Published in final edited form as:

Int J Radiat Biol. 2018 August ; 94(8): 782–788. doi:10.1080/09553002.2017.1364801.

Focus small to find big – the microbeam story

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Abstract

Purpose—Even though the first ultraviolet microbeam was described by S. Tschachotin back in 1912, the development of sophisticated micro-irradiation facilities only began to flourish in the late 1980s. In this article, we highlight significant microbeam experiments, describe the latest microbeam irradiator configurations and critical discoveries made by using the microbeam apparatus.

Materials and methods—Modern radiological microbeams facilities are capable of producing a beam size of a few micrometers, or even tens of nanometers in size, and can deposit radiation with high precision within a cellular target. In the past three decades, a variety of microbeams have been developed to deliver a range of radiations including charged particles, X-rays, and electrons. Despite the original intention for their development to measure the effects of a single radiation track, the ability to target radiation with microbeams at sub-cellular targets has been extensively used to investigate radiation-induced biological responses within cells.

Results—Studies conducted using microbeams to target specific cells in a tissue have elucidated bystander responses, and further studies have shown reactive oxygen species (ROS) and reactive nitrogen species (RNS) play critical roles in the process. The radiation-induced abscopal effect, which has a profound impact on cancer radiotherapy, further reaffirmed the importance of bystander effects. Finally, by targeting sub-cellular compartments with a microbeam, we have reported cytoplasmic-specific biological responses. Despite the common dogma that nuclear DNA is the primary target for radiation-induced cell death and carcinogenesis, studies conducted using microbeam suggested that targeted cytoplasmic irradiation induces mitochondrial dysfunction, cellular stress, and genomic instability. A more recent development in microbeam technology includes application of mouse models to visualize *in vivo* DNA double-strand breaks.

Conclusions—Microbeams are making important contributions towards our understanding of radiation responses in cells and tissue models.

Keywords

Radiation; microbeam irradiator; bystander effect; cytoplasmic irradiation; abscopal effect

Conflict of Interest declaration. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Introduction

Shortly after Wilhelm Rontgen published his report about X-rays in December 1895 (Rontgen 1896), the first use of X-rays under clinical conditions was performed by John Hall-Edwards in Birmingham, England in January 1896 to radiograph a needle stuck in the hand of an associate. Since then, radiation has been applied to the medical field, including medical imaging, radiation oncology, and many more areas. Modern radiation therapy uses high-energy radiation to kill cancer cells and control tumor size (Lawrence 2008), and about half of all cancer patients receive radiation therapy during the course of their treatment. Even though radiation therapy efficiently kills cancer cells by damaging DNA and causing mitotic cell death, potential damage to surrounding normal tissue by radiation also increase the probability of side effects, including fibrosis, fatigue, or even development of secondary malignancy. Based on epidemiological data from atomic bomb survivors and other radiation-related cohorts, radiation contributes to increased incidence of leukemia and solid tumors. It is clear that radiation functions as a double-edged sword in cancer treatment. Hence, precision irradiators provide essential platforms to study the properties of radiation in different experimental systems.

Many different types of radiation machines have been developed for research purposes. Cabinet X-ray machines, gamma irradiators, particle accelerators, and image-guided small animal radiation research platform are the most common types of radiation-producing machines in research laboratories. Extensive studies have been done to focus on the properties of different beams, doses, and dose rates to provide fundamental support to clinical radiation therapy. However, for other studies such as space radiation research involving the effect of high linear energy transfer (LET) low dose rate heavy ion beams, or environmental radon effects investigating the role of alpha-particles released during radon decay, these irradiators were not capable of providing the suitable experimental configurations. Microbeam irradiators were generated to solve problems such as irradiation precision, low dose radiation, and accuracy. In this review, we describe the timeline of important microbeam experiments, the most up-to-date microbeam irradiator configurations, and critical discoveries made using the microbeam apparatus.

What is microbeam?

A microbeam is a micrometer or sub-micrometer diameter beam of radiation that allows damage to be precisely deposited at specific locations within a biological target. Because of the beam size, it is easy to focus on a very small target and deliver radiation without damaging surrounding tissues. Therefore, microbeam irradiators are of valuable use to understanding the communication between radiated cells or tissues to the surrounding environments. Another advantage of microbeam is it allows delivery of low doses of radiation. The modern charged particle microbeam delivers a precise number of ions to individual targets with a micrometer lateral resolution, which offers low and controlled doses of radiation that cannot be achieved by other irradiators. Because of these unique features, microbeam irradiators have been used at various institutions, contributed to many important basic science discoveries, and now is being developed into a new radiation therapy approach.

Historical microbeam experiments

First microbeam apparatus in 1912

In 1912, S. Tschachotin used quartz lenses to reduce the image of an ultraviolet source to microscopic dimensions (Tschachotin 1912). In Tschachotin's device, the quartz reducing system also served as condenser for visible light in a microscope system with which the target preparation was viewed. However, the apparatus setting of irradiation and imaging/viewing were performed from opposite sides of the preparation, and this type of setting limited the usage in biology (Uretz and Perry 1957).

First microbeam experiment in 1953

The first microbeam experiment was performed by Zirkle and Bloom in 1953 (Zirkle and Bloom 1953). A 2 MV Van de Graaf accelerator was used to generate protons. Microcollimators which consisted of two metal plates with a groove etched on one were clamped together to achieve a beam size of 2.5 μm . This microbeam setting was used to study the process of cell division after proton exposure. The earliest microbeam experiments observed and recorded the effects of radiation on chromosomal changes during mitosis, including temporary and permanent chromosome bridges, inhibition of anaphase movements, inhibition of metaphase configuration, unequal distribution of chromosomes in daughter nuclei, and rapid and repeated protrusion of abnormally large cytoplasmic 'bubbles'. These findings confirmed that radiation induces cell death through mitotic catastrophe.

Pioneer microneedle experiments in 1970

Other attempts to focus on partial irradiation using a different apparatus was also performed in early 1970s (Munro 1970). Munro irradiated Chinese hamster fibroblasts with alpha particles from a polonium-tipped microneedle. Based on careful calculations, determined doses of alpha-radiation were delivered selectively to the cytoplasm or nuclear area of the cells. It was discovered that large doses of cytoplasmic irradiation (over 250 Gy) had no effect on proliferation, while nuclear alpha-irradiation killed cells via mitotic cell death. These conclusions were further confirmed by modern microbeam facilities twenty years later.

Modern microbeam

The prototype

The development of modern microbeams was initiated in the early 1990s. A 25- μm microbeam using the cyclotron facility at the Brookhaven National Laboratories with 11 MeV/nucleon protons or 22 MeV/nucleon deuterons was one of the first being developed (Slatkin et al. 1992) and introduced the usage of the microbeam in radiation therapy. In this microbeam setup, micrometer diameter beam size was achieved by using the collimator approach. The disadvantage of this setup was that the radiation was delivered in relatively high doses, and the number of particles traversing biological samples was not controlled. To solve these issues, the GSI Darmstadt microbeam connected to a UNILAC accelerator to collimate a variety of heavy ion beams from carbon to uranium was constructed (Kraske et

al. 1990). It used a plastic track detector to monitor the particles crossing and a posteriori detector to determine the particles encountered by each sample. Although the accuracy increased significantly, this approach was very time-consuming and only 20 cells could be irradiated with a single ion in 10 hours (Prise 2014).

Key components of modern microbeam

Based on the microbeam approaches mentioned above, many of the current generation of microbeams has been established worldwide (Gerardi 2006). In general, a microbeam facility consists of the following components (Figure 1): 1) radiation source, mostly particle accelerators, in some cases x-ray and electron; 2) focusing lens or collimator to produce a stable radiation beam of micrometer diameter; 3) moveable stage to align the samples with radiation beam with high spatial resolution; 4) particle detector able to detect the dose delivered to samples; 5) imaging system to visualize the location of target cell and/or capture images for living cell experiments; and 6) computer system to control beam shutter, focusing lens, cell stage, particle detector, and imaging system which integrates all information with a user-friendly interface.

Physical settings

The most important aspect for a microbeam facility is the capability to confine the beam to a micrometer or even sub-micrometer diameter size. The two most popular approaches are collimation and magnetic focusing.

The micro-collimator was used in early microbeams. It is the most straightforward method and its advantages include easy location of beam and accurate dose delivery. Facilities that employed collimators were able to achieve a beam size of a few microns. However, due to scattering, the biological samples were required to be placed very close to the collimator. The particle scattering also reduced beam energy delivered to biological samples. Collimators of various materials and thicknesses were tested to minimize the scattering and to achieve a better accuracy (Folkard et al. 1997). An alternative collimation system contains a pair of aligned microapertures: the first one defines beam size and the second one placed closer to samples functions as an anti-scattering device for the first aperture. Many laboratories used this system, including JAERI (Takasaki, Japan) (Kobayashi et al. 2009), the INFN-LNL (Legnaro, Italy) (Gerardi, Galeazzi and Cherubini 2005) and Columbia University (New York, USA) (Randers-Pehrson et al. 2001). Figure 2A illustrates the two-microaperture collimation system.

Due to increased demand for narrower beam to investigate subcellular targets such as cytoplasm irradiation (Zhang et al. 2013), magnetic focusing systems were used to achieved submicron beam size. Under vacuum conditions, magnetic or electrostatic focusing devices can narrow down charged particle beams to a few tens of nanometers since they do not have the same scattering problems as collimators. Another advantage of magnetic focusing, especially the electronically focused system, is that the field strength required for focusing is independent of particle mass and charge for the accelerator (Marino 2017). Hence the magnetic focusing systems are the preferable choice for accelerator based heavy ion irradiators. Facilities using focusing systems with charged particle microbeams includes

PTB (Braunschweig, Germany) (Greif et al. 2006), GSI (Darmstadt, Germany) (Heiss et al. 2006), NIRS (Chiba, Japan) (Imaseki et al. 2007) and Columbia University (Bigelow et al. 2009). Figure 2B illustrated the electrostatic lens focusing system.

Discoveries made using microbeam

As discussed previously, the purpose of establishing modern microbeam facilities is to open new avenues for radiation biology studies. Many studies focused on biological effects of exact number of particles traversal or simulation of low dose environmental and occupational radiation have benefited from microbeam facilities.

Low-dose risk estimation

The first discovery made by microbeam facilities was the estimation of low dose radiation effects with high accuracy. Before the establishment of microbeam facilities, the occupational low dose of radiation affecting mainly uranium miners was evaluated using the Poisson distribution based on atomic bomb survivor data. The quality factor of low dose radon-progeny alpha particles were carefully calculated and reduced based on *in vitro* data. However, even using the updated quality factor, radon-induced mortality was still higher than the actual value (Brenner et al. 1995). Using the charged particle microbeam at the Columbia University, the oncogenic transforming efficiency of the nuclear traversal of one alpha particle was evaluated (Miller et al. 1999). Comparing to the prediction based on the Poisson distribution, the microbeam results of oncogenic transforming efficiency were significantly lower. These findings suggested that in a Poisson distribution, delivery of one alpha particle on average, multiple particle traversals contribute more to the oncogenic the transforming effect. Therefore, extrapolation from the average of one alpha particle traversals may overestimate the single traversal risk. On the other hand, the same experiment settings were used to explore toxic and mutagenic probabilities of a single alpha particle traversal. In A_L human-hamster hybrid cells, although one alpha particle traversal was only slightly cytotoxic, it was highly mutagenic, and the induced mutant fraction averaged 110 mutants per 10⁵ survivors (Hei et al. 1997). Consistent results have been reported in various laboratories using the microbeam irradiator. In human T-lymphocyte cells, a single alpha particle traversal was demonstrated to increase aberration 12–13 population doublings after exposure (Kadhim et al. 2001). These data suggested that even though the effect of low dose environmental radiation was overestimated using atomic bomb or Poisson distribution data, the low dose single alpha particle through cell nucleus can induce genomic instability.

Cytoplasmic irradiation

Other than studying a single alpha particle traversal of the nucleus, another advantage of the microbeam irradiator is the ability to target areas outside of the cell nucleus with great accuracy. Even though back in the 1970s experiments using the polonium needle showed no cytotoxic effect of cytoplasmic irradiation (Munro 1970), the underlying biological response, if any, was not understood. Using A_L hybrid cells, Wu et al. reported induced mutant fraction in both targeted nuclear and cytoplasmic irradiations (Wu et al. 1999). Moreover, based on the CD95⁻ mutant fraction, the principal class of mutations induced by cytoplasmic irradiation was similar to those of spontaneous origin and was entirely different

from those of nuclear irradiation, suggesting that different mutagenic mechanisms may be involved. DNA damage responses were observed after cytoplasmic irradiation, although at a lower extent when compared to direct nuclear irradiation (Tartier et al. 2007). Reactive oxygen species have been reported as the mediator of biological responses triggered by cytoplasmic irradiation, and mitochondria play an important role as well (Tartier et al. 2007; Zhang et al. 2013). Using an *in situ* live imaging system, Walsh et al. also recorded mitochondrial membrane potential loss after cytoplasmic heavy ion irradiation (Walsh et al. 2017). More recent studies showed cytoplasmic irradiation induces biological responses distinct from that of nuclear irradiation. Using human small airway epithelial cells, Wu et al. reported cytoplasmic irradiation induces mitophagy and autophagy in a AMPK/ERK-dependent manner (Wu, Zhang, Wu, Davidson, et al. 2017). Further studies comparing gene expression differences induced by cytoplasmic and nuclear irradiation suggested upregulation of glycolysis-related genes and enhanced glycolysis after radiation specifically targeting the cytoplasm (Wu, Zhang, Wu, Zou, et al. 2017). Taking together, these results suggested a unique role of cytoplasm in radiation-induced biological responses that cannot be discovered without microbeam irradiators.

Bystander effect

As early as 1992, the bystander effect has been reported in Chinese hamster ovary cells after an extremely low dose of alpha particle radiation (Nagasawa and Little 1992). When less than 1% of the cell nuclei were traversed by an alpha particle, 30% of the cells showed an increased frequency of sister chromatid exchanges. This was the first study to raise the question that non-irradiated cells may still respond to radiation. Even though this study could be performed using a basic shielding system, precise targeting and dose delivery to individual cells may provide a more comprehensive understanding of the underlying mechanism. By selectively irradiating only a few cells, the microbeam approach is particularly suitable to investigate bystander effect (Hei et al. 2008).

The first studies with microbeams showed that the bystander effect can be observed in primary human fibroblasts (Prise et al. 1998). When only four individual cells were chosen to be traversed by multiple alpha particles in a microbeam dish, the ratio of micronucleated cells increased by 3-fold when compared to control cells. Subsequent studies tested the bystander effect of irradiated cells using different experimental settings and end points. Using A_L cells, Zhou et al. observed a 3-fold increase of mutation on CD59 locus when 20% of cells were randomly selected and irradiated with 20 alpha particles (Zhou et al. 2000). By using oncogenic transformation ratio in C3H 10T1/2 cells, Sawant et al. also reported that when 10% cells were irradiated through nuclei, the transformation frequency was no less than that observed when every cell on the dish is exposed to alpha particle (Sawant et al. 2001).

With microbeam facilities, the role of cytoplasmic radiation in bystander responses has also been explored. Shao et al. reported the bystander effect in radioresistant glioma cells when only the cytoplasm was irradiated (Shao et al. 2004). Mitochondria signaling (Hei et al. 2008; Chen et al. 2008; Tartier et al. 2007), membrane rafts (Shao et al. 2004) or gap-junction (Azzam, de Toledo and Little 2001) have all been connected to the bystander effect.

Even though direct DNA damage was not required to initiate the bystander effect, DNA damage was observed in bystander cells after microbeam irradiation either by reactive oxygen species (Zhou et al. 2000) or reactive nitrogen species (Shao et al. 2002). Secondary messengers such as calcium (Shao et al. 2006) and TGF β (Shao, Folkard and Prise 2008) were reported to be key players in response to the bystander effect as well.

With the observation of abscopal effect and application to immunotherapy, the bystander studies now have been extended to tissue models. New generation of microbeams, with longer radiation range and deeper tissue imaging techniques, may offer a unique contribution to understanding the mechanisms and therefore a better treatment plan and higher efficiency in immunotherapy.

Investigation of DNA repair

The first application of the microbeam by Zirkle back in 1950s was to look at the role of radiation during mitosis (Zirkle and Bloom 1953). With modern microbeam settings and an advanced imaging system, spatiotemporal analysis of DNA damage repair can be studied with a live imaging system. Using a charged particle accelerator, Tobias et al. visualized the kinetics of repair-related proteins being recruited to DNA damage sites after irradiation (Tobias et al. 2010). Fast recruited proteins like DNA-PK or XRCC1 or slower recruited proteins like 53BP1 or MDC1 were classified by microbeam microscopy, and this classification helped to establish the hierarchical organization of damage recognition and subsequent repair events. Due to the subnuclear dose deposition by the microbeam and the development of fluorescent protein tags, the accumulation of different repair proteins to DNA damage sites and their mobility can be efficiently analyzed. With the combination of microbeam and fluorescence tagged proteins, roles of proteins involved in various biological processes can be analyzed in a real-time manner.

Application of microbeam in radiation therapy

Microbeam radiation therapy (MRT), a novel form of spatially fractionated radiotherapy, uses arrays of kilovoltage-energy X-ray microbeams (size approximately 25–50 μm , spaced at 200–400 μm). MRT was developed in a preclinical environment initially at the National Synchrotron Light Source at Brookhaven National Laboratory, Upton, USA, and later at the European Synchrotron Radiation Facility in France and Image and Medical Beamline in Australia. MRT is a promising treatment concept especially for malignant central nervous system tumors. Preliminary experiments using rodents (Bouchet et al. 2014) and piglets (Laissue et al. 2001) showed that small and large animals can tolerate much higher radiation doses delivered by spatially separated microbeams than those delivered by a single and macroscopically wide beam. MRT with high dose and high precision decreased normal tissue toxicity and can potentially improve therapeutic outcome.

Future studies

With the increased accuracy in beam confining and improvement of the imaging system, the goal of microbeam is to expand the biological targets from single cells to tissue and finally to *in vivo* studies. The microbeam has been reported in 3D model systems; however, the

approaches used were still conventional. These studies used microbeams to irradiate a localized region and measured biological responses at later time points in fixed tissues (Sedelnikova et al. 2007). Inclusion of a live imaging system into microbeam facilities will provide more comprehensive understanding to the radiological responses, especially in *in vivo* studies.

Experiments using model systems involving plants and small animals such as *Arabidopsis thaliana*, *C. elegans*, and zebra fish were also performed on the microbeam platform to study radiation induced biological responses. In combination with staining of target genes or GFP tagged response proteins with microbeam irradiation, bystander effects were detected in those systems mentioned above (Li et al. 2010; Buonanno et al. 2013; Choi and Yu 2013). More recently, a mouse ear model was used to investigate distant bystander effects from the radiated region (Buonanno et al. 2015). Further applications of microbeam in *in vivo* studies will offer assistance in understanding long-range bystander responses, its relation to the abscopal effect, and may lead to improvement of radiation in immunotherapy.

Acknowledgments

The authors thank Dr. Sherry Yan and Mr. Yen-Ruh Wu for helpful discussion and editorial assistance.

Sources of support

This research was supported by the NIH grants 5P01-CA49062-23 and 5R01-ES12888-09. The Radiological Research Accelerator Facilities is an NIH sponsored Resource Center through grant EB-002033 (National Institute of Biomedical Imaging and Bioengineering).

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Figure 1.

Essential components for a modern microbeam apparatus. When a radiation beam was produced, it went through **A.** beam deflector/shutter, which was controlled by a computer to determine when to irradiate biological samples. The radiation beam then passed through **B.** focusing lens or collimator to achieve required micro-meter diameter beam size. **C.** moveable stage and **E.** imaging system were included to precisely locate biological targets to the radiation site. Particle counts were accomplished using **D.** particle detector. The particle counts were then sent back to a computer and fed back into **A.** Beam deflector/shutter.

Figure 2.

Diagram of collimation system and electrostatic lens focusing system. **A.** Two-microaperture collimation system. The collimator system consists of a pair of apertures laser-drilled in 12.5-mm-thick stainless steel foils and separated by a 300-mm spacer. The limiting aperture is a 5-mm-diameter hole in the first foil. The second aperture, which is 6 mm in diameter, acts as an anti-scatter element (Randers-Pehrson et al. 2001). **B.** Electrostatic lens focusing system (Russian quadruplet configuration). The geometry of ion microprobe lens is illustrated in the upper panel. The compound lens consisting of two electrostatic quadrupole triplets with ‘Russian symmetry’ was to ensure a circular beam spot. The lenses used four 1 cm diameter ceramic rods gold plated in bands to create positive and negative electrodes whose strengths are arranged following the (+A, -B, +B, -A) pattern for each quadruplet and (+A, -B, +C), (-C, +B, -A) for the triplets with voltage up to 15 kV. Each triplet is 0.3 m long and they are separated by 1.9 m (Dymnikov et al. 2000; Bigelow et al. 2010).