each element for the photo-electric effect and the ionization, or to 10 eV for the bremsstrahlung.

The simulation package has been developed on an Intel Pentium 4 (3 GHz) personal computer running under Fedora Core 5 Linux system with installed GCC compiler 4.0.1, where the last version (4.8 patch 1) of Geant4 and its low energy extension have been installed. OpenGL and VRML drivers have also been installed for the visualization of the simulated interactions.

Results of the performed simulations. The main goal of simulation is to determine the correspondence between the irradiating number of protons and the dose deposited in the cell nucleus as well as in the whole cell. The obtained results are presented in Fig. 3.36. The assumed cell dimensions were of 68 x 18 x 3.5 µm and nucleus dimensions of 18 x 10 x 3 µm. The cell nucleus, situated in the centre of the cell, was selected as the target of irradiation.
The advantage of the simulations is not only a simple recalculation of a ratio between the number of protons to the absorbed dose. It also brings more detailed information about the energy (dose) deposited by every single proton in the specific place. Such information is very important when the beam spot size is less than the nucleus size. In this case, different entry points of protons and the corresponding different nucleus thickness cause different length of proton tracks (Fig. 3.37).

Fig. 3.36. Dependency of the dose deposited in the cell nucleus and the whole cell on the number of irradiating protons.

Fig. 3.37. The side view of the simulated passage of protons (blue lines) through the cell nucleus: (a) 50 simulated proton tracks and (b) 200 simulated proton tracks.
Thus, energy deposited in the cell nucleus by single protons has some distribution (Fig. 3.38). Therefore, cell (or nucleus) thickness becomes an important factor, as it determines the number of events along the track. Finally, it is worth to notice that simulations performed for 10000 protons aimed to the cell nucleus showed that 395 protons missed the target.

Fig. 3.38. The distributions of the energy deposited by single proton in the cell nucleus, cytoplasm and whole cell.

The energy loss of a proton in cell, obtained using the simulation, was about 47.5 keV. This value was in good agreement with the experimentally determined energy deposited in cells (about 45 keV). The experimental value was obtained as a difference between proton energy measured behind cells (992.0 ± 27.1 keV) and in empty places in close proximity of every cell (1036.8 ± 18.5 keV).

The dependencies presented in Fig. 3.36 have been used for dose estimates in our radiobiological experiments as is described in Chapter 4. As most of protons hit the nucleus, the mass of the cell nucleus was taken for the dose calculations.

One should mention, however, that traditional dose calculations might be misleading in the case of the targeted irradiation. The same number of particles
traversing the cell body or cell nucleus gives different dose values with regard to the object that has been taken into consideration. In case when the cell nucleus is a target for precise irradiation, dose values are calculated relative to average cell nucleus. If targeting accuracy is lower, it is reasonable to choose the data corresponding to a whole cell, as not only nucleus becomes a target. However, the whole concept of the energy deposited in a certain mass, elaborated initially for X–ray irradiations, seems to be doubtful in case of the targeted particle. Therefore, direct comparison of the results between different radiations (and different facilities) in terms of the dose (in Greys) is difficult. Therefore, it seems plausible to focus on trends and tendencies rather and not to rely too much on direct dose comparisons.

The developed simulation code can be easily modified and adapted for the dose estimate in application to other radiobiological experiments performed under various conditions. The following parameters can be varied: the type of irradiating particles, beam energy, beam form and size, materials and constructions of the SIHF elements, types of targets, their size and shape.
CHAPTER 4. SIHF APPLICATION FOR LIVING CELL IRRADIATION

4.1 Materials and Methods

4.1.1 Cell culturing and preparing to experiment

Experiments for the determination of living cell response to the proton irradiation have been carried out with normal human skin fibroblasts (CCL-110) and well differentiated gastric adenocarcinoma (MKN-7) cell lines (for details see Section 4.1.2). The CCL-110 cells were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS), L-glutamine and antibiotics. The MKN-7 cells were grown in RPMI-1640 medium supplemented with 10% FBS and antibiotics. All cells were incubated at 37°C in an atmosphere containing 5% CO₂. Once a week, cells were passaged using standard cell culturing procedures (Freshney et al. 2000).

For the irradiation experiment, the cells were deposited on a Si₃N₄ window (500 nm thick, with the size of either 1.5 x 1.5 mm, or 3 x 3 mm) or on thin (2 µm) Mylar foil, both glued over a hole perforated in a standard Petri dish (see Section 2.3). Two days before the experiment, the dishes were sterilized: they were incubated in 70% ethanol for 10 minutes and afterwards exposed to UV light for 15 minutes. One day before the experiment, dishes with Mylar bottom were additionally pre-treated with medium solutions (DMEM or RPMI depending on the type of cells) and then stored in the thermostat. On the same day, the cells were trypsinised and seeded on the Petri dishes.

4.1.2 Materials

Cell lines: normal human skin fibroblasts (CCL-110) and differentiated gastric adenocarcinoma (MKN-7) were purchased from Promochem (www.lgepromochem.com) and Riken Bioresource Centre (www.brc.riken.jp) correspondingly. Medium solutions: Dulbecco Minimum Essential Medium (DMEM) and RPMI-1640 and fetal bovine serum (FBS) and L-glutamine were bought from Sigma. The following fluorescent dyes were used: Propidium Iodine (Fluka), Hoechst 33342
(Sigma), Anti-Phospho-Histon H2A-X (Usptate), and AlexaFluor 488 (Molecular). β-cytochalasin was purchased from Sigma.

DMEM medium was supplemented with 10% FBS, L-glutamine and antibiotics. RPMI-1640 medium was supplemented with 10% FBS and antibiotics.

4.1.3 Cell dying

Visualization of interphase cell death. Living and dead cells are distinguished using two fluorescent dyes: Hoechst 33342 (used concentration 10 µg/ml; \( \lambda_{\text{emitted}} = 480 \text{ nm, blue colour} \)) and Propidium Iodine (used concentration 0.5 µg/ml; \( \lambda_{\text{emitted}} = 675 \text{ nm, red colour} \)). Both the Hoechst 33342 and Propidium Iodine fluorescent dyes selectively labels DNA molecules of cells. Propidium Iodine does not penetrate the membrane of living cells, therefore it is used as a label for dead (apoptotic and necrotic) cells.

Visualization of mitotic cell death. Mitotic dead cells can be observed via visualization of their micronuclei (see mitotic death part). β-cytochalasin (2 µg/ml) is used to stop cell division process in the moment after finishing cell nucleus division but before complete cell division. After incubation in β-cytochalasin (72 hours), the cells are fixed with methanol (20 minutes) and acetic acid, and rinsed with demineralized water. Such fixed cells are labelled with Hoechst 33342 (10 µg/ml) to visualize cell nuclear material – both nuclei and micronuclei.

Visualization of DSB. After cell irradiation, cells are fixed with 2% formaldehyde and rinsed with phosphate buffered saline (PBS). The detergent TRITON-X 100 (0.5 %) is used to destroy cell membrane in order to provide access of the fluorescent dye to the cell nucleus. The first added label, Anti-Phospho-Histon H2A-X (0.02 %), binds to histones (proteins being the compounds of chromosomes) phosphorilated in the case of double strand breaks. Next, the fluorescent dye Alexa Fluor 488 (0.02 %, \( \lambda_{\text{emitted}} = 488 \text{ nm, green colour} \)) is added. It binds to the first label and enables the observation of double strand breaks location.
4.1.4 Experiment performance

Irradiation experiments have been carried out for two cell lines (CCL-110 and MKN-7) separately. The observation of the following radiation-induced effects have been performed: cell interphase death, cell mitotic death (including micronuclei formation) and the induced DSB. Cells have been dyed before or after irradiation according to the procedures described above. The cells seeded on the Petri dishes have been imaged after irradiation with the fluorescence microscope (Olympus BX51 microscope equipped with a 100 W mercury lamp). Three filters have been used: U-MNG2 ($\lambda_{\text{exit}} = 470–490$ nm, $\lambda_{\text{emit}} > 510$ nm), U-MNU2 ($\lambda_{\text{exit}} = 360–370$ nm, $\lambda_{\text{emit}} > 425$ nm), and U-MNG2 ($\lambda_{\text{exit}} = 530–550$ nm, $\lambda_{\text{emit}} > 580$ nm).

Immediately before irradiation, most of the medium has been removed from the Petri dish. The dish has been covered with Mylar foil (2.5 µm) and placed on the moving stage (see Section 2.3). The cell irradiation has been carried out by 2 MeV protons according to the algorithm described in the Section 3.4.3. In order to exclude the spontaneous cell death, the duration of a single experiment has been kept below 10 minutes.

For the cell irradiation, the number of irradiating protons has been recalculated into dose per nucleus, according to the Fig. 3.36. Thus, the used numbers of protons 10, 20, 40, 80, 500, 3000, 5000 correspond to the doses 0.06, 0.13, 0.29, 0.56, 3.30, 20.20, 33.40 Gy.

After cell irradiation, the dish has been filled back with the medium, and the second part of cell dyeing procedure has been performed, if necessary. After that, next fluorescence images have been taken. The fluorescence microscopy images taken before and after irradiation have been compared to find out the radiation-induced changes.
4.2 Results

4.2.1 Observation of interphase cell death in irradiated cells

In order to observe the effect of the radiation-induced cell interphase death, only the differentiated gastric adenocarcinoma (MKN-7) cells have been irradiated. The main aim of the experiment is to show the qualitative difference of the ratio living/dead cells in irradiated and non-irradiated (control) regions of the studied group of cells.

The cells have been treated according to the procedure “Visualization of interphase cell death” (see Section 4.1.3). A part of the Petri dish with seeded cells has been automatically irradiated using a defined number of protons (3000, 20.2 Gy) per a single cell. The cells deposited on another (control) part of the dish have not been irradiated. The fluorescence pictures of both irradiated and non-irradiated regions have been taken after 30 minutes (Fig. 4.1). To gather statistically significant data, the experiment has been repeated for several Petri-dishes with the cells.

![Fluorescence microscopy images](image_url)

Fig. 4.1. The fluorescence microscopy images of (a) non-irradiated (i.e. control) and (b) irradiated regions of a Petri dish with the seeded differentiated gastric adenocarcinoma (MKN-7) cells. Pairs of the fluorescent images (one taken using the U-MNU2 filter, and another one using the U-MNG2 filter) have been superimposed. The nuclei of living cells (Hoechst 33342 labelled) are blue coloured, while the nuclei of dead cells (Propidium Iodine labelled) are red.
Figure 4.1 presents an example of the fluorescence images obtained for the non-irradiated (Fig. 4.1 a) and irradiated regions (Fig. 4.1 b) of the same Petri dish. The images taken using the U-MNG2 filter have been superimposed to those taken with the U-MNU2 one. The blue-coloured (Hoechst 33342 labelled) nuclei of living cells prevailed in the non-irradiated cell group (Fig. 4.1 a) indicate that living cells are the majority in the non-irradiated group. Oppositely, a large portion of red-coloured (Propidium Iodine–labelled) nuclei of dead cells appears in the irradiated group (Fig. 4.1 b), showing that radiation induces the interphase death of the cells. Thus, a difference of living/dead cells ratio between the irradiated and non-irradiated (control) regions becomes evident.

4.2.2 *Observation of cell mitotic death via visualisation of micronuclei formation in irradiated cells*

For both CCL-110 and MKN-7 cell lines, the effect of mitotic cell death has been observed indirectly, via the visualisation of the micronuclei formation. The Petri-dishes with seeded cells have been automatically irradiated with a defined number of protons (500 and 3000, corresponding to the dose 3.3 Gy and 20.2 Gy per nucleus). After irradiation, cells have been treated according to the procedure “Visualization of mitotic cell death” (see *Section 4.1.3*). Then, the fluorescence pictures of the mitotic cells have been taken (Fig. 4.2). To improve statistics, the experiment has been repeated for several cell dishes. For control experiments, the other Petri-dishes with seeded cells have been treated exactly in the same manner, excluding the irradiation step only.

Figures 4.2 a and b present two examples of the fluorescence images taken from the irradiated Petri dishes with the MKN-7 (left) and CCL-110 (right) cells. Both images have been taken using the UV filter U-MNU2, therefore, the blue-coloured Hoechst 33342-labeled cell nuclei and micronuclei can be seen.
Fig. 4.2. The fluorescence microscopy images of Petri-dishes region with irradiated MKN-7 (a) and CCL-110 (b) cells. Blue-coloured structures are cell nuclei and micronuclei (indicated with arrows) labelled with Hoechst 33342.

Analyzing the series of such fluorescence images, a percentage of mitotic cells possessing micronuclei related to a whole number of mitotic cells has been estimated for both irradiated and non-irradiated cells (see Table 4.1). For both studied cell lines (MKN-7 and CCL-110), the cells with one, two or three micronuclei have been observed.

Table 4.1. The percentage of the mitotic cells possessing micronuclei for different number of irradiating protons.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>CCL-110</th>
<th>MKN-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-irradiated (control)</td>
<td>1.6 %</td>
<td>12.2 %</td>
</tr>
<tr>
<td>500 protons (3.3 Gy)</td>
<td>8.8 %</td>
<td>21.8 %</td>
</tr>
<tr>
<td>3000 protons (20.2 Gy)</td>
<td>13.9 %</td>
<td>34.9 %</td>
</tr>
</tbody>
</table>

The percentage of the mitotic cells possessing micronuclei correlates with a number of irradiating protons, as Table 4.1 presents.

4.2.3 Study of the DNA double strand breaks formation in irradiated cells

Cells of both the MKN-7 and CCL-110 lines have been automatically irradiated using the defined dose values (corresponding to the defined number of protons). For the
MKN-7 cell line, the dose of 20.2 Gy (3000 protons) has been used, while for the CCL-110 line, the higher and the lower dose ranges have been applied. The high dose range is 3.3-33.4 Gy (corresponding to 500-5000 protons), the low one is 0.06-0.56 Gy (corresponding to 10-80 protons). After irradiation, cells have been treated according to the procedure “Visualization of DSB” (see Section 4.1.3). The obtained fluorescence images of cell nuclei of the MKN-7 line are presented in Fig. 4.3, while those of the cell nuclei of the CCL-110 line are in Fig. 4.4 and Fig. 4.5, for high and low ranges, correspondingly. For control experiments, the non-irradiated Petri-dishes with seeded cells have been treated in the same manner, but they have not been irradiated.

Fig. 4.3 The fluorescent microscopy images of MKN-7 cells – non-irradiated and irradiated with 20.2 Gy (3000 protons). Light green-coloured spots indicate the location of Alexa Fluor 488–labelled double strand breaks of DNA in cell nuclei.

The appearance of double strand breaks of DNA was observed in the fluorescence images corresponding to both irradiated cell lines. The locations of DSB were visible as the light green-coloured spots (due to Alexa Fluor 488 labelling).
Fig. 4.4 Fluorescence images of CCL-110 cells – non-irradiated (control) and irradiated with a defined number of protons per cell from 3.3 to 33.4 (500 to 5000 Gy). Light green-coloured spots indicate the location of Alexa Fluor 488–labelled double strand breaks of DNA in cell nuclei.

The intensity and density of light green spots visible in cell nuclei correspond to the number of induced DSB. Thus, the control images showing no light green spots indicate that no spontaneous DSB events have occurred. The growing intensity of green colour from the smaller dose value (3.3 Gy) to the higher one (33.4 Gy) shows qualitatively that the number of the induced DSB changes increased with increasing of the dose (the number of irradiating protons).

Fig. 4.5. The fluorescent microscopy images of the CCL-110 cells – non-irradiated (control) and irradiated with a defined number of protons per cell from 10 to 80 (corresponding to the dose range from 0.06 to 0.56 Gy). Light green-coloured spots indicate the location of Alexa Fluor 488–labelled double strand breaks of DNA in cell nuclei.
For the lower dose range applied, the presented fluorescence images (Fig. 4.5) show that the number of the induced DSB changes increases with increasing of the irradiating protons number.

4.2.4 Additional control experiment

In all experiments presented in the work, the maximal time needed to carry out the preparation of cells and cell irradiation was 10-15 minutes. A special control experiment was carried out to find out if any changes (any events of cell interphase death) appear in the control Petri-dishes without irradiation. The control dishes with seeded cells were processed as usually except the final irradiation, while the control experimental conditions were maintained exactly the same as in the above-described study of cells interphase death (room temperature, temporary removal of the most of medium, vertical positioning in the irradiation stage for certain time, and cell dyeing). At the end of the control experiment, the cells were imaged with the fluorescence microscopy, subsequently after 5, 10, 15, 20, 25, and 30 minutes. No dead cells were observed in this time range. Thus, it was concluded that in our case, the time needed for the cell preparation and irradiation was much shorter than time limitation existing in experiments with living objects.

4.2.5 Conclusions

The successively performed qualitative studies of the radiation-induced effects of cell interphase death, mitotic death and DNA DSB formation justifies the future possibility of carrying out the same studies in dependence on the number of irradiating protons (radiation dose). It has also verified the applicability of the developed SIHF for automatic cell irradiation.

It has been shown that the effects induced by the irradiation of single cells with the single protons, such as micronuclei and DSBs formation correlate with the dose increase for both higher and lower dose ranges used. However, more irradiation experiments should be carried out in order to determine the quantitative dependencies
and to find out if they are linear, and if there is the difference between the dependencies obtained for high and low dose ranges.
CONCLUSIONS

In the summary of the performed works the following conclusions can be drawn:

1. Several modifications have been introduced in the construction of the Cracow microbeam facility to upgrade it and enable the single ion operation mode for irradiations of living cells with targeted protons using optical automatic recognition. Upgrading includes the development of cell visualisation system, cell positioning system, blanking system, and certain modifications of data acquisition system.

2. Two methods of unstained, living cells visualization (brightfield and Quantitative Phase) have been developed using the illumination source placed inside the irradiation chamber. Both methods have shown satisfactory image quality in routine experiments, allowing cells observation in the transmitted light without the necessity to stain cell cultures nor to use harmful UV illumination.

3. Specialized software package has been designed for automatic cell recognition. The program allows easy, on-line modification of the recognition process and thus may be tailored to different types of cell images. Moreover, the software can be used independently for various image processing tasks.

4. New control system has been especially developed for precise automatic cell irradiation by the chosen number of protons. This system links together all parts of the hardware responsible for automatic cells recognition, positioning, beam blanking, and data acquisition. Thus, individual cells (in cell population) can be targeted with precisely defined number of single ions. The high degree of automatisation enables several experiments to be carried out in sequence and, in that way, assures statistically significant results.

5. Protocols (algorithms) have been designed, tested, and introduced into routine use for the on-line optical system and moving stage calibrations, beam position determination, beam profiling, and cell irradiation. These protocols may be applied separately or may constitute the compound protocol used for different types of radiobiological experiments in future.
8. The main functional characteristics of the developed facility, such as the targeting precision and the precision of the dose control have been determined and positively verified in relation to experimental requirements for cells irradiation.

9. The simulation of proton-cell interaction has been performed using the GEANT4 tool-kit. The results of simulations have been used to find the dependency between the dose adsorbed by the target (nucleus, cell) and the number of irradiating protons. Moreover, the simulated model of the proton pathway through a biological cell may be further extended for more advanced cases of the proton-cell interaction.

10. The developed SIHF, the only facility of such type in Poland, was successfully applied for irradiation of living cell (MKN-7 and CCL-110 cell lines) with different doses (in range of 0.06 – 33 Gy) that allowed observation of radiation-induced effects as cell interphase death, mitotic death and DNA DSB formation.

12. Since the developed SIHF is based on the very short focusing, the obtained results may be considered as the essential step on the pathway of the vertical focused SIHF development, where cells can be irradiated in more preferable, horizontal position.
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