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Chapter XXIV: In Situ Correlations of Radiation Damage: From the Single Molecule to the Whole Animal

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Microscope based imaging approaches. Seeing things as they are.

Over the last 15 years microscope based imaging approaches have changed from a principally descriptive set of technologies which relied on the eye of the pathologist to interpret what was going on to a truly sophisticated armamentarium of tools which can be used in association with endogenous fluorescent proteins and other markers to dissect molecular behavior in living and fixed tissues in real time. There are few pathologies more appropriate for the use of these approaches than understanding the sequelae following an acute but specific trauma like radiation damage. In experimental models the approach can be quantitative and specific in dose and region, in clinical situations there is a clear temporal event which can be associated with the time of irradiation. However, experience has shown that in animal studies while the dose of the radiation may be quantified non-lethal complex pathologies are apparent, where some tissue regions show dramatic damage and other regions are spared. This is principally because of tissue shadowing or inconsistent positioning of the animal relative to the radiation source. Regardless it is essential to use multiple different technologies to gain a true understanding of what is actually happening in the tissue following the assault. In this unit we will discuss multiple approaches which are currently applied including simple sectioning methods to large massive tissue approaches that allow us to build images or entire organs in three dimensions with multiple reporters to define the downstream tissue pathology following the initial radiation event. Beyond the need to gain a "big picture" view of the entire tissue discussed above multi-dimensional approaches allow quantitative dissection of the number, position and abundance of the different cellular and molecular players in the pathology. So for example the ability to determine in 3-dimensions the structures, and interaction between cell populations within a tissue or organ that has been freshly removed from an experimental animal or human has great value (1). But beyond the static approaches that form the bulk of this article it is also possible to image living systems in multiple dimensions. At low resolution this may include MRI, PET or luciferase based approaches, however if we extend the methods to include multiphoton microscopy we are able to image events within living systems at the diffraction limit of the selected optical system. Essentially with a 200-400nm resolution generally this is considered 4-dimensional" imaging (2). Equivalently we use these approaches in multiple other models, though most directly in models of tissue transplantation and immune cell migration (camarind/watkins and others). Generally, these methods employ cells labelled with multiple different fluorescent proteins or cells that are harvested from syngeneic animals, colored with fluorescent dyes (for example cell tracker dyes) and then introduced into the subject animals. We routinely do bone marrow transplantation of green fluorescent protein positive (GFP+) or m-cherry (red) transgenic fluorochrome labeled cells into animals that are then irradiated such that we can image the movement of bone marrow derived cells throughout an irradiated tissue and quantitate interactions in real time in an extravasated section of that organ. The effects of adding a radiation protector or mitigator drug to the system can then be quantitated with respect to modifying such real time interactions.

In this chapter, we will review and discuss multiple published techniques and methodologies and highlight the strengths and weaknesses of each, from the more traditional to the current within the experimental forum of radio-biology.

Examples of How <u>In</u> <u>Situ</u> Imaging is the Preferred Approach to Answer Specific Questions

A recent challenge for researchers has been the study of induction of senescence compared to death of cells in tissues by ionizing irradiation. Experiments to study these effects require the use of in situ imaging.

Irradiated cells have multiple options for the response to irradiation including: 1) quiescence (stabilization in a non-dividing, but viable state); 2) cell division; 3) unsuccessful recovery of proliferative capacity after DNA repair leading to cell death; 4) malignant transformation; and 5) senescence. Senescence is defined as the state in which cells no longer have capacity for proliferation, but remain in situ and continue to produce both RNA and secreted mediators including proteins. Many proteins produced or secreted into the microenvironment, which includes pro-inflammatory cytokines, growth factors, and proteases, are called the senescent cell secretory phenotype (3). The study of age-related accumulation of senescent cells and the effect of irradiation on this phenomenon must use in situ imaging. The markers currently used for senescence are intracellular not in the cell surface. Historically this was performed using β-gal staining (4). Quantitative techniques for automated scoring of β-gal stained or labeled cells have been published (5). Other biomarkers for senescent cells include p16, p21, telomere length, senescence associated heterochromatic foci, and p53, although these do not always correlate with β-gal (5).

Other techniques for study of irradiation effects on senescent cells have required explant of cells from tissues, and then irradiation in vitro, where they then accumulate beta-galactosidase positive sub-populations. However, the secretory phenotype of these cells in vitro may not necessarily be the same as that of the same senescent cells in situ. An obvious solution to the problem would be to sort senescent cells and concentrate them from the tissue for in vitro study on injection into animals. The problem with this approach is that the biomarkers for senescent cells are intracellular and their identification requires staining or treatment of the cells in the tissue using techniques that make them lose viability. To sort cells, one needs biomarkers on the cell membrane. Attempts to use published techniques for sorting senescent cells are largely unsuccessful and result in very low recovery rates (6).

As Beta-galactosidase staining is an enzymatic reaction, it is possible to "over stain" which leads to measurements which are essentially over estimated. So rigorous approaches generally use other biomarkers of senescence including antibody staining for accumulation of p16 and p21 (5). Yet, other reports indicate that senescent cells accumulation p53 (5). Staining single cells with each of multiple antibodies for two or more cell markers adds strength to the diagnosis of a senescent cells.

There are other examples of the need for in situ imaging in specific experimental procedures, which require removal of organs, tissues, and cell populations before they can be studied or quantitated for the irradiation effect. However, analysis of the magnitude of irradiation damage has limited usefulness if quantitated by imaging histopathological sections from removed and non-viable tissues.

Imaging Techniques to Quantitate Radiation Acute and Late Effects in Viable Tissue, and in Living Animals.

As an example, acute radiation damage to the lung is associated with swelling of endothelial cells, accumulation of fluid in alveoli, and infiltration of inflammatory cells including polymorphonuclear leukocytes (7). Imaging of histopathologic sections of acute lung radiation damage allows scoring of these cell populations by visual counting or also staining with monoclonal antibodies for each of these cell phenotypes. Radiation-induced fibrosis (late effect), which occurs in C57BL/6J mice can be quantitated by removing lung and preparing whole sagittal sections of each of the five lobes of the mouse lung, then staining for areas demonstrating accumulation of fibrotic cells (8). The Mallory Trichrome stain for fibrosis has been used in conjunction with an Optimus Imaging System (9) to quantitate the relative percentage of each lung lobe that contains fibrosis. By examining and quantitating 10 sagittal sections from each mouse lobe (a total of 50 sections), very reliable quantitation of the percent fibrosis can be obtained (9).

In situ imaging of cell populations involved in the acute irradiation response (days to weeks) and late effects of fibrosis (months to years) is aided by use of monoclonal antibodies to stain specific cell populations within the fibrotic area. Antibodies to endothelial cells, compared to macrophages, can be used to determine what populations from each of these phenotypes arrive in the lung before the onset of fibrosis measuring and quantitating these cell numbers at days 80 and 90 after 20 Gy irradiation (10). The fibrotic areas that contain concentrations of collagen that accumulates by day 100 after thoracic irradiation allows counting of the cell phenotypes that are seen at the onset of fibrosis, which can be both diagnosed and quantitated (11).

In situ imaging of the intestine is critical to determine the kinetics of epithelial damage, movement of microbes into the circulation, and appearance of inflammatory cells (12).

In Situ Imaging of Radiation Damage to the Brain and Spinal Cord.

Irradiation damage to the spinal cord is associated with gliosis or accumulation of glial cells. Specific antibodies staining for M1 (inflammatory microglia) compared to M2 (nerve growth factor secreting and reparative glial cells) can be quantitated in sequence over time after irradiation (13). Matching quantitative data (counting numbers of glial cell subsets) with other data quantitating the depletion of endothelial cells, neurons, and arrival at the irradiated tissue of astrocytes and other inflammatory cells can be done efficiently with specific in situ imaging.

Novel 3-Dimensional Techniques for Quantifying Whole Organ Effects of Irradiation.
The effects of irradiation damage can be found in multiple organs and involve heterogenous effects throughout any individual organ. These effects can include differential pathology and infiltration of immune cells. The extent to which these vary is difficult to quantify through tradition microscopy which is generally limited to snapshots of sub millimeter areas and depths due to the opacity of tissue. This is complicated further by the relatively slow speed of imaging system that can require days if months to acquire multi-cubic millimeter areas that are required to address questions of complex disease pathology. Recent developments in tissue clearing (19), optics and confocal microscopy have improved the depth, resolution and speed at which images can be acquired. These innovations have made it possible to acquire thousands of cubic millimeters of tissue at micron and sub-micron resolution, making it possible to comprehensively

image whole organs to identify cell types, pathology and gene expression using all the tools available for fluorescence microscopy. Ultimately the speed at which imagery can be acquired is dependent in the imaging modality that is chosen.

Light sheet microscopy has reached the mainstream in recent years (20), allowing for rapid acquisition of very large volumes at micron resolution. Light sheet microscopes generally offer high sensitivity and superior reduction in photobleaching due to the use of CMOS camera technology to acquire imagery with high sensitivity and the lateral delivery and axial collection of light. Light sheet microscopy is available to a wide range of researchers as an active community has developed sophisticated open source software and plans for building custom instruments (http://openspim.org). Additionally, the recent availability of refined commercial products has recently become available. However, that question that this technique can address are limited by the size of the samples which is restricted by the properties of the objectives that are available. Consequently, the optics, generally air objectives, also limit the resolution that can be achieved in the largest samples as a function of numeric aperture and working distance but also the ability to deliver a uniform Z axis illumination which is greatly dependent on the optical properties of the tissue. To this end, micron resolution is the limit in very large samples which can make it difficult to pinpoint cell-specific characteristics and small infiltrating immune cells.

More traditional confocal techniques (line scanning single photon, multiphoton, and wide field based spinning disk and swept field modalities) can be used for large volume acquisition. Each has its advantages in sensitivity, speed, depth and the number of parameters per sample. However, the biggest advantage of these systems is that the only limitation on the size of the samples that can be acquired are the robotic stages and optics that can be fitted to the microscope – stages can always be made larger and optics continue to evolve. The best clearing optics currently enable depths approaching 1 centimeter with NA that enable near diffraction limited resolution (~300nm). Still the most complicating factor is speed. For most if these techniques, assessing radiation induced damage in the intestinal illium would require weeks of imaging for a single sample at submicron resolution as potentially millions of individual images must be acquired over the volume. The authors have used the Caliber ID RSG4 (Rochester, NY) to increase the imaging speed of large volumes up to 40 times. Known as ribbon scanning, this confocal uses constant movement of the sample under a resonant line scanner which enables it to acquire a single image in the vertical axis, reducing the need to acquire thousands of individual snapshots per z-plane and as a result abbreviating acquisition times (21). Massive volume acquisition is informative in isolation, enabling comprehensive sampling of a large tissue, but it can also be used as a guide to inform more traditional analysis including pinpointing regions for sectioning where further, more specific analysis can be focused. Some examples of this are shown below.

Intestinal Lupus Extravasation and Study of Migration of Inflammatory Cells

Intestinal irradiation damage results in a prolonged and systematic inflammatory event that attracts proinflammatory cell infiltrates and exacerbates damage to the intestinal walls characterized by thinning and destruction of villus structure. An example if this is seen in figure 1 where the tissue was harvested two days following irritation, cleared and imaged using the

ribbon scanning confocal described above. This single image as a complete reconstruction of the illeal bowel segment. The bowel was taken from mice expressing green florescent protein in their neutrophils.

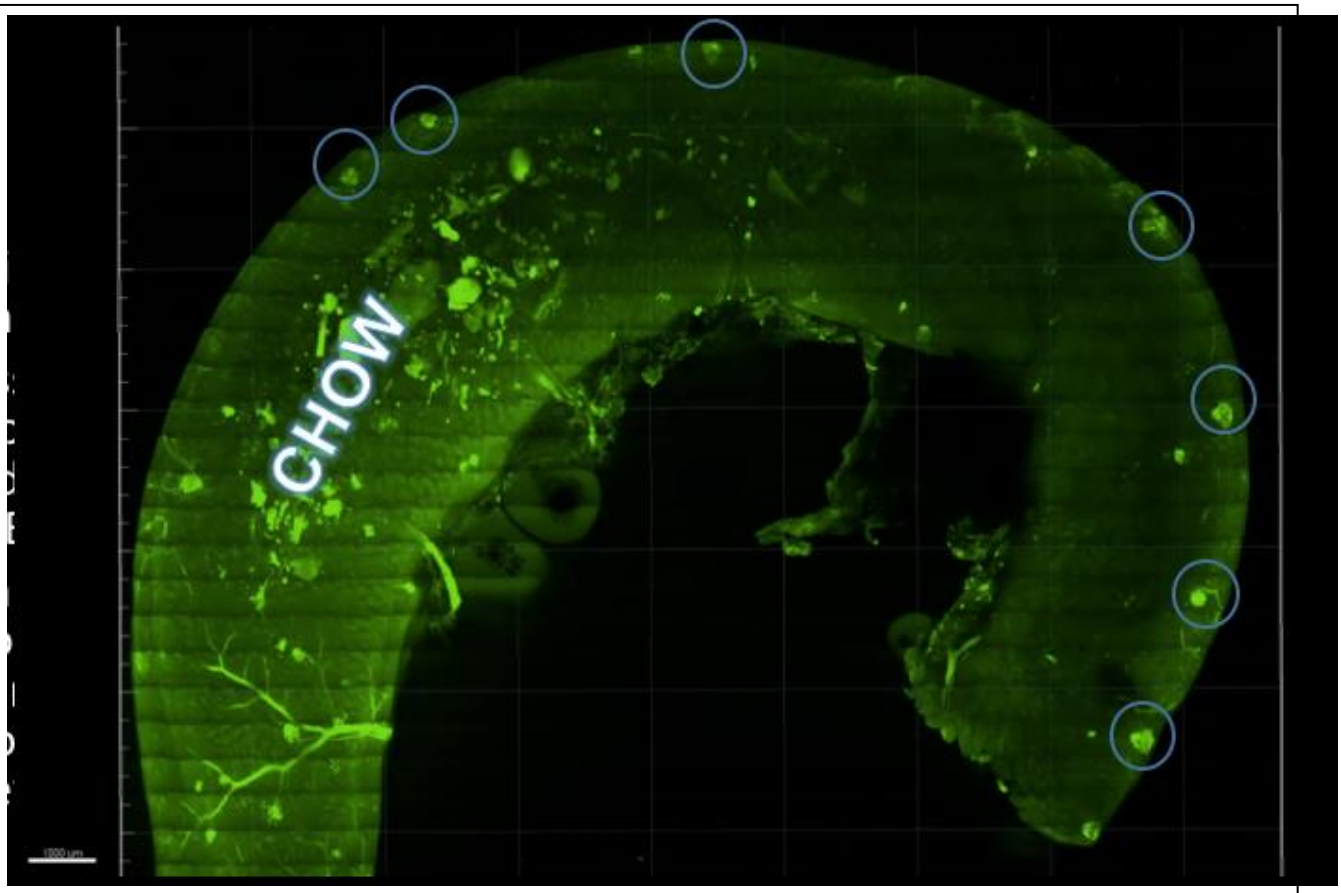

Figure 1

The autofluorescent material in the bowel is chow. The surprising and immediate finding was that the Peyers patches (circled in blue) are heavily loaded with neutrophils as an immediate sequelae of irradiation. These findings then guided section based investigation and indeed when we imaged individual sections looking specifically for Peyers patches we defined the same effect was evident. This is shown in figure 2, panel A shows the presence of abundant neutrophils in the villi and lamina propria, panel B shows the concentration of neutrophils within the Peyers patches.

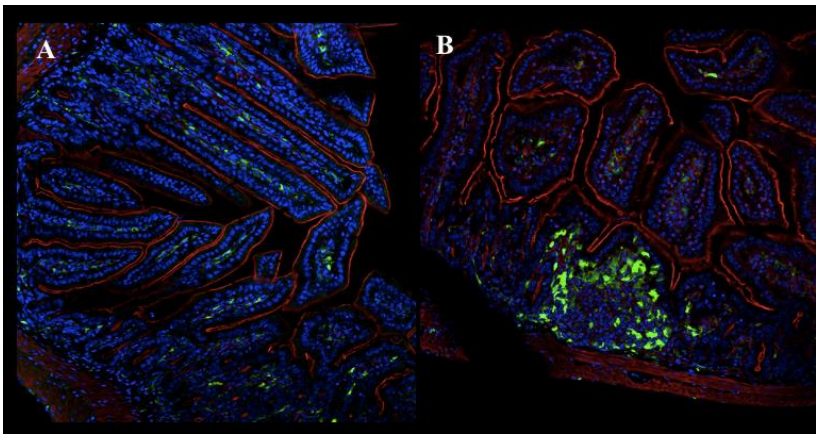

Figure 2

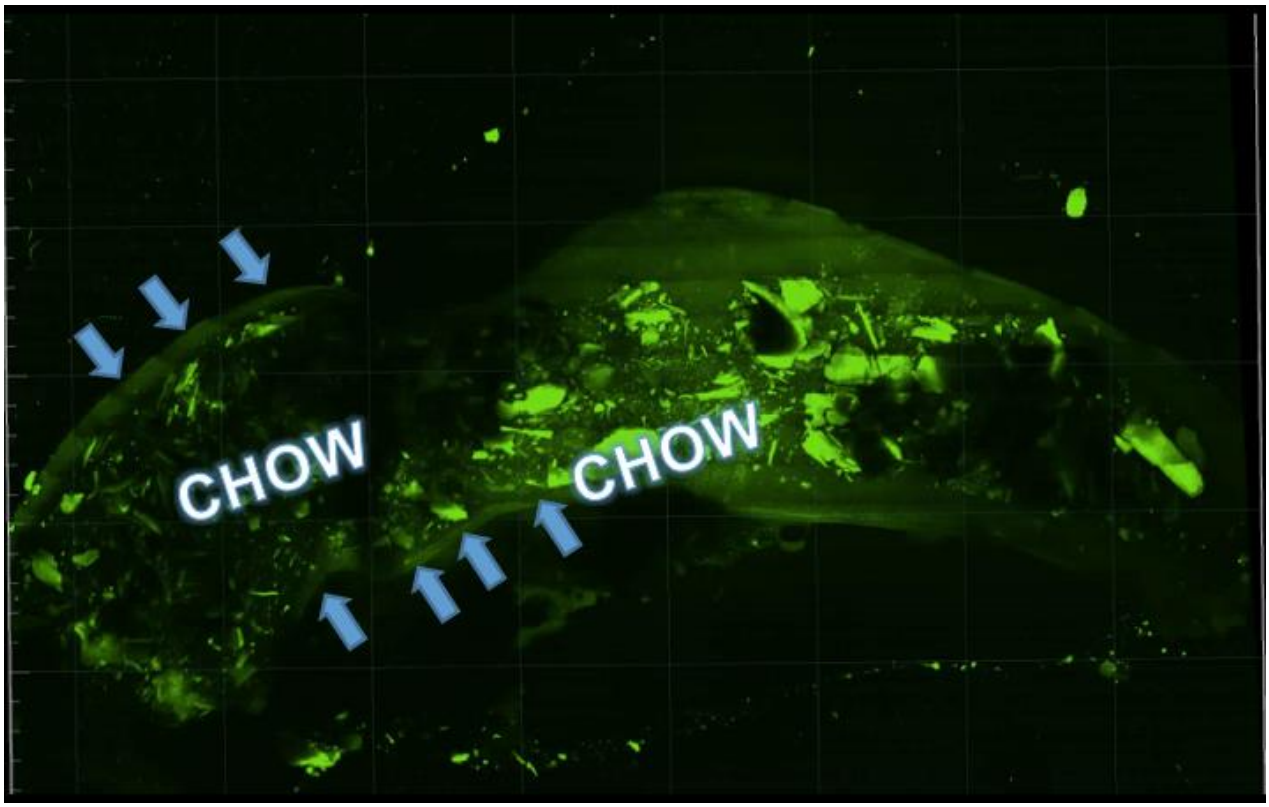

Figure 3

When we used the same 3D clearing method to image bowel 5-7 days after irradiation (Figure 3), there is almost no villus structure left at all. The blue arrows show that the gut wall is thinned significantly, and apoptosis is ubuiquitous. This was expected and confirmed on sectioned material, the red labelling in figure 4 highlights the apoptotic cells in this bowel using nick end labelling tools (TUNEL).

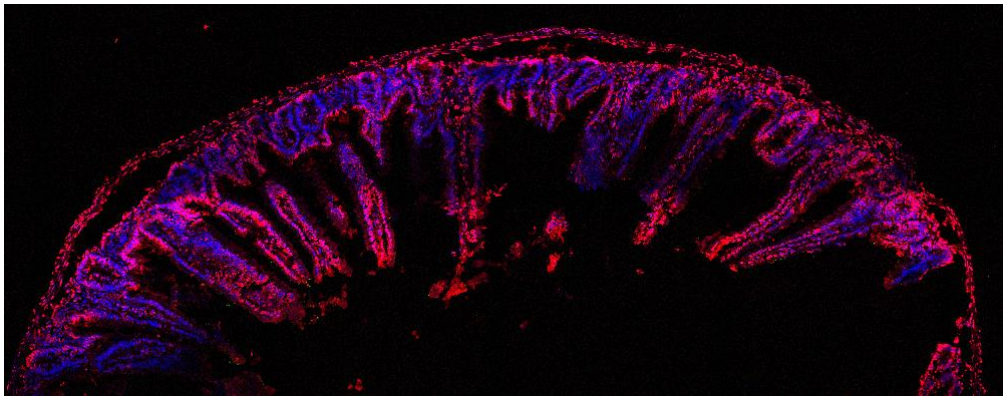

Figure 4

Continuous imaging (motion picture) imaging of the interaction of pseudomonas aeruginosa with pulmonary epithelial cells has recently been demonstrated. Two populations can be imaged in an interaction with each other watching the accumulation of proliferating bacteria moving over pulmonary epithelial cells has recently been demonstrated (14). Studies of the intestinal microbiome with sequential imaging studies of cross-sections of ilium after 9.25 Gy irradiation demonstrates the time course of disappearance of the intestinal villi, breakdown of the intestinal barrier, and movement of 2 micron labeled microspheres inserted into the intestinal lumen by gavage into the mice, penetrating through the disturbed intestinal barrier, getting into the villi, and then into the circulation.

A time course of movement of microspheres from lumen into the intestinal villi can then be correlated with reduction of intestinal crypt cells, villus length, and matched to time course for disappearance of specific cell phenotypes such as mucin producing goblet cells, and also by sequential in situ labeling. Study of bacteria interaction with the intestinal lumen is possible as well. M-Cherry positive E Coli bacteria can be inserted into the intestine and watched for movement through the damaged intestinal barrier into the villi, and then into the circulation. Imaging techniques are available for both study of the pulmonary and intestinal microbiome interaction with epithelial cells of that organ.

Other Imaging Techniques of Value in Radiation Biology

At the cellular level, study of movement of specific molecules involved in irradiation-induced apoptosis, necroptosis, and ferroptosis utilizes fluorochrome labeled monoclonal antibodies that can decipher the site in the cell, where irradiation-induced molecules accumulate, whether in mitochondria, endoplasmic reticulum, nuclear membrane, or cell membrane. Sequential imaging studies over time can plot the genetics of induction of specific proteins and movement from nucleus to cytoplasmic organelles (15).

Electron Microscopy Imaging Techniques

Radiation-induced death of specific cell populations can be confirmed by electron microscopy. There are specific patterns associated with cell death by apoptosis, necroptosis, and lysosomal necrosis. The latter pathway of cell death is associated with groups of intestinal cells that form necrotizing enterocolitis. Serpins (serine-theronine proteases) can be visualized in situ (16-18). Lysosomes have a specific electron microscopic appearance on high resolution electron microscopy. Determining the mode of cell death after irradiation of cells from specific knockout or transgenic mice can identify a specific cell pathway of death, and then led investigators to confirm that pathway by analysis of subcellular molecular markers. When this work is carried out with cell lines, the EM appearance can be followed by studies collecting micrograms of cells and analyzing for specific proteins by Western blot, RNA stress response genes associated with that death pathway by rt-PCR, and ultimately, by identification of specific proteins and sequencing specific peptides.

Conclusions:

There are instances in which in situ imaging is greatly desirable to define and/or confirm a specific mechanism of ionizing irradiation damage and response. Such data is critical to document the therapeutic action of new radiation protector or mitigator agents. In particular, the interaction of different cell phenotypes in situ in their natural environment may uncover mechanisms of irradiation-induced cell death that are different from those studied in cell lines in vitro, even if those lines are derived from each of these cell phenotypes. When a tissue is irradiated, interaction of one cell phenotype if remaining adjacent to another may blunt the response of one latter and exacerbate the response of the other. Since the application of radiation countermeasures will undoubtedly require in vivo testing, there will be a need for in situ imaging to elucidate the mechanism of drug action. Such knowledge will guide subsequent in vivo experiments.

References:

1. Wenzel SE, Tyurina YY, Zhao J, St Croix CM, Dar HH, Mao G, Tyurin VA, Anthonymuthu TS, Kapralov AA, Amoscato AA, Mikulska-Ruminska K, Shrivastava IH,Kenny EM, Yang Q, Rosenbaum JC, Sparvero LJ, Emlet DR, Wen X, Minami Y, Qu F,Watkins SC, Holman TR, VanDemark AP, Kellum JA, Bahar I, Bayır H, Kagan VE. PEBP1 Wardens Ferroptosis by Enabling Lipoxygenase Generation of Lipid Death Signals. Cell. 2017 Oct 19;171(3):628-641.e26. doi: 10.1016/j.cell.2017.09.044. PubMedPMID: 29053969.

2. Camirand G, Li Q, Demetris AJ, Watkins SC, Shlomchik WD, Rothstein DM, Lakkis FG. Multiphoton Intravital Microscopy of the Transplanted Mouse Kidney. Am J Transplant. 2011 Aug 11. doi: 10.1111/j.1600-6143.2011.03671.x. [Epub ahead ofprint] PubMed PMID: 21834913

3. Munoz-Espin D, and Serrano M. Cellular senescence: from physiology to pathology. Nat Rev Mol Cell Biol, 15(7): 482-496, 2014.

4. Lee BY, Han JA, Im JS, Morrone A, Johung K, Goodwin C, et al. Senescence-associated β-galactosidase is lysosomal β-galactosidase. Aging Cell, 5(2): 187-195, 2006.

5. Farr JN, Weivoda MM, Monroe DG, Fraser DG, Onken JL, et al. Targeting cellular senescence prevents age-related bone loss in mice. Nature Medicine, 23(9): 1072-1081, 2017.

6. Chen J, Chen K-H, Fu B-Q, Zhang W, Dai H, Lin L-R, et al. Isolation and identification of senescent renal tubular epithelial cells using immunomagnetic beads based on DcR2. Exp Geron, 95: 116-127, 2017.

7. Epperly MW, Travis EL, Whitsett JA, Epstein CJ, and Greenberger JS. Overexpression of manganese superoxide dismutase (MnSOD) in whole lung or alveolar type II (AT-II) cells of MnSOD transgenic mice does not provide intrinsic lung irradiation protection. Radiat Oncol Invest, 96: 11-21, 2001.

8. Epperly MW, Bray JA, Kraeger S, Zwacka R, Engelhardt J, Travis E, and Greenberger JS. Prevention of late effects of irradiation lung damage by manganese superoxide dismutase gene therapy. Gene Therapy, 5: 196-208, 1998.

9. Epperly MW, Travis EL, Sikora C, and Greenberger JS. Magnesium superoxide dismutase (MnSOD) plasmid/liposome pulmonary radioprotective gene therapy: Modulation of irradiation-induced mRNA for IL-1, TNF-α, and TGF-β correlates with delay of organizing alveolitis/fibrosis. Biology of Blood and Marrow Transplantation, 5: 204-214, 1999.

10. Epperly MW, Sikora CA, Defilippi S, Gretton JE, and Greenberger JS. Bone marrow origin of myofibroblasts in irradiation pulmonary fibrosis. Am J Resp Molecular Cell Biology, 29: 213-224, 2003.

11. Kalash R, Epperly MW, Goff J, Dixon T, Sprachman MM, Zhang X, Shields D, Cao S, Wipf P, Franicola D, Berhane H, and Greenberger JS. Amelioration of irradiation pulmonary fibrosis by a water-soluble bi-functional sulfoxide radiation mitigator (MMS350). Radiat Res, 180: 474-490, 2013.

12. Wei L, Leibowitz BJ, Wang X, Epperly M, Greenberger J, Zhang L, and Yu J. Inhibition of CDK4/6 protects against radiation-induced intestinal injury in mice. Journal of Clinical Investigation, 126(11): 4076-4086, 2016.

13. Vogel DY, Vereyken EJ, Glim JE, and Dijkstra CD. Macrophages in inflammatory multiple sclerosis lesions have an intermediate activation status. Journal of Neuroinflammation, 10(1): 35-50, 2013.

14. Dar HH, Tyurina YY, Mikulska KA, Shriastava I, Tyurin VA, Krieger J, St. Croix C, Watkins S, Bayir E, Ting H-C, Mao G, Ogunsola AF, Flitter BA, Freedman C, Gaston JR, Holman T, Pilewski J, Greenberger JS, Mallampalli R, Bahar I, Bomberger J, Bayir H, and Kagan VE. Theft-ferroptosis by Pseudomonas aeruginosa pLoxA via AA-PE oxidation occurs in cystic fibrosis and persistent lower respiratory tract infections. J Clinical Investigations (in press).

15. messenger RNA movement in cells, nucleus to mitochondria

16. Thermozier S, Epperly MW, Franicola D, Zhang X, Fisher R, Shields D, Wang H, Willis JA, Luke C, Silverman GA, and Greenberger JS. Hematopoietic progenitor cells form the bone marrow of Serpin3A-/- mice are radioresistant. ASH Meeting, San Diego, CA, December, 2016, Blood, 128: 2680, 2016.

17. Niehaus JZ, Miedel MT, Good M, Wyatt AN, Pak SC, Silverman GA, and Luke CJ. SERPINB12 is a slow-binding inhibitor of Granzyme A and Hepsin. Biochemistry, 54: 1 – 18, 2015.

18. Niehaus JZ, Good M, Jackson LE, Ozolek JA, Silverman GA, and Luke CJ. SERPINB12 is an abundant intracellular Serpin expressed in most surface and glandular epithelia. J Histochem Cytochem, 63(11): 854-870, 2015.

19. Richardson, D.S., and Lichtman, J.W. (2015). Clarifying Tissue Clearing. Cell *162*, 246–257.

20. Watkins, S.C., and St Croix, C.M. (2018). Light sheet imaging comes of age. J. Cell Biol. *217*, 1567–1569.

21. Watson, A.M., Rose, A.H., Gibson, G.A., Gardner, C.L., Sun, C., Reed, D.S., Lam, L.K.M., Croix, C.M.S., Strick, P.L., Klimstra, W.B., et al. (2017). Ribbon scanning confocal for high-speed high-resolution volume imaging of brain. PLOS ONE *12*, e0180486.