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Short Communication

Short-term response of melanoma spheroids and melanocytes to FLASH proton therapy - colorimetric and FTIR microscopy study

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Abstract

Introduction: Melanoma, an aggressive and highly immunogenic cancer, arises from uncontrolled melanocyte growth. FLASH radiotherapy, a breakthrough technique, delivers ultra-high radiation doses, offering the potential for improved cancer treatment while minimizing harm to healthy tissue.

Material and Methods: To study the short-term response of spheroids to FLASH radiotherapy, 3D cultures of melanocytes and melanoma were used. Spheroids were irradiated using the FLASH method with the total doses of 3, 20, and 40 Gy, and conventionally with a dose of 3 Gy. After 8 days from irradiation, the measurements were taken using an imaging cytometer, FTIR and colorimetric microscopy (C-Microscopy).

Results: Studies conducted on melanocytes showed that doses of 20 and 40 Gy are toxic to them and cause cell necrosis. In contrast, for melanoma, these two doses resulted in tumor growth inhibition. IR measurements revealed spectral changes in lipids, proteins, and DNA/RNA, indicating similarities between the effects of the FLASH method and conventional radiotherapy for both spheroid models (i.e., cancerous and normal). The spheroid quantitative color analysis allowed for the differentiation between different irradiated and control groups.

Conclusion: Both colorimetric and infrared microscopy can be used to analyse the response of tumors to radiation.

Keywords: FLASH proton radiotherapy; melanoma spheroids; 3D cell culture; infrared microscopy; colorimetric microscopy

Introduction

Melanoma is an aggressive cancer, accounting for about 5% of all malignant tumors, arising from the uncontrolled proliferation of melanocytes.[1,2] Due to its typically pronounced lymphatic infiltration, it is considered an exceptionally immunogenic cancer.[3] The ultimate goal of radiotherapy, required in about 60–70% of cancer patients during treatment, is to eliminate cancer cells without toxic effects on normal tissues.[4,5] Ultra-high-dose rate radiotherapy (FLASH) is recognized as one of the most promising breakthroughs in cancer treatment. This method involves delivering radiation at an ultra-high dose rate (>40 Gy/s), several orders of magnitude higher than the dose rates currently used in conventional clinical radiotherapy (0.5–5 Gy/min) (**Figure 1A**).[6,7] Melanoma spheroids are a research model that has found widespread use in biomedical studies. Actually, the following types of treatment are used for melanoma: surgery, radiation therapy, chemotherapy, immunotherapy (PD-1, PD-L1, and CTLA-4 inhibitors) and targeted therapy (BRAF/MEK inhibition).[8] Research conducted by Jasińska-Konior et al. comparing the effects of proton and X-ray beam irradiation of melanoma cells showed a similar level of survival of cells for both types of radiation. However, only proton beam irradiation led to long-term inhibition of melanoma cell migration.[9] Therefore, the aim of this study is to evaluate the effect of FLASH proton therapy on melanoma.

Authors' contribution: A – Research concept and design, B – Collection and/or assembly of data, C – Data analysis and interpretation, D – Writing the article, E – Critical revision of the article, F – Final approval of the article.

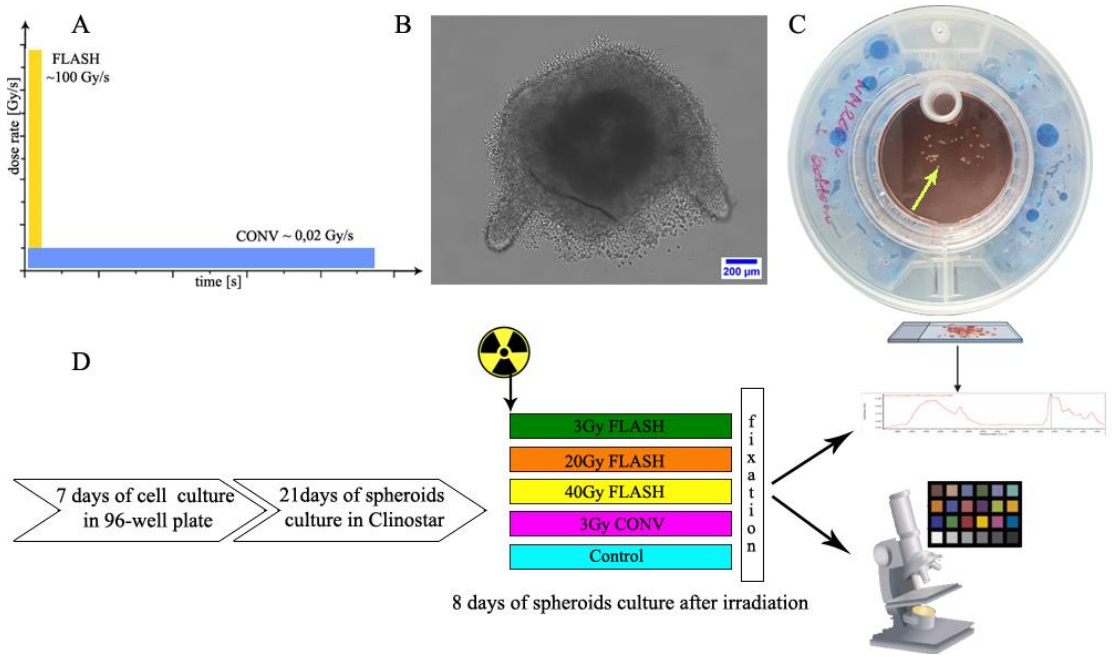

Figure 1. A- Dose rate comparison between FLASH/ CONV. B- Melanoma spheroid image. C- ClinoStar reactor with spheroids marked with a yellow arrow. D- Experiment Scheme.

Material and Methods

Cell lines

Malignant human melanoma cancer derived cells (Wm266-4) originated from the ESTDAB Melanoma Cell Bank, Tübingen, Germany. Cells were cultured in RPMI 1640 medium (Cat. purchased No. 21875091 Gibco™ Paisley, UK) supplemented with 10% Fetal Bovine Serum (Cat. No. 10500064 Gibco™ Paisley, UK), 2 mM L-Glutamine (Cat. No. 25030081 Gibco™ Paisley, UK), 100 U/ml Penicillin and 100 µg/ml Streptomycin (Cat. No. 15140122 Gibco™ Paisley, UK) seeded into T75 cm^2 dish and incubated in a humidified atmosphere with 5% CO_2 at 37°C. The culture medium was replaced every second day.

HEMA-LP (Cat. No. C0245C, Life Technologies, Paisley, UK) originated from lightly pigmented adult skin's epidermal melanocytes. Cells were cultured in Medium 254 (Cat. No. M254500 Gibco™ Paisley, UK) supplemented with Human Melanocyte Growth Supplement-2 (Cat. No. S0165 Gibco™ Paisley, UK), 100 U/ml Penicillin and 100 µg/ml Streptomycin (Cat. No. 15140122 Gibco™), seeded into T75 cm^2 dish and cultured at 37°C and 5% CO_2 atmosphere. The culture medium was replaced every other day.

Spheroids preincubation

Seven days before the experiment WM266-4 cells (2×10^3 cells in 250 µL/per well) were seeded into 96-well U-bottom SPL3D™ Cell Floater plates (SPL Life Sciences Co., Ltd., Pocheon-si, South Korea). The goal of this step was to improve model reproducibility by pre-cultivating the cells until they form spheroids before transferring them to the bioreactor. During this initial period of incubation (37°C, 5% CO_2), medium renewal was performed every two days.

Spheroid growing in ClinoStar incubator

Culture of large-sized spheroids was performed using the ClinoStar® (CelVivo, Inc, Chevy Chase, MD, USA) system. The rotor for the ClinoStar was prepared for cultivation and calibrated according to the manufacturer's instructions. The spheroids generated in the 96-well plate were transferred to the generator (**Figure 1C**). During the experiment, the rotation speed ranged from 15 to 30 rpm, and the adjustment depended on the size of the tumorspheres separately for each reactor placed in the incubator. The day after the spheroid transfer, all aggregates detached from the tumor spheres were removed from the chamber to maintain homogeneity and optimal growing conditions. The medium was replaced every 2 days and the bioreactor after 14 days, according to the manufacturer's recommendations. After 21 days, the spheroids were transferred to a 96-well plate to separate them into a control group and a group subjected to radiation.

For post-experimental sectioning, spheroids were fixed in 2.5% glutaraldehyde (ThermoFisher Scientific) and embedded in OCT Embedding Matrix for Frozen Sections (Cat. No. 6478.1, Carl-Roth, Karlsruhe, Germany). Then, 8 µm cryosections were cut on a CM3000 Leica cryostat (Leica Microsystems) and placed on the MirrIR low-e microscope slides (Kevley Technologies).

Spheroid irradiation and dosimetry

Spheroids were irradiated with conventional (CONV) proton radiotherapy at a final dose of around 3 Gy (dose rate about 0.140 Gy/s) and with FLASH radiation at a final dose around 3, 20 and 40 Gy (dose rate > 60 Gy/s).[10]

For both CONV and FLASH irradiation, the PTW Unidos Romeo electrometer with PTW Pinpoint (type 31023) ionization chamber was used. The ionization chamber was placed in an Eppendorf tube in a water environment, and the Eppendorf was then placed in a special holder. For CONV irradiation, the PTW Monitor Chamber (type 786) and for FLASH irradiation PTW Semiflex (type 31010) ionization chamber were used as monitors. In FLASH regime irradiation, the monitor chamber was placed 4 cm from the proton beam center before the collimator. The chamber collected signal from scattered protons without obstructing the sample. Before irradiating the samples, dosimetric measurements were performed to determine the calibration factor from the monitor units to the dose at the location of the PTW PinPoint ionization chamber. For irradiations, the Eppendorf tube with the ionization chamber was replaced by Eppendorf tubes containing the samples. The dose was determined based on the signal from the monitoring chamber and the previously defined calibration factor. For detailed time measurement in FLASH irradiations, the POTROS system[11] with radioluminescent crystal of LiMgPO4 was applied. The crystal was connected via optic fiber to a photomultiplier with a set of filters. In this research, a radioluminescent signal was read every 1 ms.

Analysis of spheroids growth using imaging cytometer

On the day of irradiation and 8 days after irradiation, pictures of the spheroids were taken using a Celigo bright-field (BF) and fluorescence imaging cytometer (Nexcelom Biosciences, Lawrence, MA, USA) (**Figure 1B**). The acquisition setup was adjusted to the cell line type, shape and size of the spheroid, and included pre-filtering to determine analysis parameters such as colony diameter, tumorsphere area and tumorsphere intensity range to exclude debris and other visible artefacts.

Colorimetric measurements

The quantitative color measurements at microscale were performed by colorimetric microscopy (C-Microscopy) [12]. The collected images were color calibrated (D65 illuminant). For the measurements, the entire fixed spheroid was transferred onto a slide with black carbon tape tape attached. After the water evaporation, images of the spheroids were taken. The analysis was performed using the C-Microscopy approach, ImageJ/FIJI and a neural network for image embeddings. The collected C-Microscopy image data are freely available from Zenodo repository.[13]

Fourier transform infrared (FTIR) microscopy

The measurements using FTIR microscopy were done in the Laboratory of Atomic and Molecular Biospectroscopy of the Faculty of Physics and Applied Computer Science, AGH University of Krakow. For this purpose, Nicolet iN10 MX (Thermo Fisher Scientific) infrared microscope was utilized. The specimens placed on the MirrIR glass slides (Kevley Technologies) were measured in the transflection mode, using the beam with a size limited with aperture to 25 μm × 25 μm. A liquid nitrogen-cooled MCT-A detector was used for the study. Typically, 32 scans were accumulated per spectrum, and the spectrum was recorded for the wavenumber range of 900-4000 cm^{-1} with a resolution of 8 cm^{-1}. The samples were raster scanned in two directions with a step size equal to 25 μm. The obtained absorption spectra were subjected to PCA.

Results

Melanoma and melanocyte spheroids were irradiated with a low dose rate (0.140-0.145 Gy/s), delivering a final dose of approximately 3 Gy (conventional radiotherapy) and with a high dose rate (45-90 Gy/s), delivering a final doses of around 3, 20 and 40 Gy (**Table 1**, **Table 2**).

Table 1. Summary of information on melanocyte radiation.

	Parameters of proton irradiation		
	Total dose [Gy]	**Time [s]**	**Dose rate [Gy/s]**
3 Gy CONV	2.99 ± 0.02	22.04 ± 5.01	0.14 ± 0.02
3 Gy FLASH	2.83 ± 1.21	0.04 ± 0.23	83.4 ± 25.08
20 Gy FLASH	21.47 ± 0.39	0.32 ± 0.01	66.79 ± 2.65
40 Gy FLASH	40.24 ± 0.72	0.62 ± 0.01	64.92 ± 0.96

Table 2. Summary of information on melanoma radiation.

	Parameters of proton irradiation		
	Total dose [Gy]	**Time [ms]**	**Dose rate [Gy/s]**
3 Gy CONV	2.98 ± 0.53	20.51 ± 0.96	0.14 ± 0.01
3 Gy FLASH	5.75 ± 1.78	0.073 ± 0.03	82.73 ± 22.52
20 Gy FLASH	20.94 ± 3.45	0.41 ± 0.06	51.17 ± 9.06
40 Gy FLASH	40.95 ± 4.95	0.87 ± 0.09	47.06 ± 5.61

After 8 days from irradiation, spheroids were measured and after that, fixed for further investigation. Analysis of percentage changes in the surface area (spheroid surface area 8 days after irradiation / spheroid surface area on the day of irradiation ×100%) of melanocyte spheroids showed that 3 Gy radiation delivered by FLASH method causes inhibition of cell proliferation compared to control spheroids. The difference between the FLASH and the CONV methods was not statistically significant (**Figure 2A**). Melanocytes irradiated with a dose of 20 and 40 Gy were destroyed after 8 days and showed viability at a level of 9 and 4.65, respectively (data from trypan blue measurements, not shown in figures).

FTIR mapping of 8 μm sections of melanocyte spheroids followed by PCA showed that spectral ranges of absorption spectra characteristic for lipids, proteins, and DNA/RNA did not differ significantly between spheroids irradiated with the FLASH and conventional method but are different from those obtained for the control group (**Figure 3A**).

Analysis of the color of whole melanocyte spheroids using colorimetric microscopy showed that linear projection of selected after scoring image embeddings from colorimetrically calibrated (D65 illuminant) images allows for the separation of the control, FLASH-irradiated and conventional spheroid populations (**Figure 4A**).

The analysis of melamona spheroid surface changes showed that both conventional radiotherapy and FLASH delivering a 3 Gy dose slowed proliferation compared to the control. However, only the 20 and 40 Gy doses inhibited proliferation and increased cell necrosis (**Figure 2B**).

IR analysis of the sections showed that changes in the spectral region characteristic of DNA/RNA were most pronounced for spheroids irradiated with 3 Gy and 20 Gy using the FLASH method. The 40 Gy dose was similar to both the control and the 3 Gy dose delivered by the conventional (CONV) method. For the protein spectrum, the 20 Gy dose also showed the greatest difference from the control. The 40 Gy and 3 Gy doses delivered by CONV did not show significant variation in the protein spectrum analysis. For lipids, the most distinct spectra compared to the control were observed in spheroids irradiated with 20 Gy and 3 Gy delivered by the FLASH method. The 40 Gy dose delivered by FLASH showed results similar to the conventional method, which may suggest that this dose is too high for treating melanoma (**Figure 3B**).

Colorimetric analysis of whole spheroids showed that there is a colorimetric marker that allows separation of all spheroid groups (i.e. control, spheroids 7 days younger than control, irradiated with CONV and with different doses of FLASH) (**Figure 4A**). Quantitative analysis of the mean RBG color signal of the spheroid surface shows statistically significant differences between the analysed groups (FLASH combined into 1 group) (**Figure 4B**).

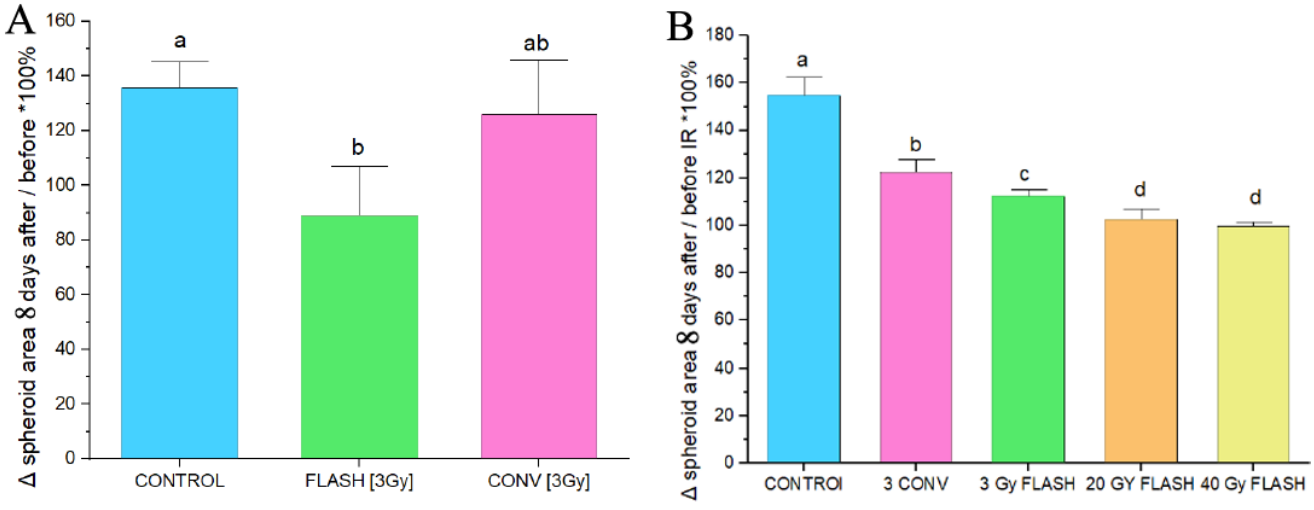

Figure 2. Changes in the surface of spheroids for melanocytes (A) and melanoma (B).

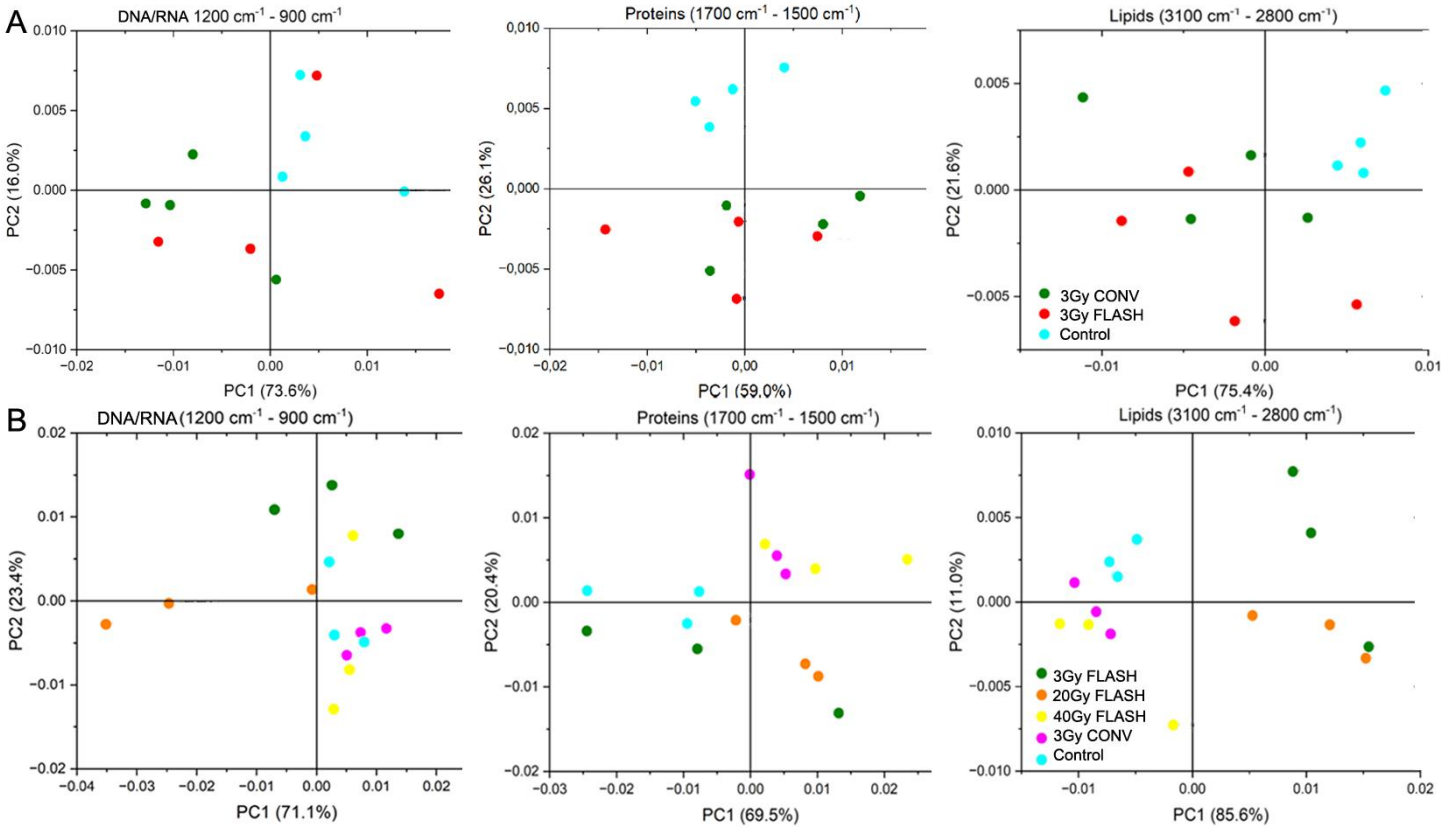

Figure 3. PCA analysis of spectrum for DNA/RNA, proteins and lipids for melanocytes (A) and melanoma (B).

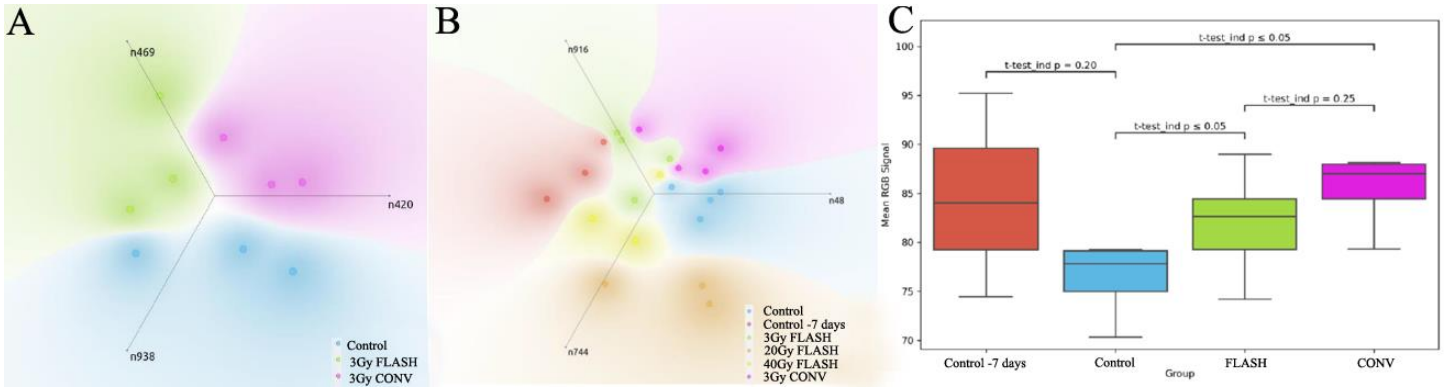

Figure 4. Results of colorimetric microscopy (C-Microscopy) imaging. Linear projection of selected after scoring image embeddings from colorimetrically calibrated (D65 illuminant) images of melocytes (A) and melanoma (B). It is seen that there is a separation between different irradiation conditions. Quantitative analysis of mean RGB color signal of melanoma spheroids surface (C). One sees a statistically significant difference between different irradiation conditions of melanoma.

Discussion

The use of 3D cell culture models as models for tumor imaging[14-15] and positronium biomarkers[16] is becoming increasingly popular. FLASH radiotherapy is still in the research stage, and the current list of clinical trials is small. This suggests the need for a more detailed study of the mechanism of FLASH's effect on normal and tumor cells, in order to confirm greater safety for healthy cells compared to the conventional method.

The use of colorimetric methods like colorimetric microscopy (C-Microscopy) is only just beginning to develop in biological research. The potential use of these methods in assessing anemia in Ghana children has been demonstrated.[17] The obtained results indicate the potential use of colorimetric microscopy, as an accessible and non-invasive method, to assess the effectiveness of radiotherapy, which could be performed in patients with skin cancers. Later, this could be coupled with more detailed studies to indicate what processes occur within the spheroid after irradiation and to enable the assessment of its effectiveness.

Conclusions

Our experiments confirm the possible use of 3D cell cultures for studying proton FLASH radiotherapy. We have shown that both modern colorimetric C-Microscopy and FTIR microscopy can be used to assess the short-term response of spheroids to radiation, providing data that can complement each other. To obtain the full view, the infrared microscopy method has to be enriched with data from other available techniques, such as spectral cytometry, viability and clonogenicity tests.

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Editor's comment

The abstract of the work was submitted to the 18th Congress of the Polish Society of Medical Physics (held in Poznań, Poland, 19-21 September 2024). The authors of the work were invited by the Scientific Committee of the Congress and the Editor-in-Chief of the Polish Journal of Medical Physics and Engineering to present their work in the form of a Short Communication in the Journal.

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