A Semi-Automated Assay for Radiation-Induced Intestinal Damage in Mice

by

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A semi-automated assay for radiation-induced intestinal damage in mice

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Abstract

**Purpose:** The effectiveness of radiotherapy to eradicate cancers is limited by normal tissue that is inevitably included in the irradiated volume. Drugs are being developed with the hope of increasing the therapeutic ratio between tumour cell eradication and normal tissue damage. Improvements in assays to measure normal tissue toxicity are needed. This thesis summarizes the development of a semi-automated intestinal assay with three analysis models - *crypt proliferation* assessing S-phase cells, *villi morphology* assessing villi size and *DNA damage* assessing DNA double strand breaks (DSBs). We use computer automation to address issues such as subjective assessment of damage.

**Methods:** Mice were irradiated with 0-15 Gy x-rays and euthanized at 48 h, 3.5 day and 1–24 h for proliferation, morphology and DNA damage models respectively. Mice received 5-Ethynyl-2’-deoxyuridine (EdU) to stain S-phase cells. A 5 cm length of the jejunum was collected, cut open, wrapped to form a swirl and frozen for cryosectioning. Cryosections (10 µm) of the jejunal swirl were stained and imaged using a robotic microscope; images were processed using ImageJ software. EdU was identified via histochemical staining and the proliferation model reports the proportion of pixels positive for EdU per length of the jejunum. Endothelial cells were identified using immunostaining for CD31, with the villi morphology model reporting the length of villi capillary beds. γH2AX was stained to quantify DSBs, with the DNA damage model reporting the amount of DSBs. A statistical power and assay sensitivity analysis were
performed to estimate the detectable effect size and robustness to variability.

**Results:** Proliferation and length of villi were inversely related to radiation dose and the intensity of γH2AX was inversely related to time post-irradiation. Applying to future drug evaluations, power analysis estimates that both proliferation and morphology models can detect EdU and villi length alterations >20%, while the DNA damage model can detect γH2AX modifications >10%. Sensitivity analysis showed that the proliferation model could withstand <30% random error while both morphology and damage models could withstand <15%.

**Conclusion:** A semi-automated intestinal assay was developed with three models applicable to assessing the effects of radiation dose-modifying drugs in intestinal tissue.
Radiotherapy is an important way to treat cancer, but damage to ‘normal’ cells included in the beam limits effectiveness. Drugs are being developed to combine with radiation and improve its cell-killing effects. However, these new drugs may also affect the ‘normal’ cells, therefore a good system to test the cell-killing effect in non-cancerous cells is needed. We have designed a system that permits examination of the effects of radiation in normal tissues. Three models were developed using the intestines of mice to assess how much radiation damage they have sustained by looking at: 1) the ability of cells in the intestines to reproduce; 2) the functional structure of the intestine; and 3) the amount of DNA damage. These models can be used in future mouse studies to test the effect of drugs in normal tissues when combined with radiotherapy.
Preface

Dr. Jennifer Baker, who designed and conducted the preliminary experiment in terms of irradiation and tissue collection, initiated this project. I took over the project starting with staining and analyzing the preliminary data. Experiments hereafter were designed, executed and analyzed by myself under the approval and guidance of Dr. Jennifer Baker.

I have received training in the usage of irradiation machine from the radiation officer and proper techniques in handling, injections and euthanization of mice from the Animal Resource Centre of BC Cancer Research Centre. For immunohistochemistry and automated imaging platform usage, I was trained by Dr. Jennifer Baker. All aspects of hands-on laboratory work were performed by myself with occasional assistance offered by our animal technician. The automated imaging platform was designed and built by Dr. Alastair Kyle. The automated analysis programs running on ImageJ were written and optimized by Dr. Alastair Kyle according to my requirements.

The University of British Columbia Animal Care Committee approved the animal studies under the ethics certificate A17-0042.
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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BrdUrd</td>
<td>5-Bromo-2’-deoxyuridine</td>
</tr>
<tr>
<td>CBC</td>
<td>Crypt base columnar cells</td>
</tr>
<tr>
<td>CD31</td>
<td>Cluster of differentiation 31</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variant</td>
</tr>
<tr>
<td>DSB</td>
<td>Double strand break</td>
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<tr>
<td>ED50</td>
<td>Effective dose 50</td>
</tr>
<tr>
<td>EdU</td>
<td>5-Ethynyl-2’-deoxyuridine</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin fixation, paraffin embedding</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>γH2AX</td>
<td>Phospho-histone H2A.X (Ser139)</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin &amp; Eosin</td>
</tr>
<tr>
<td>H2AX</td>
<td>H2A histone family member X</td>
</tr>
<tr>
<td>IGRT</td>
<td>Image-guided radiotherapy</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IMRT</td>
<td>Intensity-modulated radiotherapy</td>
</tr>
<tr>
<td>IP</td>
<td>Intra-peritoneal</td>
</tr>
<tr>
<td>IR</td>
<td>Ionizing radiation</td>
</tr>
<tr>
<td>ISC</td>
<td>Intestinal stem cell</td>
</tr>
<tr>
<td>LI</td>
<td>Labeling index</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non-homologous end joining</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimum cutting temperature medium</td>
</tr>
<tr>
<td>---------</td>
<td>-------------------------------------------------</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffered saline + 10% Tween</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PECAM</td>
<td>Platelet endothelial cell adhesion molecule</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PIKK</td>
<td>Phosphoinositide 3-kinase related kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>Radiotherapy</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SI</td>
<td>Small intestine</td>
</tr>
<tr>
<td>SSB</td>
<td>Single strand break</td>
</tr>
<tr>
<td>TA</td>
<td>Transit-amplifying cells</td>
</tr>
<tr>
<td>TI</td>
<td>Therapeutic index</td>
</tr>
<tr>
<td>WBI</td>
<td>Whole body irradiation</td>
</tr>
<tr>
<td>4DRT</td>
<td>4-dimensional radiotherapy</td>
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</tbody>
</table>
Acknowledgements

First and foremost, I would like to thank Dr. Andrew Minchinton for accepting me as a graduate student and giving me a chance to participate in the pre-clinical studies of a novel DNA damage repair inhibitor with some of the top scientists in the field.

I owe particular thanks to Dr. Jennifer Baker who taught and guided me through the research design, execution, and data analysis of my thesis as well as patiently provided timely suggestions to my written reports. She has enlarged my visions for scientific research, inspired me to question more deeply and improve my skills in academic writing.

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Last but not least, I’d like to thank my family for their continuous love and support, particularly my husband Yuanxi Wang, through even the most difficult times.
To my dearest mother, Liu Shan, and the love of my life, Wang Yuanxi.
Chapter 1: Introduction

1.1 Radiotherapy: historical advancements in mitigating normal tissue toxicity

The global burden of cancer is predicted to continually increase, stabilizing its position among the leading causes of death worldwide\(^1,2\). The International Agency for Research on Cancer estimated that 18.1 million incidences would be revealed in 2018 and it was projected that 29.5 million incidences will be found in 2040\(^2\). In 2017, the Canadian Cancer Society proposed that nearly half of all Canadians will develop cancer at some time during their lifetime and one quarter of these Canadians will die from cancer\(^3\). Over the past decades, progress in treatment development diminished the overall risk of dying from cancer in Canada. Patients surviving longer also means that acute normal tissue toxicity that may develop into long-term toxicity affecting patient’s quality of life requires attention\(^3\).

Among current treatment modalities, radiotherapy remains a major contributor to curative and palliative cancer treatment. Approximately 50% of all diagnosed patients receive and benefit from radiotherapy as their treatment regimen and 40% of patients who received radiation treatment are cured\(^1,4-6\). However, challenges remain in increasing the relative radiation dose delivered to tumour tissues while protecting surrounding normal tissue. Although advancements in technology have allowed increased precision in radiation dose delivery, normal tissue at close proximity is inevitably irradiated. As a result, radiation-induced toxicity in normal tissue narrows the
therapeutic index (Figure 1.1) and has historically been a key limitation to curative radiation treatment.

Figure 1.1 Therapeutic index of radiotherapy. As the probability of tumour control increases with increase in radiation dose, the probability of normal tissue injury increases as well, creating a narrow therapeutic window.
1.1.1 Advancements in radiotherapy to improve the therapeutic index

Soon after the discovery of x-rays by Wilhelm Conrad Roentgen in 1895, its therapeutic use in cancer commenced\(^7\). However limited penetration into tissue layers made low energy x-rays (<50 keV) applicable for superficial tumour treatments while deeper tumours within the body could not be efficiently eradicated. Moreover, the placement of cathode ray tubes at close proximity to tumours resulted in extensive normal tissue toxicity. Claudius Regaud later showed that skin toxicity was mitigated when full external beam radiation doses were separated into 3 days spaced 15 days apart, marking the introduction of the fractionation concept\(^7\). The success in eradicating tumours and the degree of normal tissue toxicity were both dependent on the total radiation dose relative to the fractionated dose, which takes advantage of the difference in repair capacity between cancerous and normal cells. Increasing total dose was, and still is, limited by the ability of the surrounding normal tissue to withstand and recover from the delivered dose.

Technological advancements in external beam therapy allowed development of a radiation beam with megavoltage and photon energy > 0.33 MeV\(^8\). The increased energy in the delivered radiation dose allowed less attenuation in the beam path compared to low-energy x-rays. Therefore, penetration into deeper tissues, such as the lung, and diminished dose deposition in the skin compared to low-energy x-ray treatment was achieved. Precision in radiation delivery has been revolutionized by imaging. Three-dimensional imaging is routinely used to plan treatments that conform
to the tumour volume, minimizing normal tissue inclusion\textsuperscript{8-10}. For example, computer tomography (CT)-assisted treatment planning is one of the standard operations in treatment planning\textsuperscript{11,12}. CT itself utilizes x-rays emitted at various angles to produce detailed images. Other modalities include magnetic resonance imaging (MRI), which relies on magnetic force and radio waves for imaging\textsuperscript{13,14}. Positron emission tomography (PET) is another imaging technique based on detecting metabolic processes in the body using a radioactive tracer\textsuperscript{15,16}. Recent advancements in hybrid-radiotherapy machines allowed concurrent imaging and radiation delivery that further contributed to treatment precision. Image-guided radiotherapy (IGRT) performs pre-treatment imaging and allows micro-adjustments relevant to tumour alterations along the course of treatment\textsuperscript{9,15}. Intensity-modulated radiation therapy (IMRT) allows irregular-shaped field planning utilizing multi-leaf collimators to deliver different intensities of radiation to different locations in the radiation field\textsuperscript{17,18}. Four-dimensional radiotherapy (4DRT) allows imaging during ventilation cycles to visualize tumour motion within the body\textsuperscript{8}. Treatments can be planned by either expanding radiation field to include tumour motion or restrict dose delivery only when tumours are confined within a pre-specified margin. However, even with these technological advancements, the inclusion of non-cancerous tissues is still inevitable. The boundaries of tumour masses are estimates and portions of the surrounding tissue are intentionally included to ensure full irradiation of the entire tumour mass. As a result, normal tissue exposure along the radiation beam path and surrounding the tumour volume can still be a limiting factor in delivering lethal doses to malignancies.
1.1.2 Cellular responses to ionizing radiation and its implications to enhancing the therapeutic index

With the discovery of DNA as hereditary material, it was widely accepted that the major target of radiation-mediated cell killing is the chromosome. The role of DNA repair, replication as well as genomic instability all contribute to the fate of the cell in induction of apoptosis upon radiation damage. Although the presence of tumour hypoxia has been observed to reduce treatment outcome, direct and indirect interactions of radiation can cause damage to most DNA components with some resulting in DNA single strand breaks (SSBs) and double strand breaks (DSBs). DSBs are most dangerous to cells because if unrepaired they often result in cell death\textsuperscript{19-21}. SSBs in close proximity can be converted to a DSB as well\textsuperscript{19}. On the one hand, the ability of cancer cells to repair DSBs is therefore believed to be one of the important mechanisms of resistance to radiation\textsuperscript{20}. The oxygen fixation hypothesis, in which the absence of oxygen molecules at the time of irradiation to “fix” the broken DNA ends, attempts to explain the increased radioresistance observed in hypoxic cells compared to oxygenated cells. On the other hand, inappropriate repair may give rise to genome instability such as mutations or chromosome aberrations. Ultimately, cellular death can be commonly provoked in the form of 1) apoptosis, also known as programmed cell death in which the cell shrinks forming fragmented DNA with intact cellular membrane; 2) mitotic catastrophe in which the formation of aberrant chromosomes result in giant cells during unsuccessful mitosis; and 3) necrosis in which organelles swells and the cell membrane rupture releasing cellular contents\textsuperscript{1}. 
Studies in pathophysiology, cellular and molecular mechanisms of DNA repair have enabled development of targeted therapeutics that can complement and enhance the effect of radiotherapy. Drugs have been developed targeting redundant DNA repair pathways, producing synthetic lethality. For example, inhibiting alternative non-homologous end-joining pathway in the presence of deficient non-homologous end-joining is a form of synthetic lethality in which the 2 genes that are both intended repair DSBs are compromised. Cancer cells that have compromised DNA repair mechanisms can be sensitized to radiation resulting in increased probability to radiation-mediated cell death. In turn, decreased radiation doses can be given in combination with a dose-modifying drug to sensitize and eradicate tumours while alleviating toxicity to surrounding normal tissue from the effects of radiotherapy. As a result, expanding the therapeutic index (Figure 1.2A). Developments of drugs that mitigate the effects of radiation in normal tissue have been investigated as well. This approach is based on the concept that lethal doses can be delivered to malignancies while the drug in combination would protect surrounding normal tissues that is included in the radiation beam. As a result, expanding the therapeutic index as well (Figure 1.2B). The goal of either sensitizing cancer cells or protecting/mitigating normal cell damage is to widen the therapeutic index of radiotherapy (Figure 1.2). For this reason, evaluating the effects of novel drugs in normal tissue is an essential process to determine the new therapeutic index modified by a drug.
Figure 1.2 Expanding the therapeutic index. Efforts in drug developments have been made in expanding the therapeutic window by either A) increasing tumour sensitivity to radiation or B) mitigating normal tissue toxicity to radiation.
1.2 Normal tissue toxicity upon irradiation: The intestinal crypts

The human body consists of a diverse population of cells that have specific functions and distinct structures with unique physiological roles. Conventional recognition of a cell type is by morphological features but cells of the same type that are resident at different locations can exhibit differing cellular activities and gene expression levels which further complicates categorization\(^{22}\). As previously mentioned, curative radiotherapy is greatly dependent on the amount and the type of normal tissue included in the radiation beam. Since the cells in the human body are variable therefore different manifestations of normal tissue injury induced by radiation can appear. Among the various types of injury, gastrointestinal damage has been well characterized and utilized to perform preclinical drug toxicity evaluations in rodent models\(^{23}\).

1.2.1 Pathophysiology of radiation-induced intestinal damage

The small intestine is composed of rapidly renewing epithelia that is supported by the intestinal stem cells (ISCs) residing in the crypts (Figure 1.3). Crypt base columnar (CBC) cells are highly proliferative stem cells while +4 stem cells (named after its position; usually position 4 found from the bottom of the crypt with the central paneth cell as position 0) are quiescent under homeostatic conditions\(^{24-27}\). Each crypt sustains its adjacent villi, which are finger-like projections into the lumen. The villus is composed of 3 major post-mitotic cell types: absorptive enterocytes, mucus-secreting
goblet cells and hormone-secreting enteroendocrine cells\(^{28-30}\). Upon differentiation from transit-amplifying cells, which are progenitors in the crypt proliferation zone, cells migrate into the villus as senescent cells are shed from the villus tip. Paneth cells are antimicrobial-peptide secreting cells that upon differentiation migrate down to the bottom of the crypt and reside for months before renewal\(^{28,30}\). The simple columnar epithelium of the villi encapsulates microvasculature that forms capillary beds. The structural organization of the villi increases surface area for efficient nutrient absorption. A newly differentiated epithelial cell would migrate from the crypt into the villus and shed into the lumen in 3 – 5 days\(^{28,31-33}\). The integrity of the intestinal epithelium relies on an equilibrium between homeostatic differentiation in the proliferation zone, apoptosis and shedding of senescent cells from the villi tip\(^{28,34}\).
Figure 1.3 The intestinal epithelium. A single-layer of columnar cells composes the intestinal epithelium. Enterocytes (orange) are most abundant in the epithelium. Transit-amplifying cells (TA; purple) are the homeostatic proliferative progenitors residing in the crypts along with quiescent stem cells (green). Crypt base columnar cells (CBC; red) are proliferative cells producing TA progenitors. Paneth cells (yellow) are immune cells residing at the bottom of the crypts. Goblet cells (blue) are sparsely located in the upper crypt and villi epithelium. Under homeostasis, cells would migrate from the crypt into the villi and eventually extrude into the lumen in 3 – 5 days after differentiation with the exception to CBC and Paneth cells which migrate downwards to the bottom of the crypt.
The pathophysiology of irradiated intestinal tissue damage can be complicated as the intestine is composed of a complex system including vascular and immune components, epithelial and stem cells as well as other cell populations. Transgenic murine studies have also demonstrated the complexity in response to radiation damage between different model systems. Whole body irradiation of 8.5 Gy result in 50% of mice survival by the 30\textsuperscript{th} day (LD\textsubscript{50/30}), but this value can be variable depending on the strain of the mice. For example, both outbred CD1 mice and CD2F1 mice have LD\textsubscript{50/30} at 8.5 Gy\textsuperscript{35,36}. BALB/c mice reached LD\textsubscript{100/30} while C57BL/6 mice reached only LD\textsubscript{10/30} when both strains were exposed to 7 Gy irradiation\textsuperscript{37}. In general, total body irradiation up to 8 Gy causes serious injuries but does not sterilize crypts, which are able to fully recover\textsuperscript{38,39}. Gastrointestinal syndrome begins at 6 - 8 Gy\textsuperscript{36}. Radiation doses above 10 Gy are considered lethal doses and can induce loss of CBCs and activate quiescent +4 stem cells in an attempt to rescue the crypts\textsuperscript{36}. Surprisingly, many early progenitors can be “recalled” into the stem zone for restoration as well\textsuperscript{24,27,40–42}. As a compensatory mechanism to the proliferation time that was lost during damage repair, the crypt stem cells hyper-proliferate in an attempt to regenerate and re-establish epithelial integrity\textsuperscript{43,44}. During the hyper-proliferative stage, the ISC\textsubscript{s} shorten their cell cycling time from 24 to 8 h, taking one third of the homeostatic time for restoration\textsuperscript{44,45}. When radiation doses increase, intestinal epithelial damage can occur due to inadequately replacing epithelial cells that does not match the rate of apoptotic or mitotic death. Consequently, epithelial lesions resulting in ulcers and bacterial infiltration can result in pro-inflammatory responses that may eventually lead
to septic shock or death.

1.2.2 Implications and applications of radiation-induced intestinal injury

Early clinical signs of radiation-induced GI toxicity can appear within days after treatment. These symptoms can include nausea, vomiting, diarrhea, decreased appetite, fluid and electrolyte loss and increased stool frequency\textsuperscript{23}. The severity of the complication is dependent on the amount of intestinal tissue that is included in the radiation beam as well as the amount of total radiation dose delivered. Radiation-induced DNA damage can delay or inhibit crypt stem cell proliferation and proliferation is a requirement for survival of the crypts and therefore of the organism. For this reason, intestinal toxicity can be indicated by attenuation in proliferative properties of crypt cells. It has been found that ISCs exhibit fluctuating proliferative potentials throughout the day\textsuperscript{46}. A synchronized sinusoidal trend was observed with roughly 20\% of $^3$H-TdR labeling index (LI; radioactive $^3$H-Thymidine incorporated into cells during S-phase to detect proliferation) at 0900 then inclines reaching roughly 35\% LI at 2400 hour then declines reaching roughly 20\% LI at 0900 again\textsuperscript{46}. The mice were in a 12/12 light-dark cycle (light =0600 - 1800; dark = 1800 - 0600) so the time of the day to irradiate the intestine could results in different degrees of proliferative toxicity\textsuperscript{47,48}. The well-characterized mechanisms of the intestinal response to radiation prompted researchers to develop strategies that either protect normal tissue from injury or specifically sensitize tumour tissues to radiation. Pre-clinical drug testing in the murine intestine has been a favorable model as both qualitative and quantitative
measurements can be made. The most utilized parameters that have been assessed historically include: crypt survival, villi length, crypt length, crypt width, DNA damage and apoptosis. The highly proliferative properties of the crypt stem cells, the structural integrity of the intestinal villi and the amount of DNA damage induced by an ionizing radiation (IR) beam can all inform acute toxicity. More importantly, acute toxicity of potential drugs in animal models is also a piece of required information in compliance with preclinical study guidelines established by many regulatory authorities.49

1.3 Historical intestinal assays in pre-clinical studies

The rapid proliferating properties of the intestinal crypts makes them highly sensitive and prompt indicators of radiation damage, allowing establishment of intestinal toxicity assays. A number of studies in the past were built to investigate treatments that would potentially mitigate the toxic effect of abdominal/pelvic radiotherapy or systemic chemotherapies in murine models.

The physiology and anatomical structures are very similar between mice and human intestines and so the mouse intestine is widely used in pre-clinical studies (Table 1.1). The overall gastrointestinal tract is composed of mouth, esophagus, stomach, small intestine, large intestine and anus for both human and mice.50. Similar tissue and cellular composition in the intestinal anatomy are observed as well. The mouse small intestinal villi are longer compared to human intestinal villi, which is believed to be a compensation for a lack of mucosal folds that are found in human
intestines\textsuperscript{50}. However, the overall function and gross anatomy are well conserved thus the mouse intestine is favored as a platform to predict potential treatment outcomes that may be seen in humans.
### Table 1.1 Anatomy of mice vs. human gastrointestinal tract

<table>
<thead>
<tr>
<th></th>
<th>Mice</th>
<th>Human</th>
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<tbody>
<tr>
<td><strong>Gross anatomy</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouth</td>
<td>present</td>
<td>present</td>
</tr>
<tr>
<td>Esophagus</td>
<td>present</td>
<td>present</td>
</tr>
<tr>
<td>Stomach</td>
<td>present</td>
<td>present</td>
</tr>
<tr>
<td>Small intestine (SI)</td>
<td>present</td>
<td>present</td>
</tr>
<tr>
<td>Cecum</td>
<td>present</td>
<td>adapted as part of large intestine</td>
</tr>
<tr>
<td>Large intestine</td>
<td>present</td>
<td>present</td>
</tr>
<tr>
<td>Appendix</td>
<td>Absent</td>
<td>present</td>
</tr>
<tr>
<td><strong>Small intestine features</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crypt</td>
<td>present</td>
<td>present</td>
</tr>
<tr>
<td>Villi</td>
<td>present; longer than human villi</td>
<td>present</td>
</tr>
<tr>
<td>Mucosal folds</td>
<td>absent</td>
<td>present</td>
</tr>
<tr>
<td>Epithelium</td>
<td>simple columnar</td>
<td>simple columnar</td>
</tr>
<tr>
<td>SI : Colon length ratio</td>
<td>2.5</td>
<td>7</td>
</tr>
<tr>
<td>Goblet cells</td>
<td>present</td>
<td>present</td>
</tr>
<tr>
<td>Paneth cells</td>
<td>present</td>
<td>present</td>
</tr>
<tr>
<td>Endocrine cells</td>
<td>present</td>
<td>present</td>
</tr>
</tbody>
</table>

Adapted from references^{50}
1.3.1 Intestinal assays and their applications

Perhaps Withers and Elkind\textsuperscript{51} described the most implemented intestinal assay – the gut microcolony assay that was established in 1970. The microcolony assay enables \textit{in vivo} sub-lethal whole body irradiation (WBI) whereas earlier \textit{in vitro} studies often required doses that would be lethal to mice\textsuperscript{51}. The mice would tolerate the radiation damage and survive for days before histological analysis on the jejunum crypts was done. Moreover, Withers and Elkind have claimed that, based on their findings, the repair capacity of the crypt cells is very similar to other tissue types therefore the model generated by the microcolony assay could also estimate responses of other normal tissues as well\textsuperscript{51,52}. The proliferative circadian rhythm as found in crypt stem cells is also found in the tongue and skin basal layers\textsuperscript{46}. Although the degree of change in proliferative capacity are not exactly the same amongst the different tissues, similar patterns may be observed. The models developed in this thesis were built based on the agreement with historical studies that the ISCs can (1) reflect early toxicity if injured and (2) estimate general responses to radiation-induced damage in other proliferative tissue types\textsuperscript{51}.

The original gut microcolony assay described by Withers and Elkind is as follows: mice were euthanized roughly 3.5 days after receiving 10 – 25 Gy WBI via x-ray. A 2 cm segment of the jejunum was excised and processed with routine histological procedures, which the details were not specified. Transverse sections of the intestinal jejunum were obtained and stained with haematoxylin and eosin (H&E).
Crypt viability was assessed via the overall appearance of the crypts. Crypts were considered regenerative if they contained 10 or more living cells as determined via cellular morphology. The number of surviving crypts was then normalized to the circumference of the jejunal transverse section. Ultimately, a dose vs. crypt survival relationship was obtained.

The gut microcolony assay has been widely implemented in the past 20 - 30 year to investigate intestinal mitigation to radio-or chemotherapies (Table 1.2). Bisht et al. 53 assessed the radiation modification effect of WR-2721 by obtaining 10 transverse paraffin-embedded sections to quantify the number of surviving crypts/circumference and found that WR-2721 treatment significantly increased surviving crypt population. Ten or more living cells scored by H&E staining defines a surviving crypt. A more recent study by Liu et al. 54 investigated the effects of WR-2721 in ameliorating intestinal damage by evaluating the number of crypts/circumference using H&E as well but without specifying the definition of a surviving crypt. Ha et al. 55 observed promoted intestinal crypt regeneration, post-irradiation, in the presence of IR protective Phloroglucinol in the small intestine; tissues were embedded in paraplast wax and sections were obtained to quantify regenerating crypts/circumference with 10 live cells or more defining a regenerating crypt. Similarly, Odawara et al. 56 evaluated the protective effect of Polaprezinc to IR in the intestinal epithelium via the gut microcolony assay as well and found that pre-treatment with Polaprezinc elicited stronger protective effects in the intestines compared to post-treatment. Lamas et al. 57 evaluated the radio-protective effects of the JNJ7777120 compound in the small
intestine by quantifying crypts/circumference scored via paraffin embedding followed by H&E staining. Qualitative assessment of proliferation in crypts was scored via a proliferating cell nuclear antigen (PCNA) antibody. They found that JNJ7777120 administration preserved villi morphology as well as the number of crypts by reducing radiation-induced apoptosis. Similarly, Zhang et al. 58 assessed the effects of Resveratrol in combination with irradiation in the intestines and concluded that Resveratrol treatment improved villi morphology and maintained cellular regeneration. They performed paraffin embedding of segments of small intestine followed by quantifying crypts/circumference in 6 transverse intestinal sections.

Several studies have utilized similar techniques that were adapted from the gut microcolony assay (Table 1.2). Tinkum et al. 59 investigated the impact of fasting upon administering etoposide in the intestinal epithelium. Intestinal segments were cut longitudinally and pinned flat for fixation followed by paraffin embedding and H&E staining. Cells/crypt as well as crypts/1mm length of intestine were quantified. Proliferation marker 5-Bromo-2'-deoxyuridine (BrdUrd) was administered prior to euthanization. The number of BrdUrd positive cells were manually counted to establish the amount of proliferation at staggered time points after treatment. Interestingly, their data shows that the percentage of BrdUrd positive cells was significantly decreased in the fasted group compared to the fed group in the absence of treatment. However, the number of BrdUrd positive cells was not significantly different between the fasted and the fed groups when both were exposed to etoposide. Lam et al. 60 also quantified cells/crypt in the intestine to assess the protective effect of PHY906 (a four-herb
Chinese medicine) in combination with Irinotecan. Paraffin embedded intestinal segments was cut to obtain transverse sections. Instead of H&E scoring, PCNA positive-cells were counted per crypt while BrdUrd incorporation was used to visualize the migration of cells from the crypt into the villi. Although PHY906 did not protect against Irinotecan-induced DNA damage, intestinal epithelium restoration was promoted by increased progenitor and stem cell regeneration. Chaudary et al.\textsuperscript{61}, Gani et al.\textsuperscript{62} and Liang et al.\textsuperscript{63} evaluated the effects of plerixafor, 12-O-tetradecanoylphorbol-13-acetate and AZD-2281, respectively, in combination with radiation in the intestinal jejunum. Proliferation marker Ki-67 was used to immuno-stain proliferating cells in paraffin embedded jejunal sections. Their definition of a surviving crypt is a crypt that contains \(>5\) Ki-67 positive-cells, reducing the objective parameter utilized by Withers and Elkind\textsuperscript{51} from 10 living cells to 5. Moreover, Ki-67 was used to determine proliferating cells instead of cellular morphological assessment with H&E. Both plerixafor and 12-O-tetradecanoylphorbol-13-acetate demonstrated reduced radiation-induced acute intestinal toxicity by promoting crypt stem cell proliferation while AZD-2281 resulted in significant tumour growth delay while did not augment intestinal toxicity. More recently, Fernandez et al.\textsuperscript{64} evaluated the effects of melatonin in combination with radiation in rat intestine using a modified microcolony assay. Similar to Chaudary et al.\textsuperscript{61}, the number of Ki-67 positive-cells were quantified but normalized to the total number of cells in the entire section. Instead of manually counting the number of cells, Ki-67 activity was measured using ImageJ with a cell counter plugin.

The length of the villi is indirectly related to the proliferative properties of the
crypt. As previously mentioned, the rapidly differentiating crypts sustain the epithelium integrity. Therefore, a reduced proliferative capacity would cause an imbalance between epithelial regeneration and extrusion. As a result, the length of the villi would decrease. For this reason, some studies evaluate villi length as an indication of toxicity (Table 1.2). Thibault et al. 65 conducted qualitative assessment of the rat intestine by visually demonstrating the change in villi length across different treatments. Briefly, bars were drawn, from the crypt-submucosal boundary to the crypt-villi junction, in the H&E stained intestinal sections to demonstrate the length of crypts. The length of villi was defined from the top of the bar to the lumen. Some studies quantified the length of at least 10 villi or the 10 longest villi from multiple transverse sections 58,59,64,66. Other studies assigned numerical scores according to the overall morphological damage of the transverse intestinal section 67.

Measuring the amount of double strand breaks to monitor radiation-induced normal tissue toxicity has also been useful. Upon radiation-induced DSB formation, it was first reported in 1998 that H2A histone family member X (H2AX) molecules are phosphorylated at the serine-139 position within the SQ motif 68. This phosphorylated form is named γH2AX for its finding after irradiation using γ-ray. Molecular studies have revealed that γH2AX can be phosphorylated by kinases within the Phosphoinositide 3-kinase family such as ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3 related (ATR) and Phosphoinositide 3-kinase related kinases (PIKK) such as DNA-dependent protein kinase (DNA-PK) within minutes post-irradiation. Foci of γH2AX form at the sites of DSB, reaching a maximum level as early as 30 minutes after
irradiation\textsuperscript{69,70}. γH2AX staining has been widely used in cell-based and tissues-based models to study radiation-induced DNA damage, including in the intestine (Table 1.2). Generally, formalin-fixed intestinal segments are paraffin embedded and immuno-stained with anti-γH2AX antibody. Rube et al.\textsuperscript{21} concluded that measuring γH2AX foci in tissue samples allowed determination of DSB repair deficiencies that may affect the radiosensitivity of normal tissues. For quantitative analysis, they recorded the number of foci in 80 cells counted by eye. They expressed the number of γH2AX foci at 0.5 – 48h following a 2 Gy irradiation dose. Tinkum et al.\textsuperscript{59} assessed 200 crypts at 1.5h and 3h to quantify the percentage of γH2AX positive-cells. Similarly, Hou et al.\textsuperscript{71} assessed γH2AX expression to quantify the radio-protecting effect of Thymoquinone in combination with radiation in the small intestine and found that Thymoquinone treatment modulated radiation-induced DNA damage and reduced γH2AX expression. The amount of DNA damage was quantified at 1, 6, and 12h following 19 Gy irradiation. They visually counted the percentage of ISCs in crypts that displayed γH2AX immuno-staining\textsuperscript{72}. The sensitivity of a novel transgenic mouse (Knock-in model to modify p53 activity) to radiation was also evaluated by assessing the expression of γH2AX in the small intestine among other normal tissues at 1h following 8 Gy irradiation\textsuperscript{73}. The above examples indicate that the use of γH2AX to study the capacity of repair after irradiation damage is widely implemented in pre-clinical settings.

In summary, pre-clinical evaluation of drugs, radiation or drug + radiation treatments have been widely studied in the small intestine of mouse models, as an indication of normal tissue toxicities. The rapidly renewing intestinal epithelium and the
resemblance between mice and human gastrointestinal anatomy render mice intestine a preferred pre-clinical model. The gut microcolony assay developed by Withers and Elkinder retains its popularity today. In fact, many recent studies implement the assay with the exact same parameters while others may deviate somewhat. Villi length measurements are popular too in taking advantage of the intestinal morphology to both illustrate and quantify changes to homeostasis. Moreover, the length of the villi signifies the capacity to facilitate nutrient absorption. Therefore, a reduction in the villi length can be interpreted as toxicity to functionality. γH2AX have been used to visualize the amount of DNA double strand breaks and monitor the capacity of repair after irradiation. Drugs that potentially modify the amount of damage or the rate of damage repair can be directly measured using γH2AX detection. All of these approaches to assess radiation-induced damage to the intestine are, importantly, in advance of animals manifesting the worst symptoms of a lethal dose, and do not require a survival outcome.
### Table 1.2 Summary of conventional intestinal assays

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Quantification</th>
<th>Time (day)</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gut Microcolony assay</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 – 25 Gy x-ray</td>
<td>Manually count the number of regenerating crypt / circumference; &gt; 10 living cells defines a regenerating crypt</td>
<td>3.5</td>
<td>Formalin fixation, paraffin embedding; H&amp;E staining of 6 – 10 transverse intestinal section;</td>
<td>Withers and Elkind.51</td>
</tr>
<tr>
<td>WR-2721 + 0 – 6 Gy γ-ray</td>
<td></td>
<td>1, 3, 7</td>
<td></td>
<td>Bisht et al.53</td>
</tr>
<tr>
<td>Phloroglucinol + 7 Gy Co-60</td>
<td></td>
<td>8.5</td>
<td></td>
<td>Liu et al.54</td>
</tr>
<tr>
<td>Polaprezinc + 15 Gy x-ray</td>
<td></td>
<td>3</td>
<td></td>
<td>Ha et al.55</td>
</tr>
<tr>
<td>JNJ7777120 + 5 Gy Cs-137</td>
<td></td>
<td>3</td>
<td></td>
<td>Odawara et al.56</td>
</tr>
<tr>
<td>Resveratrol + 7Gy Cs-137</td>
<td></td>
<td>6</td>
<td></td>
<td>Lamas et al.57</td>
</tr>
<tr>
<td><strong>Qualitative assessment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JNJ7777120 + 5 Gy Cs-137</td>
<td>Visualize difference in PCNA positive cells across treatments</td>
<td>3</td>
<td>PCNA antibody stained proliferation</td>
<td>Lamas et al.57</td>
</tr>
<tr>
<td>PHY906 + Irinotecan</td>
<td>Visualize migration of cells from crypt to villi</td>
<td>4</td>
<td>Formalin fixation, paraffin embedding; BrdUrd staining</td>
<td>Lam et al.60</td>
</tr>
<tr>
<td>CDK4/6 inhibitors</td>
<td>Visualize change in villi length across treatments</td>
<td>15</td>
<td>Formalin fixation, paraffin embedding; H&amp;E staining</td>
<td>Thibault et al.65</td>
</tr>
<tr>
<td><strong>Modified gut microcolony assays</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting + etoposide</td>
<td>Manually count cells / crypts; Crypts / 1mm length of intestine</td>
<td>5</td>
<td>Formalin fixation, paraffin embedding; H&amp;E, BrdUrd staining</td>
<td>Tinkum et al.59</td>
</tr>
<tr>
<td>PHY906 + Irinotecan</td>
<td>Manually count PCNA positive cells / crypt;</td>
<td>4</td>
<td>Formalin fixation, paraffin embedding; H&amp;E, PCNA staining</td>
<td>Lam et al.60</td>
</tr>
<tr>
<td>Plerixafor + x-ray</td>
<td>Manually count surviving crypt / circumference; &gt; 5 Ki-67 positive cells define a surviving crypt</td>
<td>3.5</td>
<td>Formalin fixation, paraffin embedding; H&amp;E, Ki-67 staining</td>
<td>Chaudary et al.61</td>
</tr>
<tr>
<td>12-O-tetradecanoylphorbol-13-acetate + 10 Gy γ-ray</td>
<td></td>
<td>3.5</td>
<td></td>
<td>Liang et al.63</td>
</tr>
<tr>
<td>AZD-2281 + 8 Gy x-ray</td>
<td></td>
<td>3</td>
<td></td>
<td>Gani et al.62</td>
</tr>
<tr>
<td>Melatonin + 7.5 Gy x-ray</td>
<td>Automatically count Ki-67 positive cells / total cell using cell counter plugin in ImageJ</td>
<td>14</td>
<td>Formalin fixation, paraffin embedding; H&amp;E, Ki-67 staining</td>
<td>Fernández-Gil et al.64</td>
</tr>
<tr>
<td>Treatment</td>
<td>Quantification</td>
<td>Time (day)</td>
<td>Method</td>
<td>Reference</td>
</tr>
<tr>
<td>-----------</td>
<td>----------------</td>
<td>------------</td>
<td>--------</td>
<td>-----------</td>
</tr>
<tr>
<td>Villi length assay</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resveratrol + 7 Gy Cs-137</td>
<td>Manually measure and average the 10 longest villi / section; 6 section / mouse</td>
<td>6</td>
<td>Formalin fixation, paraffin embedding; H&amp;E staining</td>
<td>Zhang et al.58</td>
</tr>
<tr>
<td>Fasting + etoposide</td>
<td>Manually measure and average 30 villi / animal</td>
<td>5</td>
<td></td>
<td>Tinkum et al. 59</td>
</tr>
<tr>
<td>Melatonin + 7.5 Gy x-ray</td>
<td>Ambiguous</td>
<td>14</td>
<td></td>
<td>Fernández-Gil et al.64</td>
</tr>
<tr>
<td>Coniferyl aldehyde + 12.5 Gy</td>
<td>Manually measure and average 10 longest villi / section; 10 sections / mouse</td>
<td>3.5 &amp; 30</td>
<td></td>
<td>Jeong et al.66</td>
</tr>
<tr>
<td>DNA damage assay</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 Gy x-ray</td>
<td>Manually count foci within 80 cells</td>
<td>0.02 – 2</td>
<td>Formalin fixation, paraffin embedding; Anti-γH2AX staining</td>
<td>Rube et al.21</td>
</tr>
<tr>
<td>Thymoquione + radiation Transgenic mouse model + 8 Gy</td>
<td>Manually quantify γH2AX expression</td>
<td>0.04</td>
<td></td>
<td>Hou et al.71, Kuser-Abali et al.73</td>
</tr>
<tr>
<td>Fasting + etoposide</td>
<td>Manually count percentage of γH2AX positive cells in 200 crypts</td>
<td>0.06 &amp; 0.125</td>
<td></td>
<td>Tinkum et al.59</td>
</tr>
<tr>
<td>19 Gy x-ray</td>
<td>Manually count percentage of intestinal stem cells positive for γH2AX staining.</td>
<td>0.04, 0.25, 0.5</td>
<td></td>
<td>Hua et al.72</td>
</tr>
</tbody>
</table>
1.3.2 Challenges associated with conventional intestinal assays

As described in earlier sections, numerous assays utilize mouse intestinal models to evaluate effects and side effects of drugs and/or radiation. Although the intestinal collection, processing and quantification methods have remained unchanged with minor deviations over the past few decades, there can be challenges and limitations associated with the assays. These labour-intensive methods can also yield subjective and non-comprehensive results.

Conventional tissue histology is performed via formalin fixation and paraffin-embedding (FFPE) for preservation and sectioning. Paraffin processing of tissue requires long hours of fixation. For optimal histology, tissues need to be adequately fixed for a minimum of 48 h, otherwise specimens may become dehydrated during processing resulting in problematic sectioning. After fixation, tissues are dehydrated using a series of ethanol baths in different concentrations. Tissues are submerged in each bath for up to 1 h. The infused ethanol solvents are then removed by a clearing agent, usually Xylene, followed by paraffin wax infiltration and embedding taking another several hours. Sections are mounted onto slides to air dry and are de-paraffinized using Xylene again followed by rehydration for Immunohistochemistry analysis. Overall, it can take up to a week for tissue processing and requiring more time until quantitative results are obtained. Although tissues embedded in paraffin blocks can be stored for years and morphological features are well preserved, the extensive labor and requirement for numerous reagents makes paraffin methods
undesirable for quick turn-around analysis. Moreover, an antigen retrieval process is often required to perform immunohistochemistry. This step is often heat-induced in which the balance between the temperature, time of incubation and pH is critical to unmask epitopes for antibody binding without damaging tissue morphology. The above described multi-stepped FFPE technique is still widely used today but the labor-intensive method is not optimal. Moreover, fixatives that dehydrate tissues may shrink tissue size therefore potentially result in biased measurements. Most importantly, the measurement of γH2AX is time sensitive and the extensive experimental procedures of FFPE may not yield trustable results. We performed cryopreservation to take advantage of its fast turn-around time and good antigenic exposure for histological analysis. A comparison between Cryopreservation and FFPE techniques is summarized in Table 1.3.
### Table 1.3 Paraffin-embedded tissue vs. frozen tissue

<table>
<thead>
<tr>
<th></th>
<th>Formalin fixation, paraffin embedding</th>
<th>Cryosection</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fixation</strong></td>
<td>Immediately after harvesting, submerge in formalin for at least 48h</td>
<td>After sectioning, submerge in formalin for 15 minutes</td>
</tr>
<tr>
<td><strong>Embedding</strong></td>
<td>Paraffin wax</td>
<td>OCT</td>
</tr>
<tr>
<td><strong>Sectioning</strong></td>
<td>5 µm thickness</td>
<td>10 µm thickness</td>
</tr>
<tr>
<td><strong>Immunostaining preparations</strong></td>
<td>Heat facilitated epitope exposure</td>
<td>None</td>
</tr>
<tr>
<td><strong>Overall time until sectioning</strong></td>
<td>Roughly 1 week</td>
<td>Roughly 3 h; same-day sectioning possible</td>
</tr>
<tr>
<td><strong>Advantages</strong></td>
<td>Good morphological conservation</td>
<td>Good antigenic/enzymatic detection</td>
</tr>
<tr>
<td><strong>Limitations</strong></td>
<td>Lengthy fixation can mask epitopes and not favorable for time sensitive antigenic labeling</td>
<td>Prone to artifacts if slowly frozen</td>
</tr>
<tr>
<td><strong>Storage</strong></td>
<td>Multiple years at room temperature</td>
<td>Approximately 1 year at -80°C</td>
</tr>
<tr>
<td><strong>Antigen retrieval needed?</strong></td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

Adapted from references\(^{77,78}\)
In conventional assays, most intestinal segments are fixed without further processing. As a result, a circular intestinal section often referred to as a transverse section is obtained. However, this format allows analysis of the intestine at just one location with roughly 50 villi and crypts for quantification at most. For this reason, 6 – 10 sections at different locations along the intestinal segment are often required for statistical analysis. A recent enhancement of the intestinal assay is described as the “Swiss-roll” method, in which the intestinal segment is longitudinally cut open and wrapped around a toothpick to form a “Swiss-roll” for FFPE. This method allows analysis along the entire intestinal segment in one section. In this thesis, the “Swiss-roll” method for tissue collection has been adapted and further enhanced.

Data acquisition has often been done by manually counting the number of cells in the various positions of the crypts, or that display particular features such as γH2AX labeling. As first suggested by Withers and Elkind, subjectivity can be included as cells of interest are determined by eye. Regardless, this manual method has been implemented in most gut microcolony assays until recent emergence of computer assistance. Withers and Elkind proposed an objective parameter that crypts containing more than 10 live cells, based on the cellular morphological features, are considered regenerating crypts. Later, proliferating cell markers such as PCNA or Ki-67 were used and the objective parameter reduced from 10 to 5 cells that are positive for a proliferation marker. However, it is debatable that whether a crypt containing 4 Ki-67 positive cells or 9 living cells can be concluded as non-regenerating. The results obtained with these objective parameters can be debatable. Finally, cell counting via a
cell counter plugin running on ImageJ have been executed as well. Although subjectivity may be reduced as a standardized operating procedure is implemented on all cells recognized by the software, cells in tissue structures can be in different sizes, shapes and orientations, and some may overlap with each other. Moreover, the intestine harbors different types of cells with different features so a system that can accurately determine units of cells can be difficult to establish.

Collectively, the concept in evaluating toxicity via alterations to intestinal homeostasis is rational. The methods in conventional intestinal assays can be further improved to reduced subjectivity while increasing efficiency for a comprehensive analysis. In this thesis, we hypothesize that the conventional intestinal assays can be further enhanced, and a comprehensive semi-automated analysis system can be utilized to develop three models for radiation-induced intestinal toxicity evaluating: proliferation, villi morphology and DNA damage. Each of the three models would be applicable in future pre-clinical studies testing radiation dose-modifying effects of drugs.
Chapter 2: Methods and materials

2.1 Animal model and irradiation

Ethics statement: Studies described in this thesis were approved by the Animal Care Committee of the University of British Columbia in accordance with the Canadian Council on Animal Care guidelines under the approved protocol A17-0042. Rag2M Mice (129S6/SvEvTac-Rag2tm1Fwa), bearing a mutation in recombination activation gene 2 leading to deficiency in mature B and T cells, were bred and housed in our institutional Specific-Pathogen-Free (SPF) Animal Resource Centre (ARC). Mice were monitored daily with continuous access to food and water.

Groups of up to 5 mice (female and male) aged 12 – 16 weeks old, received total body irradiation while within a rectangular plexiglass jig allowing only horizontal movements, and were supplied with air flow. Radiation was delivered using Pantak-Siefert XRAD320, 300 KeV and 10 mA with 1.5 mm A1, 0.25 mm Cu and 0.55 Sn filter. The dose rate was 1.27 Gy/min for all radiation treatments. Mice received 1000 mg/kg 5-Bromo-2’-deoxyuridine (BrdUrd; Sigma-Aldrich, St. Louis, USA) or 200 mg/kg 5-Ethynyl-2’-deoxyuridine (EdU; Sigma-Aldrich, St. Louis, USA) via IP injection 2 h prior to euthanization to stain S-phase cells. Intestines were collected for histology at 1) 48 h after receiving 7, 10, 12 and 15 Gy to develop the proliferation model. Mice were euthanized at 2) 3.5 days after receiving 2, 5, 7, 10, 12 and 15 Gy to develop the villi morphology model, and at 3) 1, 3, 6 and 24 h after receiving 8 Gy irradiation to develop the DNA damage model (Figure 2.1).
2.2 Isolation of mice jejunum

Mice were anesthetized using inhaled isoflurane then euthanized via CO₂ asphyxiation followed by cervical dislocation as a secondary physical euthanasia\(^7\). Euthanized mice were laid on their dorsal side and the ventral side disinfected with 70% ethanol. The intestinal-stomach junction was excised, and the intestine slowly removed by gentle pulling. The first 5-7 cm was discarded to ensure complete elimination of the duodenum and the proceeding 5-7 cm was collected (Figure 2.2). The collected segment of jejunum was placed in a petri dish submerged in PBS to prevent moisture loss. A blunt ended gavage needle was inserted into the lumen of the jejunum and phosphate buffered saline (PBS) was flushed through the tissue to expel food particles. A second gavage needle was used to fill the intestinal lumen with 30% Tissue-Tek OCT (Sakura Finetek, California, USA) homogenized in PBS. The jejunum was cut open longitudinally using scissors to open and lay flat. A hemostat clamped the end of the jejunal segment and was gently turned such that the jejunum was wrapped around the hemostat tip to roll and form a swirl. The intestinal swirl was then immediately placed on a frozen aluminum block for rapid freezing and temporary storage in a -20°C freezer. Up to 6 frozen swirls were organized into arrays and embedded with OCT for optimal cryosectioning and storage in a -20°C freezer.
2.3 Cryosection and immunohistochemistry staining

Cryosections at 10 µm thickness were cut with Cryostar NX70 (ThermoFisher Scientific, Waltham, USA) and adhered onto a microscope slide (Figure 2.2). The slides were immediately submerged in 10% buffered formalin phosphate (Fisher Scientific, Hampton, USA) for 15 min and in PBS bath for 2 mins followed by PBS + 10% Tween (PBST) bath for 10 min. Once cryosections were obtained for all the tissues in an experiment, fixed and rinsed slides were paired with empty slides such that PBST and other reagents could be wicked up between the two slides using the capillary gap system. Pairs of slides were collected into a handle for batch processing.

BrdUrd was detected via immuno-staining. Slides containing tissues were submerged in Sodium citrate (10 mM; BioShop, Burlington, Canada) with 0.05% Tween and autoclaved at 125 – 130°C for 25 min. Slides removed from the autoclave were quickly cooled down in an ice water bath then washed with PBS and loaded into the capillary systems. The detection of BrdUrd was by rat anti-BrdUrd (1 µg/ml; Bio-Rad, California, USA) incubation for 1 h followed by chicken anti-rat IgG secondary antibody, Alexa Flour 488 (4 µg/ml; ThermoFisher Scientific, Waltham, USA) for 30 min with 3x PBST wash in between.

EdU was detected via EdU click chemistry cocktail: L-ascorbic acid (20 mg/ml; Bioshop, Burlington, Canada), CuSO₄ (4 mM; Fisher Scientific, Hampton, USA) and Sulfo-Cy3-Azide (4 µg/ml; Jenna Bioscience, Labsteder, Germany), was wicked up into
the capillary system, and incubated at room temperature on a shaker (300 rpm) for 30 min. Reagents were then removed via blotting and slides rinsed 3x with PBST.

Tissues were stained for purified rat-anti-mouse CD31 (1 µg/ml stock; BD Pharmingen, San Jose, California) for 1 h, chicken-anti-rat Alexa Fluor 488 (4 µg/ml; ThermoFisher Scientific, Waltham, USA) for 30 min followed by 15 min of formalin incubation with PBST washes in between.

Tissues were stained for anti-phospho-Histone H2A.X Ser 139 (2 µg/ml; Millipore, Burlington, USA) for 1 h, goat-anti-mouse secondary Alexa Fluor 647(4 µg/ml; ThermoFisher Scientific, Waltham, USA) for 30 min followed by 15 min of formalin incubation with 3x PBST washes in between.

Hoechst labeling of nuclei was done to visualize tissue morphology. Tissues were incubated with bis-benzamide H33342 trihydrochloride (0.02 mg/ml; Hoechst 33342; Sigma-Aldrich, St. Louis, USA) for 30 min followed by 3x PBST wash. Paired slides were then taken apart that slides containing tissues were thoroughly washed by submerging in PBS bath with magnetic stir bar for 10-15 min.

2.4 Automated image acquisition and analysis

Slides in capillary systems were taken apart and the slides with tissues attached are thoroughly rinsed with PBS then loaded onto an imaging system platform consisting of a Robotic Fluorescent microscope (Zeiss Imager Z1), Nikon Plan Fluorite Imaging 10X objective (N10X-PF), sCMOS camera (pco.edge 4.2; PCO Photonics).
Customized ImageJ software operational on Macintosh computer creates tiling of adjacent microscope fields. Jejunal cryosections, roughly 1cm², were captured at 1.3 μm resolution/pixel (Figure 2.2).

Sectioning and staining artifacts were manually removed using ImageJ. Quantification of proliferation was done by obtaining a ratio between the number of BrdUrd or EdU positive pixel to the length of jejunum analyzed. BrdUrd or EdU positive pixels were determined via a threshold system such that pixels with intensities above the threshold was positive. The length of jejunum was determined by drawing a border along the lamina propria – submucosa boundary of the intestinal segment and the number of pixels that compose the length of the border represents the length of the jejunum. Villi length quantification was done by measuring the length of 30 villi per image. The length of each villi was denoted by the length of the CD31 stained capillary from the crypt-villi junction to the tip of the capillary bed. Quantification of DNA damage was done by obtaining the average γH2AX staining intensity. Pixels representing tissues were first distinguished from background via Hoechst 33342 staining then these pixels were used to obtain the staining intensity in the γH2AX layer.

2.5 Statistical analysis

All statistical analysis was performed using Graphpad prism 7. Non-linear regression curve fitting was done for all 3 models. The inter-assay coefficient of variation (CV) represents the variability between mice that received the same treatment.
The intra-assay CV represents the variability between sections of the same intestinal swirl. Intra-assay CV was calculated using 4 tissue sections per animal for each model. The non-parametric t-test: Mann-Whitney test was used to determine significance between two independent treatments. The non-parametric one-way ANOVA: Kruskal-Wallis test was used to determine significance across more than two independent treatments. A p < 0.05 was taken as significant. A sample size power calculation, using the mean of experimental results with a level of 0.05 significance, 80% power and assuming similar sizes of error variability, was done to make predictions of the sample size required to detect differences between two groups. Sensitivity analyses were done by artificially and randomly introducing errors to data points, followed by comparing the new models to the 95% CI of the original model as well as comparing model parameters. New models that did not reside within the 95% CI of the original model were considered significantly different. Parameters that did not lie within the 95% CI of the original model’s parameters or sustained ambiguous values were determined as significantly different.
**Figure 2.1 Intestinal toxicity assay – experimental scheme.**

1) **DNA damage model** – mice received 8 Gy whole body irradiation and were euthanized at 1, 3, 6 and 24 h post-irradiation. Staining: gamma-H2AX, Hoechst 33342. Quantify: staining intensity.

2) **Crypt proliferation model** – mice received 0–15 Gy whole body radiation and 200 mg/kg EdU via IP injection at 46 h. Tissues were collected at 48 h for model establishment. IP injection: 200 mg/kg EdU at 46h. Euthanize: 48h. Staining: EdU, Hoechst 33342. Quantify: proliferation / length.

3) **Villi morphology model** – mice received 0–15 Gy whole body irradiation and were euthanized at 84 h post-irradiation for model establishment. Euthanize: 84h (3.5 day). Staining: CD31, Hoechst 33342. Quantify: Villi length.
Figure 2.2 Overview of the intestinal toxicity assay. The first ~5 cm of the small intestine is discarded and the following ~5 cm collected. The segment is flushed with PBS, filled with OCT and cut longitudinally to open flat. The flat intestinal segment is wrapped into a swirl using a hemostat and placed on an aluminum block for rapid freezing. The swirls are organized into an array and embedded using OCT. Using a cryostat, 10 µm sections are cut and adhered onto a microscope slide. Slides are paired with an empty slide and combined for batch processing. Tissues are Immuno-stained with EdU, CD31 and γH2AX. Slides are taken apart for imaging. Artifacts are removed and followed by quantification to develop the Crypt proliferation, villi morphology and DNA damage models.
Chapter 3: Establishment of a semi-automated immunohistochemistry-based intestinal toxicity assay

3.1 The crypt proliferation model

The crypt proliferation analysis outputs the percentage of proliferation (normalized to intestinal length) relative to the radiation dose (Gy). Our preliminary experiment quantified crypt proliferation via BrdUrd staining. Instead of counting and recording the number of cells that stains positively for BrdUrd that has been described in conventional assays, our automated system evaluated every pixel in the image. All pixels with intensities above a user-specified threshold were recorded. Since the crypts along the length of the jejunum are similar in size and proliferation only occurs in the crypts, therefore expressing the fraction of positive pixels in the crypts relative to the length of the jejunum is representative of proliferation (Figure 3.3). Figure 3.5A shows that at 24 h post-irradiation, the proliferation profiles are not significantly different between 2 – 10 Gy treatment groups (p=0.096). This outcome may indicate that the 24 h time point is too early to observe a proliferative response. Therefore, a follow up experiment collected tissues at 48 h post-irradiation. As shown in Figure 3.5B, there was a proliferation surge at 5 Gy that was significantly different compared to the non-irradiated control (p=0.016). The surge at 5 Gy has previously been reported to be the result of the hyper-proliferative phase responding to radiation injury: activation of quiescent stem cells as well as recalling differentiated cells back into the proliferation pool. To confirm the relationship between radiation dose, time post-irradiation and...
proliferation surge, a third experiment collecting tissues at 3.5 days to quantify crypt proliferation for the same 2 - 15 Gy range was conducted (Figure 3.5 C). The proliferation surge shifted from 2 - 7 Gy at 48 h to 7 - 10 Gy at 3.5 days. Proliferation at both 7 and 10 Gy were significantly higher compared to the non-irradiated control (p=0.0079 for both). Radiation treatment at 15 Gy yielded proliferation that was significantly decreased compared to an non-irradiated control (p=0.0159). Our results indicated that the higher the radiation dose, the longer the time required for the hyper-proliferation phase to appear in the intestinal crypts which is in agreement with previous literature\textsuperscript{43,44}.

For model development, 7 - 15 Gy was chosen as the suitable dose range to quantify crypt proliferation at 48 h post-irradiation. Control and irradiated (>10 Gy) intestinal segments sustained observational differences. Non-irradiated intestines are opaque with a uniform pink colour and blood vessels distributed along the segment. Segments of the lumen contained digested food particles in yellow (same colour as their food). The lumen could be expanded and stayed expanded with OCT filling and the epithelium fell into a flat sheet when cut open. At radiation doses above 10 Gy, the intestinal segments appeared pale yellow in colour. The epithelium was more transparent, and the lumen was narrower compared to the control. The mice generally had a stomach filled with food while the intestinal lumen was either empty or contained yellow liquid. Ulcers were frequently noticed when PBS was flushed and leaked from these ulcers. Upon OCT insertion, the intestinal lumen did not expand well compared to the control and shrieveled when cut open.
According to the preliminary study, the 7 - 15 Gy range should allow establishment of a monotonic relationship between proliferation and radiation dose. Instead of BrdUrd, the proliferation marker was switched to EdU. Similar to BrdUrd, EdU was injected into mice 2 h prior to euthanization at 200 mg/kg. However, EdU was stained via click chemistry, which eliminated the heat-induced epitope exposure procedure that was necessary for immuno-staining of BrdUrd. The usage of EdU reduced the overall staining process by 2 h and produced images with a higher signal-to-background ratio (Figure 3.1 & 3.2). Mice in groups of 5 were quantified for crypt proliferation that, as expected, monotonically decreased as radiation increased from 7 to 15 Gy (Figure 3.4 & 3.5 D). By contrast, compared to a non-irradiated control, 7 Gy irradiation resulted in a similar proliferation that was not significantly different (p=0.69). On the other hand, 10, 12, and 15 Gy treatments were all significantly different from the non-irradiated control (p = 0.0079 for each). The percentage of proliferation exponentially dropped from 42.6% at 10 Gy to 10.7% at 15 Gy. The crypt proliferation model, for the range 7 - 15 Gy, demonstrated exponential decline in proliferation as radiation dose linearly increased, and was thus fitted with a one-phase decay curve. The inter-assay CV was 14.2% while the intra-assay CV was 9.78%.

3.1.1 Power and sensitivity analysis

Based on the crypt proliferation model (7 - 15 Gy at 48 h), using 5 animals per treatment (a drug + radiation) and assuming a similar variability compared to the untreated (radiation alone), we estimate, using the sample size calculation\(^80\), that the
proliferation assay would allow us to detect a difference of 20% or greater between two treatments. For example, using the assay to elucidate the effects of radiation dose-modifying drugs that may enhance the effect of radiation, we would expect dose-modifying effects corresponding to an increase in radiation dose, and the anticipated outcome would be a decrease in proliferation. The ability to detect an effect of -20% or greater in crypt proliferation would translate to the ability in detecting a dose modification equivalent to approximately +0.8 Gy or higher. In other words, if the drug exerts with only a decrease of 10% in proliferation, equivalent to a dose modifying effect of +0.3 Gy, our assay would conclude there is no difference between the drug + radiation treatment compared to radiation alone. If the desired effect size of an inhibitor is less than 20%, an increase in sample size is necessary to detect the smaller effects. For example, a sample size power calculation estimates that 24 mice are needed for each treatment group to detect a 10% reduction in proliferation effect at 7 Gy, which is equivalent to a +0.3 Gy dose modifying effect. Similar calculations predicting effect sizes detectable by the relative sample size are shown in Figure 3.6 A. Future experiments for dose-modifying drugs can be designed based on these calculations.

A sensitivity analysis provided in Figure 3.6 B & C shows that the addition of 30% random variability yielded 90% of the trials to be within the original 95% CI. Use of 40% of random variability yielded estimation that 10% of the trials are contained within the original 95% CI. Therefore, the crypt proliferation model was roughly robust up to 30% random variability. In other words, when the crypt proliferation model is applied in future drug evaluations, there can be random variability of up to 30% in the
data collected. Therefore, any conclusions need systematic determination that in fact a significance exists when effects are within 30% difference between two treatments. Adequate sample size analysis should be conducted to eliminate probabilities of mistakenly concluding significance as a result of random error.

**Figure 3.1 Comparing BrdUrd with EdU staining.** BrdUrd stained images had a lower signal-to-background ratio compared to EdU as shown. BrdUrd sustained non-specific antibody staining and therefore the non-proliferative villi and submucosal tissue were apparent while Edu did not stain them. As a result threshold selection in distinguishing positively stained pixels from negative ones was more problematic with BrdUrd compared to EdU.
Figure 3.2 An intestinal swirl with EdU staining. An unprocessed, complete EdU stained jejunal swirl is shown. Proliferating cells that are in or have completed the S-phase of the cell cycle are stained with EdU (Black). Enlarged image of a segment of the intestinal swirl shows that cells in the crypts are stained while cells in the villi are not (blue dashed lines show where some villi are located).
Figure 3.3 Quantification of intestinal proliferation. EdU stained layer (black) is overlapped with Hoechst 33342 layer (light grey). The number of pixels above a threshold (EdU-positive-pixels) in the EdU layer is recorded automatically using ImageJ. In the Hoechst 33342 layer, a drawn border, at the submucosal-lamina propria border, in dashed pink represents the length of the jejunal segment analyzed for EdU.

Figure 3.4 Dose response of jejunal crypt proliferation. Representative images show that as radiation dose increased, the proliferation decreased. EdU stained jejunal segments are shown. The amount of EdU staining at 7 Gy was very similar to that of an non-irradiated control. All of 10, 12 and 15 Gy visually demonstrate shrinking proliferation populations.
Figure 3.5 Quantification of the crypt proliferation model. Preliminary experiments were done with radiation doses at 0 - 15 Gy and jejunal swirls collected for quantification at A) 24 h, B) 48 h (BrdU) and C) 3.5 day. The finalized crypt proliferation model was developed at 7 – 15 Gy collected at D) 48 h (EdU) with E) experimental scheme as shown. The proliferation at 7 – 15 Gy exponentially decreased and also was fitted to a one-phase exponential decay curve. Data are Median ± IQR; n=5 mice per group (treatment or control).
Figure 3.6 Effect size and sensitivity of the crypt proliferation model. A) Dashed lines in light grey represent the minimum required effect size to conclude significance with the relative sample sizes (n=1, 5, and 25). Effect sizes that are above the upper and below the lower dashed lines can be concluded as significant with 80% power and 0.05 significance. B) 10 models (blue lines) with 40% random variability in the data points. C) 10 models (blue lines) with 30% random variability. Black dashed lines represent the 95% CI of the original model in B&C. All models were fitted with one-phase decay curve.
3.2 The villi morphology model

The proliferation surge as seen in jejunal crypts prevents establishment of a monotonic curve covering the entire 0 - 15 Gy range, and therefore complicated interpretation of results between 2 - 7 Gy. In an attempt to produce the full dose response curve including the 2 - 7 Gy range, villi morphology was examined. As the length of villi is dependent on the intestinal crypt proliferation, the villi morphology model can indirectly reflect proliferation properties upon radiation-induced damage, while directly reflect the outcome as a result of radiation-induced damage to the organ. Since the major function of the villi is to increase surface area for efficient nutrient absorption, shortened villi have reduced functional capacity and therefore are direct indications of toxicity.

Unlike the crypt proliferation model in which data acquisition was fully automated, the quantification method for the villi morphology model was partially automated with human intervention. Intestinal swirls stained with CD31 are shown in Figure 3.7. Some portions of villi are discontinuous, because the rest of the villi and the connecting crypts reside in a different plane of section (Figure 3.7 blue box). Moreover, villi are not rigid, and they can freely bend toward different directions. The difference between a short villus and one that is cut off due to sectioning conditions is difficult for software to detect. Therefore, establishment of a fully automated quantifying system was problematic.
Objective parameters in villi length quantification was developed: each measured villus was spaced 10 villi apart from the next measured villus and a total of 30 measurements were made per image. Measurements always began at the outer terminal of the swirl. Initially, roughly 15 villi were measured per image, but sections of the same tissue could double in the average villi length measured. Therefore, sets of 30, 40, and 50 villi were measured per image to find the optimal number (Figure 3.7). Systematic determination show that the mean ± SEM cease to fluctuate between 30 - 50 villi measurements, therefore, 30 villi was concluded as an optimal number per section for 4 swirl replicates per animal and 5 animals per group, generating 120 data points per animal and 600 data points per treatment group.

Villi length was initially determined as the length from the crypt-villi junction to the tip of the villi epithelium extruding into the lumen. However, as measurements were made for 7 - 10 Gy irradiation treatments, it became difficult to determine where the villi epithelium tip was as a result of epithelial lesions (Figure 3.8). The ISCs, which proliferate as a survival requirement to maintain epithelial integrity, are more sensitive to radiation damage compared to the non-proliferating epithelial cells24,28,38. Therefore, ISCs that failed to differentiate into daughter epithelial cells at the rate that they were shed into the lumen resulted in lesions of the epithelium. To overcome this issue, CD31/PECAM, which is a marker expressed on endothelial cells, was stained to visualize villi capillary (Figure 3.9). As expected, the length of the capillary bed was explicit with a defined tip even in the presence of epithelial lesion and the bottom remains to be the crypt-villi junction. A straight line was manually drawn on the image.
from the top to the bottom of the villi capillary bed in ImageJ then customized software operating on ImageJ determined the length of the lines in pixels. By using the resolution of the image as a conversion factor (1.3 μm resolution/pixel), we obtained the length of each villus in terms of millimeters (mm). The villi morphology model, therefore, measured the length of villi as determined by the length of the villi capillary bed at each radiation dose (Gy).

Figure 3.10B shows that as radiation dose increased, villi length decreased. The average villi length of an non-irradiated control was 0.607 mm (mean; SD = 0.039 mm) reducing in length as radiation increased to a length of 0.131 mm (mean; SD = 0.009 mm) for 15 Gy treated mice. The relationship between villi length and radiation dose was monotonic, as expected, without a surge. However, when radiation dose linearly increases, the reduction in villi length was non-linear. A typical “S” shaped curve was shown demonstrating the rate of villi reduction relative to radiation dose and this “S” shaped curve was usually seen in a logistic dose inhibition model. As a result, the data points are fitted with a four-parameter logistic curve (Figure 3.10C) with the upper asymptote at 0.598 mm, lower asymptote hitting the constraint at 0 mm, hill-slope at 1.69 and the Effective dose 50 (ED50; the dose that reduced average villi length by 50%) at 6.9 Gy. The inter-assay CV was 12.7% and the intra-assay CV was 6.6%.

3.2.1 Power and sensitivity analysis

Similar to the crypt proliferation model, using 5 mice per group and assuming similar sizes in error bars between a treated group (drug + radiation) to an untreated
group (radiation alone), a sample size power calculation estimates that our assay was capable of detecting at minimum a 20% difference between two groups. For example, if a drug was administered in combination with 5 Gy and reduced the length of villi from 0.379 mm to 0.303 mm for a -20% effect, translating to +1.9 Gy in dose modification, our assay would be able to conclude that the two treatments were significantly different from each other. However, for drugs that are expected to exert smaller effects such as reducing villi length from 0.379 mm at 5 Gy to 0.341 mm for a -10% effect, translating to +0.8 Gy modification, our assay may conclude that the two treatments are not distinct from each other, resulting in a type II error (concluding insignificance when in fact there is a significance). A sample size power calculation estimated that a minimum of 25 mice per group were needed to detect a 10% effect with a level of 0.05 significance and 80% power. The ability to detect small differences between treatments could be improved by increasing sample size (Figure 3.11A).

As shown in Figure 3.11B&C, a sensitivity analysis revealed that 15% random variability and yielded 100% of the trials residing within the original 95% CI. While 30% of random variability yielded 40% of the trials residing within the original 95% CI. Therefore, the villi morphology model is robust up to roughly 15% of random variability. If the sample size is not enough to support statistical parameters such as significance and power, a difference between treatments that may be a result of random error could be mistakenly determined as significant.
Figure 3.7 Optimization of the number of villi measurements. Measurements were made from the outer end of the jejunal segment measuring every tenth villus into the swirl. Measuring 30 villi was determined as the suitable number for model establishment. Data are mean±SEM.

Figure 3.8 Villi epithelial lesions at 7 Gy radiation treatment. Hoechst 33342 stained jejunal section at 3.5 days after 7 Gy irradiation is shown. Notice every villus in the image on the left sustained epithelial lesion close to the villi tip. This condition makes villi length measurements difficult as the location of the top of the villi is debatable.
Figure 3.9 An intestinal swirl with CD31 staining. Jejunal capillaries are stained with CD31 (red). The CD31 represents a schematic of the villi that allows measurement of the length of the villi. An enlarged image is shown for more detail (bottom right). Notice there were incomplete villi portions that were detached from the epithelium (blue box shows enlarged image of the blushed dashed box).
**Figure 3.10 The villi morphology model.** A) Mice were irradiated with 0 – 15 Gy x-ray and euthanized at 3.5 days post-irradiation. B) As radiation dose increased, the length of the villi decreased. Bars (from crypt-villi junction to the tip of the villi capillary bed) denote the length of the villi that would be measured for quantification. C) Data points are fitted with a 4-parameter logistic curve. Data are Mean±SD; 30 villi / section; 4 sections / mouse; n=5 mice.
Figure 3.11 Effect size and sensitivity of the villi morphology model. A) Dashed lines in light grey represent the minimum required effect size to conclude significance with the relative sample sizes (n=1, 5, and 25). Effect sizes that are above the upper and below the lower dashed lines can be concluded as significant with 80% power and 0.05 significance. B) 10 models (blue lines) with 15% random variability in the data points. C) 10 models (blue lines) with 30% random variability. Black dashed lines represent the 95% CI of the original model in B&C. All models were fitted with a four-parameter logistic curve.
3.3 The DNA damage model

The DNA damage model measures normal tissue toxicity to radiation in terms of the amount of DNA damage with respect to time post-irradiation. To visualize the amount of DNA damage, early time points such as hours post-irradiation is optimal as repair mechanisms can begin as early as 30 min post-irradiation\textsuperscript{21,70}. Moreover, to evaluate the effects of dose-modifying drugs in altering the amount of damage at a time point or altering the overall repair rate, a model that encompasses a range of time visualizing DNA damage is desirable.

γH2AX is a marker for DNA double strand breaks (DSBs). When DSBs are repaired γH2AX is de-phosphorylated to H2AX, and therefore the amount of detectable γH2AX would be expected to decrease with time. Since the entire intestinal epithelium, including both proliferating and non-proliferating cells, sustains radiation-induced DNA damage, customized software was developed to analyze and quantify the entire tissue section (Figure 3.12). All pixels representing cellular nuclei were selected via detection of pixels positive for Hoechst 33342 dye using a threshold, then these pixels were evaluated for γH2AX labeling (Figure 3.13C). This method allows comprehensive quantification of all tissues while excluding non-specific background staining. As a result, an average intensity of γH2AX in intestinal tissue, with arbitrary units, was obtained (Figure 3.13A). The DNA damage model demonstrates the amount of DNA damage at staggered time points post-irradiation and shows that the rate at which γH2AX is de-phosphorylated, or in other words the rate of DSB repair, followed an
exponential reduction manner with time (Figure 3.13B&D). An exponential decay relationship between γH2AX intensity and time post-irradiation indicated that the rate of DNA damage repair was proportional to the amount of DSB. There was a baseline amount of γH2AX visible in the proliferating cells located in the crypts of non-irradiated mice. ISCs constantly replicate their DNA for progeny production, and also there was consistent DNA double strand break formation, which formed γH2AX foci. At 1 h post-irradiation, γH2AX peaked in our model then exponentially decreased and plateau at our end point, 24 h. The average γH2AX staining at 24 h was not significantly different from that of an non-irradiated control (p=0.69) while at 1, 3, and 6 h there was significant detection in the intensity of γH2AX compared to a non-irradiated control (p=0.008 for each). The Inter-assay CV was 9.44% and the intra-assay CV was 3.85%.

3.3.1 Power and sensitivity analysis

A power calculation estimated that the DNA damage model could detect a difference between 2 groups that was 10% or greater. For example, if a drug is given in combination with 8 Gy and quantified for average γH2AX intensity at 6 h post-irradiation, our assay could conclude a significance between an intensity of 7.94 at 6 h (radiation only) and 8.73 at 6 h (drug + radiation). This +10% effect in γH2AX intensity translates to a -0.9 h modification, from 6 h to 5.1 h, meaning that the drug + radiation treatment delays the repair time by 0.9 h compared to radiation alone treatment. This model directly demonstrates alterations to DNA DSB repair induced by radiation dose-modifying drugs in terms of preventing/delaying repair. Effect sizes required by dose-
modifying drugs to conclude significance for the DNA damage model is shown in Figure 3.14A. However, if the desired modification effect of a drug is less than 10% from radiation alone, this assay may not be able to conclude its significance. For example, if a 5% difference is to be detected at the 6 h time point between a radiation alone treatment and a drug + radiation treatment, with γH2AX intensity at 7.94 and 8.34, which translates to 6 h and 5.6 h respectively, it is estimated that at least 19 mice per group is required to conclude significance at a level of 0.05 significance and 80% power.

A sensitivity analysis is shown in Figure 3.14B&C, and 90% of the trials are contained within the original 95% CI when 15% random variability was added to the data. About 50% of the trials were within the original 95% CI, when 30% random variability was added. Therefore, when the DNA damage model is applied in future DNA damage repair inhibitor evaluations, effects within 15% should be systematically confirmed that the sample size is adequate in providing a level of 0.05 significance and 80% power.
Figure 3.12 An intestinal swirl with γH2AX staining at 1 h post 8 Gy radiation. The entire jejunal segment sustains radiation induced DNA damage as shown by staining for γH2AX. Image at bottom right is an enlarged image of the dashed box on the left. DNA damage can be induced in both mitotic and post-mitotic cells.
Figure 3.13 The DNA damage model. A) The measured peak of the intensity and amount of γH2AX foci is seen at 1 h post-irradiation, decreasing as time passes and approaching control levels at 24 h post-irradiation. B) The DNA damage model is developed with mice euthanized at 1, 3, 6, and 24 h after 8 Gy x-ray irradiation. C) Cell nuclei were first selected using a threshold in the Hoechst 33342 stained layer. These pixels were then evaluated in the γH2AX layer to quantify the staining intensity in cell nuclei. D) The rate at which γH2AX intensity decreases from 1 h to 24 h follows an exponential trend. Data are median±IQR; n=5; a non-irradiated control is shown at 0 h.
**Figure 3.14 Effect size and sensitivity of the DNA damage model.** A) Dashed lines in light grey represent the minimum required effect size to conclude significance with the relative sample sizes (n=1, 5, and 25). Effect sizes that are above the upper and below the lower dashed lines can be concluded as significant with 80% power and 0.05 significance. B) 10 models (blue lines) with 15% random variability in the data points. C) 10 models (blue lines) with 30% random variability. Black dashed lines represent the 95% CI of the original model in B&C. All models were fitted with one-phase decay curve.
3.4 Discussion

3.4.1 Enhancing the jejunal tissue collection method

Handling the murine intestinal tissue is often problematic as it is soft and fragile. A single layer of columnar epithelial cell separates the lumen to the lamina propria and so the epithelium can be easily damaged causing artificial lesions during tissue processing. Moreover, the mice intestinal tissue is remarkably thin, so moisture retention requires extra attention. To maximally preserve intestinal morphology in conventional methods, harvested intestinal segments are often left undisturbed in the cylindrical format and are fixed immediately in formalin before embedding in paraffin. In this thesis, the intestinal segments harvested from mice were immediately submerged in PBS to prevent moisture loss and downstream processing was conducted within the PBS bath. Food particles that are sometimes inevitably included in the segment of harvested intestine that was preserved through formalin fixation were removed in our approach by insertion of a gavage needle and gentle flushing using PBS. The usage of a blunt-end gavage needle prevents tearing of the intestinal epithelium, as the tip is smooth. A PBS flush generates enough force to remove food particles without damaging the epithelium. The use of PBS also preserves the moisture content of the cells avoiding rupture or shrinkage similar to techniques mentioned in the “Swiss-rolling” method\textsuperscript{74}.

Preserving the intact intestinal segment in cylindrical format limits accessibility to one location for analysis in one tissue section (Figure 3.15). Often 5 - 10 sections are
obtained to ensure enough crypts and villi are assessed for statistical analysis. To improve the amount of tissue for analysis per image and efficiency, we have longitudinally cut open the intestine forming a flat sheet as suggested with the “Swiss-rolling” technique\textsuperscript{74}. To prevent artificial damage introduced by scissors, 30% OCT diluted in PBS was filled into the lumen using a gavage needle again. The OCT medium is viscous, and it acts as an expanding agent creating space for insertion of scissors without touching and scraping the intestinal epithelium. Once cut open, the OCT medium also provides enough force to naturally flatten the intestinal segment into a rectangular sheet. It is also perfusing the villi with the same OCT that is used to embed and preserve the tissue for freezing. The entire process of flushing with PBS, expanding with OCT and cutting open with scissors to form a flat sheet requires less than 30s per sample, to be a quick and efficient technique with practice.

The “Swiss-roll” method described by Bialkowska\textit{ et al.} \textsuperscript{74} utilizes a toothpick as a platform for wrapping the intestine. In our trials, the use of a toothpick was undesirable, because the wooden toothpick became slippery against the intestinal tissue. Therefore, wrapping was difficult unless the intestinal tissue was manually pressed against the toothpick for attachment. This process significantly cost time and became increasingly difficult as more intestines were collected and risked increased damage to the tissue through over-handling. We have adapted the technique to use a hemostat clamped onto the end and tissue wrapped with the villi facing outwards onto the hemostat tip. The clamping is minimized to a small area and with the protection of the OCT medium, the hemostat only moderately affects the epithelium. The hemostat
is then turned vertically and slightly retracted for the intestinal swirl to glide off onto a frozen aluminum block. The OCT was a key factor allowing smooth separation between the tissue and the hemostat with minimal damage to the fragile epithelium.

Rapid freezing allowed preservation of the swirl format then assembling them into arrays before embedding was achieved. Prompt embedding is critical for obtaining smooth and continuous cryosections. Generally, tissues were embedded within 3 days after they have been rapidly frozen on the aluminum block. Samples are embedded promptly to avoid ice crystal formation on the surface of the tissue that can result in suboptimal sectioning conditions. Samples embedded in arrays at approximately 1 week after harvesting resulted in poor sectioning and reduced the amount of tissue available for analysis (Figure 3.16). Once tissues with surface ice crystals were organized in arrays and submerged in OCT (room temperature) for embedding, the ice crystals were melted. Once the OCT medium freeze again, the ice forms again. Not only stretching and damaging the swirl surface tissues, a sheet of ice uniformly forms at the boundary against OCT. During cryosectioning, the tissues were unable to attach to the rest of the array and the rim of the swirl appears to be shredded. As a consequence, the number of artifacts increases and reduces the amount of quantifiable tissue. For this reason, same-day embedding in OCT was preferred to avoid surface ice formation and poor cryosections. The array was customized such that sections containing 4 - 5 intestinal swirls obtained from the cryostat would fit on one microscope slide. For a study of 50 intestinal swirls, our methods require roughly 10 slides with 175 µl of reagents per slide (1.75 ml reagent in total) compared to a
conventional study where 50 slides (1 tissue section per slide) and up to 200 µl reagent per slide (10 ml reagent in total) may be necessary.

The collective improvements relative to conventional methods in intestinal tissue collection, processing and staining reduced the overall processing time from weeks to days, minimized material and reagents used, and relieved some of the more labour-intensive work.

Figure 3.15 Intestinal swirl vs. intestinal transverse section. The intestinal swirl (left) allows analysis over a 5 – 7 cm length along the jejunum while a transverse section of the intestine (right) provides roughly 1 cm length of jejunum at one location for qualitative and quantitative analysis.
Figure 3.16 Suboptimal cryosection due to freezer burn. The intestinal swirl to the left was embedded after sitting in the freezer for 1 week. The intestinal swirl to the right was embedded on the same day that it was harvested. Notice the outermost circumference of the left swirl have shredded, resulting in artifacts and cannot be used for quantification analysis.

3.4.2 Advantages and disadvantages of each computer-assisted quantification method

Proliferating cells are exclusively found in the crypts, villi length measurements require boundary determinations between crypt and villi and DNA damage post-irradiation can be sustained throughout the intestinal tissue. As a consequence, different analytical methods had to be developed to quantify each of the models separately. Both crypt proliferation and DNA damage were quantified using customized softwares applications while the villi length analysis utilized a combination of human intervention and computer assistance.
By using an automated image analysis approach, every pixel in the image was examined and evaluated based on user-specific commands. On average, 4 minutes were spent per image for cropping and drawing borders to develop the proliferation model, 1 minute was devoted per image to measure 30 villi lengths for the villi morphology model and less than 30 seconds were spent per image for removing artifacts to develop the DNA damage model. The quantification macros, for crypt proliferation and DNA damage, took only 20 - 30 seconds per image to evaluate every pixel and the size of each image was roughly 15000 × 15000 pixels (225,000,000 pixels in total). The numerical output representing the villi lengths were simultaneously produced as bars are drawn against villi in the image for developing the villi morphology model. Not only did the automated method improve efficiency, comprehensive analysis of the entire image was achieved.

The concept of measuring the amount of proliferation by quantifying pixels positive for EdU is similar to conventional methods that measure the number of cells positive for proliferation markers. The assessed target remained to be the proliferating population within the intestine. However, evaluating the number of cells can be subjective and may vary between operators. Setting up a series of code for a computer to determine the number of cells can be complicated as well. Cells range in different sizes, shapes and can sometimes overlap, and so a precise standard is difficult to be determined. A simple approach is to evaluate the fundamental units within an image - the pixels. In a black and white image, the intensity of each pixel can be quantified and reflects the amount of fluorescence from the stain, which reflects the relative amount of
antigen or target. Our DNA damage model was developed using this method - by evaluating and averaging the intensity of each pixel. Other than quantifying intensities of each pixel within an image, we can also command the computer to investigate every pixel and decide whether that pixel is above or below a threshold. The threshold is pre-determined by examining multiple images and deciding an intensity value that is between intensities of signal and background noise. As long as the pixel is above the pre-determined threshold, the computer records it as signal regardless of by how much. Pixels with intensities below the pre-determined threshold are not included in the quantification as they are regarded as noise. As a consequence, the robustness of the staining agent and tissue environment is critical in obtaining accurate and consistent results. Elements such as signal to noise ratio is a critical factor when using the threshold quantification method. Disadvantages of the system arise if the positive signal range overlaps with the noise intensity, then false positive or false negatives may be mistakenly recorded or disregarded, respectively.

The villi length quantification method involves more human intervention compared to the proliferation and DNA damage quantification methods. As previously described in Section 3.2 of Chapter 3, a fully-automated quantification method is problematic due to 1) the miscellaneous floating tissue artifacts; and 2) precise determination between the boundary of crypt and villi. Each of these elements is complicated because artifacts appear in numerous quantities, in different sizes and in close proximity to quantifiable tissue. Epithelial lesions and varying sizes and widths of villi cause difficulty in deciding a common setting classifying the boundary. As a result,
the use of an automated system may be more problematic compared to directly and manually measuring each villus. For this reason, we have retained quantification by manually determining the length of each villi with a line drawn over the villus of interest determined by our objective parameter in ImageJ. Our customized analysis macro running on ImageJ would then automatically record the length of the line drawn representing the length of the villi. As a result, simultaneous line drawing and length recording can be achieved. To further minimize subjectivity, we developed objective parameters such that measurements are spaced apart by 10 villi along the entire segment of the jejunum rather than choosing, by eye, for measurements. However, some subjectivity is inevitable. An operator drawing a line to represent the length of the villi is a way for the computer to indirectly recognize the length of a particular villus. Since the computer is recognizing the line that was manually drawn, it does not verify if the line represents the true length of the villi in the image. As a result, error may be introduced if a line is carelessly drawn longer or shorter than the villi it is meant to represent. To minimize this problem, we obtained 4 cryosections per intestinal swirl for a total of 600 villi measurements per treatment to obtain representative average villi lengths for model development.

Quantification of DNA double strand breaks was fully automated with a standard threshold similar to the proliferation quantification method. All cells, proliferating or non-proliferating, are subject to radiation damage if irradiated. Therefore, an analysis system that evaluates all tissues contained within the image was preferred. Again, we commanded the computer to evaluate every pixel within the image for positive Hoechst
33342 staining. Hoechst 33342 stains nuclear contents, which signified pixels containing DNA. The pixels that were positive for nuclear content were then evaluated for DNA damage marked by γH2AX. This step was important, as we could not positively select pixels for γH2AX analysis using γH2AX staining itself. The amount of DNA damage sustained can vary between cells, so some cells could sustain less intense staining compared to others. For this reason, we customized an analysis program to obtain an average intensity of γH2AX in the Hoechst 33342-positive pixels. The benefit of thresholding Hoechst 33342 was to gate for tissues or pixels containing DNA. The intensity of γH2AX in the background is between 0 – 2, while positive signals were typically between 20 - 60, therefore if no selection for DNA was made, then the γH2AX intensity could be upset and the degree of deviation was dependent on the amount of non-tissue pixels which can also vary between samples. As a consequence, the results become meaningless. Disadvantages of this model arise if the labeling of nuclear staining agent, Hoechst 33342, is inconsistent. For example, a common threshold cannot be determined to accurately distinguish pixels containing DNA for γH2AX intensity quantification. DNA-containing pixels may be lost, or DNA-free pixels may be false positively included. Therefore, good staining technique is required for the DNA damage quantification methods to succeed.
3.4.3 Applicability and limitations of each model

Our intestinal assay is widely applicable. For studies that intend to analyze histological sections for antigenic contents in the intestines, our tissue collection techniques can be readily adapted. Our models were developed using cryosections of the jejunum, but the techniques in retrieval and processing tissues can be used to collect any section in the small or large intestines. Cryopreserved tissues have better antigenic exposure, but a shorter shelf life compared to FFPE tissues, and therefore our assay is recommended to those studies intended for quick result output with quick turn-around time and for tissues that are not intended for long-term storage.

Each of the crypt proliferation, villi morphology and DNA damage model is developed for the purpose of evaluating effects of radiation dose modifying drugs. The jejunum is a good model representing normal tissue as both proliferating and non-proliferation cells are present for qualitative and quantitative analysis. To begin evaluating the effect of a dose modifying drug in cellular proliferation, morphology and DNA damage repair, it is recommended to re-establish the full curves as described in each model in combination with radiation. This way, effects of drugs from low to high doses can be visualized. For instance, drugs may only be toxic at certain radiation doses while non-toxic in another dose range. Drugs may also be uniformly toxic or non-toxic at all radiation doses. Once a single or a few radiation doses of interest is determined, further detailed experimentation could be executed.
The proliferation model can report toxicity in terms of proliferation caused by dose modifying drugs combined with irradiation. For example, if the fraction of S-phase cells was significantly reduced when administering drug + radiation, then our model indicates that the drug of interest is toxic to normal tissues and further enhances the effects of radiation in normal tissue by reducing proliferation. This indicates that the drug is enhancing sensitivity of normal tissue to radiation, specifically further damaging proliferative properties. As a result, conclusions can be drawn with the notion that the drug could not expand the therapeutic index. Further tweaking and testing of the drug would be needed to diminish the normal tissue toxicity. Therefore, our proliferation model is informative in the effects of radiation dose modifying drugs in proliferating cells and can be readily applied in pre-clinical screening of drug toxicity.

The villi morphology model can report the change in villi length as caused by radiation dose modifying drugs. As the model showed, the length of villi decreased as radiation dose increased, indicating toxicity is in the form of shrinking villi size. If a drug + radiation treatment further reduced villi length as compared to radiation alone, then our model indicates that the drug of interest is toxic to normal tissue. Specifically, reducing villi length means decreased surface area and, as a consequence, decreased capacity in nutrient absorption. Our model is limited in evaluating drugs that exert less than 20% effect. When there is a 15% effect, for example, our model would conclude from the results that there was no effect. This can be a typical statistical type II error (concluding insignificant when in fact there is a significant difference) but may be fixed
by increasing sample size. For this reason, the assay basics such as sample size, power, and desired effect size should be tailored to complement each study.

The DNA damage model can report the alterations to the rate of DNA damage repair but can become unreliable when the drug of interest interferes with γH2AX activities. Naturally, γH2AX is phosphorylated by multiple upstream proteins that are response factors to DNA double strand breaks. As a result, if an inhibitor is designed to target and inhibit upstream proteins relating to γH2AX phosphorylation, then the amount of γH2AX does not represent the rate the double strand break repair anymore. For example, γH2AX foci form at sites of DSB induced by irradiation but a drug that prevents phosphorylation of γH2AX may show that there are less γH2AX foci compared to irradiation alone. Results may be mistakenly interpreted as the drug mitigates radiation injury by preventing radiation-induced DSB formations. Nevertheless, the γH2AX formation is simply inhibited by the drug and does not truly reflect the amount of DNA DSB. However, if the dose modifying drug interferes DNA double strand break repair but does not hindering γH2AX phosphorylation, then our model can be readily applied.

The intestinal swirl assay and the 3 analyses along with their corresponding models have a wide applicability in pre-clinical studies. However, the limitations should be understood and taken into consideration during experimental planning to ensure that meaningful and interpretive results can be concluded for future studies.
<table>
<thead>
<tr>
<th></th>
<th>Crypt proliferation</th>
<th>Villi length</th>
<th>DNA damage</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mice / group</strong></td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td><strong>Marker injection</strong></td>
<td>200 mg/kg EdU</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td><strong>X-ray radiation</strong></td>
<td>7 – 15 Gy</td>
<td>0 – 15 Gy</td>
<td>8 Gy</td>
</tr>
<tr>
<td><strong>Time</strong></td>
<td>48 h</td>
<td>3.5 day</td>
<td>1 – 24 h</td>
</tr>
<tr>
<td><strong>Staining</strong></td>
<td>EdU; Click chemistry</td>
<td>Anti-CD31; immuno-staining</td>
<td>Anti-γH2AX; immuno-staining</td>
</tr>
<tr>
<td><strong>Portion of jejunum for analysis</strong></td>
<td>All</td>
<td>Villi only</td>
<td>All</td>
</tr>
<tr>
<td><strong>Quantification method</strong></td>
<td># of EdU-positive pixels / length of jejunum</td>
<td>Length of 30 villi spaced 10 villi apart / section</td>
<td>Average staining intensity / section</td>
</tr>
<tr>
<td><strong>Computer assistance</strong></td>
<td>Automated</td>
<td>Semi-automated</td>
<td>Automated</td>
</tr>
<tr>
<td><strong>Intra-assay CV</strong></td>
<td>9.78%</td>
<td>6.55%</td>
<td>3.85%</td>
</tr>
<tr>
<td><strong>Inter-assay CV</strong></td>
<td>14.2%</td>
<td>12.7%</td>
<td>9.44%</td>
</tr>
<tr>
<td><strong>Effect size detectable between 2 treatments</strong></td>
<td>&gt; 20%</td>
<td>&gt; 20%</td>
<td>&gt; 10%</td>
</tr>
<tr>
<td><strong>Model sensitivity (random error allowed)</strong></td>
<td>&lt; 30%</td>
<td>&lt; 15%</td>
<td>&lt; 15%</td>
</tr>
<tr>
<td><strong>Can be used to evaluate dose-modifying drugs?</strong></td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Advantages</strong></td>
<td>Comprehensive analysis of entire intestinal sections</td>
<td>Efficient data acquisition</td>
<td>Comprehensive analysis of entire intestinal sections</td>
</tr>
<tr>
<td><strong>Limitations</strong></td>
<td>Complicated interpretation between 2 – 7 Gy</td>
<td>Subjectivity not completely eliminated compared to past assays</td>
<td>Cannot be used to test inhibitors interrupting activities of γH2AX</td>
</tr>
</tbody>
</table>

**Note that the effect size is related to the random error allowed. If the random error is lowered, a smaller effect size may be detected.**
Chapter 4: Conclusion and future directions

4.1 Summary of research

The narrow therapeutic index of radiotherapy remains a challenge in effectively eradicating cancer while mitigating normal tissue toxicity. Increased understanding in the molecular mechanism and interaction between radiation and cancer cells give rise to developments of radiation dose-modifying drugs. These drugs are intended to be administered in combination with radiation and sensitize cancerous tissue. As a result, lower radiation doses can be given to mitigate normal tissue toxicity while tumours may be effectively eradicated. Overall, achieving higher cure rates while patient quality of life may be well conserved compared to radiation treatment alone. However, conventional assays evaluating normal tissue toxicity in the intestines can be non-comprehensive and subjective. In this thesis, I have demonstrated that a semi-automated intestinal assay has been established along with 3 different analysis methods yielding 3 models. The models collectively can inform normal tissue response to radiation in terms of intestinal crypt proliferation, villi morphology and DNA damage. The rapidly renewing intestinal epithelium is an ideal representation of normal tissue providing both proliferating and non-proliferating cells for assessment of their relative response to radiation damage. Moreover, as a highly proliferative organ, acute toxicity induced by drugs in combination with radiation can be detected. The similarity of the intestinal circadian rhythm relative to the epidermis and the tongue indicate that the intestinal models may be used to predict the response of similar proliferative tissues to
radiation injury. Our models can assess radiation dose modifying drugs that may cause a population reduction in S-phase cells, which may indicate that the drug is toxic to proliferating cells. A drug may also further reduce the length of villi, which could reveal toxicity to structural integrity. Finally, a drug that manipulates DNA damage mechanisms may cause alterations to γH2AX intensity and show impaired DNA double strand break repair.

Intestinal swirls were collected and rapidly frozen for cryosectioning. EdU, CD31 and γH2AX were stained to visualize proliferation, villi length and DNA double strand breaks, respectively. The proliferation quantification method automatically evaluated EdU positive pixels and was normalized to the length of the jejunal segment. Analyses reveal that proliferation was related to radiation dose, decreasing exponentially as radiation dose increased. The villi morphology quantification method semi-automatically measured the length of villi capillary beds. Analyses reveal that villi length decreased as radiation dose increased. Finally, the DNA damage quantification methods automatically evaluated the intensity of γH2AX. Analyses revealed that the amount of DNA damage peaked within one-hour post-irradiation and exponentially decreased as time pass. The automated quantification programs were written by Dr. Alastair Kyle using Java and the programs were performed on ImageJ.

Our study demonstrates that efficient tissue processing and comprehensive image analysis with computer automation can be achieved. Our jejunal collection method allowed processing of 30-40 mice within 1.5 h, while immediate freezing and embedding in an array allowed rapid preservation of γH2AX expression levels while
maximizing the amount of tissue on each slide. This minimized sectioning time and the amount of antibodies used as well as reduced microscope slide usage during same-day sectioning and staining. Automatically acquiring images at 1.3µm/pixel resolution was done overnight using the custom robotic microscope previously developed by our staff scientist, Dr. Kyle. Automated analyses using ImageJ, while less specific than counting the number of cells affected by proliferation or DNA damage, allowed comprehensive analysis of every pixel in each image, and reduces the amount of labour required for image processing and data acquisition while also minimizing potentially subjective determination of results. Analysis standards, such as thresholds, can be adjusted each time to account for staining variations between experiments. Overall, our semi-automated intestinal assay is estimated to have shortened the entire tissue collection, processing, sectioning, staining, imaging and analysis protocol from weeks to days compared to conventional FFPE and manual data collection methods.

Each of the crypt proliferation, villi morphology and DNA damage quantification methods has advantages and disadvantages. The three models are intended to be applied in future pre-clinical studies evaluating acute intestinal toxicity induced by radiation dose-modifying drugs when combine with radiation. Specifically, the three models can complement each other by demonstrating alterations to DNA DSB damage repair at hours post-irradiation, crypt proliferation at 2 days post-irradiation and villi length at 3.5 days post-irradiation. The time points are selected in compliance with major regulatory authorities for acute toxicity evaluations in pre-clinical drug studies.
The corresponding sample size calculation, power calculation and sensitivity tests indicate the sample size, effect sizes as well as random variability that each model withholds. Both the crypt proliferation and villi morphology models are capable in detecting effect sizes greater than 20% while the DNA damage model can detect effect size that this greater than 10%. Both the villi morphology and DNA damage models are robust up to 15% random error while the crypt proliferation model can sustain up to 30% random variability in data. Detection of smaller effect-sizes as well as increased sensitivity can be achieved by increasing sample size. Our enhanced intestinal assay as well as each of the crypt proliferation, villi morphology and DNA damage models can be readily applied in future pre-clinical studies with considerations to their applicability and limitations.
4.2 Future directions

The automated analyses software can be further improved and optimized. Currently, the crypt proliferation analysis requires the computer to automatically determine pixels that are above a certain threshold. This threshold is pre-determined by human intervention and this determination is critical in the final data. Moreover, although the computer measures the length of the jejunum, the segment to be measured must be pre-determined by a human as well. If an intestinal segment was recorded for the amount of EdU but the length was unintentionally dismissed, errors would be introduced. The villi morphology quantification system can be further improved as well. As previously described, the length of each villus was determined by human visual inspection. Discrepancy between the true length of villi and manually determined length can be introduced as a result of human error and can generate increased variability in the final data as well. Again, efforts can be focused primarily in determining the length of villi in a less subjective method. Lastly, the DNA damage quantification method is the least dependant on manual settings as compared to both the proliferation and villi morphology models. However, the portion of the image to be evaluated relies on a threshold system as well. For example, errors can be introduced if the threshold was set such that fractions of the background was mistakenly included as tissue due to wrongfully determining a value for the threshold. The degree of falsely included background can be variable between images if staining was inconsistent, therefore technical skills in staining is crucial as well. Further enhancing the automated
quantification systems can be done to decrease variability, random error and man-made error during analysis. The use of artificial intelligence (AI) is slowly finding its position in biomedical research. AI could be incorporated to benefit our current quantification systems. For example, AI could automatically recognize the amount of proliferation, the length of each villi and the amount of the double strand breaks. Variability and error may be further lowered, and detection of smaller effect sizes induced by drugs in combination with radiation may be achieved.

Our intestinal swirl assay and the three automated analyses methods with their corresponding models can be readily applied in assessing novel DNA damage repair inhibitors, also known as radiation dose modifying drugs. Currently, an non-irradiated control and several radiation treatments compose each model. When drugs are available for normal tissue toxicity evaluation, an untreated (control), drug only (irradiation free), radiation only (drug free) and a radiation + drug cohort is recommended in the experimental plan. The effects of the drug alone should be compared to an untreated group to conclude effects of the drug in modifying proliferation, villi length or DNA damage in the absence of radiation. This comparison provides an idea of the toxicity of the drug as radiation dose modifying drugs should not exert effects on its own and would be futile if it negatively affects normal tissues when administered alone. Moreover, different concentrations of the drug should also be tested to determine if there exists a maximum concentration that can be administered until introduction of normal tissue toxicity. The crypt proliferation model may demonstrate toxicity in terms of decreased percentage of proliferation and the villi
morphology model demonstrates toxicity in terms of decreased villi length. The DNA damage model may demonstrate toxicity in compromised or delayed DNA DSB repair. Provided that the nature of each drug may be different in reducing proliferation, damaging structural integrity and inhibiting/delaying DNA DSB repair, model settings such as radiation dose and time of tissue harvest could be adjusted based on our models to elucidate meaningful and relevant results contributing to pre-clinical studies.
Bibliography


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