Ion microbeam irradiation for radiobiology and radical chemistry: status and prospect

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Abstract. Ion microbeams are commonly used to study local irradiation effects in living cells, as it has been established that ion beam irradiations can lead to deleterious changes in cells that are not struck directly by the microbeam. Such changes, which take place over distances long compared to the size of the irradiation spot and for times long compared to the time of irradiation, are collectively termed radiation-induced bystander effect or RIBE. Free-radical chemistry is frequently invoked to explain the RIBE but no unified model is available at present. Ion microbeams when coupled with advanced methods for observing free radicals are the tools of choice for investigating the chemistry and biological processes governing RIBE.

1. Introduction
Intense efforts have been made over the two last decades to clarify the ways that living cells, tissues, and organisms respond to low dose radiations. Indeed, many experiments have shown that a low dose of ionizing radiation produces deleterious effects not only directly in the cells targeted, but also indirectly in cells that receive no direct dose. Such changes are known collectively as the radiation induced bystander effect (RIBE). The RIBE has practical implications for radiation protection and for the administration of radiotherapy. Today, the RIBE seems to be uncontroversial for large linear energy transfer (LET) irradiations when the dose is administered with light ions (protons, α-particles). These irradiations are representative of environmental radiation hazards, and in certain cases are used for radiotherapy purposes. The term ‘bystander effect’ is now also employed to describe secondary effects on neighboring cells induced by other kinds of stresses, namely, chemical [1], heat shock [2], and nanoparticle absorption [3]...). Today many laboratories around the world are engaged in investigating the chemical mechanisms and kinetics of the RIBE. Often, the experiments include a device for producing a microbeam of irradiation [4] for its ability of delivering a controlled dose in a spatially restricted area.

Several lines of experimental evidence demonstrate the essential role of free radicals in the appearance and propagation of RIBE. Although neither the underlying mechanisms nor the full range of observed changes are fully understood today, the study of free-radical chemistry has progressed during these last years [5], and has led to an improved description of radical formation and evolution in solutions following an irradiation event. Particularly, interactions between radical species are well understood and models with a high reproducibility have been developed. Both experimental methods and efficient simulation tools are available today and should contribute to the emergence of comprehensive models for the RIBE.
2. Radiation induced Bystander effect
Several comprehensive reviews regarding RIBE are available in the literature [6-8]. The existence of RIBE was first demonstrated nearly 20 years ago [9] when it was reported that after irradiating with 3.7 MeV α-particles only 1% of cell nuclei, more than 30% of the cell population exhibited sister chromatid exchanges, a mutagenic event. This work showed that a direct interaction between energetic particles and nucleus is not needed to induce deleterious effects in cells. Later it was established that even in the case of cytoplasmic irradiations, targeted cells were damaged and RIBE was observed in neighboring cells [10,11]. Recently, it was demonstrated that pre-cancerous cells may be selectively directed to apoptosis (genetically programmed suicide) through RIBE by irradiating neighboring normal cells at relatively low doses [12]. Other experiments demonstrated that cells located up to 1 mm from the initial impact of ionizing radiation exhibit DNA damages [13,14]. Today the RIBE has been demonstrated in a large panel of cell lines and has been observe with different types of radiations, including x-rays, γ-rays and heavy ions, but recent reports have questioned its effectiveness when irradiating with x-rays and heavy ions [15,16]. It seems however uncontroversial that RIBE is clearly induced when using α particles [15].

Quantitative and semi-quantitative methods are applied to measure the induction of deleterious effects on cells and identify associated pathways, and are generally based on fluorometric measurements: fluorescence of DNA repair protein binding antibodies, observation of cellular radical induction and amplification by direct or indirect (scavenging) techniques. Modulation of gene expression is also employed to identify the biological mechanisms governing the RIBE: deficient cell lines in terms of DNA repair or inter-cellular junction potency have been exposed to microbeam irradiations, suggesting an intercellular communication process through gap junctions [17]. A typical RIBE experiment is presented in figure 1.

![Figure 1](image)

**Figure 1.** Typical RIBE in cultured cells. (a): incoming ionizing radiation, (b) RIBE in neighboring cells mediated through culture medium, (c) RIBE in distant cells mediated through gap junctions.

Very early, radical species were suspected to trigger and also mediate the observed RIBE. The introduction of radical scavengers such as DMSO inhibits the RIBE. Scavengers however interfere with normal signaling activity among cells. In some experiments [18,19], “direct” observation of radical species, based on fluorescent radical probes confirmed the link between radical and RIBE spatial distributions. However fully quantitative evaluations using radical fluorescent probes are controversial as it has been shown that probe oxidation induces intermediate radical creation, leading to emergence of a self-amplified signal [20].
3. Microbeams for RIBE investigation

Interactions between the ion beam projectiles (e.g. protons) and atomic nuclei in a target produce recoil ions and nuclear fragments and ionize bound electrons. Spectroscopy of all these processes is used to obtain information about element concentrations. Initially, ion microbeams (mainly protons and less often deuterons, alpha particles, and heavier ions accelerated to energies of ~1 MeV/nucleon) were employed for elemental analysis of solid samples with dimensions of tens of micrometers [21]. As the methods improved, elemental analysis of solid samples with dimensions of tens of micrometers became possible. Ion microbeams are a good tool to induce the RIBE and to control its magnitude. The beams can be chosen and tuned to generate ion tracks in tiny regions (typically less than few microns) near cell surfaces. The pioneering work was done from 1953-1963 (see [22]). During the 1990s, groups at Gray Cancer Institute [23] and Radiological Research Accelerator Facility [24] built facilities which demonstrated the interest of microbeam setups for RIBE investigations. Since then, about twenty laboratories started investigating this new field. Recent reviews of the facilities used for cellular irradiation are given in [25,26]. Most workers use experimental setups that ideally would include the following elements (see figure 2): (i) a sub-micrometer ion beam; (ii) a horizontal sample stage that can be operated under atmospheric pressure; (iii) targets consisting of a cell monolayer cultured with fresh medium and located in a standard culture environment, (iv) a device for precisely controlling the number of charged particles delivered to the target; (v) a microscope for observing the targets optically; (vi) tools for observing changes in the target.

![Figure 2. An ideal experimental setup for RIBE investigation. For explanations of parts (i)-(vi), see text.](image)

System elements (iv-vi) must have minimal impact on biochemical processes. The listed requirements are however difficult to reach concomitantly and are frequently conflicting. Sub-micrometer beams are available only under vacuum. If the target is to be irradiated in air, passage of the beam through a window introduces unavoidable radial and angular scattering and inevitably enlarges the impact area. The smallest reported beam spots on target are about 1 µm [27]. Many ion beam facilities were originally devoted to ion beam analysis with beamlines mounted horizontally, requiring targets to be mounted vertically. A growing number of facilities have built the complex hardware needed to bend the beam in order to irradiate horizontal targets [28,29]. Different strategies are used to achieve the unusual culture conditions, either by the use of moisturized atmosphere or micro-environmental
chamber [30,31]. Ion counting can be performed by detecting ion crossing through a scintillator thin film located between the exit window of the accelerator and the target cells, but at the cost of increasing beam scattering and consequently reducing the hit precision of the beam on target. Alternatively, ions can be counted by using a charged particle detector positioned ‘downstream’, i.e., behind the targeted cells. This approach requires the removal of the culture medium, which otherwise would prevent the beam particles from reaching the detector. Removal of the culture medium may induce potential parasitic stresses on the cells.

Cell observation is intrinsically a disturbing process, and a simple operation such as tagging a nucleus with a fluorescent probe in order to make it observable, can lead to misinterpretation if used without great care [32]. As already pointed out, fluorometric behavior of radical probes has been intensively discussed. Some probes such as the popular 2’,7’-dichlorofluorescein are suspected to induce radical generation leading to signal amplification or are known to be photosensitive [20,33].

4. Radical chemistry: local levels

The aqueous chemistry of radical species produced in cells by ionizing radiation is important in many fields, particularly in radiobiology. These radical species evolve with complex spatial and temporal dependencies, owing to the large range of reaction rates ($10^{-3}$ to $10^1$ M$^{-1}$ s$^{-1}$) and diffusion coefficients ($10^{-5}$ to $10^{-6}$ cm$^2$ s$^{-1}$) [34]. Radical chemistry has been intensively investigated for decades, and many experimental and simulation tools have been developed to elucidate the impact of radicals on molecules of interest in biology (DNA, proteins). The systems studied include both dry extracts or solutions. Although the “direct/indirect effect” terminology employed in this literature could evoke RIBE, such studies are in fact conducted over very small distance scales, typically less than 1 µm, i.e., for the immediate molecular environment of the interacting species. The temporal behavior of generated species has been investigated by the combined techniques of pulse radiolysis and radical scavenging. A clear dependency of main generated radical species type with radiation quality is evidenced [35]. Spatial recombination of the different generated free radicals species, as far as we know, has not yet been observed experimentally. Numerous simulation reports treat the interactions of free radicals, but the maximum interaction distance considered to date is less than 1 µm.

In the radical chemistry approach, DNA and protein damages are treated as a consequence of interactions between the molecules in the sample and the incoming particle during its deceleration. The swift particles (primary ions or secondary electrons) can affect chemical bonds in the target by ejecting bonding electrons or by exciting them to states in which molecules may rearrange in undesirable ways. Typically, damages as double strand breaks or even more complex damages such tandem damages are considered as main deleterious cell nucleus damages as repair systems are not always fully efficient. Obviously, these effects are inherently spatially limited to the track volume. This approach is far too limited in spatial extent to explain the RIBE effect. The energy of secondary electrons emitted along an ion track is governed by the maximum energy transfer rule:

\[
E_{e}^{Max} = 4 \times E_i \frac{m_e m_i}{(m_e + m_i)^2}
\]

where $E_{e}^{Max}$ is the maximum transferred energy to electrons, $E_i$ is the ion kinetic energy, $m_e$ and $m_i$ being electron and ion masses, respectively. Typically, this leads to transferred electron kinetic energies lower than 2 keV. Electrons with energies in this range cannot reach neighboring cells or even the cell nucleus in case of cytoplasmic irradiation. Pimblott et al [36] calculated that for various ion projectiles (H$^+$, α, C$^{6+}$), the most probable secondary electron energy is 10 eV, with the mean at 60 eV, leading to a projected range of approximately 1 nm in water.

An exhaustive study regarding spatial and temporal distributions of radical species induced by a scanning electron beam with water was reported by Royall et al [37]; they conclude that the remaining
radical species are hydrogen peroxide and hydroxyl for dwell times up to 1 ms. For longer dwell times, a quasi-steady state is reached for which spatial repartition is governed by recombination and diffusion processes. In this regime, radical species are found much farther from the track vicinity (up to 15 µm from central track with 25 keV electron beam). The main contributors to radical production are electrons from the primary beam that have slowed to thermal velocities, but continue to diffuse through water. It is interesting to note that the relatively large spatial extent of the radical distribution is not associated experimentally with RIBE induction with electron beams [38]. In a tentative Monte-Carlo investigation of RIBE, Muroya et al [39] investigated time and spatial distributions of species generated following ion irradiation with 7-MeV α-particles. The dominant species, hydrogen peroxide, is found uniformly distributed in a 1 µm-diameter cylindrical tube 10 µs following the arrival of the ion. It is however important to note that the study was limited here to a 1 µm-section of the track. Plante et al [40] looked recently at radical and dose radial distributions for heavy ion tracks (10 to 170 keV/µm) by means of Monte-Carlo simulations; no detectable events were found outside a 100 nm cylinder.

The most probable contribution to the production of radical species outside the immediate zone of interaction has to be associated with the lateral straggling that affects the primary ion path in the Bragg region where nuclear collisions are dominant. This leads to an uncertainty of the track path localization. Typically, in the case of a 3 MeV α-particle beam, lateral ion straggling in water estimated using SRIM software [41] is 430 nm, while no significant additional contribution is associated with secondary electrons emitted in this part of the track owing to the low energy transfer occurring at the end of the path. These data confirm that radical generation through radiolysis process cannot directly induce signaling on neighboring cells. Consequently, deleterious effects observed in non-targeted cells by the RIBE process are necessarily initiated by biochemical radical species and are highly dose distribution dependent.

5. Future directions
Today huge amounts of data regarding RIBE in numerous cell lines have been accumulated by means of microbeam techniques. Although radical species are clearly identified as major actors in RIBE, physical chemical considerations exclude bystander induction by purely chemical processes, implying mandatory biological steps. Experimental protocols vary, however, and it has not yet been possible to create a unified, quantitative model of RIBE. Joint efforts from radiobiology and radiation chemistry communities are needed. Recent progress in detecting small amounts of radical species in small regions should open new perspectives. In particular, fluorescent nanomaterials, such as nanoparticles [42] and nanotubes [43] are promising tools, as signals from a single molecule now can be detected. Encoded fluorescent proteins can be also used to map the distribution of radical species in cells [44]. Improvements in beam targeting precision, with minimal parasitic ion detection should be helpful in controlling the dose microdistribution in cells. In situ observation methods for cell nucleus identification should be effective in a near future provided that they can be engineered not to have side effects. In parallel, microdosimetry simulations, (see [45]) might give hints about the effective cellular target where transition between chemical and biological stages occurs. As indicated above, RIBE is not systematically observed with different particles having the same LET. Thus, 3D modeling of the interactions between incoming ion and target material, particularly the end of the track during the last stages of ion deceleration, should be of major importance in understanding the underlying processes.

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References
[1] Cogan N, Baird D M, Phillips R, Crompton L A, Caldwell M A, Rubio M A, Newson R, Lyng F and Case C P 2010 Mutat. Res.-Fundam. Mol. Mech. Mutag. 683 1.
[2] Purschke M, Laubach H-J, Anderson R R and Manstein D 2009 J Invest Dermatol 130 86.
[3] Bhabra G, Sood A, Fisher B, Cartwright L, Saunders M, Evans W H, Surprenant A, Lopez-Castejon G, Mann S, Davis S A, Hails L A, Ingham E, Verkade P, Lane J, Heesom K, Newson R and Case C P 2009 Nat Nano 4 876.

[4] Schettino G, Al Rashid S T and Prise K M 2010 Mutat. Res. - Rev. Mut. Res. 704 68.

[5] Aitken C E, Marshall R A and Pulglisti J D 2008 Biophys. J. 94 1826-35

[6] Matsumoto H, Hamada N, Takahashi A, Kobayashi Y and Ohnishi T 2007 J. Radiat. Res. 48 97.

[7] Prise K M and O'Sullivan J M 2009 Nat Rev Cancer 9 351.

[8] Averbeck D 2010 Mutat. Res.-Fundam. Mol. Mech. Mutag. 687 7.

[9] Nagasawa H and Little J B 1992 Cancer Res. 52 6394.

[10] Shao C, Folkard M, Michael B D and Prise K M 2004 Proc. Natl. Acad. Sci. USA 101 13495.

[11] Tartier L, Gilchrist S, Burdak-Rothkamm S, Folkard M and Prise K M 2007 Cancer Res. 67 5872.

[12] Bauer G 2007 Int. J. Radiat Biol. 83 873.

[13] Belyakov O V, Mitchell S A, Parikh D, Randers-Pehrson G, Marino S A, Amundson S A, Geard C R and Brenner D J 2005 Proc. Natl. Acad. Sci. USA 102 14203-8

[14] Kashino G, Kondoh T, Nariyama N, Umetani K, Ohigashi T, Shinohara K, Kurihara A, Fukumoto M, Tanaka H, Maruhashi A, Suzuki M, Kinashi Y, Liu Y, Masunaga S-i, Watanabe M and Ono K 2009 Int. J. Radiat. Oncol. Biol. Phys. 74 229-36

[15] Sowa M B, Goetz W, Baulch J E, Pyles D N, Dziegielewski J, Yovino S, Snyder A R, de Toledo S M, Azzam E I and Morgan W F 2010 Int. J. Radiat Biol. 86 102-13

[16] Fournier C, Barberet P, Pouthier T, Ritter S, Fischer B, Voss K O, Funayama T, Hamada N, Kobayashi Y and Taucher-Scholz G 2009 Radiat. Res. 171 530.

[17] Azzam E I, de Toledo S M and Little J B 2001 Proc. Natl. Acad. Sci. USA 98 473

[18] Lyng F M, Maguire P, Kilnurray N, Mothersill C, Shao C, Folkard M and Prise K M 2006 Int. J. Radiat. Biol. 82 393.

[19] Hanot M, Hoarau J, Carrière M, Angulo J F and Khodja H 2009 Int. J. Radiat. Oncol. Biol. Phys. 75 1247.

[20] Wardman P 2007 Free Radical Biol. Med. 43 995.

[21] Pierce T B, Peck P F and Cuff D R A 1969 Nucl Instrum Methods 67 1.

[22] Zirkle R E and Bloom W 1953 Science 117 487.

[23] Folkard M, Vojnovic B, Prise K M, Bowey A G, Locke R J, Schettino G and Michael B D 1997 Int. J. Radiat. Biol. 72 375.

[24] Randers-Pehrson G, Geard C R, Johnson G, Elliston C D and Brenner D J 2001 Radiat. Res. 156 210-4

[25] Bigelow A W, Brenner D J, Garty G and Randers-Pehrson G 2008 IEEE Trans. Plasm. Sci. 36 1424.

[26] Gerardi S 2009 J. Radiat. Res. 50 A13.

[27] Randers-Pehrson G, Johnson G W, Marino S A, Xu Y, Dymnikov A D and Brenner D J 2009 Nucl. Instrum. Methods Phys. Res., Sect. A 609 294.

[28] Daudin L, Carrière M, Gouget B, Hoarau J and Khodja H 2006 Radiat. Prot. Dosim. 122 310.

[29] Kobayashi Y, Funayama T, Hamada N, Sakashita T, Konishi T, Imaseki H, Yasuda K, Hatashita M, Takagi K, Hatori S, Suzuki K, Yamauchi M, Yamashita S, Tomita M, Maeda M, Kobayashi K, Usami N and Wu L 2009 J. Radiat. Res. 50 A29.

[30] Hable V, Greubel C, Bergmaier A, Reichart P, Hauptner A, Krücken R, Strickfaden H, Dietzel S, Cremer T, Drexler G A, Friedl A A and Dollinger G 2009 Nucl. Instrum. Methods Phys. Res., Sect. B 267 2090.

[31] Khodja H, Hanot M, Carrière M, Hoarau J and Angulo J F 2009 Nucl. Instrum. Methods Phys. Res., Sect. B 267 1999.

[32] Gault N, Rigaud O, Poncy J-L and Lefaix J-L 2007 Radiat. Res. 167 551.
[33] McArdle F, Pattwell D M, Vasilaki A, McArdle A and Jackson M J 2005 Free Radical Biol. Med. 39 651.
[34] Hill M A and Smith F A 1994 Radiat. Phys. Chem. 43 265.
[35] Baldaechino G 2008 Radiat. Phys. Chem. 77 1218.
[36] Pimblott S M and LaVerne J A 2007 Radiat. Phys. Chem. 76 1244.
[37] Royall C P, Thiel B L and Donald A M 2001 J. Microsc. 204 185.
[38] Morgan W F, Goetz W and Sowa M B 2009 J. Radiat. Res. 50 A81.
[39] Muroya Y, Plante I, Azzam E I, Meesungnoen J, Katsumura Y and Jay-Gerin J-P 2006 Radiat. Res. 165 485.
[40] Plante I and Cucinotta F A 2010 Radiat. Environ. Biophys. 49 5.
[41] Ziegler J F, Biersack J P and Littmark U 1985 The stopping and range of ions in solids (New York: Pergamon)
[42] Casanova D, Bouzigues C, Nguyen T-L, Ramodiharilafy R O, Bouzhir-Sima L, Gacoin T, Boilot J-P, Tharaux P-L and Alexandrou A 2009 Nat Nano 4 581.
[43] Jin H, Heller D A, Kalbacova M, Kim J-H, Zhang J, Boghossian A A, Maheshri N and Strano M S 2010 Nat Nano 5 302.
[44] Belousov V V, Fradkov A F, Lukyanov K A, Staroverov D B, Shakhbazov K S, Terskikh A V and Lukyanov S 2006 Nat Methods 3 281.
[45] Incerti S, Seznec H, Simon M, Barberet P, Habchi C and Moretto P 2009 Radiat. Prot. Dosim. 133 2.