

Chapter XV: Methods for Study of Radiobiology: Overview

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New Approaches to Radiobiology Research

It is the purpose of this chapter to give an overview of both classic methods of radiobiology, which will be covered in Chapter XVI, and new methods in Radiation Biology, which will be covered in Chapter XVII.

Thus, the goal of this chapter is to be a historic link between classic radiobiology and the new technology as it applies to study of many topics in what is now called Radiobiology. These new topics include a spectrum of disciplines all the way from phylogeny of DNA repair and response to environmental stress, leading up to management of high radiation doses, acutely experienced by victims of radiation terrorism, and late effects of irradiation. There is a current challenge for managing both those long-term survivors of combined modality cancer therapy (including radiation therapy), and the individuals suffering total body irradiation from a radiation terrorist event.

The purpose of this chapter is to provide information for scientists seeking to enter the field of Radiation Biology and to alert both young scientists and veteran scientists of the challenges involved in understanding Modern Radiation Biology. Modern Radiation Biology must take into consideration the classic radiobiologic methods (since these were designed and implemented at times when the basics of radiobiology were first discussed including: the oxygen effect, linear energy transfer, which is between particle and photon beam irradiation, and clonogenic survival curves) were first utilized. The modern radiation biology must also include topics addressed in other chapters in this textbook including: DNA double and single strand break repair, RNA transcription, other moieties of RNA, silent regions of DNA, epigenetics, as well as, local tissue and distant abscopal effects *in vivo*. The methodologies for studying each of the “modern” areas of basic molecular biology has changed greatly in the past decades. Other sections of this textbook include focus on the individual components of modern radiobiologic techniques. For graduate students, post-doctoral fellows, and radiation oncology residents, as well as, medical students seeking a research experience in radiobiology, the entry point into this field will determine how one integrates classical radiobiological methods with the new methods.

It is the purpose of this chapter to provide some guidance and understanding the history of radiation biology, as well as, the new advances that are occurring yearly.

Approaches to the Classical Methods in Radiation Biology

If the student or established scientist decides to enter the field of Radiation Biology, the entry point will be critical to eventual understanding of the entire field. If one begins research in a laboratory that carries out total body irradiation experiments in mice looking at the metrics of survival, and damage to bone marrow and intestine, one will have entered the so-called “classic” radiobiology. The textbook by Hall and Gaccia (26) provides entry level radiobiology at the beginning chapters of the textbook and includes understanding of the basic principles of the oxygen effect, linear energy transfer components of irradiation showing that the DNA damage is greater by particle beam irradiation compared to photons, understanding of the RBE (relative biological effect), understanding partial body irradiation, and looking at principles such as dose rate, fractionation, and tissue effects including repopulation.

Alternatively, if one enters the field of Radiation Biology at the level of sophisticated DNA sequencing, measurement of hypermethylation or hypomethylation of DNA, or changes in non-coded or silent segments of DNA (essentially entering Radiobiology from the Molecular Biology side of the spectrum, and may be using ionizing irradiation as a “tool” to produce DNA damage; this person will be entering the field through a rapidly changing dimension. For example, studies in the molecular biology of DNA repair now include not only DNA sequencing techniques, but the kinetics of DNA sequence changes over time, individual cell DNA strand break measurements, individual cell RNA profiling (single cell RNA Seq), and use of techniques for single cell proteomics. If one starts the study of radiation damage to tissue with methodologies that can perform immunologic stain for 10 – 100 different proteins, such an approach provides an entry point at a level of microscopic imaging and tissue science far beyond classic radiation biology. One may be using irradiation as a “tool” to produce the immunohistochemical staining changes.

If one enters the field of radiation biology through studies of genetics (mutants of *C. Elegans* mutants, *Drosophila*, or Zebra fish), one may be using ionizing irradiation as a “tool” to induce genetic changes leading to gene induced phenotypic modifications.

It is important to understand at what level one is entering the field of radiobiology, so as not to delete parts of the spectrum of the discipline.

Entering Radiobiology by the Classic Methods Approach

Radiation Oncology Residents, doing lab year research, as well as, medical students seeking a fellowship to carry out basic radiobiology may find it useful to learn the classic techniques of the clonogenic radiation survival curve, H2AX DNA strand break measurement, measurement of chromosome aberrations in irradiated cells in culture, and, finally, measurement of total levels of antioxidant stores in irradiated cells. These “classic” radiobiology methods serve a valuable purpose toward understanding of the sophisticated molecular biologic techniques are also required in advancing the field.

It may also be possible for students to enter the field through Radiation Physics. There is a clear need for “dose harmonization” comparing radiation generated data between different laboratories and using different sources to deliver ionizing irradiation. The chapter (Chapter XXVIII) by Ke Sheng in Physical Dosimetry addresses the challenges in measuring radiation dose. Despite the availability of ionization chambers, thermoluminescent dosimeters, and other techniques for measuring radiation dose, the importance of other parameters are not usually considered and include: dose rate, complexity of the irradiation beam, x-ray spectrum, and possibility of mixed beam (particle beam, and x-ray photon) involvement in the delivery system.

Calibration of radiotherapy machines utilizing isotope generated x-rays (Cesium units, Cobalt 60 units, and others) may require more attention to detail with respect to dose rate since isotope decay lowers the dose rate over months to years. In contrast, radiobiologists using x-ray sources including orthovoltage or megavoltage irradiation beams usually rely on clinical physicists in clinical radiation oncology departments to carry out the dose calibrations. With clinical radiation

beams, attention should be given to the requirement for bolus (tissue equivalent material placed over the site to be irradiated, whether this is *C. Elegans*, *Drosophila*, Zebra fish or rodent experimental subjects, or cells in culture) to produce the required radiation dose. Delivery of x-ray or electron beam to the isolated limb of a rodent may require attention to detail for an experimental radiobiology experiment, a very different requirement than that which clinical physicists consider in the daily radiobiology of patient care.

Integrating New Molecular Biological Methodologies with Classic Radiobiology

Most scientists currently entering the field of radiation biology, will enter through molecular biology. The number of pathways for DNA repair is increasing incrementally with the advancement of techniques to measure single and double DNA strand breaks. The number of proteins and protein networks involved in the scaffold for binding of DNA repair enzyme increases every year (for example, the number of Fanconi Anemia pathway associated proteins now approaches 25, and each year at the Fanconi Anemia Research Fund meeting, there is usually another appointed candidate gene for the FA pathway). The overlapping pathways for DNA repair and other signaling pathways have increased in complexity. One should consult the chapters (Chapter XII and Chapter XIII) by Bing Liu on interacting pathways, and the multiple death pathways for irradiation damage. Direct DNA strand break damage caused by irradiation leading to cell death is quite distinct from the secondary cell death induced by release of inflammatory cytokines and the action of stress response genes induced in cells outside the irradiated field. Total body irradiation from exposure to a radiation terrorist device will not be as uniform as clinical total body irradiation, which is carried out in preparing a patient for marrow transplantation. In the clinical situation, TLD and ionization chamber measurements are carried out at multiple areas of the body to be certain that the dose delivered to the head or distal extremities is equivalent to that delivered to the torso. In a case of a radiation terrorist event, some areas of the body may be shielded and bone marrow in these shielded areas may survive and reseed areas of irradiated bone marrow. Furthermore, in the non-clinical scenario, abdominal irradiation doses may differ significantly from that to areas of bone marrow. Assessing irradiation dose is extremely important, and this area is covered in chapters in this textbook by John Chute (Animal Models for Total Body Irradiation – Chapter XXV) and Nelson Chao (Clinical Total Body Irradiation – Chapter XXVI).

The techniques for measuring *in vivo* radiation damage are becoming more sophisticated each year. Measuring the effects of hemibody, partial body, as well as, total body irradiation can use peripheral blood by quantitating nucleic acid circulating as a result of the death and breakdown of irradiated cells, as well as, markers of the irradiation-induced cell death pathways, such as circulating cardiolipin from radiation associated mitophagy, mitochondria breakdown, and apoptosis. These are sophisticated techniques that were not available 40 – 50 years ago at the time of first reports of what is now classical radiobiology. Techniques are available for surveying circulating nucleated cells for both classic radiobiologic dosimetry measures (Heterocyclic, ring form, chromosome aberrations, or fragmented nuclei), as well as, new biologic monitors for cell damage (removing single cells, and carrying out RNA Seq for irradiation-induced stress response genes in subpopulations of circulating cells including: lymphocytes, monocytes, and neutrophils). There are chapters in this textbook, which deal with methods for studying each

category of radiobiology that are available at the present time. Update of these methodologies on a frequent basis should be carried out by scientists entering the field.

It is easy to be persuaded to use the elegant resources available to approach new scientific questions in radiobiology. The availability of sophisticated computer software for mining gigabytes of data in genomics, transcriptomics, proteomics, and metabolomics using computer programs to present data facilitates a very impressive way to present data. Furthermore, the animal's model and *in vitro* cell culture systems now available have become so broadly available and sophisticated that it is difficult to know what resources to use and what systems to apply to any research question. The present chapter in this web-based textbook is designed to help investigators focus on current important topics in radiobiology and alleviate some of the confusion faced by scientists wishing to enter the field of Radiobiology. This chapter in this textbook will describe a strategy for determining, which methodologies to apply to answer relevant questions. It is the goal of this chapter to be an introduction to the complex, but specific chapters that follow dealing with specific areas in Radiobiology. This chapter will be an overview to guide the scientists entering the field of Radiobiology and also scientists transferring their vast knowledge into Radiobiology with respect to approaching major questions. This chapter will attempt to simplify daunting challenge of how to design experiments, how to formulate hypothesis, and to clearly determine what resources are needed, and which are best avoided.

Computational Biology and Molecular Biology Resources

The sophistication of current methods of study in DNA repair, mechanisms of DNA damage, and transmission of damage not only in the nucleus, but throughout the cytoplasm, and cytoplasmic organelles when applied to irradiation damage and repair may appear daunting (1). The state-of-the-art with respect to understanding RNA transcription also presents a new complexity, involving interaction of translated and spliced RNA with small interfering RNA, and microRNA (2). The availability of resources to amplify and study specific miRNA moieties that are related to the radiation response has been widely reported (3). Single cells studies of RNA represent state-of-the-art (4-6).

The field of proteomics has exploded from basic knowledge of purification of specific proteins in quantitative analysis (7). The original method of Western Blot, which was state-of-the-art techniques in the 1970s (8) has evolved to now protein array experiments to measure the hundreds or thousands of proteins and peptides produced within individual cells (9). New fields of research have merged with Radiobiology, most dramatically the field of oxidative lipidomics (10), and the new language of oxidized lipids (11) revealed a pathway, which communicates radiation damage first within single cells, then within cells in a tissue, and ultimately throughout the body of irradiated animals. Lipid signaling (through oxidized lipids and phospholipids) is now a major part of Radiobiology. Systems of signal transduction, and interactions of pathways require computational biology resources to understand the interactions of different cellular responses to ionizing irradiation and how they can enhance or neutralize each other (12).

New molecular biology and computational biology tools are available to the radiation biologist (12).

Animal Model Systems

Many transgenic and homologous deletion recombinant negative (knockout) mice as well as conditional knockout mice or conditional knock-in mice are available. In these “conditional” mice the addition of a specific agent to the diet or drinking water – such as Tetracycline or Tamoxifen, can turn on or shut off a gene in animals engineered by the Cre-Lox System (13). This technology allows radiation biology to enter the fourth dimension of time and determine at what stage of embryo gestation, but during the life of an animal, and during response to irradiation a specific pathway can be regulated. Scientists must utilize a transgenic core facility and be certain that the conditional animal genetics are not leaking (14) and are under tight control.

Formulating Hypothesis, Testing Hypothesis, and Knowing Which Resources to Use.

The basic principles in Science have not changed despite the wealth of available new methodologies and resources. Radiobiologists may each have different answers to the question of: What are the most important questions to be answered? The Center for Medical Countermeasures Against Radiation Consortium (CMCR-C of NIAID/NIH) has a two-fold mission: 1) The development of reliable radiation dosimeters to determine the level of radiation sustained by individuals in the event of a mass irradiation exposure from radiation terrorism or a nuclear reactor accident; and 2) the discovery and development of new radiation mitigator agents, which can be easily and safely delivered to large numbers of individuals, 24 hrs. or later after irradiation exposure. Radiobiologists seeking to fulfill these goals should first structure questions, test hypothesis, then validate, and present reliable results. The basic principles of scientific discovery have not changed since the first administration of ionizing irradiation-induced tissue damage.

Some of the Most Important Current Questions in Current Radiobiology are the Following:

1. What are the reliable biomarkers that can be used to provide strong evidence that an individual has been exposed to a “significant” level of ionizing irradiation?

Several chapters in this textbook will address the challenges of the researcher attempting to answer this question. Exposure to ionizing irradiation depends upon radiation dose, dose rate, and the relative volume of the body exposed. Controlled laboratory situations in which of these parameters can be tightly controlled are required. The physics of radiation should be precisely controlled (15). There will still be individual experimental and animal variations, even with tight control of gender, weight, age of animals, and even in the setting of genetically identical inbred experimental animals.

Physical measurements of irradiation dose sustained have been complicated by the need to measure a residual radiation effect 24 hrs. or later after exposure. Techniques by which to quantitate irradiation exposure using samples from hair, teeth, fingernails, and skin are complicated by disappearance of physical signatures for this 24 hr. time point (16). Therefore, researchers have focused on biological metrics of irradiation exposure.

Animals respond to ionizing irradiation by activating multiple stress response and inflammatory responses. These responses lead to changes in physiology (functioning of specific organs and production of products), as well as changes in metabolism.

The field of metabolomics (looking at biomarkers of metabolic changes that follow irradiation) requires multiple measures over time and represents the challenge of determining what changes were induced by ionizing irradiation, and which are common to multiple other forms of stress (17). Advances in this area have been dramatic and are discussed in the chapters by David Brenner and colleagues. The issue of a biological dosimeter for mass casualties also presents the challenge of high throughput. If metabolomics will be the measure of radiation dose sustained, then multiple time points must be tested and the evolution of production of specific biomarkers must be measured. Preparing for 10 – 100 patients, is very different than caring for a 1000 – 10,000 if one is using metabolomics as the signature for radiation dose sustained. Therefore, the issue of fast and reliable tests, as well as specificity of the results of each test for ionizing irradiation exposure, are a major goal for this area of research. Once the goal is established, then hypothesis driven research can follow. Radiobiologists should determine whether they wish to study urine metabolites, fecal metabolites, serum/plasma signatures of metabolic changes, or other tissues in sites of measurement. Clearly, the ease of obtaining urine or saliva specimens, and analyzing irradiation dose by metabolomics signatures would be preferable. Scientists should determine how difficult it will be to obtain answers and how practical their research results will be. For example, a metabolic signature, which required biopsy of the liver 24 hrs. after irradiation exposure would not be practical, safe, or acceptable.

Once the hypothesis are established, and the system is designed, then one can think about, which of the multiple assay systems that are available is useful.

The chapter in this textbook dealing with oxidative lipidomics addresses the molecular mechanisms and elegant signaling pathways in irradiation-induced oxidized lipids. However, the technology required to make the measurements is expensive and sophisticated. Few laboratories in the world can now identify specific lipid signatures of irradiation damage and how new radiation mitigators modulate these biomarkers of damage. However, if the results for identification of a specific oxidized phospholipid are clearly ionizing irradiation specific, quantitative, reliable, and reproducible, then the device may be constructed to focus on detection of this particular phospholipid as a radiation biomarker. The use of specific measuring devices should follow the questions.

- 2) What is a safe and effective radiation mitigator that can be administered 24 hours or later, after irradiation exposure?

A mission of the Center for Medical Countermeasures Consortium is the discovery and development of safe and efficient radiation mitigator agents. Advances in this field have been equally challenging and represent yet another complication for the radiation biologist. Unlike research into the development of measuring devices and quantitating results in samples collected from an individual, the discovery and development radiation

mitigators requires administration of agents to suspected irradiated individuals. This second area requires an overwhelming consideration for safety. Biological Dosimetry will not be perfect. There will be situations in which individuals may be diagnosed as receiving a higher radiation dose than what they actually received, and a decision to give these individuals a radiation mitigator agent, which might in fact be unnecessary. However, at the time of administration, the necessity may not be known so the agent must be safe.

Other Chapters in this textbook will address the multiple areas of concern in designing a radiation mitigator. Safety, being the primary concern, must apply to individuals of all ages, in all medical conditions, consider polypharmacy (what drugs and agents are the individuals already taking in the complex medical care environment), is the agent safe for pregnant women, the elderly, and is it safe for the developing fetus and in children. Is it safe in the setting of combined injury in which individuals, who may show the metabolomics signatures of a specific radiation dose, are also suffering thermal burns, concussion, trauma, and other evidence of radiation terrorist event.

To achieve the goal of providing radiation mitigator agent for use in the National stockpile, radiobiologists should, of course, be guided by the science. However, at the outset, some thoughts about practical considerations are relevant. The discovery of a small molecule that can be administered by injection into the skin, muscle, or applied topically to the skin will be desirable, compared to intraoral administration (when victims may be suffering nausea and vomiting), intravenous administration in which medical care personnel trained to deliver I.V. drugs may not be available (18). Once the small molecule is discovered, then formulations to achieve these goals will follow. The situation is much more complex if the radiation mitigator agent is a protein, lipid, nucleic acid (RNA or cDNA), or a large protein, carbohydrate, or complex glycoprotein. While there is concern about side effect administration of any agent, the larger and more complex the pharmaceutical agent to be delivered, the more potential for a complicated response.

Also, the expense of production, storage, and readiness for administration to mass casualties is not a trivial matter. One goal of the CMCR Consortium is to deliver a practical radiation mitigator that can be applied to the National Stockpile, production costs will be relevant.

Other chapters in this textbook describe the product development pathway, the FDA requirements for approving a clinical trial, and the “animal rule”, which describes testing in the appropriate animal model for the indication, and in this case radiation mitigation.

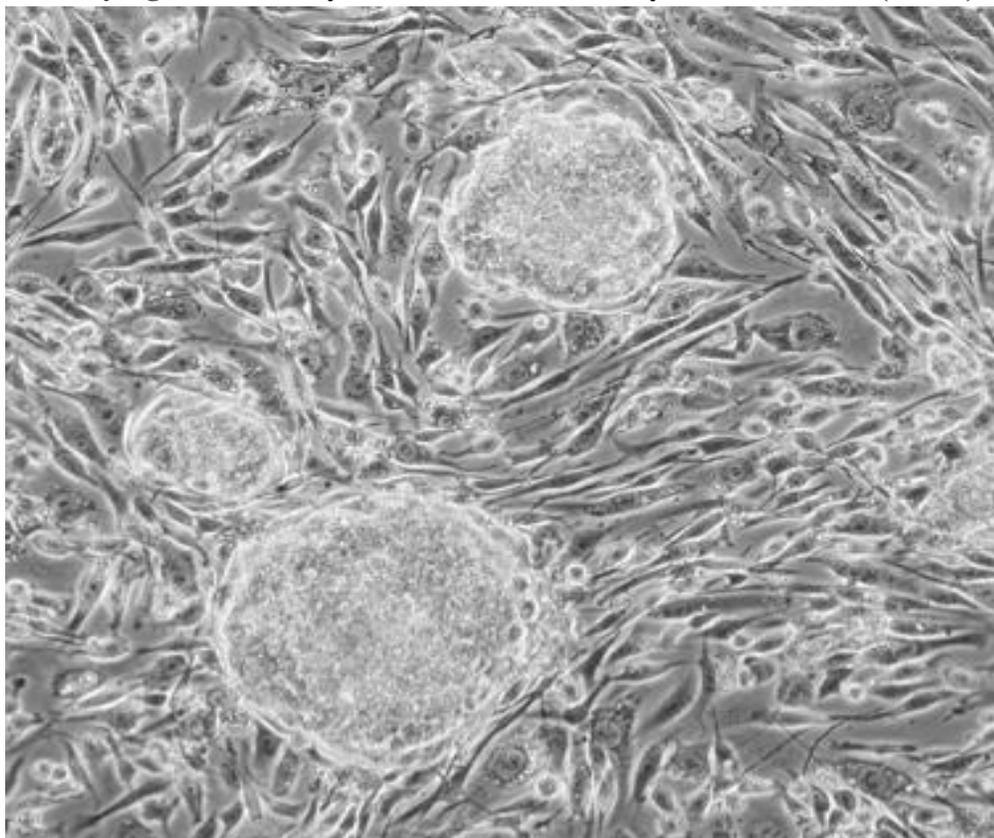
How to Formulate Questions, Design Experiments, and Utilize the Tools Available in Modern Radiobiology

The concern for developing radiation countermeasures has brought with it vast and exciting new opportunities for research in Radiobiology. Those individuals entering Radiobiology from the novice level will first receive training in Basic Radiobiology, and then move into the

sophisticated areas of DNA repair, transcriptomics, proteomics, radiation death pathway analysis, and radiation chemistry. Those individuals designing experiments in Radiobiology will come into the field with pre-existing knowledge, and expertise in other scientific disciplines.

Almost every discipline in biology has utilized ionizing irradiation in some form for their experiments. These prior experiences, may now be readdressed with an eye toward understanding of challenges in Radiobiology. For example, scientists have used irradiated “feeder layers” of cells in culture to provide nutrients, adhesion surfaces, or extracellular matrix for the study of a second population of growing cells placed in the culture medium above the irradiated cells. Feeder layer experiments utilize the irradiated cells to produce cytokines required by the second population of cells, and also the adhesion molecules secreted extracellular matrix, and cell surface properties of the irradiated cells to promote survival and allow study of the second population. This technique is particularly useful in growing embryonic stem cells, ESCs (Fig. 1).

Fig. 1: Inverted microscopic photo of round mouse embryonic stem cells grown in clusters overlying a “feeder layer” of stellate mesenchymal fibroblasts (X 100).



The irradiated feeder layer is itself a major radiobiological phenomenon. Other chapters in this textbook will address the concept of senescence of irradiated cells, the senescent secretory phenotype of such irradiated cells, and the biological properties of irradiated adherent cells, which represent the microenvironment of irradiated tissues in situ in experimental animals and

humans. Cells, which are contact-inhibited and do not divide when irradiated (19), continue to express irradiation damage, which is revealed in many forms including delayed or incomplete repair capacity (20) and genomic instability (21-25).

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