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Date 30 April 1997
ABSTRACT

Traditionally, cell survival following x-irradiation has been assumed to follow a monotonic dose response, even at very low doses. Recent improvements to the low dose assay have revealed that many cell lines exhibit a complex response whereby cells are hyper-radiosensitive to X-rays at these doses (HRS) followed by an increased radioresistance (IRR) as dose approaches 1 Gy. This hypersensitivity may be eliminated by pre-treatment with small priming doses of x-rays, and there is evidence that the increased radioresistance may be a reflection of an inducible repair mechanism. Because molecular evidence strongly suggests a coupling of DNA repair and apoptosis, or programmed cell death, a hypothesis was put forth that HRS/IRR would be reflected in changes in the levels of apoptotic cell death over this dose region. To test this hypothesis, a very large cell population would be required.

To overcome the technical and statistical problems associated with such measurements, an automated image cytometric method of apoptotic cell classification was developed. Image acquisition software was adapted to gather double-stained cell images from slides prepared using cell fixation and staining methods which emphasised apoptotic morphology. Chinese hamster ovary cells were classified individually by discriminant analysis of morphological and nuclear texture features calculated for each image. Discriminant functions were constructed from a manually classified set of over 60,000 cell images categorised as “normal”, “apoptotic”, “cell doublets” or “debris” and all subsequent cell images collected were classified using these functions. Application of this technique resulted in a 99.8% accuracy in classification of the normal cell population, and 81.7% classification accuracy for apoptotic cells. This method was then
applied to study the time course of the apoptotic response of CHO cells following x-irradiation.

Following irradiation with 5 Gy, no increase above control levels of apoptosis was noted until 18 hours post-irradiation, which corresponded to the release of the G2-block as determined by DNA-content analysis. Apoptotic frequency increased to a peak level of 12.1%±4.6 at 42 hours post-irradiation. CHO cells irradiated with 0.25 or 1.0 Gy also exhibited peak levels at 42 hours, although no cell cycle perturbations were noted following irradiation. A secondary peak in apoptosis was noted 60 hours post-irradiation for these doses. Cells exposed to 0.5 Gy however, showed no distinct peak in apoptosis frequency. Analysis of the cumulative amounts of apoptosis observed at 6 hour time intervals over a 72 hour period following irradiation showed greater levels of apoptosis in the 0.25 Gy irradiated population than in the cells exposed to 0.5 Gy. These results were not statistically significant when subjected to Student’s t-test analysis. Experiments using small priming doses of x-rays 6 hours prior to challenge doses failed to show a reduction in apoptotic frequency as would be expected if apoptosis were directly responsible for the HRS/IRR phenomenon.

While a direct involvement of apoptosis in HRS/IRR cannot be ruled out, these results do not generally support the original hypothesis. The post-mitotic nature of apoptosis in CHO cells, several generations following low dose irradiation, obscures the relationship of these results to cell survival data. However, there may be some implications for cell survival measurements due to effects on resulting colony size. These studies suggest that the characterisation of the low dose apoptotic response requires further investigation. The automated techniques developed here will aid significantly in this pursuit.
# TABLE OF CONTENTS

ABSTRACT .............................................................................................................. ii

TABLE OF CONTENTS ............................................................................................ iv

LIST OF TABLES ...................................................................................................... vii

LIST OF FIGURES .................................................................................................... viii

LIST OF ABBREVIATIONS ........................................................................................ x

ACKNOWLEDGEMENTS ........................................................................................... xi

DEDICATION .............................................................................................................. xiii

1. INTRODUCTION .................................................................................................. 2

1.1 THE RESPONSE OF CELLS TO LOW DOSE IRRADIATION ............................... 2

1.1.1 Absorption of X-rays in Biological Systems.................................................... 2

1.1.2 Mammalian Cell Survival Curves ................................................................. 4

1.1.3 Cell Survival following Low Dose Irradiation .............................................. 6

1.1.4 Implications of Low Dose Hyper-Radiosensitivity ...................................... 10

1.1.5 HRS/IRR: An Inducible Response? ............................................................... 12

1.1.6 Possible Mechanisms for HRS/IRR ............................................................ 13

1.2 RADIATION-INDUCED APOPTOSIS ................................................................ 17

1.2.1 Cell Suicide: A Brief History ....................................................................... 17

1.2.2 General Features and Incidence of Apoptosis ............................................. 19

1.2.3 Radiation-induced Apoptosis ....................................................................... 22
4.1 METHODOLOGICAL CONSIDERATIONS ........................................ 97
  4.1.1 Characteristics of Misclassified Cells .................................. 97
  4.1.2 Learning Set Characteristics for Future Studies..................... 99

4.2 APOPTOSIS FOLLOWING HIGH VERSUS LOW DOSE IRRADIATION ....... 101
  4.2.1 Apoptosis Following 5 Gy is a Post-Mitotic Event in CHO Cells .... 101
  4.2.2 Low Doses of X-rays Result in "Delayed" Apoptotic cell death ... 104
  4.2.3 Apoptosis in the Progeny of Irradiated Cells ...................... 105

4.3 NON-LINEAR APOPTOTIC RESPONSE AT LOW DOSES .................... 107
  4.3.1 Is it Real? ......................................................................... 107
  4.3.2 Relationship of Apoptosis to Cell Survival at Low Doses ........ 110
  4.3.3 Non-linearity of the Apoptotic Response to Low Doses ........... 114

4.4 SUGGESTED FUTURE WORK ..................................................... 116
  4.4.1 Further Definition of the Low Dose Response ....................... 116
  4.4.2 Time-lapse Studies of Apoptotic Response .......................... 117
  4.4.3 Other Uses for Two-Colour Fluorescence Hardware/Software .... 117

4.5 CONCLUSIONS ...................................................................... 119

REFERENCES .............................................................................. 121

APPENDIX A .............................................................................. 142

APPENDIX B .............................................................................. 147
LIST OF TABLES

Table 2.1: Summary of fixation protocols .................................................. 37
Table 2.2: Summary of cell cycle analysis .................................................... 39
Table 2.3: Effects of fixatives on apoptotic morphology ............................... 41
Table 2.4: Classification criteria for learning and test sets ........................... 61
Table 2.5: Classification accuracy of discriminant function DSF3-1 .............. 67
Table 2.6: Classification accuracy of discriminant function DSF4-1 .............. 68
Table 2.7: Classification accuracy of discriminant function DSF1-1 ............... 68
Table 2.8: Features used in classification functions for DSF1-1 ..................... 69
Table 2.9: Classification accuracy of discriminant function DSF1-2a .......... 71
Table 2.10: Features used in classification functions for DSF1-2a ................. 71
Table 2.11: Classification accuracy of discriminant function DSF1-2b .......... 72
Table 2.12: Features used in classification functions for DSF1-2b ................. 73
Table 2.13: Overall classification matrix for learning set ............................ 74
Table 2.14: Overall classification matrix for test set ................................... 75
Table 2.15: Intra-slide variance for apoptotic frequency measurements .......... 77
Table 3.1: Sample t-test results, calculated for 42 hour data ...................... 89
Table 3.2: Sample t-test results, calculated for 72 hour cumulative data ......... 92
Table 3.3: Effect of priming doses on subsequent apoptosis frequencies ......... 93
Table A.1: Cell cycle parameters after low doses over 72 hours ................. 142
Table B.1: Number of experiments required for statistical validation .......... 150
LIST OF FIGURES

Figure 1.1: The direct and indirect actions of radiation ........................................ 3
Figure 1.2: Typical mammalian cell survival curve ............................................. 5
Figure 1.3: Low dose survival of cells as measured by DMIPS assay ...................... 7
Figure 1.4: HRS/IRR in human tumour cell lines ............................................... 8
Figure 1.5: Morphology of apoptosis versus necrosis ......................................... 20
Figure 1.6: The effect of low dose priming on subsequent high dose apoptosis ....... 28
Figure 1.7: Apoptotic response of radioresistant lymphocytes ............................. 29
Figure 1.8: DNA-end fragment patterns formed during apoptosis ....................... 32
Figure 2.1: Sample images of cells fixed by PfM method .................................. 42
Figure 2.2: Fluorescence-modified CytoSavant automated image cytometer ....... 43
Figure 2.3: Typical epi-illumination configuration for fluorescence microscopy .... 45
Figure 2.4: Flow chart describing AcquireDL software procedures .................... 48
Figure 2.5: Simple discriminant analysis .......................................................... 56
Figure 2.6: Schematic depiction of binary discriminant trees tested .................... 64
Figure 3.1: Variation of apoptotic frequency with time following 5.0 Gy X-rays .... 83
Figure 3.2: Variation of cell cycle parameters with time following 5.0 Gy .......... 84
Figure 3.3: Raw apoptotic frequencies .............................................................. 86
Figure 3.4: Apoptosis as a function of time following irradiation for low doses of X-rays 87
Figure 3.5: Cumulative plot of x-ray-induced apoptosis ................................... 90
Figure 3.6: Effect of priming doses on apoptosis frequency ............................. 94
Figure 4.1: All low dose apoptotic fraction data plotted from each experiment .... 109
Figure 4.2: Mean size of surviving x-irradiated colonies .................................. 113
Figure A.1: Variation of cell cycle parameters with time for control cells ......................... 143

Figure A.2: Variation of cell cycle parameters with time for 0.25 Gy irradiated cells..... 144

Figure A.3: Variation of cell cycle parameters with time for 0.5 Gy irradiated cells....... 145

Figure A.4: Variation of cell cycle parameters with time for 1 Gy irradiated cells........ 146
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>3-AB</td>
<td>3-aminobenzimide</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AR</td>
<td>adaptive response (of human lymphocytes)</td>
</tr>
<tr>
<td>bcl</td>
<td>B-cell lymphoma</td>
</tr>
<tr>
<td>CCD</td>
<td>charge-coupled device</td>
</tr>
<tr>
<td>ced</td>
<td><em>Caenorhabditis Elegans</em> death</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CV</td>
<td>coefficient of variation</td>
</tr>
<tr>
<td>DMIPS</td>
<td>Dynamic Microscope Image Processing System</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DSB</td>
<td>(DNA) double-strand break</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>EM</td>
<td>electron microscopy</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>HRS</td>
<td>hyper-radiosensitivity</td>
</tr>
<tr>
<td>HVL</td>
<td>half-value layer</td>
</tr>
<tr>
<td>ICE</td>
<td>interleukin-converting enzyme</td>
</tr>
<tr>
<td>IOI</td>
<td>integrated optical intensity</td>
</tr>
<tr>
<td>IRR</td>
<td>increased radioresistance</td>
</tr>
<tr>
<td>ISEL</td>
<td><em>in situ</em> end-labelling</td>
</tr>
<tr>
<td>LET</td>
<td>linear energy transfer</td>
</tr>
<tr>
<td>LQ</td>
<td>linear quadratic (model)</td>
</tr>
<tr>
<td>LUT</td>
<td>look-up table</td>
</tr>
<tr>
<td>MACs</td>
<td>malignancy associated changes</td>
</tr>
<tr>
<td>MEM</td>
<td>minimum essential medium</td>
</tr>
<tr>
<td>MeOH</td>
<td>methanol</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>OER</td>
<td>oxygen enhancement ratio</td>
</tr>
<tr>
<td>PARP</td>
<td>poly(ADP ribose)-polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>plating efficiency</td>
</tr>
<tr>
<td>Pf</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SF</td>
<td>surviving fraction</td>
</tr>
<tr>
<td>SSB</td>
<td>(DNA) single-strand break</td>
</tr>
<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase-mediated UTP nick end-labelling</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

When I started this project nearly five years ago, it seemed like it would be a fairly straight-forward task: figure out what you want to do, figure out how, and then do it. Just like anything else, though, it's never as easy as you think. However, I've been very fortunate to have had the help and support of many people, each of whom has contributed in their own way to this thesis. Some have contributed by providing technical assistance, others through providing unique insights on this work, and still others through their friendship and encouragement. To each, I would like to express my sincere gratitude for making this project a more pleasurable experience.

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For my parents,

whose love,

and many sacrifices

have made this possible.
Section 1

Introduction

"Printing is so dull. There is nothing exquisite about it at present. In my next publication I am hoping to give examples of something more satisfying in this way. The letters shall be of a rare design; the commas will be sunflowers, and the semicolons pomegranates."

- Oscar Wilde
1. INTRODUCTION

It is an inescapable fact of life that all living organisms, including humans, are continuously exposed to ionising radiation. Each one of us is constantly bombarded by cosmic rays from outer space. Our cells and tissues absorb energy from the decay of naturally occurring radioactive materials in the earth and from radioactive elements in the air we breathe. However, our greatest threat from radiation comes from man himself. Intentional exposures may come from diagnostic x-rays, nuclear medicine and radiotherapy of cancer, and occupational exposures are not uncommon. For instance, exposures from natural sources of radiation are typically less than 1 cGy per year. Doses of a few cGy to several Gy are experienced in accidental exposures to ionising radiation and in cancer radiotherapy, daily dose fractions between 1 and 3 Gy are generally used, with a total dose of 40-60 Gy delivered to the tumour.

Since radiation plays such a prominent role in our daily lives, it is pertinent that the medical risks and consequences of such exposures are fully understood. Fundamental to this understanding is the characterisation of the basic biological effects of radiation absorption, at the molecular and cellular levels.

1.1 THE RESPONSE OF CELLS TO LOW DOSE IRRADIATION

1.1.1 ABSORPTION OF X-RAYS IN BIOLOGICAL SYSTEMS

While there are many different forms of radiation, the current discussion will concentrate on X-rays, as this is the primary type utilised in the clinical management of cancer. Other forms of ionising radiation, such as charged particles, have sufficient
kinetic energy that they may physically disrupt the atomic structure of the biomolecules through which they pass, in their wake leaving chemical and biological alterations. X and γ-radiations in general do not possess sufficient energy to cause such damage. Instead, the x-ray photons interact with loosely bound outer-shell electrons of the medium through which they pass via Compton interactions. The result is a large number of fast electrons which themselves may interact with biologically important molecules. This mode of damage is known as the "direct effect" of ionising radiation (see Figure 1.1). However, the majority of the ejected electrons interact with the absorbing medium, which in the case of biological systems, consists of mostly water. For instance, as a result of these interactions, the water molecules will become ionised such that,

\[ \text{H}_2\text{O} \rightarrow \text{H}_2\text{O}^+ + e^- \]

The \(\text{H}_2\text{O}^+\) radical is a highly reactive species with an extremely short lifetime, on the order of \(10^{-10}\) seconds, and thus has a tendency to interact with neighbouring water molecules,

\[ \text{H}_2\text{O}^+ + \text{H}_2\text{O} \rightarrow \text{H}_3\text{O}^+ + \text{OH}^- \]
The hydroxyl radical, OH\(^*\), may diffuse a short distance (~20 Å), where it may interact with important macromolecules. It is estimated that it is responsible for almost two-thirds of x-ray damage to DNA in mammalian cells, the assumed ultimate "target" for the deleterious effects of radiation. Combined with other highly reactive species created by the radiolysis of water (i.e. H\(^*\), HO\(_2\)^*, H\(_2\)O\(_2\)^*), the indirect effect of radiation typically accounts for 80% of x-ray damage in aerobic cells [1].

1.1.2 MAMMALIAN CELL SURVIVAL CURVES

While the physical processes involved in radiation absorption only require approximately \(10^{-5}\) seconds for completion, the biological expression of this damage can take hours, days, months and in some cases, even years to appear. The amount of time is generally related to the level of biologic organisation, beginning with macromolecular changes, followed by alterations at the cellular level and possible long-term effects at the tissue and systemic levels.

One of the most widely used gross measurement of radiation effects at the cellular level is its toxicity to mammalian cells, expressed in terms of colony-forming ability (or "cell survival"). The standard method of its measurement was first described in 1955 by Puck and Marcus to study the inactivation of cells following irradiation [2], and this technique continues to be used today. Cells grown in culture are exposed to radiation and while in suspension, their concentration is determined via automated counting methods or by haemocytometer. A volume of this suspension is then diluted into petri dishes, such that an approximate known number of cells are seeded. Following incubation for 1-2 weeks under appropriate conditions, the dishes are stained and the number of resulting colonies, each assumed to be the progeny of a single cell, are
counted. The "plating efficiency" (PE) can then be calculated as the number of colonies counted divided by the number of original cells seeded. Normalisation of this value by the PE of unirradiated control cells gives the "surviving fraction" (SF), the value typically reported. A typical experiment would involve irradiation of the cell culture with several incremental doses, which produces a "cell survival curve," such as that shown in Figure 1.2.

There have been many attempts to develop mathematical models of the cell survival curve: some based on physical parameters of radiation absorption [3], others based on damage repair processes inherent in cells [4, 5] and still others determined empirically. Of these, the most widely used model is the "Dual-action Theory" [6] described by the poorly-named "Linear Quadratic Equation" (or LQ model),

\[ SF = e^{-\alpha D - \beta D^2} \]

where D is the absorbed dose and \( \alpha \) and \( \beta \) are constants. The theory of dual radiation action stems from early chromosomal studies in which the myriad of chromosomal aberrations observed following irradiation of cells were clearly the work of two separate

\[ \text{Figure 1.2: Typical mammalian cell survival curve generated by the Puck and Marcus colony-forming assay. The linear quadratic model adequately describes the shape of the curve at doses for which this assay is useful.} \]
breaks in the chromatin. The $\beta D^2$ term of the LQ model represents the interaction of breaks caused by two separate events, the $\alpha D$ term represents those breaks caused by a single event. The result is a continuously bending survival curve, often containing an initial "shoulder" region at lower doses, or higher SF (see Figure 1.2). This equation has been found to adequately describe the majority of mammalian cell survival curves at doses above 1 Gy and has the added attractive feature of requiring only two parameters.

For the study of survival due to low doses of radiation, the Puck and Marcus colony-formation assay has severe limitations [7-9]. In general, it is not sufficiently precise to measure the very small reduction in survival resulting from absorbed doses of less than 1 Gy. Foremost is the uncertainty in the number of cells plated, due to random errors in cell counting and dilution. For this reason, low dose survival has traditionally been determined by extrapolation of data obtained at higher doses. The validity of the assumptions contained within such procedures had often been questioned [10, 11] until the advent of more precise methods for the assessment of cell survival.

1.1.3 Cell Survival Following Low Dose Irradiation

Within the last several years, two different technical innovations have been employed to overcome the limitations of the traditional cell survival assay. The first employs computer-assisted imaging (the "Dynamic Microscope Image Processing System", or DMIPS) to locate a known number of individual cells on a petri dish and revisit their precise location on the dish after several days incubation [11, 12]. The second uses fluorescence-activated cell sorting (FACS) to dispense a precisely determined number of cells directly into the petries to minimise any errors in the number
of cells seeded [13, 14]. In each case, these have allowed highly accurate measurement of the low-dose portion of the mammalian cell survival curve.

In what has now become a landmark paper for radiobiologists interested in the effects of low doses, Marples and Joiner, using the microscopic assay and V-79 Chinese hamster cells, showed a divergence from the LQ model at doses of less than 0.5 Gy [15]. As illustrated in Figure 1.3, the LQ model significantly over-predicted the level of survival following low-dose irradiation. In effect, X-ray doses in the range of 0-0.3 Gy exhibited a greater lethal effect per unit dose than higher doses of X-rays. Moreover, this effectiveness approached that of neutrons, a much more lethal mode of irradiation. Between doses of 0.5 - 1 Gy, the survival curve appears to recover to levels represented by the LQ model, an apparent "increased radioresistance" (IRR). Although the immediate reaction to this study was one of scepticism, subsequent studies by other investigators, and a rekindling of interest in past studies using non-mammalian systems now suggest that this low dose hyper-radiosensitivity (HRS) phenomenon is widespread.

Figure 1.3: Low dose survival of cells as measured by DMIPS assay for neutron (N) and X-irradiated V79 cells. The linear quadratic model over predicts survival at low doses, as shown in inset. Reproduced from Marples and Joiner [15], with permission.
Since their initial publication Marples, Joiner and others have expanded upon the work to show that the HRS/IRR phenomenon is present under both well-oxygenated and hypoxic conditions in V79 cells, resulting in a generally decreasing Oxygen Enhancement Ratio (OER) as dose decreases [17]. This is in agreement with earlier studies which demonstrated a reduced OER at low doses [11, 18]. Further support for the presence of the HRS/IRR regions of the cell survival curve comes from a series of studies performed on cultured human tumour cell lines. These studies were performed primarily by two separate groups, each using different techniques previously shown to yield quantitatively similar results [19, 20]: the microscopic DMIPS method [21-24], or the FACS method [25-27]. The cell lines studied were of varied origins and varied radiosensitivity: melanomas (U1, Be11, MeWo), colorectal carcinomas (HT29, SW48), a prostate and bladder (RT112) carcinoma, lung adenocarcinoma, a cervical squamous carcinoma (SiHa), and a neuroblastoma cell line (HX142). Interestingly, the most radioresistant of those cell lines (as traditionally assessed by survival at 2 Gy) demonstrated the most marked HRS region.

**Figure 1.4:** HRS/IRR in human tumour cell lines. The more radioresistant cell lines exhibit the most pronounced IRR below 1 Gy. In contrast, there is no evidence of deviation from the LQ model of survival (dashed line) in the more radiosensitive lines. Reproduced from Joiner et al. [16].
specifically, the HT29, U1, Be11 and RT112 lines. Furthermore, the most sensitive of these lines (HX142, SiHa and SW48) showed no HRS/IRR. Some of these data are featured in Figure 1.4 for comparison. Together these data indicate that the intrinsic radiosensitivity of a cell line may vary in a dose dependent manner, and provide compelling evidence that the HRS/IRR phenomenon is not an experimental artefact.

In actuality, there is a wealth of evidence lending support to the existence of HRS/IRR, which comes from earlier studies of non-mammalian cell systems. Data presented as early as 1967 showed an increase in the survival of the protozoa Tetrahymena pyriformis with increasing doses [28]. HRS/IRR-like survival curves had also been reported in budding yeast [29], algae [30], and pollen grains [31, 32]. In a series of elegant studies of a highly radioresistant lepidopteran insect cell line, TN-368, Koval [33-35] demonstrated HRS/IRR when irradiated in either the presence or absence of oxygen and concluded that this phenomenon was indeed a result of increasing radioresistance with dose. Thus, the appearance of HRS/IRR in both lower and higher cell systems indicates this may be a well-conserved response to toxic insult.

A different phenomenon which has been under study for a number of years, and which has gained wide acceptance within the radiobiology community is the adaptive response (AR) of human lymphocytes [36-38]. The AR describes a phenomenon by which small “priming” doses of radiation confer a radioprotective effect to higher “challenge” doses of radiation delivered 4 to 6 hours after the initial insult. The effect was only seen for priming doses between 0.5 to 20 cGy [39]. The traditional end-point of these studies has been the measurement of chromosome aberrations, although the effect has been noted as a reduction in hprt mutation frequency [40] and an increase in
Section 1 - Introduction

survival [41]. The AR has also been characterised in lower cell systems, such as algae [42], fern spores [43], and yeast [44]. Such adaptation has now been found to occur in irradiated fibroblasts as measured by survival [45] and micronucleus frequency [46]. The application of priming doses leads to the abolition of the HRS region in cell survival curves measured due to subsequent doses (following priming with either x-rays or H₂O₂) [47], leading to speculation that the AR and HRS/IRR phenomena are manifestations of the same inducible processes [16].

While such *in vitro* data presents an intriguing biological concept, the *in vivo* consequences of HRS/IRR remain largely undiscovered. However, evidence for its effect *in vivo* has been noted in studies of hyper-fractionation. A cornerstone of modern radiotherapy is the fact that smaller fractionated doses (typically ~2 Gy), delivered over time, allow a greater total dose delivered to the tumour than a single acute dose. This technique serves to spare normal tissues from unwanted damage. The efficacy of fractionation is based in the repair of sublethal damage in the normal tissues between fractions, while allowing re-oxygenation of the tumour prior to the next dose. In an experimental tumour system, when the dose was hyper-fractionated to up to 126 dose fractions, the total dose necessary was found to actually decrease [48]. Other *in vivo* studies with end-points other than survival, showed a similar result in skin [49], kidney [50] and lung [51]. These results are consistent with the hyper-radiosensitivity observed *in vitro* and imply that HRS/IRR could have significant consequences.

1.1.4 Implications of Low Dose Hyper-Radiosensitivity

Apart from the benefits which could be achieved through the use of hyper-fractionated radiation treatment schedules, as described above, the existence of a low
dose hypersensitivity to radiation is important for several reasons. In radiotherapy for instance, normal tissue in the beam path and on the margins of the tumour volume invariably receive at least some small dose of radiation. For instance, for a typical 2 cm wide $^{60}$Co treatment field, the penumbra of the beam is approximately 1.5 cm wide (measured from 80% to 20% of the peak dose). With a typical 2 Gy fraction as currently applied, surrounding normal tissue in this area will receive doses ranging from 0.4-1.6 Gy, and yet more distant areas will receive even lower doses. While this is unavoidable, existence of hyper-radiosensitivity at low doses suggests that a greater than expected level of damage may be produced within this tissue, an important factor especially when the treatment area is juxtaposed near sensitive organs (e.g. the spinal cord). In fact, a recent publication by Hamilton et al. [52] which showed a more pronounced skin erythema than expected following low dose fractionation, has underscored the important implications of the low dose hypersensitivity phenomenon for radiotherapy treatment planning [53].

The carcinogenic risk from low-level radiation exposure is the basis upon which maximum permissible dose (MPD) limits have been determined. The International Committee on Radiation Protection (ICRP) has defined the MPD as “the dose of ionising radiation that... is not expected to cause appreciable bodily injury to a person at any time during his lifetime.” Their estimates are based largely upon data obtained from Japanese atomic bomb survivors, but include medical and occupational exposures and nuclear accident data. In the majority of cases, the doses received were significant. In those cases where relatively low doses were received, the data remains inconclusive due to a variety of compounding factors such as uncertainty in biological dosimetry and the long latent period for cancer induction. As such, the carcinogenic risk of low dose radiation
exposures is obtained by extrapolation from a rather narrow range of higher dose data. In the absence of a means of measurement of low dose carcinogenic risk, it is therefore imperative that the effects of low dose irradiation be better understood and that the mechanisms of action are well characterised. Low dose hyper-radiosensitivity offers an exciting possibility then, of producing new and more accurate estimates of carcinogenic risk of radiation exposure. To do so, however, a more complete explanation of the mechanisms underlying this phenomenon is required.

1.1.5 HRS/IRR: An Inducible Response?

It comes as no surprise that living cells, intent on passing on their genome to their progeny, should have evolved mechanisms which would act in response to toxic insult. In fact radiobiologists have known and accepted for a number of years that cells temporarily suspend passage through their life cycle following irradiation, as a means of coping with the damage they incurred. Should it then be surprising that cells would have other weapons at their disposal which could be called upon when needed? Is this what HRS/IRR represents?

Sceptics have argued that the substructure in the survival curve observed at low doses can be modelled by a population of cells which contains two distinct subpopulations differing in their inherent radiosensitivity. However, there have now been descriptions of cell lines (HT29 and Be11, Figure 1.4) which exhibit IRR regions of positive slope (i.e. there is an increase in survival with increasing dose) [21, 22, 26]. Clearly, should the HRS/IRR phenomenon be caused by the subpopulation explanation, one of the populations would need to exhibit a greater than 100% survival [29]. Furthermore, excepting these cases, the more sensitive population would have to be
Section 1 - Introduction

over 10-fold more sensitive than the most radiosensitive cell line known to exist [21]. In addition, the difference in radiosensitivity between the two would be approximately 80-fold, far greater than the 10-fold difference in radiosensitivity observed between different phases of the cell cycle [54]. That the HRS/IRR is caused by different phases of the cell cycle present in asynchronous populations was addressed by both Marples [15] and Wouters [27], who showed that survival curves for partially synchronised populations still exhibit the HRS/IRR regions. It is therefore apparent that the recovery of survival in the IRR region represents an inducible radioresistance of individual cells; an inherent protective mechanism which acts in response to radiation damage.

1.1.6 Possible Mechanisms for HRS/IRR

While there is no direct evidence which specifies the precise mechanisms of IRR or AR, there are indirect data accumulating which help illuminate some of the features of these processes. These studies are, in general, of two distinct natures:

1) identification of the nature of the “trigger” for increased radioresistance

2) identification of the mode of action of the response.

In the first case, a significant contribution has been made by Skov et al. through the study of other agents and their effects upon the HRS/IRR and AR [55]. While the original study by Marples and Joiner [15] indicated that cells exposed to neutrons exhibit no HRS, it now appears that the magnitude of the IRR is inversely related to the linear energy transfer (LET) of the radiation [56]. Furthermore, adaptation was found to occur through small priming doses of neutrons and $\pi^-$-mesons [56, 57], in addition to x-rays. Because differences exist in the spectrum of DNA lesions caused by different LET
radiations, these studies led to the hypothesis that DNA strand breaks might be the trigger for increased radioresistance. Cells receiving incremental doses of x-rays or peak or plateau pions showed different levels of single- and double-strand breaks, although the response appeared linear over the doses studied [57]. Supporting the case of DNA strand breaks is the notion that H$_2$O$_2$ pre-treatment, which causes primarily single-strand breaks (SSB) through OH$^-$ damage (similar in nature to the indirect action of x-rays), eliminates the HRS phenomenon in mammalian cells [47]. H$_2$O$_2$ can also induce the adaptive response in lymphocytes [38] and induces radioresistance in other cell lines [44, 58]. Incorporation of radio-labelled $^{14}$C or $^9$H thymidine, whose damage is primarily localised to DNA, has also been found to induce radioprotection in cells [36, 59]. However, in all these cases, the relative numbers of breaks expected from each treatment do not result in a single threshold (i.e. a specific number of breaks) beyond which adaptation occurs. Compounding this is the fact that the chemotherapeutic agent cisplatin, which delivers its toxic effects primarily due to DNA-adduct formation and cross-linking, also induces radioresistance [60, 61]. This has led to the incorporation into the DNA damage hypothesis of a qualitative component, where the location or chemical specificity of the DNA damage influence the triggering of IRR [60]. Alternately, it has been surmised that the DNA breaks produced by constitutive enzymes involved in excision repair of the DNA-damage may themselves provide the signal for induction of the radioresistant response [59, 60]. It is also possible that the trigger mechanism is initiated by radiation damage at the cell membrane and acts through signal transduction mechanisms [62], although there is little information in this regard.

These data naturally lead to the question "Just what is being induced?" Conceptually, the most attractive explanation is that the increased radioresistance is a
DNA repair process which acts in response to damage, through an increase in quantity or quality (fidelity) of repair. And there is mounting indirect evidence that supports this notion. Cycloheximide, an inhibitor of protein synthesis, can prevent the development of IRR [15] and blocks the adaptive response when present in the interval 4-6 hours following the priming treatment [63]. In insect cells, inhibitors of RNA synthesis were shown to have a similar effect [34]. Newly synthesised RNA and proteins are therefore implicated in the response. More specifically, the effects of inhibitors of DNA repair processes have been studied [64]. Of these, only 3-aminobenzimide (3-AB) was found to inhibit the induction of IRR. 3-AB is a potent inhibitor of poly(ADP ribose)-polymerase [65], which is thought to inhibit the ligation step involved in DNA excision repair [66] of damage caused by x-rays and cisplatin. Other convincing arguments for the involvement of DNA repair in IRR, come from studies of host-cell viral reactivation following radiation. Host cells treated with conditioning doses of radiation [67], chemicals [68] and UV [69] reactivate, or repair, damaged viral DNA subsequently infected into the hosts. As well, Skov et al. have examined the low dose survival of cell lines known to be deficient in DNA repair processes compared to their normal parent cell lines (AA-8 and CHO) [70, Skov, unpubl. results]. The X-V15B line derived from V79 cells and deficient in DSB repair ability exhibits a purely exponential response (HRS), with no evidence of IRR [70]. The Chinese hamster ovary-derived UV-20 line, defective in an incision step of excision repair, showed a similar survival curve [70]. A single-strand break repair-deficient CHO cell line, EM-9 exhibited both HRS and IRR, albeit somewhat altered [70]. Together, these data paint a complex picture where DSB and excision repair might both contribute to IRR, while SSB repair takes place in the HRS region. That DSB repair is at least partly
Section 1 - Introduction

responsible for IRR is supported by evidence that conditioning doses of x-rays leading to faster DSB rejoining following subsequent challenge doses [71, 72].

If indeed repair is implicated in the development of induced resistance, it can be envisioned that DNA-repair enzymes would be up-regulated, or manufactured, upon irradiation with a sufficient dose. In actuality, new repair enzymes might appear several steps downstream from the initiating signal, or could represent secondary reactions stemming from post-translational modifications [73, 74]. Several laboratories have reported the induction of a plethora of gene products following toxic insult at high doses: DNA-repair genes, such as the O⁶-methylguanine-DNA methyl-transferase [75]; transcription factors, such as XIP175 [76]; proto-oncogenes such as c-fos [77] and c-jun [78]; cell-cycle and growth-related proteins such as PCNA [79] and protein kinase C [80]; and numerous others. A complete treatment of this subject is well beyond the scope of this introduction and the reader is referred elsewhere [81-84].

Much of the molecular evidence for radiation induced resistance involves the regulation of cell cycle delays in response to radiation. It is believed that temporary suspension of normal cellular activity allows time for the cell to repair DNA damage before progression through DNA synthesis and/or mitosis. In fact, the induction of checkpoints at the G1-S [85] and G2-M [86] transitions has been shown to be an active and not accidental response to radiation damage. Of particular note in this regard is the accumulation of the p53 protein following radiation, which functions to arrest cells prior to entry into S phase [85] through induction of cell-cycle control genes such as GADD45 [87] and WAF1/CIP1 [88, 89]. Mutations in p53 can result in failure of radiation-induced G1-arrest [85, 90] and are related to a greater overall radiation resistance [91-94].
Although little is known regarding genes involved in the G2/M checkpoint, cells which exhibit a longer G2 arrest following irradiation are generally more radioresistant, and radiation-induced changes in cyclin B mRNA and protein levels have been implicated [95, 96]. The majority of evidence for the importance of the G2-delay comes from its inhibition by caffeine, which renders cells more sensitive to radiation [97, 98]. However, no such cell cycle delays have so far been detected following doses in the HRS/IRR range [99].

Thus, there is significant evidence that the low-dose hyper-radiosensitivity and increased radioresistance phenomena are a reflection of an inducible repair mechanism, and that this mechanism could be molecularly coupled to internal control of the cell cycle check-points. However, in the past several years, there has been the startling discovery that nearly all living cells have yet another weapon at their disposal: an extreme measure of last resort, and one which is intimately related to both of these processes.

1.2 RADIATION-INDUCED APOPTOSIS

1.2.1 CELL SUICIDE: A BRIEF HISTORY

In 1858, a German physician by the name of Rudolph Virchow first proposed the theory of "biogenesis". It was the idea that all living cells arise from pre-existing cells, contradicting the popular belief (even among scientists) in spontaneous creation. With convincing proof for this theory provided only four years later by Louis Pasteur, a scientific revolution occurred. Backed by the strength of Darwin's theory of evolution [100], which essentially defines the driving force behind life as the perpetuation of its genome, scientists subsequently paid scarce attention to the mechanisms involved in the
death of cells, preferring to concentrate their efforts on unravelling the mysteries of how cells propagate. All cell death was generally accepted as pathologic and studies which hinted at the contrary [101-103] went largely unnoticed throughout most of the scientific community.

In 1971, John Kerr of the University of Queensland Medical School in Australia described a mode of cell death in hepatocytes of the rat which he termed “shrinkage necrosis” [104]. Different in its microscopic appearance from classic pathologic death (which he referred to as “coagulation necrosis”), Kerr attributed this “new” mode of death to the “moderately noxious environment” surrounding the cells. Less than a year later, Kerr, working with colleagues Wyllie and Currie, would come to realise the full significance of this discovery and would decide the process warranted its own distinct name. They proposed the name “apoptosis”, from the Greek for “falling off”, in reference to the seasonal shedding of leaves from trees [105]. Evidence that apoptosis was far from accidental cell death came in 1980, when Andrew Wyllie showed that DNA from apoptotic cells was cleaved into discrete fragments in multiples of 185 base pairs [106]. This, he hypothesised, was the result of the activation of an endogenous nuclease that cleaves the DNA between nucleosomes: the cell’s own machinery was responsible for its demise.

Still largely unnoticed by most in the scientific community, save for some developmental biologists, the first genetic evidence for active cell death came from studies of Caenorhabditis Elegans, a nematode in which 131 of its 1090 cells consistently die at specified periods during its development. Yuan and Horvitz, identified two genes in 1986, ced-3 and ced-4, which were an absolute requirement for the death
of these cells [107]. Shortly thereafter, in 1990, David Hockenberry, Stanley Korsmeyer and colleagues, studying the human \textit{bcl-2} proto-oncogene, determined that it encodes for a membrane-associated protein that blocks programmed cell death in human B-cells [108]. With the realisation that there was now a genetic basis for active, cell-mediated death in human cells and that the inhibition of this process was implicated in the development of human cancers, the study of apoptosis rapidly gathered momentum. Only three years later, Horvitz gathered the final piece of evidence to convert the non-believers: he showed that the newly cloned, apoptosis-inhibiting \textit{ced-9} gene of \textit{C. Elegans} shares significant homology to the human \textit{bcl-2} proto-oncogene [109-111]. Apoptosis, the alternative to pathologic cell death, was not to be overlooked any further: its genetic blueprint had survived the long evolutionary journey from the smallest of multicellular organisms, to the complex multi-tissue systems of man.

\section*{1.2.2 General Features and Incidence of Apoptosis}

What Kerr saw under his microscope in 1971 was no different from what numerous others had described for the previous 100 years (e.g. [101]). And since his landmark paper of 1972, the basic morphological characteristics of apoptotic cells have proved to have remarkable consistency across a spectrum of cell types from any number of species.

Apoptosis is distinct both morphologically and biochemically from necrosis [112]. Necrosis is generally regarded as the result of severe insult to a cell or group of cells. It is a degenerative process characterised by the loss of membrane integrity, cell swelling and lysis. Accompanying this is the release of a host of chemicals and lysosomes into the surrounding tissues, thereby eliciting a significant inflammatory response [113, 114].
On the other hand, apoptosis is an active process which occurs discreetly in individual cells and induces little or no inflammatory response [115]. Cell death by apoptosis is believed to be initiated by a signal transduction mechanism in a cascade of several known oncogenes, although the various cellular triggers are not yet fully characterised. Shortly thereafter, the distinct cleavage of nuclear DNA, first observed by Wyllie, occurs. Concurrently, the DNA becomes markedly more condensed in areas adjacent to the nuclear membrane and the nucleus may fragment into two or more pieces. As the degradation of the DNA continues, these pieces may break away from the cell and be digested by neighbouring cells by phagocytosis.

Hence there is a distinct morphological appearance of apoptotic cells which is identifiable by microscopy [116]. The consistency of the morphological changes in apoptosis have lead some investigators to argue in favour of a "Final Common Pathway" [117], although the molecular events leading to this morphological signature and indeed, the events which trigger its onset, vary widely between cell and tissue types. The defining quality of apoptosis then, is one based on the morphology, or appearance of the

![Figure 1.5: Morphology of apoptosis versus necrosis. (A) An apoptotic body with four nuclear fragments, illustrating condensed chromatin, and well preserved organelles. (B) Necrotic cell illustrating ill-defined edges of chromatin clumps, mitochondrial swelling and membrane dissolution (from Kerr et al. [118])](image)
dying cells. Figure 1.5 illustrates the differences observed between necrosis and apoptosis under electron microscopy.

The ubiquitous nature of apoptosis has made it readily apparent that it is of extreme importance in a number of fundamental biological processes. As predicted by the early research on C. Elegans, apoptosis plays a crucial role in embryogenesis and development in higher vertebrates. It is involved in such phenomena as terminal differentiation of in the retina [119] and the deletion of interdigital webs [120, 121]. One of its foremost responsibilities is the maintenance of tissue homeostasis, or regulation of cell numbers. While cell growth and division provide the "input" of cells into tissues, cell death by apoptosis balances the equation by ensuring there is adequate cell "output". This fact is evidenced by several studies which show that, over time, apoptosis balances out mitosis [106], in that many of the same growth factors which stimulate mitotic division of cells also serve to control the suppression of apoptosis [122, 123]. For instance, apoptosis accounts for cell deletion observed in the regression of the lactating breast following weaning [124, 125] and the involution of the endometrium in the latter stages of menses [126]. Deregulation of apoptosis may adversely affect the maintenance of tissue homeostasis and therefore has important implications towards the development of neoplasia [127]. Mutations required for multi-hit carcinogenesis could also accumulate with loss of normal apoptotic function [128]. As well, apoptosis is believed to be the putative mechanism by which cytotoxic chemotherapy, radiation and endocrine therapy, the mainstays of cancer treatment, exert their therapeutic effects [129]. Inhibition of the normal apoptotic response is regarded as an important mechanism to explain the resistance of some tumours to chemotherapeutic agents [130-133].
1.2.3 Radiation-induced Apoptosis

That the discovery of apoptosis is highly significant to radiobiology is best illustrated in the interpretation of cell survival data. The convention for scoring surviving colonies has remained consistent since Puck and Marcus first described the technique: those cells leading to colonies with greater than 50 cells are "survivors". Thus, the clonogenic, or "survival" assay does not measure cell survival in its truest sense, but rather the reproductive integrity (clonogenicity) of cells following radiation exposure. The "reproductive death" of cells has long been associated with the development of chromosome aberrations, and thus is linked with the passage of cells through mitosis [134]. However, radiobiologists have long been aware that another type of cell death exists which can appear at an early stage following irradiation and is independent of passage through mitosis. This form of death was termed "interphase death", which we now know to be apoptosis [135]. For example, in 1961, Fliedner reported an early death which occurred in bone marrow 4-6 hours post-irradiation with 5-10 Gy, which was followed by mitotic-linked death several hours later [136]. A more precise description of apoptosis as a form of radiation-induced cell death appeared in 1982 in studies of crypt cells of the small intestine [137]. Cells which had not passed through mitosis and with apoptotic morphology were detected in the period 3-6 hours following irradiation [138].

Since then, there have been numerous reports of apoptosis as a result of ionising radiation in both cultured cells and animal tumour models (for a review, see Nakano and Shinohara [139]). With several exceptions (e.g. mature lymphocytes), radiation does not generally induce apoptosis in most mature, non-proliferating cells [140]. Instead, apoptosis has been noted as a significant response of immature, proliferating cells from, for instance, the cerebellum [141], the kidney [142], and salivary glands [143].
Specifically, apoptosis may be preferentially induced during the S phase of the cell cycle [144], although exceptions have been noted. The importance of the association of apoptosis with cell proliferation cannot be underestimated, for it is this very quality which is the centrepiece of our long-standing definition of cancer: an aberrant deregulation of cell proliferation. However, in a study of a panel of 14 murine tumours, including mammary and adenocarcinomas, sarcomas, squamous cell carcinomas, a hepatocarcinoma and a lymphoma, Meyn et al. observed a very heterogeneous apoptotic response to irradiation [145]. The findings indicated that those tumours prone to apoptosis exhibited longer growth delays, suggesting that apoptosis was an important pathway in radiation-induced tumour cell death.

1.2.4 Molecular Features of Apoptosis

The list of genes involved in the regulation of apoptosis is long. Despite the concerted effort to unravel the biochemical pathways responsible for apoptosis regulation, the research is still in its infancy and thus no complete picture has emerged. For this reason, descriptions of only those genes with special relevance to the current context of low-dose radiation response will be provided here.

A critical regulator of many aspects of cellular responses to ionising radiation, including apoptosis, is the transcription factor encoded by the p53 tumour suppressor gene [90]. The normal p53 genotype has been found to promote apoptosis following irradiation, while cells with the mutant version are unable to undergo apoptosis, contributing to their increased viability [146, 147]. Stabilisation of the p53 protein following radiation and other DNA-damaging agents may therefore induce either growth arrest in G1 (as discussed in Section 1.1.6) or apoptosis [90], as does forced expression
of p53 in unirradiated cells [148-150]. It is believed that the dual nature of p53 function uses apoptosis to prevent mutation inheritance in cells which have bypassed the G1/S checkpoint, possibly due to de-regulation of the c-myc oncogene [146], a common occurrence in the early stages of carcinogenesis.

Although not a universal pathway, p53 is thought to exert some of its control over apoptosis by regulation of bcl-2-family proteins. Bcl-2 was originally isolated from the t(14:18) translocation frequently found in B-cell lymphomas, and codes for a common membrane associated protein found throughout the cell [151]. In vitro, its deregulation leads directly to the cessation of normal apoptotic cell death and the extension of cellular viability [108]. In a wide variety of cell types, it has been shown that bcl-2 expression may also confer resistance to treatment with several chemotherapeutic agents [130, 152] and ionising radiation [153] as a direct result of its suppression of apoptosis. However, the apoptosis-inhibitory effects of bcl-2 are not universal. Some forms of apoptosis may take place irrespective of bcl-2 protein levels [154-156], quite possibly due to its interactions with other bcl-2 family proteins with which it may heterodimerise. Other apoptosis inhibiting proteins in the bcl-2 family include bcl-xL, bcl-xS and mcl-1 and apoptosis-promoting proteins include bad, bak, bcl-xS and bax [157]. It has now been shown that in at least some cell types, ionising radiation acts to down-regulate bcl-2 and bcl-X mRNA [158], while up-regulating expression of bax [159], causing an increase in apoptosis. The exact mechanism of bcl-2 inhibition of apoptosis is unclear, and there is conflicting evidence suggesting that it down-regulates the activity of Nuclear factor-xB, a known apoptosis-inducer [160, 161]. Interestingly, bcl-2 over-expression inhibits the
apoptotic program of p53, but appears to have little effect upon the p53-induced G1/S checkpoint [162].

P53 is also implicated in the regulation of the interleukin converting enzyme (ICE), which shares significant homology to the C. Elegans ced-3 gene [163]. ICE activates pro-interleukin 1β and another unknown structural substrate, leading to apoptosis. This function is known to be inhibited by bcl-2. Another related protein, prlICE has been found to cleave the nuclear enzyme poly(ADP-ribose) polymerase (PARP), at a site identical to that which ICE cleaves prointerleukin 1β [164]. There is some suggestion that prlICE plays a pivotal role in initiating apoptosis in mammalian cells, as loss of its expression results in the absence of morphological features or DNA fragmentation characteristic of apoptosis [164]. This cleavage of PARP has been found to be a response to ionising radiation and some chemotherapy agents [165].

As noted in Section 1.1.6, PARP is involved in the ligation step of DNA repair. The destruction of this protein as a first-step in apoptosis implies that the cell is making the life-or-death decision, surrendering its capacity for repair and instead choosing self-destruction. The dual nature of radiation-induced bcl-2 down-regulation, and cell cycle control by p53 underscores the possible coupling of DNA repair, cell cycle checkpoints and apoptosis. Furthermore, the involvement in apoptosis of a number of the same genes and gene products found to be induced in response to low dose radiation (e.g. c-fos, c-jun, NF-κB, WAF1/CIP1, GADD45, etc.) suggests that any hypotheses regarding a low dose-induced response to radiation must incorporate the effects of apoptosis.
1.2.5 **Low Dose Hypersensitivity and Apoptosis: A Working Hypothesis**

The coupling of DNA repair and apoptosis presents significant implications for low dose hyper-radiosensitivity. Indeed, much of the evidence for IRR as an induced-repair phenomenon would be consistent with a dose-dependence in the apoptotic response. In fact, the active nature of the apoptotic process itself offers proof of the existence of inducible phenomena by radiation. An elegant hypothesis thus emerges:

**Hypothesis:** At low doses, where only little damage is produced, the apoptotic mechanism is activated in order to lower the probability of mutation inheritance through sacrifice of those cells at risk, rather than expend the considerable energy required to identify and repair all potentially lethal lesions. At higher doses, however, where the entire population is at risk, and thus self-sacrifice is not an option, the cells choose to delay their progress through the cell cycle and/or simultaneously increase their repair capacity.

**Corollary:** A dose dependent decrease in the apoptotic frequency occurs in the dose range spanned by induced radioresistance. The primary mode of cell death in the HRS region is apoptosis, while the most prominent form of cell death at higher doses is necrosis.

This hypothesis receives support from several instances of similarities in characteristics of IRR and apoptosis induction. Some of the most convincing evidence that DNA-repair induction is responsible for HRS/IRR involves the effect of 3-AB, an inhibitor of PARP. 3-AB has previously been found to potentiate apoptosis following γ-
Section 1 - Introduction

Irradiation [166], which would most likely result in the abolition of IRR, as has been noted. In other studies, the inhibition of PARP has been found to inhibit the development of necrosis, while allowing apoptosis to proceed normally [167]. The requirement of protein synthesis for induction of IRR (as demonstrated by cycloheximide inhibition), is consistent with the de novo synthesis of the DNA-cutting endonuclease required for apoptosis [168]. Apoptosis may be induced by other agents which in priming studies, have been proven capable of inducing IRR, such as cisplatin [169], H₂O₂ [170], and irradiation with neutrons [171, 172], although these results vary with the cell line studied [169, 173]. Furthermore, the possibility that DNA double-strand breaks act as the trigger mechanism of IRR also provides evidence of an apoptotic mechanism. Studies with H₂O₂-induced apoptosis have revealed that while it may be induced by either SSBs or DSBs, that the presence of DSBs enhances apoptosis [174]. Murine lymphoma cells devoid of apoptotic function exhibit greater capacity for DSB repair compared to similar apoptosis-permissive cells where DSB repair and apoptosis were found to be independent, the apoptosis-pathway apparently stimulated by initial damage [175].

However, for the same reasons that the low-dose hypersensitivity phenomenon went unnoticed for many years, there is little information regarding apoptotic response following low dose irradiation. An exception is recent work by Hyun et al. [176] who investigated the apoptotic response in relation to the adaptive response of lymphocytes. As reproduced in Figure 1.6, the authors described the reduction in apoptotic levels caused by 2 or 8 Gy challenge doses when a 0.01 Gy priming dose was applied 8 hours previously. In lymphoma cells, this response was found to be independent of p53 function, in contrast to the p53-dependence of apoptosis in normal lymphocytes. This
study therefore suggests the involvement of apoptosis in the AR, and therefore apoptosis may be implicated in the HRS/IRR.

Payne et al. have studied the low-dose apoptotic response in radioresistant lymphocytes and as reproduced in Figure 1.7, their results showed a non-linear response, and indicate that the predominant form of cell death where cell survival is high (i.e. > 90%) is indeed apoptosis as opposed to necrosis [177]. While these studies generally support the hypothesis that apoptosis acts as an inducible response to low-dose irradiation, it should be noted that cells of haemopoetic origin generally differ significantly in their apoptotic response from fibroblast cell lines, in that they have a much greater propensity for apoptosis. The challenge of quantitation of apoptosis due to low doses in fibroblast cell lines is therefore compounded, and special considerations must be given to experimental techniques.
Section 1 - Introduction

Figure 1.7: Apoptotic response of radioresistant lymphocytes in relation to overall cell death. Apoptosis levels peak at a dose of 500 rads (5Gy), in the region where total cell survival is > 90%. Apoptosis represents the prominent form of cell death at these high survival levels, but decreases as a percentage of the whole following doses > 5Gy. Reproduced from Payne et al. [177].

1.3 METHODS OF QUANTITATION OF APOPTOSIS

In their original work, Kerr et al. [105] defined apoptosis by the sequence of morphological changes observed during the process. Since then, despite the vast amount of information obtained regarding the apoptotic process at the subcellular and biochemical levels, the most reliable definition remains these distinct and characteristic morphological features.

The original method employed by Kerr for the detection of apoptosis employed electron microscopic techniques [105] and in subsequent papers, conventional light microscopy. The characteristic DNA condensation, cytoplasmic and nuclear shrinking and nuclear blebbing (forming membrane-enclosed apoptotic bodies) are all detectable
under normal brightfield microscopy, and quite often can be distinguished from the hallmarks of necrosis, namely cellular swelling, nuclear and cytoplasmic disorganisation and loss of cytoplasmic membrane integrity [178]. While adequate for analysis in cultured cells, electron microscopy (EM) is more suitable for analysis of in situ apoptosis as it allows the detection of more subtle features of apoptotic cells such as the appearance of intact cellular organelles such as mitochondria, endoplasmic reticulum and double-layered cytoplasmic membranes, in contrast to the general anarchy observed in necrotic cells, and the frequently compromised membranes (see Figure 1.5). As such, morphological assessment by EM is still regarded as the "gold-standard" of apoptosis detection. However, the vast time expenditure required for EM makes it unsuitable for studies where a large number of samples is required or for routine clinical application.

Wyllie [106] demonstrated the unique inter-nucleosomal cleavage caused by the action of endogenous endonucleases in the early stages of the apoptotic process. When the DNA of these cells is subjected to agarose gel electrophoresis, a characteristic "ladder" pattern appears due to the regular-length DNA fragments of 185 base pair multiples. Several protocols for this technique have been developed to demonstrate the existence of apoptosis, although it is qualitative in essence, as it requires a cell homogenate to be prepared in preparation for electrophoresis [179, 180]. Several investigators have noted that this digestion of the DNA results in a decreased fluorescence intensity of incorporated fluorochromes, as measured by flow cytometry [181, 182], as a result of progressive loss of DNA fragments from apoptotic cells [183]. Resulting DNA histograms exhibit a sub-G1 peak whose cells are regarded as apoptotic. However, apoptosis results in the complete fragmentation of the nucleus and cytoplasm,
and this debris can affect quantitative analysis by this method. Other investigators have utilised forward light scatter to differentiate between sub-G1 debris and apoptosis [184], however this method has been shown to prove effective in only a very few cases [185]. Another approach to this problem exploits a feature characteristic of apoptotic cells: the integrity of the plasma membrane remains intact until the very latter stages of apoptosis. This is in contrast to the early breakdown of membrane function observed in necrosis [186]. The ability of apoptotic cells to exclude propidium iodide (PI) provides discrimination between necrotic and apoptotic cells and staining with Hoechst 33342 provides DNA content information and detection of sub-G1 cells [187, 188]. While this method provides adequate quantitative results where apoptotic cells represent a significant fraction of the whole population, measurements where apoptosis is a relatively rare event are subject to the significant uncertainties in the application of floating thresholds over the continuous and overlapping populations of G1 and sub-G1 cells. This, in fact is a problem encountered with all flow cytometric methods, including those based on mitochondrial transmembrane potential [187], protein and RNA content [189], light scatter [190], and acridine orange assessment of DNA susceptibility to denaturation [191].

More recent methods which exploit the unique nature of the DNA end-fragments caused by the endogenous endonuclease in apoptosis have been developed [115, 192]. The first of these, the *in situ* end-labelling (ISEL) method is used to detect the peculiar recessed 3' ends of double-stranded-DNA fragments (as shown in Figure 1.8) formed during apoptosis [193]. The overhanging 5' ends are used as templates for the synthesis of new DNA strands, where biotinylated nucleotides are incorporated via DNA polymerase action. These biotinylated fragments may then be detected via avidin-biotin
immunohistochemical methods. A second method, known as the TUNEL assay, has also been developed which labels the 3'-OH termini of DNA fragments with biotinylated d-UTP by exogenous terminal deoxynucleotidyl transferase (TdT) [194]. Originally developed for use in tissue section analysis, these techniques have been modified for use with flow cytometry, and thus can be applied to apoptosis in cell suspensions [195]. Possibly due to differences in the specificity of the end fragments each of these detect (Figure 1.8), the two assays may produce dissimilar apoptotic indices measured over the same cell population [196]. The assumption that the end-fragments detected are unique only to apoptotic cells, and that the more stochastic degradation during necrosis causes only relatively few fragments of these types, is contradicted by reports that elevated staining levels can be observed in necrotic cells, calling into doubt the specificity of these assays [197, 198]. Furthermore, it now appears that DNA fragmentation is not an absolute requirement for apoptosis and that the morphological changes may appear in the absence of DNA cleavage [199, 200]. For these reasons, it has been recommended that assessment of DNA fragmentation by these methods or others (e.g. comet assay [201]), should only be used as a supplement to morphological assessment of apoptosis [202, 203].

Figure 1.8: DNA-end fragment patterns formed during apoptosis as a result of endonuclease action. (A) blunt ends; (B) 5' recessed ends; (C) 3' recessed ends. The ISEL method detects only C-type fragments, whereas the TUNEL assay may detect all three.

\[
\begin{align*}
5' \text{ PO}_4 & \quad \text{OH} \quad 3' \\
3' \text{ OH} & \quad \text{PO}_4 \quad 5' \\
5' \text{ PO}_4 & \quad \text{OH} \quad 3' \\
3' \text{ OH} & \quad \text{PO}_4 \quad 5' \\
5' \text{ PO}_4 & \quad \text{OH} \quad 3' \\
3' \text{ OH} & \quad \text{PO}_4 \quad 5'
\end{align*}
\]
Section 1 - Introduction

Therefore, despite the availability of numerous other methods, the most reliable method of apoptosis measurement remains based upon the distinctive morphology of the cells. EM assessment involves an inordinate time-expenditure and thus is unsuitable for studies of apoptosis induced by low doses of radiation, where a large number of samples are required. Ordinary light and fluorescence microscopy suffer from the same shortcoming, although to a lesser degree. They still, would require numerous investigators to classify the thousands of cells necessary for a study of apoptosis at very high survival levels, and the subjectivity introduced could mask any low dose effects.

A similar problem has been faced in cervical cytology. In British Columbia alone, there are over 700,000 annual cervical smears performed, each of which must be screened by cytologists searching for the sometimes rare anomalous-appearing nuclei characteristic of dysplasia or carcinoma. The variability between cytologists is well documented, and the human resource requirements are enormous. There are several systems now under development to automate the screening process, most of which rely upon pattern recognition techniques. A somewhat different approach to the problem has been taken by Palcic et al., who use mathematical descriptions of the nuclear chromatin distribution (texture features) to characterise each cell [204]. Cell classification is performed by the construction of discriminant functions based on these features from a teaching set of known diagnoses [205]. In some of these cells, they have been able to measure very small perturbations in chromatin organisation, invisible to the human eye, which may indicate the presence of malignancies in the vicinity of the cell (these are known as Malignancy Associated Changes, or "MACs" [206, 207]). To perform these measurements on a routine basis, an automated image cytometry device has been
constructed, the CytoSavant™, which automatically loads slides, identifies and focuses individual cells, and calculates texture features for each [205].

The use of texture features to characterise the very subtle chromatin changes defined as MACs, indicates that the comparatively gross morphological changes observed during apoptosis would be easily detected using similar methods. The use of discriminant analysis for classification would provide a consistent basis of apoptotic cell classification which could not be achieved by multiple human observers. Furthermore, the automated nature of the slide scanning process would allow a high throughput of samples to aid in overcoming the statistical problems associated with measurement of low dose effects.

1.4 EXPERIMENTAL OBJECTIVES

In order to test the hypothesis stated in Section 1.2.5, the following experimental objectives were set out:

I. To develop and characterise an automated method for image acquisition and classification of apoptotic cells based on a high resolution image cytometer

II. To characterise the apoptotic response of mammalian cells due to low doses of x-rays through measurement of the frequency of apoptotic cells.
Section 2 - Technical Developments

Section 2

Technical Developments

"Sir, as your mandate did request,
I send you here a faithfu' list
O' guids an' gear an' a' my gaith,
To which I'm clear to gie my aith"

- Robert Burns, "The Inventory"
2. TECHNICAL DEVELOPMENTS

2.1 CELL STAINING AND FIXATION PROTOCOLS

Development of a technique to allow characterisation of apoptotic cells based on image cytometric measurement of nuclear texture features necessitates a thorough consideration of sample preparation techniques. The most considerable influence upon nuclear texture within these procedures is the process of cell fixation. Chemicals used in the process of fixation can affect the cells in both a physical and a chemical way, by dissolving or precipitating certain cellular constituents, coagulating proteins, etc. Because of this, a number of studies have demonstrated a significant variation in fluorescent stain accessibility to DNA using different protocols for fixation [208, 209]. While the end-point chosen by other investigators has been the degree of stoichiometry of staining, this would be inappropriate in the current context where differences in nuclear texture between normal and apoptotic cells wish to be preserved.

Thus, an investigation into the effect of fixation on morphology and texture was undertaken, where the most suitable was a compromise between the following conditions, listed in order of importance:

1) optimal visual discrimination of normal versus apoptotic nuclei
2) degree of accessibility to the fluorescent dye
3) variability and degree of background staining

While it is acknowledged that a greater visual discriminatory power is a less quantitative means of comparing fixation effects than perhaps comparison of nuclear texture
differences, it should be noted that the learning set of images (visually classified) is the basis for all discriminant analysis and thus the accuracy of visual discrimination is of utmost importance. Therefore, a high degree of visual resolving power between the two groups will be reflected in a greater degree of accuracy in the classification function developed using the nuclear texture features.

Table 2.1 lists several fixatives which were studied and the protocols which were applied. Briefly, the fixatives studied may be subdivided according to their actions within cells. Alcohol-based fixatives (i.e. ethanol and methanol) and acetone cause a significant decrease in cell volume due to dehydration. The foremost properties of these fixatives is that they extract lipids and coagulate proteins, while having little direct effect upon nucleic acids thereby generally allowing greater stain access to DNA. However, the coagulative effect they have on proteins can cause a distortion of cellular structures. Non-coagulating fixatives include formaldehyde, paraformaldehyde and potassium

Table 2.1: Summary of fixation protocols. The composition, temperature and duration of fixation are listed for each fixative are listed together with the abbreviation used throughout the text. The formaldehyde solution used was not buffered and consisted of 37% formaldehyde in H$_2$O with 10-15% methanol added as a preservative.

<table>
<thead>
<tr>
<th>Fixative</th>
<th>Abbr</th>
<th>Composition</th>
<th>Time (min.)</th>
<th>Temp °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regaud's</td>
<td>REG</td>
<td>85% ddH$_2$O, 15% formaldehyde, 1M K$_2$Cr$_2$O$_7$</td>
<td>30</td>
<td>22</td>
</tr>
<tr>
<td>Boehm-Sprenger</td>
<td>BS</td>
<td>80% Methanol, 15% Formaldehyde, 5% Acetic Acid</td>
<td>30</td>
<td>22</td>
</tr>
<tr>
<td>Carnoy's</td>
<td>CAR</td>
<td>75% ethanol, 25% Acetic Acid</td>
<td>30</td>
<td>4</td>
</tr>
<tr>
<td>Methanol</td>
<td>MeOH</td>
<td>100% Methanol</td>
<td>30</td>
<td>4</td>
</tr>
<tr>
<td>Air Dried</td>
<td>AIR</td>
<td>N/A</td>
<td>N/A</td>
<td>22</td>
</tr>
<tr>
<td>Ethanol</td>
<td>EtOH</td>
<td>90% Ethanol, 10% ddH$_2$O</td>
<td>30</td>
<td>22</td>
</tr>
<tr>
<td>Acetone/Methanol</td>
<td>AM</td>
<td>50% Acetone, 50% Methanol</td>
<td>120</td>
<td>-20</td>
</tr>
<tr>
<td>Paraformaldehyde</td>
<td>Pf</td>
<td>0.5% Paraformaldehyde in PBS</td>
<td>30</td>
<td>22</td>
</tr>
<tr>
<td>Pf + 70% Methanol</td>
<td>PfM</td>
<td>0.5% Paraformaldehyde, post-fix in 70% Methanol</td>
<td>30,15</td>
<td>22,4</td>
</tr>
</tbody>
</table>
Section 2 - Technical Developments

dichromate, which actually cross-link structural proteins within the cell and therefore preserve cellular structure well, but because of this generally limit stain accessibility to DNA. Furthermore, a slight extraction of nucleic acids can result. Acetic acid represents an example of a third class of fixatives that actually precipitates nucleic acids, hence its use to preserve chromosomes. However, it produces a drastic increase in cell volume and therefore is generally used as an additive to oppose the cell shrinkage produced by alcohols.

As a first test, cells fixed as in Table 2.1 were assayed for DNA content, measured by image cytometry and subsequently analysed to determine cell cycle parameters. Human HCT-8 colorectal adenocarcinoma cells were grown in monolayer culture in RPMI 1640 medium, supplemented with 10% foetal calf serum, 100 units/ml PenStrep (Gibco, Burlington, Ont.) and 1 mg/ml sodium bicarbonate. Cells had an approximate doubling time of 26 hr and were harvested with 0.25% trypsin/EDTA prior to reaching confluence. These cells were chosen for cell cycle analysis due to their very stable karyotype, such that they would provide the opportunity to measure more accurately, differences in cell cycle parameters between the different fixatives. Cells were placed on microscope slides and allowed to attach for several hours prior to rinsing in two separate washes of phosphate buffered saline at 4°C. Fixatives were then applied according to the protocols listed in Table 2.1, removed from the fixative and allowed to air dry. Cells were permeablised by immersion in a 0.5% solution of Tween 20 (Sigma, St. Louis, MO) in PBS for 15 minutes at room temperature. Slides were incubated in a 0.2 mg/ml solution of RNAse A (Sigma, Type IV) in PBS for 1 hour at 37°C, followed by a single PBS rinse and stained overnight in 100 μg/ml propidium iodide at 4°C. Slides were then rinsed in three washes of PBS/Tween 20 for a total of 15 minutes. Coverslips
were mounted in glycergel (DAKO, Santa Barbara, CA) and the slides were stored at 4°C until use.

Each slide was scanned automatically using a modified CytoSavant™ automated image cytometry device (Oncometrics Imaging Corp., Vancouver, B.C.), operated in single-colour fluorescence mode. This system is described in detail in section 2.2.1. For each of ten slides per fixative, 1000 cell nuclei were sampled and the integrated optical intensity and area features calculated. From the resulting DNA histograms, cell cycle parameters were calculated using MultiCycle (Phoenix Flow Systems, San Diego, CA). Table 2.2 shows the summary of mean intensities and cell cycle parameters obtained for each fixative.

Table 2.2: Summary of cell cycle analysis. For each fixative the number of slides analysed (n) is listed together with the mean intensity of G1 cells (IOI_{G1}), the coefficient of variation of the G1 peak (CV_{G1}), and the percentage of the total cells in G1 phase averaged over the n slides. The average correlation coefficient (r) is given for correlations between area and IOI for cells within the G1 peak. The stoichiometric ratio (SI) is also listed.

<table>
<thead>
<tr>
<th>Fixative</th>
<th>n</th>
<th>IOI_{G1}</th>
<th>CV_{G1}</th>
<th>%G1</th>
<th>r</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>REG</td>
<td>9</td>
<td>67.3</td>
<td>8.4</td>
<td>53</td>
<td>0.334</td>
<td>2.04</td>
</tr>
<tr>
<td>BS</td>
<td>9</td>
<td>74.3</td>
<td>6.5</td>
<td>56</td>
<td>0.241</td>
<td>2.04</td>
</tr>
<tr>
<td>CAR</td>
<td>11</td>
<td>122</td>
<td>5.1</td>
<td>61</td>
<td>-0.143</td>
<td>2.09</td>
</tr>
<tr>
<td>MeOH</td>
<td>12</td>
<td>126</td>
<td>3.9</td>
<td>54</td>
<td>0.197</td>
<td>2.03</td>
</tr>
<tr>
<td>AIR</td>
<td>11</td>
<td>151</td>
<td>5.4</td>
<td>57</td>
<td>0.387</td>
<td>2.05</td>
</tr>
<tr>
<td>EtOH</td>
<td>10</td>
<td>157</td>
<td>3.4</td>
<td>53</td>
<td>0.327</td>
<td>2.02</td>
</tr>
<tr>
<td>AM</td>
<td>12</td>
<td>182</td>
<td>3.7</td>
<td>56</td>
<td>0.006</td>
<td>2.05</td>
</tr>
<tr>
<td>Pf</td>
<td>10</td>
<td>72.1</td>
<td>7.0</td>
<td>55</td>
<td>0.233</td>
<td>2.03</td>
</tr>
<tr>
<td>PFM</td>
<td>10</td>
<td>110.5</td>
<td>4.9</td>
<td>57</td>
<td>0.234</td>
<td>2.04</td>
</tr>
</tbody>
</table>

Table 2.2 shows that those fixatives whose primary mode of action is the cross-linking of proteins generally produce a higher CV of the G1 peak and result in lower overall staining intensity. This has been explained previously in terms of the
insolubilisation of nuclear proteins (e.g. nuclear matrix proteins, histones) and the immobilisation of DNA within the nuclear matrix, thus leading to a decreased permeability to the dye [210]. Those fixatives which act to solubilise nuclear proteins (CAR, AM, EtOH, MeOH) generally resulted in a greater staining intensity.

A second test was performed on exponentially growing Chinese hamster ovary cells irradiated with 2 Gy x-rays and grown in culture for 24 hours. These cells were fixed using six of the fixation protocols from Table 2.1. Regaud’s fixative was eliminated due to the poor stoichiometry of DNA staining and acetone/methanol was not pursued further because there was significant alterations in the HCT8 cells such that even mitotic cells were not visible. Slides were prepared as previously described using the remaining protocols, and were scored for the ability to visually distinguish apoptotic cells from other cells (+ → ++++, from most difficult to most obvious). Table 2.3 lists the results obtained for the remaining protocols. No apoptotic cells were noted following ethanol fixation, most likely a result of the severe shrinkage induced by alcohol dehydration. Similarly, BS fixation resulted in the apparent degradation of the apoptotic morphology, primarily as a result of cellular shrinkage. Of the protocols tested, Carnoy’s solution provided the best visual discrimination between apoptotic and normal cells, where the chromatin edges were sharply defined and clumps were well separated resulting in almost exaggerated apoptotic morphology. However, the large increase in size of the cells caused many normal cells to exceed the cell size limits introduced by buffer size limitations in Microsoft DOS operating system (available on the CytoSavant at that time). In fact, imaging of these cells using a 16x objective (compared to 20x) alleviated the problem somewhat, although a number of cells examined still exceeded the size threshold. Furthermore, significant variations in background intensity were found within individual frames (a
Section 2 - Technical Developments

problem which could not be rectified by further slide rinsing) which would result in unreliable segmentation of cytoplasmic boundaries. Fixation with paraformaldehyde followed by methanol post-fixation (PfM) resulted in a similar degree of visual discriminatory power to that of Carnoy fixation. A slightly smaller average area of normal cells with PfM fixation alleviated the problem of cell size when used with a 16x objective and variations in background staining intensity were small. For these reasons, PfM fixation was chosen as the most suitable fixation protocol for the remaining studies. Problems with clumping of cells during the paraformaldehyde fixation were overcome by pre-fixing the cells in 0.05% paraformaldehyde (10% of the subsequent concentration used) for 15 minutes on ice. Pre-fixation also resulted in the need for less agitation of the samples thereby producing slides which contained much less debris.

Table 2.3: Effects of fixatives on apoptotic morphology. Average normal cell area is provided (± standard deviation) with the score describing the ability to distinguish apoptotic cells (+ = poor, ++ = fair; +++ = excellent). A short description of apoptotic morphology using the given fixative is also provided. * indicates many cells exceeded size limits of acquisition.

<table>
<thead>
<tr>
<th>Fixative</th>
<th>Cell Area</th>
<th>Score</th>
<th>Description of apoptotic cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>BS</td>
<td>2000 ± 500</td>
<td>+</td>
<td>High degree of shrinkage, difficult to see chromatin clumps</td>
</tr>
<tr>
<td>CAR</td>
<td>3000 ± 800*</td>
<td>++++</td>
<td>Distinctive, chromatin clumps obvious &amp; very well separated</td>
</tr>
<tr>
<td>EtOH</td>
<td>1800 ± 400</td>
<td>N/A</td>
<td>No apoptotic cells visible</td>
</tr>
<tr>
<td>MeOH</td>
<td>2800 ± 700</td>
<td>+</td>
<td>Chromatin clumps more diffuse, not sharply defined</td>
</tr>
<tr>
<td>PF</td>
<td>2600 ± 700</td>
<td>++</td>
<td>Distinctive appearance, chromatin clumping obvious</td>
</tr>
<tr>
<td>PfM</td>
<td>2600 ± 700</td>
<td>++++</td>
<td>Distinctive, chromatin clumping obvious &amp; well separated</td>
</tr>
</tbody>
</table>
Figure 2.1: Sample images of cells fixed by PfM method. (A) Propidium iodide stained DNA; (B) FITC stained cytoplasm; (C) Combined DNA/cytoplasm image. PfM fixation resulted in excellent visual discriminatory power between normal and apoptotic cells.
2.2 AUTOMATED IMAGE CYTOMETRY SYSTEM

2.2.1 HARDWARE

The most significant requirement for a system capable of obtaining large sample sizes in a relatively short time period is that it function in an automated fashion. Working under the stipulation of microscopic evaluation then, an automated image cytometry system would be suitable. The Oncometrics CytoSavant is an image cytometry platform which, with several modifications as shown in Figure 2.2, was found appropriate for this application.

![Image of CytoSavant automated image cytometer](image)

Figure 2.2: Fluorescence-modified CytoSavant automated image cytometer. The CytoSavant used in this study is equipped with a 100 W stabilised mercury arc illumination system (1), a computer-controlled filter wheel (2), a triple-band dichroic mirror (3), a programmable motorised stage assembly (4) and a high resolution scientific CCD-based digital camera (5) interfaced to a PC.

The CytoSavant, centred around a Nikon Optiphot II research microscope, contains a unique digital camera (MicroImager 1400, Xillix Technologies Corp., Richmond, B.C.) [211] which is capable of acquisition in both fluorescence and absorbance configurations, producing images at the limit of theoretical and spatial
resolutions. The superior image quality is obtained through the use of a scientific charge-coupled device (CCD) mounted in the primary image plane of the microscope, containing over 1.4 million picture elements and with 100% fill-factor and high signal-to-noise ratio (100:1). The signal from the CCD is digitised using double-correlated sampling at the camera level to 12-bits (4096 grey levels) and is subsequently mapped to 8-bits for image processing and display purposes. Changes in the shape and offsets of this mapping function may be used to effectively control the camera gain. Intensity of signal may be controlled through the programmable shutter which allows programmed control of integration time. Illumination of the samples in fluorescence mode is provided by a stabilised 100 Watt Mercury arc lamp epi-illumination system (Nikon) designed for Koehler illumination, allowing bright and even illumination across the field of view. A schematic depiction of the epi-fluorescence illumination system is provided in Figure 2.3. In this present study, a tri-band dichroic mirror was used with cut-offs at 490 nm and 570, in combination with excitation filters of 530 ± 10 nm and 465 ± 10 nm (corresponding to propidium iodide and FITC excitation) mounted in the filter wheel assembly. A single emission (barrier) filter with bands corresponding to 630 ± 10 nm (for PI) and 530 ± 10 nm (for FITC) was used.

Motion of the microscope stage in three dimensions is provided by a Mertzhauler stage and controller which has typical repeatability of revisiting the same stage position of ± 5 μm. A computer-controlled filter wheel assembly (LUDL Electronics, Hawthorne, New York) was mounted in the illumination pathway such that up to six illumination frequencies could be selected. The filter wheel also contained a programmable shutter to minimise sample exposure duration.
Figure 2.3: Typical epi-illumination configuration for fluorescence microscopy. The above example shows filters specific for rhodamine excitation, which may also be used for propidium iodide measurements. Using the CytoSavant, excitation filters are contained within a programmable filter wheel assembly, and the dichroic mirror has a three-band emission; a single low-pass barrier filter is used for all stains studied.

The camera, stage and filter-wheel for the modified CytoSavant were interfaced with a 486-based computer equipped with a Matrox 1280 Image series processing board (Matrox, Montreal, Quebec). Since the Microlmager is a digital camera, the Matrox board was used solely for image processing applications (e.g. histogram functions, labelling, display, etc.) and to a certain extent, camera control. All programs were written in Microsoft C 6.0 operating under the MS DOS operating system.
2.2.2 *CytoSavant Software*

The standard CytoSavant is equipped with software which controls the system in roughly three stages: set-up and calibration, cell image acquisition and feature calculation. It was developed primarily for cells detected with absorbance stains, and for images to be acquired using brightfield microscopy. For the purposes of this work, the use of fluorescence staining would offer significant advantages to absorbance staining, and therefore several software modifications were necessary.

The fundamental difference between absorbance and fluorescence microscopy lies in the interpretation and manipulation of the pixel intensity output of the camera. Output of the camera is mapped at the pixel level to an 8-bit value (from 0-255 units) by the imaging board. For absorbance purposes, since light is absorbed by the sample, the amount of stain present within the pixel area, termed the “optical density”, is related through Beer’s law to the camera output. Thus, all pixel intensities are converted to optical densities by:

\[
OD_{i,j} = \left( \log_{10} I_{i,j} - \log_{10} I_B \right) = \log_{10} \left( \frac{I_B}{I_{i,j}} \right)
\]

where \( I_B \) is the intensity of the local background and \( I_{i,j} \) is the intensity of the \( i,j \)th pixel. Fluorochromes on the other hand, radiate the light in equal magnitudes in all directions and in direct proportion to the illumination intensity. This intensity is affected little by the presence or absence of stained material, and therefore the amount of light detected by the camera is directly proportional to the amount of fluorochrome in the sample. Therefore, measurements in fluorescence are more appropriately referred to as “optical intensities”.

46
The original versions of the CytoSavant "Acquire" software used a single predefined mapping function (LUT) to convert camera output levels to optical density. This mapping function can be changed to fluorescence mode (i.e. optical intensity) by changing a single parameter in the set-up file, which converts all feature measurements to optical intensity measurements rather than optical density. However, within the data acquisition and calibration components of the software, there were a number of functions for display, thresholding, and focus, which were capable of absorbance mode calculations only. Description of each individual program modification required to achieve this would be voluminous. Briefly, the majority of these changes were accomplished by, in each case, including a decision point based on the fluorescence mode parameter in the set-up file, to alter mapping of the camera output to an inverse linear function.

Once the program was adapted to handle fluorescence signals capably, the remaining task was to alter the flow of the program to process the two-colour fluorescence signals. Again, description of the individual modifications required would be lengthy and therefore will not be included here. Within the following description of the final, modified program (hereafter referred to as AcquireDL, "DL for double-labelled") those places where significant alterations were necessary will be readily apparent.

### 2.2.3 AcquireDL Software and Procedures

Figure 2.4 graphically illustrates the flow of the AcquireDL program, corresponding to the detailed description that follows.

**Initialisation:** At the start of the AcquireDL program, three parameter files are loaded which control the acquisition features of the program. The first file contains variables such as those related to camera settings (e.g. LUT mapping variables, integration time,
Section 2 - Technical Developments

etc.), thresholding and masking variables, and filter wheel positions. The second contains all stage-related variables, including slide stage co-ordinates, and the scannable area. A third file contains several variables which limit the number, size and other features of cell images. The majority of these parameters were disabled in this version to ensure all cell images were saved.

All accessory hardware is then initialised: the frame grabber and camera initialised and set to continuous acquisition mode with the given integration time (IT) and LUT values; the stage calibrated to its (0,0) co-ordinates and returned to the scan start position; and the filter wheel sent to the “home” position and its shutter opened. If present, a calibration image is loaded from disk for subsequent use during acquisition.

**Calibration Image Acquisition:** For quantitative measurement of

---

Figure 2.4: Flow chart describing AcquireDL software procedures. A complete description is contained within the text.
fluorescence-labelled cells, it is imperative that images are corrected for non-uniformities of the arc-lamp illumination over the microscope field, since spatial variations in excitation intensity translate directly into spatial variations in emitted fluorescence levels.

Before the first slide of each session was sampled, and following every 6 continuous hours of operation, a calibration image was acquired. The calibration image is a single image of a uniformly fluorescing field acquired under the exact lighting conditions as sample acquisition. This image may be used as a template which describes the spatial variations in light intensity across the field and therefore, further images acquired may be compared against it to adjust fluorescence intensities accordingly. Practically, this is achieved by division of a microscope field image by the calibration image (on a pixel-by-pixel basis) followed by multiplication of this ratio image by the mode of the calibration image pixel intensity histogram (as a scaling factor). In AcquireDL, each cell image was calibrated in this manner before feature calculations proceeded.

A uniformly-fluorescing sample was prepared by placing a drop of saturated rhodamine-110-perchlorate (Exciton Inc., Dayton OH) solution on a microscope slide, applying a coverslip and allowing it to spread to a thin film. With the slide mounted on the stage, the edge of the microscope field diaphragm (which is in a conjugate focal plane to the sample in Koehler illumination) was brought into focus. To compensate for any variations in the film thickness, eight separate fields were sampled from the slide, and averaged to create a single calibration image. Each of these eight images was the average of 16 consecutive images to reduce effects of camera noise and illumination fluctuations. The final calibration image was stored on disk and in one frame buffer for
subsequent image calibration procedures, and is automatically loaded into memory at the beginning of subsequent program sessions.

**Automated Image Acquisition:** Automated acquisition entails scanning the slides for usable frames in either a diagonal search pattern across the slide or in a raster pattern as indicated by settings within the parameter file. At the beginning of each focus search, the filter wheel is returned to the cytoplasm filter position and the shutter opened. A frame is considered usable if any signal (i.e. a cell) above the background mode of the pixel intensity histogram of the image is detected. A rough-focus image of the entire field (in ± 3 μm stage steps) is first obtained by maximisation of the integrated intensity of the frame image. At this focal plane, a threshold segmentation is performed, and all objects whose area lies within preset levels (typically 20-10000 pixels) are labelled as cells. This threshold level was preset to a value of 9 grey levels above the calculated background mode (determined empirically) and remained constant thereafter.

Because not all nuclei lay on a single focal plane, even in monolayer samples, a further fine-focus step is performed. The system captures up to 10 successive images above and below the original rough focus plane at 0.5 μm intervals. Since the variance in the object pixel intensity histogram has been found to have a maximum at the plane of best focus, for each object, the image with the greatest value of this variable was taken as the image of best focus and saved in memory for further processing. Those objects which did not achieve best focus (i.e. the variance did not reach a maximum or the gradient of the focus curve was too shallow) were discarded, and all remaining objects were renumbered accordingly. The effect of the fine-focus process is to essentially place all objects to be considered further onto a virtual focal plane.
Section 2 - Technical Developments

Typically in the CytoSavant acquisition process, the images and thresholded masks are then reprocessed to more accurately identify nuclear boundaries by an edge relocation algorithm which creates a single pixel chain around the object at the highest local gradient values [212]. However, this algorithm was developed and tested on nuclear images. For cytoplasmic images, this algorithm often placed the mask boundary well within the visible edges of the cell. For this reason, the edge relocation was disabled, and a second threshold was applied to the best focus image, at a preset level above the local background intensity (defined as the mode intensity of all non-object pixels within the immediate vicinity of the cell). The new threshold image was then processed to remove any holes from the binary mask by a series of painting procedures, and the cellular masks and co-ordinates were stored in a frame buffer for quick recall.

With the microscope stage locked in place, the filter position is changed to the DNA filter setting and the camera presets for DNA image acquisition are loaded and the camera re-initialised to these settings. The focus plane is then adjusted to 1μm below the rough focus plane determined for the cytoplasm (the mean focus plane of nuclei was determined to be typically below that of the cytoplasm due different wavelengths of the emitted light) and a single image is acquired of the DNA stain. The entire field is then thresholded using another preset value greater than the field mode background intensity. Those objects which overlap the overlaid boundaries of the cytoplasm masks are assigned a numeric label (corresponding to their cytoplasm mask), all others are discarded. The remaining DNA masks are then processed to remove any holes. A second, nuclear fine focus step is performed in the same fashion as the cytoplasm fine focus. The image with the highest variance in intensity of pixels falling within the nuclear mask is then taken as the image of best focus. These images are stored in memory.
Section 2 - Technical Developments

together with their cytoplasm mask, all others, those not reaching best focus and all the nuclear masks are discarded.

All images are then calibrated through pixel-by-pixel division by the saved calibration image. Pixel intensities are subsequently corrected for each image by subtracting the mode of a smoothed histogram of non-object pixels (from the DNA image) around the cytoplasm mask boundaries. Feature calculation then proceeds before a new frame search begins.

**Feature Calculation:** Once the image/mask pairs have been acquired, a variety of features of the chromatin distribution are calculated, 104 in total. While a complete treatment of all the calculated features can be found elsewhere (see Doudkine et al. [204]), a description of the seven basic classes of feature types used by the CytoSavant, together with an example for each is provided in the following section.

### 2.3 TEXTURE FEATURES

I. **Morphological features:** Such features are used as a numerical description of the object's shape, and thus in the current context are calculated entirely from cytoplasm information, *i.e.* the mask. Examples include the total number of object pixels ("area") and the "sphericity", a measure of the object's roundness, calculated as:

\[
\text{sphericity} = \frac{\text{min}_\text{radius}}{\text{max}_\text{radius}}
\]

The minimum radius is the radius of the largest circle which may be fully inscribed inside the object perimeter, and the maximum radius is the radius of the smallest circle which fully encompasses the object. Such features are particularly useful in discrimination between doublet and single cells.
II. Photometric features: These features are descriptive statistics of the pixel intensity histogram of an object. Thus, the summation of all pixel intensities gives a measure of the amount of DNA present within the cell (the integrated optical intensity or "IOI"). Other statistics, such as maximum intensity, and other moments of the distribution are also calculated. These features are particularly useful for discrimination between those cells which have lost DNA stainability, such as frequently occurs in apoptotic or necrotic cells due to degradation of the DNA.

III. Discrete Texture Features: These features are based upon the segmentation of the object into regions of low, medium and high intensity. In the current case, these levels were equally distributed across the range of intensity values for each object (i.e. scaled to 0-33%, 34-66% and 67-100% of the range of pixel intensities over the first 50 cell images captured per slide). A simple example of the discrete texture features is percentage of the total object area which is classified as low, medium or high intensity. These features could be expected to provide very different values for apoptotic versus normal cells due to differences in chromatin compactness.

IV. Local Extreme Features: These features calculated by first, smoothing the image by application of a 3x3 unit matrix and subsequently identifying all local maxima and minima within the filtered image. These maxima and minima are then used to calculate a variety of features describing, for instance, the average number of minima or maxima per unit area or the difference in intensities between the absolute maximum and minimum. These features produce discriminating power between images with finely versus coarsely clumped DNA.

V. Fractal texture features: These represent measurements performed on the three dimensional surface created by the optical density function. This function can be viewed
as a three-dimensional bar graph where the spatial x and y co-ordinates are plotted in the horizontal plane and optical density is associated with the vertical axis. For instance, the “fractal area” of the three dimensional intensity surface can be calculated by:

\[
fractal\_area = \sum_{i,j} \left( |I_{i,j} - I_{i,j-1}| + |I_{i,j} - I_{i-1,j}| + 1 \right)
\]

where \(I_{i,j}\) is the intensity of the \(i,j\)th pixel. These features aid in discrimination between images with large areas of similar intensities versus those with frequently varying intensities.

**VI. Markovian texture features:** Markovian features attempt to characterise grey level variation between adjacent pixels in an image. Practically, this is accomplished by compression of the range of image intensities into 40 grey levels, followed by computation of the sum and difference histograms over the entire image. As an example, the contrast of the image is based upon estimation of intensity differences between pixels and therefore is calculated as:

\[
contrast = \sum_{m} m^2 P_d(m)
\]

where \(P_d(m)\) is the probability that two adjacent pixels have intensities which differ by \(m\).

**VII. Run-length features:** Run length features describe chromatin distribution in terms of the length of lines which may be drawn through pixels with the same grey level. These “run lengths” are computed in each of four directions, 0°, 45°, 90° and 135°, as in the example of short_run_emphasis:

\[
short\_run\_emphasis(\theta) = \frac{\sum R_{p,q}(\theta)}{\sum R_{p,q}(\theta)}
\]
and the value $R_{p,q}(\theta)$ is calculated as the number of times the image has a run length of $p$ for an intensity level $q$. This feature produces large values for images where short runs dominate, that is, where the chromatin organisation is disorganised.

Following feature calculation, all image/mask pairs and features are stored to disk, and summary statistics of the images acquired to that point are prepared and displayed. Following acquisition of a preset number of cells, a file containing summary information is created which lists the number of cells acquired, total scanning time, and run parameters. Application of discriminant functions to the images takes place using a stand-alone program.

2.4 AUTOMATED CLASSIFICATION

2.4.1 DISCRIMINANT FUNCTION ANALYSIS

The cornerstone of the automated recognition of apoptotic cells from their normal counterparts, and both of these from any cellular debris or artefacts, is the discriminant functions. These are constructed based upon a "learning set" of images which have previously been manually classified according to their representative type. Thus, by providing a veritable template based upon the average apoptotic cell's texture features, it is possible to develop a mathematical function using any other cell's features which will return a value indicative of it's classification. In considering the optimal characteristics for the learning set, it may therefore be instructive to provide a brief background on linear discriminant function analysis.

As a simple case, imagine we have two variables, Feature 1 ($X_1$) and Feature 2 ($X_2$), measured over two classification groups, class I and class II. Figure 2.5 shows a
plot of the observations. For this case of two variables, simple inspection would allow one to draw a line which best separates the two classes. This line is termed the "decision boundary" and is based upon the original data of known classification (or "learning set"). Any further observations may then be plotted against this line and depending on their position relative to this line could be classified accordingly. However, the question arises of how to determine the best placement of the line to allow most accurate classification of the original learning set and of any further observations.

Figure 2.5: Simple discriminant analysis: discrimination of two classes based on the values of two features. The decision boundary is drawn as the bisector of the line connecting the feature means (discriminant axis) for class I (closed circles) and class II (open circles).

For our two parameter case this problem is trivial. One may simply calculate the bivariate means for each category and connect the two by a straight line. The decision
boundary may then be chosen as the bisector at a point on this line which minimises the probability of misclassification and therefore is based upon the difference between class means (as in Figure 2.5). However, this technique will function properly only in cases where the variances in the measured parameters are similar for both classes and where no correlations exist between the parameters. Alternately, one uses a normalisation factor based on the covariance matrix which, in effect, will rotate the decision boundary of the linear discriminant function [213].

If one views the decision boundary as a line drawn in Cartesian co-ordinates, then the two parameters defining this line are the slope and the intercept, here referred to as the threshold. The threshold can be calculated as the midpoint of the line between the two means may be defined by:

\[ X_{II} = \frac{\bar{X}_1 - \bar{X}_2}{2}, \quad i = 1, 2, \]

where \( \bar{X}_1 \) and \( \bar{X}_2 \) are the parameter \( i \) means for class I and II respectively. In the case of unequal variances, a weighting factor is then assessed for each variable which in essence shifts the intersection point of the decision boundary with the line drawn between the two means:

\[ w_i = \frac{2(\bar{X}_1 - \bar{X}_2)}{s_1^2 + s_2^2}, \quad i = 1, 2, \]

where \( s_1^2 \) and \( s_2^2 \) are the variances of class I and II, and the denominator is the "pooled" variance. Note that as the variance of one variable increases, its corresponding
weight is decreased, producing the effect that smaller weight is placed on those variables which have a greater overlap of values. The threshold is then calculated as:

\[ T = w_1 X_{1T} + w_2 X_{2T} \]

Similarly, the slope of this line may be calculated by first calculating the difference between mean values, \( a_i \), and calculating similarly variance normalised weight factors:

\[ w_i = \frac{2a_i}{s_i^2 + s_j^2} \]

\( i = 1, 2 \)

The slope of the decision boundary is then simply \(-w_1/w_2\). The rules for classification are as follows: for any observation with values \( X_1 \) and \( X_2 \),

\[ w_1 X_1 + w_2 X_2 \leq T \Rightarrow \text{Class I} \]

\[ w_1 X_1 + w_2 X_2 > T \Rightarrow \text{Class II} \]

While the construction of discriminant functions for the case of two variables is relatively simple, the current application utilises up to 104 nuclear texture features as its bases. Since the problem becomes quite computationally large with a feature space this large, it is important to include only those features which provide discriminating power between classes, eliminating others from the analysis. Practically this is achieved numerically by a technique known as "step-wise discriminant analysis". The fundamental principle of this technique is the acceptance or elimination of variables from the discriminant analysis based upon the F-statistic, which is essentially the ratio of the between-groups variance in the data over the pooled (or average) within-group variance. For a given feature then, if the between-group variance is significantly larger then it is likely that there is a significant difference in between-group means. For many variables, the procedure is similar except that the F-statistic is calculated from the matrix of total
Section 2 - Technical Developments

variances and co-variances. By pre-defining limits on F-values, one may effectively control the number of features which are included in the discriminant function analysis.

For some cases, the classification of more than two groups may be necessary. In such instances, one calculates several discriminant functions which are then applied in turn. For example, if three groups are present, one may first apply discriminant functions to separate a single group from the remaining two, and subsequently apply a second function to separate the remaining groups. The choice of which group to isolate first is largely dependent upon the group characteristics and will be specific to the problem under consideration.

2.4.2 Discriminant Analysis for Apoptotic Cells

As should be apparent from the preceding discussion, it is critical that a representative learning set be used for construction of the discriminant functions, as the ability to discriminate any further samples will be dependent upon these data. Two critical issues which must be addressed are the size of the learning set as well as the method of binary tree construction which is dependent upon the number of classes. Binary discriminant analysis as opposed to four-group analysis was performed in order to insure compatibility with CytoSavant analysis software.

Learning Set Characteristics: The size of the learning set is of special importance particularly when the cells of interest are infrequent, which is the anticipated scenario with apoptosis due to low doses of radiation. It is possible to develop statistical methods (see Section 4.1.2 for further discussion), based on the means and variances of features used in the discriminant functions, which would allow estimation of the sample sizes necessary for satisfactory discrimination (i.e. the sample size beyond which no further
Section 2 - Technical Developments

discriminatory power is gained). As this was the first evaluation of these protocols however, it was unclear which of the 104 features would be most useful. Furthermore, it was necessary to test a number of binary trees and classification schemes. Therefore, a large learning set was classified prior to discriminant analysis, and sample size was studied empirically.

It should be noted that a large ratio of normal cells versus apoptotic cells, as expected in this case, yields different values of the constant threshold term in the discriminant functions than for the case where the two are present in equal amounts. Therefore, in development of the learning set, it was important to have a normal cell to apoptotic cell ratio which was of the same order of magnitude as one would expect from the data to be automatically classified later. Thus, exponentially growing Chinese hamster ovary cells were X-irradiated in spinner culture with "low" doses ranging from 0 to 5.0 Gy, the dose range of primary interest. Following 24 hours at 37°C, the cells were harvested and fixed onto slides by the paraformaldehyde/methanol method described in Section 2.1. A detailed description of all cell culture and irradiation protocols may be found in Section 3.1. Using the automated scanning procedures outlined in Section 2.2.3, a series of 80 slides were scanned with approximately 1000 cells sampled per slide, the entire set of texture features calculated for each image. Images from a random sample of 60 of these slides comprised the full learning set, and the remaining 20 were excluded from the construction of the discriminant trees for testing the functions on a unique data set.

Classification Scheme: Both the learning set and test set of images were manually classified prior to construction of the discriminant functions. For each slide, the images
Section 2 - Technical Developments

and their corresponding masks were examined and obvious normal or double cells were classified into two separate groups. Using CytoSavant "Revisit" software, which sends the stage to the co-ordinates of any specified image, the cells corresponding to all remaining images were examined microscopically using a 40x objective and both FITC and rhodamine filter sets. Initially, all cells were classified as either normal, apoptotic or other, according to the rules described in Table 2.4. The classification criteria for apoptotic cells is patterned after the typically accepted conditions contained in publications on apoptosis [178]. A further condition for classification as apoptotic or normal was also adopted.

Table 2.4: Classification criteria for learning and test sets. The abbreviations for each used in further discussion are given along with criteria for entry into each group. Each characteristic followed by the logical "and" is a strict rule for classification, those with logical "or" as a possible characteristic. The total number of cells of each class in both the learning and test sets are included.

<table>
<thead>
<tr>
<th>Class</th>
<th>Abbr</th>
<th>Characteristics</th>
<th>Learning Set</th>
<th>Test Set</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>N</td>
<td>Normal interphase or metaphase appearance and at least 15% diploid DNA content and appreciable cytoplasm</td>
<td>57567</td>
<td>18741</td>
</tr>
<tr>
<td>Apoptotic</td>
<td>A</td>
<td>Nuclear condensation and decreased nuclear size and nuclear blebbing and at least 15% diploid DNA content and appreciable cytoplasm</td>
<td>455</td>
<td>153</td>
</tr>
<tr>
<td>Doubles</td>
<td>D</td>
<td>Double nuclei with overlapping/touching cytoplasm</td>
<td>2295</td>
<td>1052</td>
</tr>
<tr>
<td>Debris (Junk)</td>
<td>J</td>
<td>Less than 15% diploid DNA content or imaging artefacts or fixation/preparation artefacts or little cytoplasm</td>
<td>246</td>
<td>45</td>
</tr>
</tbody>
</table>

As discussed in the introduction (Section 1.2.2), well after the process of apoptosis begins, the cell may fragment into several pieces, each of which contains nuclear DNA surrounded by an intact cytoplasmic membrane. Thus, a single apoptotic
cell could give rise to several apoptotic bodies, which if counted could cause a considerable increase in the observed apoptotic fraction. While it is impossible to associate each apoptotic body with its original cell, some precautionary measure was warranted. To reduce the impact of apoptotic body formation on apoptotic frequency measurements, only those cells with a DNA content greater than 15% of the normal diploid amount were classified as apoptotic. The remainder were classified as debris. A similar rule was applied to the normal cell population as there was a tendency for several normal-appearing cells per slide to have a very low DNA content, which made their classification rather vague. It should be acknowledged however, that the 15% figure was arrived at in an arbitrary fashion. Increasing or lowering this figure would effectively act to increase or decrease the apoptotic frequency measurements, assuming that the number of fragments produced per cell has no dose dependence. As there is no evidence indicating that the mechanism of apoptosis differs in a dose dependent manner, this should be a valid assumption. Furthermore, the problem of apoptotic fragments is one which is not unique to this particular assay. In fact, all methods of measurement of apoptosis, including various flow cytometric methods and the TUNEL assay performed upon any tissue disaggregates or in vitro samples suffer this problem. While it may be envisioned that cells grown, fixed and stained directly upon the slides may allow one to associate apoptotic bodies with their parent cells, it is widely acknowledged that apoptotic cells frequently lose adherence to the slides and become “floaters” [214].

After an initial review, it was determined that the “other” category contained cells of two distinct subcategories, which differed significantly in their visible features. Therefore, this category was further subdivided into “doublets” and “debris”, which
Section 2 - Technical Developments

represent more homogeneous groups of cells, and therefore would be more easily
discriminated from the other categories. Table 2.4 lists all final categories for
classifications, characteristics necessary for their inclusion into each group, and the
number of images of each class present in the learning and test sets. It should be noted
that classification of necrotic cells proved difficult, and as a result no attempts were
made to develop a classification scheme for cells of this nature. Some cells were noted
which displayed a high degree of nuclear disintegration which was not apoptotic in
morphology and were possibly necrotic, but were classified as normal. Those cells which
displayed a loss of cytoplasm or disintegration of the cytoplasm were categorised as
debris regardless of other characteristics.

Binary Discriminant Trees: For a set of four classifications, there are a number of
possible binary discriminant trees which could be used. In determining which should be
tested, it is helpful to first analyse the characteristics of each group to decide which
strategies offer the best chance for good discriminatory power. Figure 2.6 illustrates the
four strategies which were tested, at least to the first level of the tree.

Tree 1 is based upon the characteristic similarities between normals and
doublets, compared to the apoptotic and debris, and thus the first decision point (DSF1-
1) functions to separate the two subcategories ND and AJ. Because apoptotic cells are
typically smaller than normal, and because the majority of the cells classified as debris
consisted of those with < 15% diploid DNA content, their average area was somewhat
smaller than either normal or double cells. Grouped together, the AJ group had a mean
area of 1400±600 (standard dev.) compared to the ND average area of 2300±600. This
difference was believed to provide a solid basis for choice of a first step in the binary
tree. A second set of decision points are then necessary to separate N and D (DSF1-2a), and A and J (DSF1-2b). As normal cells are typically more rounded, while double cells more elongated, shape features were believed to offer good discriminatory power between these cells. The separation of A and J cells could then be achieved based upon differences in the DNA content (due to the 15% DNA content limitations), but since the J cells are a very heterogeneous group, the outcome of DSF1-2b was unpredictable. Further discussion of the results of tree 1 is found below.

Figure 2.6: Schematic depiction of binary discriminant trees tested.
Section 2 - Technical Developments

Tree 2 was designed to exploit visible differences in contrast between apoptotic cells and all other classes. It was noted upon visual inspection that large areas of bright, condensed chromatin present in apoptotic cells was rarely present in the other classes and therefore might be useful to isolate all apoptotic cells in the first step of the tree. For instance, the mean value of the cluster_shade feature (a Markovian feature which produces a large value for bright areas on a dark background) for apoptotic cells was 1.3±0.5 while it was 0.35±.29 for all other cells. Furthermore, differences in size and/or shape of the cells might allow discrimination of normal cells from the remaining groups in the second step.

Since the normal cell population represented a visually homogeneous group, tree 3 was tested, where normals were first separated from all other groups. It was hoped that with this design, an accurate estimate of the total normal population, the largest of the groups, could be removed such that a lower misclassification rate would be present in the apoptotic category at the second level.

Tree 4 was tested with a slightly different motive: removal of the cells not used in the calculation of apoptotic frequency (J and D) would result in a more consistent measure of the number of normals versus apoptotics in the second level. Since the number of double cells and debris is largely dependent upon slide preparation factors, it was hoped that this tree would aid in removal of this effect by preventing a large amount of misclassification in DSF4-2.

Discriminant Analysis: The discriminant functions for the four binary trees listed in Figure 2.6 and described above were constructed using BMDP (BMDP Statistical Software Inc., Los Angeles, CA). BMDP uses forward step-wise discriminant analysis as
described in this section. For each analysis, F-values to enter and remove features from consideration were determined empirically by first starting with very low values, which results in a large number of features included in the classification functions. Stepwise increases in the F-values were then tested until discriminatory power decreased by a significant amount.

For each of the four trees, the ability to discriminate at the first branch of the tree was tested. As expected, each performed with varying degrees of success. The first step of tree 2, DSF2-1 was particularly unsuccessful. While 99.5% of the 60,108 non-apoptotic cells were classified correctly, only 71.6% of the 455 apoptotic cells were in fact classified as apoptotic. Features included in the calculation beyond the 16 used in the preceding analysis caused no further increase in classification accuracy. The majority of those apoptotic cells classified incorrectly tended to be slightly larger than average and of lower contrast. Furthermore, more than a quarter of those cells classified as apoptotic belonged to one of the other categories. Because the correct classification of apoptotic cells is the highest priority, no further analysis of binary tree 2 was conducted.

The first stage of tree 3, DSF3-1, attempted to isolate the much larger normal cell population from the other three classes. The overall classification accuracy for normal cells was 99.8%, while for the remaining population, 87.8% were correctly classified into the "other" category. No further increase in classification accuracy was obtained by addition of more than 25 features into the classification function. At first appearances, this is an acceptable result. However, closer inspection of the classification results indicated that a significant proportion (59.5%) of the apoptotic cells were classified
incorrectly as "normal" (see Table 2.5). The high classification accuracy of "others" was therefore due to the correct classification of "doublets", which comprised the largest subpopulation of the "others" category. The overwhelming majority of this type of cell therefore dominated the discriminant analysis and as a result, the classification of apoptotic and debris groups actually performed worse than completely random classification (which would yield 50% accuracy). The features included in the classification functions were primarily shape-related features, reflecting the elongated nature of the cell doublets versus the roundness of the normal population. These results indicated however, that a high degree of classification accuracy might be obtained in separating normals and doublets only. Tree 3 was pursued no further.

Table 2.5: Classification accuracy of discriminant function DSF3-1, to separate normal cells from the entire learning set population. Actual number of cells in each group (classified visually) are listed together the number of each class which were automatically classified into that group. The total number of cells classified (automatically) into each group is provided in the last column. Shaded areas represent correct classification.

<table>
<thead>
<tr>
<th></th>
<th>True #</th>
<th>Manual Classification</th>
<th>Automated Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>57567</td>
<td>57443 271 127 81</td>
<td>57922</td>
</tr>
<tr>
<td>Other</td>
<td>2996</td>
<td>124 184 119 2214</td>
<td>2641</td>
</tr>
</tbody>
</table>

Application of the first step in tree 4 to the learning set produced overall, the highest classification accuracy using 15 texture features. Normal and apoptotic cells taken together were classified with 99.8% correct, while the debris and doublets were classified 89.0% correctly. Further description of the actual classes comprising the groups is provided in Table 2.6. While in DSF2-1 it was noted that the presence of a much larger subgroup (here, Normal) might dominate the discriminant function and result in the misclassification of a smaller subgroup, this was not the case for separation of
Normal and Apoptotic groups. In fact, the classification accuracy of these two groups were 99.8% and 96.4% respectively. However, there was significant misclassification (75%) of debris as "NA".

Table 2.6: Classification accuracy of discriminant function DSF4-1, to separate normal cells from the entire learning set population. Actual number of cells in each group (classified visually; NA = normal or apoptotic; JD = debris (junk) or doublets) are listed together the number of each class which were automatically classified into that group. The total number of cells automatically classified into each group is provided in the last column. Shaded areas represent correct classification.

<table>
<thead>
<tr>
<th>True #</th>
<th>Manual Classification</th>
<th>Automated</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>A</td>
<td>J</td>
</tr>
<tr>
<td>N/A</td>
<td>58022</td>
<td>57479</td>
<td>439</td>
</tr>
<tr>
<td>J/D</td>
<td>2541</td>
<td>88</td>
<td>16</td>
</tr>
</tbody>
</table>

The results of DSF1-1 yielded 99.5% correct classification of normals and doublets grouped together and 81% correct classification of apoptotic cells and debris. A full 99.7% of normal cells, 85% of apoptotic cells and 93.5% of double cells were correctly classified. The debris had a correct classification rate of 73.1%. Again, due to the size of the populations, there was a comparably large number of normal and double cells classified as AJ (as shown in Table 2.7). This might be expected to have deleterious effects on the classification functions at the second level of the tree. However, analysis of the second level of tree 1 proceeded as described below.

Table 2.7 Classification accuracy of discriminant function DSF1-1, to separate normal cells from the entire learning set population. Actual number of cells in each group (classified visually; ND= normal or double; AJ = apoptotic or debris) are listed together the number of each class which were automatically classified into that group. The total number of cells automatically classified into each group is provided in the last column. Shaded areas represent correct classification.

<table>
<thead>
<tr>
<th>True #</th>
<th>Actual Classification</th>
<th>Automated</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>A</td>
<td>J</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ND</td>
<td>59862</td>
<td>57407</td>
<td>67</td>
</tr>
<tr>
<td>AJ</td>
<td>701</td>
<td>160</td>
<td>388</td>
</tr>
</tbody>
</table>
Table 2.8 lists the texture features which were used in the classification functions for DSF 1-1, in order of their level of significance. As might be expected, the majority of the features used were Markovian features, such as the cluster shade, correlation and contrast. The utility of these features represents the differences between the brighter, more condensed nuclei of the apoptotic cells as compared to either normal cells or doublets. For example, the most significant feature, the cluster shade returns a large value for images which have few uniform intensity clumps in high contrast to the rest of the image, as is observed in apoptotic cells, but generally absent from normal cells or doublets.

Table 2.8: Features used in classification functions for DSF1-1. Feature means for each group in the learning set are listed with their standard deviations and the standardised canonical variable (weighted by within group variances as described in section 2.4.1) are listed.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Group Means</th>
<th>Standard Canonical Var.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. cluster_shade</td>
<td>0.35 ± 0.28</td>
<td>1.3 ± 0.7</td>
</tr>
<tr>
<td>2. contrast</td>
<td>5.7 ± 1.6</td>
<td>8.7 ± 6.5</td>
</tr>
<tr>
<td>3. intensity_var</td>
<td>230 ± 190</td>
<td>800 ± 900</td>
</tr>
<tr>
<td>4. correlation</td>
<td>190 ± 40</td>
<td>160 ± 50</td>
</tr>
<tr>
<td>5. radius_fft_low</td>
<td>8.9 ± 22.9</td>
<td>65 ± 110</td>
</tr>
<tr>
<td>6. radius_var</td>
<td>30 ± 18</td>
<td>25 ± 16</td>
</tr>
<tr>
<td>7. fft_harmonic_2</td>
<td>3.9 ± 5.4</td>
<td>9.4 ± 8.8</td>
</tr>
<tr>
<td>CONSTANT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The remaining steps were then taken by developing the discriminant functions based upon the data sets of classified cells from the first level classification functions. That is, those cells which were misclassified in the first stage of the tree were included in the analysis, as opposed to developing the classification functions based on the manually classified cells themselves. This step was taken for two specific reasons: 1) a more precise analysis of the overall classification accuracy could be performed; and 2)
the second level discriminant functions would be based on a more realistic data set. For
instance, in tree 1, the second steps are: DSF1-2a, to separate normals from doublets;
and DSF1-2b, to separate apoptotics from debris. Because only the normal and
apoptotic cells are of interest for the apoptotic frequency, our end-point, this effectively
offers an "escape route" by which those misclassified stragglers might be grouped into
one of the inconsequential classes. Tree 4 did not offer such an escape route for
minimising the effects of these stragglers, and as such was not pursued further.

Table 2.9 lists the numbers of cells classified into either the normal or double
categories, together with their true classification. Normal cells were classified correctly in
99.9% of the cases, while the double cells were classified correctly 95.6% of the time. In
building DSF1-2a, the remaining apoptotic cells or debris incorrectly classified as ND in
DSF1-1 were treated as doublets with the hope that they might be assigned to this
category. This was not the case. In fact, nearly all of these populations were classified as
normal. The much greater proportion of true doublets served to dominate the
discriminant analysis. This fact is reflected in the features comprising the classification
functions, as listed in Table 2.10 The two most significant features, the variance in
radius, elongation and the 2nd harmonic of the fast Fourier transform are significantly
elevated in the doublets, which in general were more oblong shaped. The presence of
the apoptotic cells is only reflected in the least significant feature, the cluster
prominence.
Section 2 - Technical Developments

Table 2.9 Classification accuracy of discriminant function DSF1-2a, to separate normal cells from the entire learning set population. Actual number of cells in each group (ND = normal or double; AJ = apoptotic or debris) are listed together the number of each class which were classified into that group. The total number of cells classified into each group is provided in the last column. Shaded areas represent correct classification.

<table>
<thead>
<tr>
<th>Actual Classification</th>
<th>N</th>
<th>A</th>
<th>J</th>
<th>D</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>57341</td>
<td>66</td>
<td>62</td>
<td>96</td>
<td>57565</td>
</tr>
<tr>
<td>D</td>
<td>66</td>
<td>1</td>
<td>4</td>
<td>2050</td>
<td>2121</td>
</tr>
</tbody>
</table>

Table 2.10: Features used in classification functions for DSF1-2a. Feature means for each group in the learning set are listed with their standard deviations and the standardised canonical variable (weighted by within group variances as described in section 2.4.1) are listed.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Group Means</th>
<th>Standard Canonical Var.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Doublets</td>
</tr>
<tr>
<td>1. radius_var</td>
<td>1.9 ± 3.1</td>
<td>87 ± 36</td>
</tr>
<tr>
<td>2. elongation</td>
<td>1.1 ± 0.1</td>
<td>2.7 ± 1.1</td>
</tr>
<tr>
<td>3. fft_harmonic2</td>
<td>2.9 ± 2.0</td>
<td>27 ± 9</td>
</tr>
<tr>
<td>4. cl_prominence</td>
<td>1.9 ± 0.4</td>
<td>2.3 ± 0.6</td>
</tr>
<tr>
<td>CONSTANT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-1.1722</td>
<td>0.5587</td>
</tr>
<tr>
<td></td>
<td>-0.2737</td>
<td>-0.1066</td>
</tr>
<tr>
<td></td>
<td>-1.0380</td>
<td></td>
</tr>
</tbody>
</table>

With regard to the separation of stragglers from the populations of interest, DSF1-2b was more successful, as shown in Table 2.11. In this step, the true apoptotic component of the remaining data set was classified with 90% accuracy, while the debris within the set was classified correctly 86.7% of the time. In this case, the straggler normal cells or doublets were treated as debris for the purposes of discriminant analysis. This was, in fact highly successful, with 85.6% of true normals, and 98.7% of true doublets being placed into the debris category. The resulting population of cells which were classified as apoptotic was therefore 88% "pure". The success of the removal of stragglers in this case compared to DSF1-2a can be attributed to the population sizes of each group. A near equal number of normal, debris and doublets comprised one group,
which in total had a total size comparable to that of the apoptotic population (489 versus 388).

Table 2.11 Classification accuracy of discriminant function DSF1-2b, to separate normal cells from the entire learning set population. Actual number of cells in each group (ND = normal or double; AJ = apoptotic or debris) are listed together the number of each class which were classified into that group. The total number of cells classified into each group is provided in the last column. Shaded areas represent correct classification.

<table>
<thead>
<tr>
<th>Actual Classification</th>
<th>N</th>
<th>A</th>
<th>J</th>
<th>D</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>23</td>
<td>348</td>
<td>24</td>
<td>2</td>
<td>397</td>
</tr>
<tr>
<td>J</td>
<td>137</td>
<td>40</td>
<td>156</td>
<td>147</td>
<td>480</td>
</tr>
</tbody>
</table>

While there was contribution to the classification functions from 24 texture features of a variety of types (see Table 2.12), a significant number were of the run length variety, indicating that the primary difference between the two populations was the in fluctuations in grey levels. For instance, the short run emphasis at an angle of 90° (short_run_90 in Table 2.12) returns larger values for those images where short runs of equal grey level dominate, while the run_percentage is the fractional area of the cell which these short runs comprise. Such short runs appear to be present more often in the apoptotic cells most likely since these features are calculated based on images massaged into 8 grey levels, and thus distinct fragments of condensed chromatin would contrast with the darker cytoplasmic component.
Table 2.12: Features used in classification functions for DSF1-2b. Feature means for each group in the learning set are listed with their standard deviations and the standardised canonical variable (weighted by within group variances as described in section 2.4.1) are listed.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Group Means</th>
<th>Standardised Canonical Var.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Debris</td>
<td>Apoptosis</td>
</tr>
<tr>
<td>sphericity</td>
<td>0.47 ± 0.21</td>
<td>0.69 ± 0.14</td>
</tr>
<tr>
<td>short_runs_90</td>
<td>0.26 ± 0.06</td>
<td>0.28 ± 0.03</td>
</tr>
<tr>
<td>run_percent_0</td>
<td>0.31 ± 0.13</td>
<td>0.28 ± 0.08</td>
</tr>
<tr>
<td>area</td>
<td>2000 ± 1100</td>
<td>1400 ± 600</td>
</tr>
<tr>
<td>short_runs_135</td>
<td>0.26 ± 0.06</td>
<td>0.28 ± 0.03</td>
</tr>
<tr>
<td>fractal_dimen</td>
<td>2.6 ± 0.3</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td>homogeneity</td>
<td>0.48 ± 0.12</td>
<td>0.51 ± 0.08</td>
</tr>
<tr>
<td>den_drk_spot</td>
<td>0.021 ± 0.037</td>
<td>0.026 ± 0.039</td>
</tr>
<tr>
<td>compactness</td>
<td>1.7 ± 0.5</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>radius_fft_low</td>
<td>130 ± 150</td>
<td>36 ± 54</td>
</tr>
<tr>
<td>range_extreme</td>
<td>240 ± 120</td>
<td>320 ± 130</td>
</tr>
<tr>
<td>DNA_index</td>
<td>1100 ± 900</td>
<td>850 ± 560</td>
</tr>
<tr>
<td>run_length_90</td>
<td>160 ± 80</td>
<td>130 ± 50</td>
</tr>
<tr>
<td>DNA_amount</td>
<td>44 ± 40</td>
<td>34 ± 23</td>
</tr>
<tr>
<td>fft_high_15</td>
<td>0.52 ± 0.55</td>
<td>0.28 ± 0.23</td>
</tr>
<tr>
<td>energy</td>
<td>0.21 ± 0.09</td>
<td>0.23 ± 0.07</td>
</tr>
<tr>
<td>correlation</td>
<td>150 ± 70</td>
<td>170 ± 45</td>
</tr>
<tr>
<td>cl_prominence</td>
<td>5.0 ± 9.0</td>
<td>4.2 ± 1.7</td>
</tr>
<tr>
<td>OD_kurtosis</td>
<td>9.0 ± 52</td>
<td>4.3 ± 1.8</td>
</tr>
<tr>
<td>cluster_shade</td>
<td>1.2 ± 0.9</td>
<td>1.4 ± 0.5</td>
</tr>
<tr>
<td>OD_skewness</td>
<td>1.4 ± 1.6</td>
<td>1.4 ± 0.5</td>
</tr>
<tr>
<td>x_centroid</td>
<td>30 ± 9</td>
<td>24 ± 6</td>
</tr>
<tr>
<td>gray_level_0</td>
<td>95 ± 53</td>
<td>58 ± 24</td>
</tr>
<tr>
<td>radius_max</td>
<td>33 ± 11</td>
<td>24 ± 7</td>
</tr>
<tr>
<td>CONSTANT</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.9296</td>
</tr>
</tbody>
</table>

Classification Accuracy: As a summary, the overall classification matrix for the binary discriminant tree applied to the learning set is shown in Table 2.13. From this table it can be deduced that using the learning set, there would be calculated an apoptotic frequency of 0.69%. The true apoptotic frequency, that calculated from the manually assigned classes, is 0.79%. This discrepancy arises due to a lower numerator in the apoptotic fraction calculation. A greater number of apoptotic cells were misclassified into other groups compared to the number from other groups which were misclassified as
Section 2 - Technical Developments

apoptotic. By comparison, in the denominator (normal cells) there was a balance in the misclassification such that as many were "lost" as were "gained.

**Table 2.13:** Overall classification matrix for learning set. Shaded areas represent the number of correctly classified events. Part I shows actual numbers of images classified, while part II relates these numbers in percentage terms.

<table>
<thead>
<tr>
<th>I. Actual Classification</th>
<th>N</th>
<th>A</th>
<th>J</th>
<th>D</th>
<th>Total #</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>57341</td>
<td>66</td>
<td>62</td>
<td>96</td>
<td>57565</td>
</tr>
<tr>
<td>A</td>
<td>23</td>
<td>348</td>
<td>24</td>
<td>2</td>
<td>397</td>
</tr>
<tr>
<td>J</td>
<td>137</td>
<td>40</td>
<td>156</td>
<td>147</td>
<td>480</td>
</tr>
<tr>
<td>D</td>
<td>66</td>
<td>1</td>
<td>4</td>
<td>2050</td>
<td>2295</td>
</tr>
<tr>
<td>Total</td>
<td>57567</td>
<td>455</td>
<td>246</td>
<td>2295</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>II. Actual Classification</th>
<th>N</th>
<th>A</th>
<th>J</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>99.6</td>
<td>14.5</td>
<td>25.2</td>
<td>4.2</td>
</tr>
<tr>
<td>A</td>
<td>0.1</td>
<td>76.5</td>
<td>9.8</td>
<td>0</td>
</tr>
<tr>
<td>J</td>
<td>0.2</td>
<td>8.8</td>
<td>63.4</td>
<td>6.4</td>
</tr>
<tr>
<td>D</td>
<td>0.1</td>
<td>0</td>
<td>1.6</td>
<td>89.3</td>
</tr>
</tbody>
</table>

Following the development and testing of the binary discriminant tree on the learning set, the tree was further analysed on a set of data which had not been included in the learning set. A complete breakdown of the classification accuracy performed on the test set of 19991 cell images' data is shown in Table 2.14. The percentage of those images correctly classified was comparable to that of the learning set for all groups. Furthermore, for the normal, apoptotic and doublets categories, the misclassifications appeared to follow similar patterns to that observed in the learning set. However, a difference appears in the misclassification of debris. Whereas in the learning set, the majority of misclassified debris (25.2%) was labelled normal, for the test set, a similar fraction was misclassified as apoptotic. In this case then the resulting apoptotic frequency was 0.78% compared to the true frequency of 0.82%. While it is not to be
expected that a greater accuracy would be observed in the test set compared to the
learning set, this can be explained by a difference in the characteristics of the debris in
the test set. Because the debris classification comprises all roughly four subgroups of
images (Table 2.4) it would be expected that features calculated for this group would
display a larger variance than features calculated for other classes. Therefore, it is to be
expected that for a sample of only 45 images, as contained in the test set, that a greater
probability of viewing a difference in proportion of the subgroups, and hence the
classification, would arise. Furthermore, the test data set is approximately 20 times
larger than the sample obtained for each slide, and thus the amount of debris
misclassified would be much lower in general, although it would have the same effect on
the apoptotic fraction since the number of apoptotic cells would also be decreased. It is
encouraging however, that the misclassification of debris accounts for only 6-8% of the
cells classified as apoptotic, which is below the expected intra-slide and inter-slide
variability.

Table 2.14: Overall classification matrix for test set. Shaded areas
represent the number of correctly classified events. Part I shows actual
numbers of images classified, while part II relates these numbers in
percentage terms.

<table>
<thead>
<tr>
<th></th>
<th>Actual Classification</th>
<th>Total #</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>A</td>
</tr>
<tr>
<td>N</td>
<td>18694</td>
<td>21</td>
</tr>
<tr>
<td>A</td>
<td>8</td>
<td>125</td>
</tr>
<tr>
<td>J</td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td>D</td>
<td>28</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>18741</td>
<td>153</td>
</tr>
</tbody>
</table>

II. Actual Classification

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>A</th>
<th>J</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>99.8</td>
<td>13.7</td>
<td>15.6</td>
<td>3.6</td>
</tr>
<tr>
<td>A</td>
<td>0</td>
<td>81.7</td>
<td>26.7</td>
<td>0.3</td>
</tr>
<tr>
<td>J</td>
<td>0.1</td>
<td>4.6</td>
<td>57.8</td>
<td>4.8</td>
</tr>
<tr>
<td>D</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
<td>91.3</td>
</tr>
</tbody>
</table>

75
Repeatability of Measurement: For the detection of small differences between the apoptotic frequencies produced by relatively low doses of radiation, it is important to consider sources of variance in the methods used. The use of the discriminant functions to automatically classify the images obtained from each slide ideally will produce a very consistent system of measurement. However, other sources of variance in this technique exist which should be assessed prior to drawing conclusions regarding the biological differences between samples: the intra-slide variance (the sampling error for a given slide); the inter-slide variance (the variance between slides prepared from the same cell sample); the biological variance (variance caused by irradiation conditions, cell growth conditions, etc.). While the inter-slide and biological variance can be expected to change during the course of several experiments, the intra-slide variation should vary little, since the properties of the acquisition system remain fixed. Therefore, two slides containing a high and a low apoptotic fraction (cells given 5 and 0.25 Gy respectively) were measured repeatedly to ascertain the level of variance entered into the results due to sampling error. Table 2.15 lists the individual measurements obtained for each slide from samples of approximately 1000 cells per scan. For the high dose case, the mean value of the apoptotic fraction was 4.53% with standard deviation 0.97, and for the low dose case, 0.109% with standard deviation 0.072. Therefore, a significant amount of variance in the measurements can be expected due to sampling error alone. Because it was unknown at the time what differences could be expected to be observed between the doses of interest, the impact of this variance was uncertain. While the relative size of the sampling error provides an argument for the sampling of more cells per slide, this was undesirable. Most fluorochromes, when exposed to visible light undergo a photobleaching process, in effect decreasing the intensity of emitted fluorescence over
the duration of the exposure. To avoid significant bleaching of the samples which could affect the results of texture feature measurements of cells sampled in the latter portion of each scan, the number of cells was limited to 1000. To reduce the sampling error, several slides were prepared from each cell population and from 3-4 of these slides were sampled 1000 cells each.

**Table 2.15:** Intra-slide variance for apoptotic frequency measurements. High and low dose slides were prepared as described in the text and scanned ten times each. For each case, the number of cells classified as normal or apoptotic is shown with the corresponding apoptotic fraction (AF). The mean and standard deviation of each case is listed in the last row.

<table>
<thead>
<tr>
<th>Scan</th>
<th>High Dose</th>
<th>Low Dose</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Norm.</td>
<td>Apop.</td>
<td>AF</td>
</tr>
<tr>
<td>1</td>
<td>915</td>
<td>49</td>
<td>5.08</td>
</tr>
<tr>
<td>2</td>
<td>898</td>
<td>59</td>
<td>6.17</td>
</tr>
<tr>
<td>3</td>
<td>907</td>
<td>35</td>
<td>3.72</td>
</tr>
<tr>
<td>4</td>
<td>908</td>
<td>33</td>
<td>3.51</td>
</tr>
<tr>
<td>5</td>
<td>886</td>
<td>40</td>
<td>4.32</td>
</tr>
<tr>
<td>6</td>
<td>905</td>
<td>39</td>
<td>4.13</td>
</tr>
<tr>
<td>7</td>
<td>890</td>
<td>56</td>
<td>5.92</td>
</tr>
<tr>
<td>8</td>
<td>902</td>
<td>37</td>
<td>3.94</td>
</tr>
<tr>
<td>9</td>
<td>914</td>
<td>33</td>
<td>3.48</td>
</tr>
<tr>
<td>10</td>
<td>907</td>
<td>48</td>
<td>5.03</td>
</tr>
</tbody>
</table>

**Mean**

<table>
<thead>
<tr>
<th></th>
<th>High Dose</th>
<th>Low Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Scan</strong></td>
<td><strong>Norm.</strong></td>
<td><strong>Apop.</strong></td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td><strong>4.53 ± 0.97</strong></td>
<td><strong>0.109 ± 0.072</strong></td>
</tr>
</tbody>
</table>
Section 3

Results

You are never given a wish
without also being given the
power to make it true.

You may
have to work for it
however

- Richard Bach, *Illusions*
3. STUDIES OF APOPTOSIS AT LOW DOSES

To study the significance of apoptosis occurring as a result of low dose irradiation, two methods were applied: the application of "priming" doses in analogy to the adaptive response (Section 1.1.3) and a detailed analysis of the extent of apoptosis occurring as a function of time following various doses of x-rays. For clarity, a summary of all cell culture and slide preparation methods used is provided in the following section. Results are summarised beginning in Section 3.2.

3.1 METHODS AND MATERIALS

3.1.1 CELL CULTURE AND IRRADIATION

For all experiments, exponentially growing Chinese hamster ovary (CHO) cells were used. CHO cells were initially grown in monolayer culture in an α-modification of Eagle's MEM (α-MEM, Gibco, Grand Island, NY), supplemented with 10% foetal calf serum, 100 Units/ml PenStrep (Gibco) and 1 mg/ml sodium bicarbonate (the medium is hereafter referred to as α-MEM). Approximately three days prior to the day of experimentation, cells were harvested with 0.25% trypsin/EDTA and placed in α-MEM in spinner culture where they were kept at 37°C until use. In order to prevent depletion of growth factors which might introduce variability in apoptosis frequency, cells were maintained by dilution at densities below 2.5x10^5 ml\(^{-1}\). Two hours prior to irradiation, cells were diluted to between 0.5-1x10^5 ml\(^{-1}\) in 250 ml flasks. Care was taken that all flasks were diluted to similar concentrations prior to all experiments and following aerating with air/5% CO\(_2\), that all flasks were tightly sealed.
Cells were irradiated at room temperature with X-ray (250 kVp, HVL 1.5 mm Cu) doses of 0.25-5 Gy. Dosimetry was performed previously using a calibrated electrometer such that the dose rate was 0.525 Gy/min. During the entire course of several experiments, dose rate varied by no more than 6%, as verified by electrometer readings. Variation in x-ray tube output during irradiation was accounted for by calibration against the x-ray unit's internal electrometer readings. Following irradiation, cells were maintained in spinner culture at 37°C for the specified duration, up to 72 hours. Every 6-12 hours, as necessary, cell suspensions were diluted to 0.5-1.0x10^5/ml to prevent overgrowth, and exposed to 5% CO_2/Air.

### 3.1.2 Microscopic Assay

Prior to microscopic assay, all cell samples were prepared using the paraformaldehyde/methanol fixation method described in Section 2.1. A brief summary is provided here for reference.

Following a specified period in suspension culture, 3x10^6 cells (as determined by Coulter counting) were removed and washed twice with ice-cold PBS. Following centrifugation at 900g for 8 minutes, pellets were dispersed by "clicking" the tube several times and 9 ml cold PBS added and vortexed. A pre-fixation step was performed by addition of 1 ml 0.5% Pf, prepared fresh one hour prior to use, for 15 minutes at ice temperature, followed by centrifugation. Again, the pellet was dispersed and 4 ml 0.5% Pf was added while vortexing to prevent cell clumping. Following 30 minutes at room temperature, the cells were washed once in cold PBS. Post-fixation was applied by addition of 4 ml of ice-cold 70% MeOH, again while vortexing. Following 15 minutes at 4°C, the cells were spun down, the supernatant discarded and 600 μl 70% MeOH added.
and vortexed at a low setting. An aliquot of 100 µl of the cell suspension was placed on each slide (Fisher SuperFrost Plus, Fisher Scientific), 6 in total, and allowed to air dry for at least one hour.

Cell staining proceeded directly on the slides in batches of 30. Cells were permeabilised in 0.5% Tween 20 in PBS, for 15 minutes at room temperature. Slides were then immersed in 0.20 mg/ml RNAse A for 1 hour at 37°C. Slides were rinsed once in PBS/Tween solution and tapped to remove excess liquid. DNA staining proceeded by immersion in 50 µg/ml propidium iodide in 0.1 mmol sodium citrate buffer, prepared before use from a stock solution of 1 mg/ml. Following overnight staining, cytoplasm was stained using a solution of 10 µM FITC in PBS, which had been adjusted to pH 9.5. After two hours at room temperature, slides were rinsed three times in PBS/Tween for a total of 15 minutes.

Individual slides were tapped to remove excess liquid, and blotted semi-dry on lint-free tissue. Coverslips were mounted with two drops of Glycergel (DAKO) mounting medium. Excess mounting medium was removed by blotting and coverslips were pressed firmly to ensure even mounting. Slides were stored at 4°C until scanning to minimise fading of fluorescence signals.

Scanning proceeded in the fashion described in Section 2.2.3 and discriminant functions were applied as previously outlined. In order to ensure incident light intensity remained stable, the 100 W Hg arc lamp was replaced following each 150 hours of use and centred in the field by aligning the arc and its reflected image in the viewfinder of the Nikon epi-fluorescence centring tool. Prior to each scanning session, a calibration slide was prepared and the described calibration procedures were followed (Section
Section 3 - Results

2.2.3). The calibration procedure was repeated every six hours. Integration times for DNA and cytoplasm were set based on trial acquisitions of 100 cells from a single slide from each batch, such that the brightest apoptotic cell was at least 10% below saturation level. Typically, these settings did not vary more than 20% over all slides from the same batch. Average scanning time varied from 25-40 minutes depending on the integration times and the density of single cells on the slide.

3.2 TIME COURSE OF APOPTOSIS INDUCTION

The frequency of apoptosis was measured for doses of 0.25, 0.5, 1.0 and 5.0 Gy along with mock irradiated controls, at intervals of 6 hours over the period ranging from immediately following irradiation up to 60 hours, and additionally at 72 hours. Three separate experiments were performed, the first of which was conducted to include post-irradiation times of up to 42 hours only. Subsequent analysis of these results indicated that longer times would be necessary, and therefore the remaining two experiments were performed up to 72 hours duration. Three to four slides were measured for each dose-time point, resulting in a total of approximately 600 slides scanned over the course of the three experiments. For each experiment, the mean of like dose-time slides was taken and standard deviations calculated (3-4 slides), which represent the sampling error over approximately 1000 cells. The inter-experimental mean for each dose-time point was measured as the mean of these values and the biological error was determined by calculation of the standard deviation and standard error in this mean.
3.2.1 Apoptosis Frequency Following 5.0 Gy Irradiation

Figure 3.1 graphically illustrates the levels of apoptosis observed over time in those cells irradiated with 5.0 Gy. All values are expressed as the percentage of apoptotic cells and error bars are presented as the standard error in the mean over two or three experiments, representing the biological error. Those time points of 48 hours or greater were the average of only two experiments, while all others are from three separate experiments. No appreciable increase in apoptosis above control levels was observed in these cells until 18 hours post-irradiation. As the apoptosis levels rose, there was an obvious peak in the apoptotic frequency which occurred at 42 hours following irradiation, followed by a decrease and levelling off which continued until the 72 hour

![Graph showing variation of apoptotic frequency](image)

**Figure 3.1**: Variation of apoptotic frequency with time following 5.0 Gy X-rays (●) compared to mock irradiated control cells (O). Error bars are the standard error in the mean over two or three experiments. Note that the x-axis is shifted from y=0 for ease of interpretation.
Figure 3.2: Variation of cell cycle parameters with time following 5.0 Gy irradiation. Values represent averages of 2 (>42 hours) or 3 (<42 hours) experiments, and error bars are standard deviation in the mean. Note the increase in G2/M population and corresponding decrease in G1 immediately following irradiation. Apoptosis levels begin to rise only following release of G2 block.

The biological coefficient of variation (standard deviation/mean over the three experimental means) ranged between 0.08 and 0.66 for the times studied, with an average value of 0.32. By comparison, the average sampling error over the three experiments for the entire 5 Gy time course was 0.37, with the largest values occurring for the cases where a very low apoptotic frequency was observed (0-18 hours).

Supplementary information was also extracted from these data concerning the cell cycle kinetics of the cells as they undergo apoptosis. The DNA content of cells classed as normal from each slide was
analysed using ModFit software, to determine the fraction of the population in each of G1, S and G2/M phase of the cell cycle. Because only 1,000 cells were sampled per slide, there was a tendency in some cases for the CV of the G1 peak to be > 15%, which caused large inaccuracies in the G2/M and S-phase fraction estimates. Data from these cases were not included in the calculations. Means for each dose-time point were taken for each experiment, and averaged to obtain the overall estimates, together with their standard deviations. Figure 3.2 shows these results for each cell cycle position over time. Immediately following 5.0 Gy irradiation, the cells begin to accumulate in G2/M, while a corresponding decrease in the G1 fraction is observed. The cells appear to return to normal cycling 24 hours post-irradiation and the half-life of this G2-block appears to be approximately 9 hours, occurring at approximately the 18 hours. In fact, the percentage of G2 cells in control and irradiated cells were compared by t-tests for each time studied. Statistically significant differences in the populations were found between 6 and 24 hours (p<0.05). By comparison with Figure 3.1, it may be noted that it is approximately at this point where the accumulation of apoptotic cells begins. A substantial increase in apoptotic frequency following release of a G2 block has been noted previously by several investigators. In contrast, cell cycle parameters for the control populations maintained fairly constant throughout the time course of the experiment. Averages over all times were: for G1, 39.3% ± 4.2; for G2, 14.2% ± 3.6; for S, 46.7% ± 5.9 (standard deviation is quoted).
3.2.2 Apoptosis Frequency Following Low Dose Irradiation

Results obtained in the low dose cases were treated somewhat differently than those for the 5.0 Gy case. In the latter, any variation over time in the apoptotic frequency of the control cells was insignificant due to the much larger magnitude of apoptotic frequency in the 5.0 Gy irradiated cells. This was not the case at lower doses (and lower apoptosis levels), where variations over time roughly corresponded to the variations found in the control population, as is illustrated by example in Figure 3.3. Similar variations would be expected under identical cell culture conditions (i.e. cell density, pH of growth medium, temperature). For this reason, for comparison of low dose measurements, all data from each experiment were normalised by subtraction of the control apoptotic frequency at the corresponding post-irradiation time.

Figure 3.3: Raw apoptotic frequencies for control (V) and 0.5 Gy irradiated cells (▲) for a single experiment. Each point represents the mean of four slides prepared from the same irradiated population. Temporal variations in control cell apoptotic fraction were generally mirrored in the low dose samples.
Figure 3.4 shows the data for the 0.25, 0.5 and 1.0 Gy cases over the 72 hour period of study. Each point represents the mean normalised apoptotic frequency over three experiments, where three to four slides were scanned for each dose point at each time post-irradiation. Error bars in Figure 3.4 were calculated as the standard error in the mean of the averaged values for each experiment and thus represent the biological uncertainty. Global maxima in apoptotic frequency appear at 42 hours for both the 0.25 Gy and 1.0 Gy populations, of magnitude 0.70% and 1.00% respectively. Secondary maxima were observed at 60 hours, of lower magnitude (0.29% and 0.70% for 0.25 and 1.0 Gy respectively). In the case of the 0.5 Gy population, the curve was much flatter than the other two doses, although two smaller maxima of approximately equivalent magnitude were observed.

![Figure 3.4: Apoptosis as a function of time following irradiation for low doses of X-rays. All values were normalised by subtraction of the control population's apoptotic frequency. Shown are the 0.25 Gy (●), 0.5 Gy (△) and 1.0 Gy (□) cases over the 72 post-irradiation period. Error bars shown represent the biological standard error in the mean of three experiments (raw data is plotted in Figure 4.1).](image-url)
magnitude were observed at 36 and 42 hours (0.21% each). Several points in the 0.5 Gy (6, 18 and 72 hours) and one of the 0.25 Gy time points (72 hours) exhibited lower apoptotic frequency than control cells and fell below zero (Figure 3.4).

Statistical analysis was performed on the low dose samples to determine possible significance of differences in apoptotic frequency which appear between 0.25 and 0.5 Gy at 42 hours, and to determine the validity of the hypothesis that low doses of x-radiation (below 0.5 Gy) may cause more apoptosis than slightly higher doses. All analyses were performed using t-tests for dependent samples (to control for global trends in data from individual experiments), utilising Statistica for Windows analysis software (StatSoft, Tulsa, OK). As illustrated by the resulting p-values in Table 3.1, of all the doses measured at 42 hours, the only significant difference found was between the 1.0 and 0.25 Gy dose points. The rest returned p-values greater than the confidence threshold of 0.05. In general, matched t-tests performed on pairs of 0.25, 0.5 and 1.0 Gy data over the range of times studied showed p-values greater than the 0.05 tolerance. Exceptions were 0.5 and 1.0 Gy measured at 36 hours (p=0.049) and 54 hours (p=0.048). In fact, these may not reflect "real" differences or similarities due to the small number of degrees of freedom caused by only three experimental data points. Underscoring this problem is the fact that only the difference between 1.0 and 0.25 Gy populations were determined to be statistically significant, despite the fact that these doses did not exhibit the greatest difference in means (cf. 0.5 and 5.0 Gy doses). This is, likely, a result of the relatively large standard deviations (in proportion to the mean values) which are listed in the final column of Table 3.1. This result seems to indicate that several more samples are needed to counter the effects of random errors at these dose levels. Further discussion of the statistical analysis may be found in Section 4.3.1.
Table 3.1: Sample t-test results, calculated for 42 hour data. Listed are the p-values for matched t-tests performed for each dose pair studied. Data were matched according to date of experiment as these values would be expected to show some correlation. Shaded areas depict those data which showed significant differences between the dose points (p <0.05). Mean % apoptosis and standard deviations over three experiments are shown in the final column for each dose.

<table>
<thead>
<tr>
<th>p-values</th>
<th>0.25 Gy</th>
<th>0.5 Gy</th>
<th>1.0 Gy</th>
<th>5.0 Gy</th>
<th>Mean % ± s.d.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25 Gy</td>
<td>1.000</td>
<td>0.059</td>
<td><strong>0.049</strong></td>
<td>0.129</td>
<td>0.70±0.24</td>
</tr>
<tr>
<td>0.5 Gy</td>
<td>1.00000</td>
<td>0.055</td>
<td></td>
<td>0.125</td>
<td>0.13±0.07</td>
</tr>
<tr>
<td>1.0 Gy</td>
<td></td>
<td>1.000</td>
<td>0.132</td>
<td></td>
<td>0.99±0.36</td>
</tr>
<tr>
<td>5.0 Gy</td>
<td></td>
<td></td>
<td>1.000</td>
<td></td>
<td>11.9±7.9</td>
</tr>
</tbody>
</table>

Of particular note in Figure 3.4 is the fact 0.25 Gy irradiated cells exhibited a greater apoptotic frequency than the higher dose of 0.5 Gy at every time point studied. This is further illustrated by comparison of the area beneath each of the three curves, as shown in Figure 3.5. In all cases, there was no apparent increase in apoptotic frequency above control until after 18 hours post-irradiation. Furthermore, to avoid biasing effects of negative values obtained for the 0.5 Gy curve, the integrals were estimated beginning with the 24 hour point, by multiplication of the apoptosis level obtained at each time by 6 hours (Δt). Because the true shape of the curves is not generally known, more precise methods of numerical integration were not used. Over the 24 to 72 hour period, the integrated apoptosis frequency for 0.25, 0.5 and 1.0 Gy were 10.9, 4.7 and 22.6 % apoptosis-hours, respectively. All three curves appear to begin to plateau following 60 hours (note again that the 66 hours point was not measured). Analysis of the apoptotic frequency beyond 72 hours was not performed. Examination of Figure 3.5 clearly shows the greater extent of accumulation of apoptosis in the 0.25 Gy-treated cells compared to the 0.5 Gy-treated population. However, one may also observe that apart from the
greater slope observed between 36-42 hours in the 0.25 Gy curve, the slopes of the 0.25 and 0.5 Gy curves appear to be quite similar.

Figure 3.5: Cumulative plot of x-ray-induced apoptosis, illustrating the progressive amount of apoptosis produced for each x-ray dose, 0.25 Gy (closed circle), 0.5 Gy (open triangle) and 1.0 Gy (open square), from 21 hours until 72 hours post-irradiation.

To determine the possible significance of the observed differences in "cumulative" apoptosis between the three doses, statistical analysis was performed. As a first test, Analysis of Variance (ANOVA) was performed on data from each time point. In each case, pending these results, pairwise t-tests were performed on each dose pair. For post-irradiation times from 24 to 36 hours, no significant differences were found between the three groups (p=0.84, 0.44, 0.12 for 24, 30 and 36 hours respectively) and thus no further breakdown was performed. However, significant differences in the three
groups were found at all post-irradiation time of 42 hours and beyond (p=0.005, 0.008, 0.011, 0.003, 0.0005, for 42, 48, 54, 60 and 72 hours, respectively). Thus further tests were performed at each of these time points. At several of these points, significant differences were observed between the 0.5 and 1.0 Gy dose points (p=.013, 0.048, 0.026 for 42, 60 and 72 hours) and there was a single instance where the 0.25 and 0.5 Gy differed significantly, occurring at 48 hours (p=0.040). As an example of the analysis performed, a list of p-values is provided in Table 3.2 for the 72 hour point, as this may be the point which most accurately depicts the total accumulation of apoptosis following irradiation. For reference, also included in this table are the p-values for comparisons against the 5 Gy data. Similar to the observations noted for Table 3.1, those data with the lowest standard deviation relative to the mean tended to yield statistically significant results. It should be cautioned however, that these tests were performed on data from only two experiments and therefore may be greatly influenced by random errors. Furthermore, caution should be exercised in the interpretation of the "cumulative" data presented in this manner. Because it is unknown how long apoptotic cells retain their morphology, it is unknown whether each data point on Figure 3.5 is independent from the previous time point. For example, if apoptotic cells are visible for 8 hours, the 42 hour time point is dependent upon the levels of apoptosis observed at 36 hours. The fact that a peak in apoptotic frequency was observed at 42 hours however, indicates that the cells do not display apoptotic morphology indefinitely, and this fact may be reflected in the presence of a "tail" between 54 and 72 hours in Figure 3.1. Therefore, the "cumulative" data plotted in Figure 3.5 should be viewed as illustrative only.
Table 3.2: Sample t-test results, calculated for 72 hour cumulative data. Listed are the p-values for matched t-tests performed for each dose pair studied. Data were matched according to date of experiment as these values would be expected to show some correlation. Shaded areas depict those data which showed significant differences between the dose points (p <0.05). The final column lists means and standard deviations over two experiments for each dose.

<table>
<thead>
<tr>
<th>p-values</th>
<th>0.25 Gy</th>
<th>0.50 Gy</th>
<th>1.0 Gy</th>
<th>5.0 Gy</th>
<th>Mean % ± s.d.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25 Gy</td>
<td>1.00</td>
<td>.075</td>
<td>.051</td>
<td>.003</td>
<td>10.7±1.4</td>
</tr>
<tr>
<td>0.5 Gy</td>
<td>1.00</td>
<td>.004</td>
<td>1.00</td>
<td>.004</td>
<td>4.3±0.7</td>
</tr>
<tr>
<td>1.0 Gy</td>
<td>1.00</td>
<td>.007</td>
<td>1.00</td>
<td>.007</td>
<td>23.9±0.3</td>
</tr>
<tr>
<td>5.0 Gy</td>
<td></td>
<td></td>
<td>1.00</td>
<td></td>
<td>235±3.0</td>
</tr>
</tbody>
</table>

As in the case of the 5.0 Gy irradiation detailed in Section 3.2.1, complete cell cycle analysis was performed on DNA content measurements from all slides. Whereas a G2 block was noted in this case, there was no such cell cycle delay noted for the case of the lower doses. To test whether any significant changes occurred in time for each of the dose points, ANOVA was performed on the experimental means of each time point for a given dose. In each case, there was no detectable statistical difference between any of the time points. If any cell cycle perturbations do indeed exist, the levels most likely lie below the limit of detection, as a result of the small sample size of only 1000 cells per slide. For reference, cell cycle data are present in Appendix A for the control, 0.25, 0.5 and 1.0 Gy cases.

3.3 PRIMING EXPERIMENTS

Because application of priming doses has been found to eliminate the HRS response due to subsequent irradiation (as described in Section 1.1.3), experiments were performed to determine whether this effect was reflected in apoptotic frequency. The cells were cultured as described and irradiated with a 0.25 Gy priming dose, and left
in culture under standard conditions for 6 hours. The second "challenge" doses were then applied to the cultures, of a further 0.25 or 5.0 Gy. The cells were maintained in spinner culture for a period of 42 hours before fixation and staining to assess the levels of apoptosis. Five slides were prepared from each flask, along with appropriate control samples, for each experiment.

Table 3.3 lists the average apoptotic frequencies observed over 1000 cells sampled. The results are graphically illustrated in Figure 3.6. For each experiment, the average of the five scanned slides was taken, the error in the mean calculated as the standard deviation. Values listed in Table 3.3 are the means over the two experiments. The uncertainties shown are the root sum of squares of the standard deviations from the two experiments, and reported as standard error.

If there is a dose threshold, above which the tendency for apoptosis would be decreased significantly, it would be expected that the priming dose would confer a protective effect, resulting in a decreased apoptotic frequency in those cells receiving both the priming and challenge doses, compared to those receiving the challenge dose only. In the case of cells irradiated with a challenge dose of 5 Gy, there was no substantial difference observed between the primed and unprimed cell populations (3.06%±0.35 versus 3.02%±0.42, respectively). Even when the effect of the 0.25 Gy...
Section 3 - Results

A priming dose is added to the apoptotic frequency observed for the 5 Gy challenge dose only, the resulting frequency (3.13%) is not substantially (nor significantly) different from the primed case. For those cells challenged with a low dose of 0.25 Gy, substantially more apoptosis was observed in the primed cells (0.214%±0.060) versus the unprimed (0.042%±0.029). No detectable effects of priming were noted, as there remains more apoptosis caused by the prime plus challenge than by the challenge alone.

It is interesting to note that approximately two-fold greater apoptosis was observed in the control cells compared to those cells irradiated with the 0.25 Gy.

Figure 3.6: Effect of priming doses on apoptosis frequency. A priming dose of 0.25 Gy was given 6 hours prior to challenge doses of 0.25 or 5.0 Gy and apoptosis frequency was measured at 42 hours following the challenge dose. No detectable effects of priming were noted. Values reported are mean and standard deviation over two experiments.
challenge dose. The priming dose itself appeared to produce little apoptosis relative to the control, as these values were statistically similar. Temporal differences in apoptotic frequencies following irradiation could affect the interpretation of these results, as the effect of the priming dose alone is assessed at 42 hours compared to 48 hours for the challenge doses. Alternately, this result could reflect the effects of random errors and therefore a large set of subsequent experiments are necessary, as was the case for the study of the time course of apoptosis induction.
"Too many pieces of music finish too long after the end."

- Igor Stravinsky
4. DISCUSSION

4.1 METHODOLOGICAL CONSIDERATIONS

The measurement of the effects of small doses of radiation on mammalian cells presents significant challenges. The problems associated with the measurement of relatively rare events, especially for the end-point of radiation-induced cell death, have been well-described over the past few decades [7-9]. The advent of the DMIPS system allowed, for the first time, detailed characterisation of the mammalian cell survival curve at doses below 1 Gy, and flow cytometry based methods have confirmed the validity of these results [15, 25]. Both of these modifications to the cell survival assay resulted in enhanced precision by both the elimination of one or more sources of error in the methodology and the integration of automated technology.

The same philosophy has been applied in the present study of apoptosis following low dose irradiation: the application of discriminant function analysis to the classification of apoptotic cells to eliminate the subjectivity of the process, and the use of automated image cytometry to increase the efficiency of data acquisition.

4.1.1 CHARACTERISTICS OF MISCLASSIFIED CELLS

The discriminant functions used to differentiate between apoptotic, normal and other cell classes were based upon a set of manually classified images. As there was frequently some doubt as to the true classification of some cells, any classification errors present within this set could affect the results of subsequent discriminant function classified cell populations. It should be noted that the guidelines for manual classification
were strictly adhered to during the classification of the learning set, and only those cells which were apoptotic for certain were included in the apoptosis classification, the remainder classified as either normal or debris depending upon their individual characteristics. However, as noted in Table 2.13, some misclassification of apoptotic cells persisted, as would be expected from a heterogeneous population. While the false negative and false positive rates for apoptotic cell classification were 18.3% and 15.5% respectively, this appears to be of little concern in the present case. The consistency of the method over the dose range of interest is of primary importance for the measurement of the relative levels of apoptosis due to incremental radiation doses. Discriminant analysis by nature, possesses this property and the apparent invariance of features over this dose range ensures the highest level of consistency.

Although no quantitative analysis was performed, the characteristics of the cells misclassified as apoptotic and those apoptotic cells classified incorrectly were noted, as these would have the most significant impact on subsequent apoptotic frequency measurements. The greatest proportion of cells misclassified as apoptotic were from the normal population. The majority of these were very round cells of reduced area and very little heterochromatin. While there was no evidence of fragmented chromatin in these cells, these might represent cells in the very early stages of apoptosis, where a rapid decrease in cell area has been noted, accompanied by cell rounding [215]. This size decrease has been shown to occur in two distinct stages, the first occurring rapidly and the second occurring over 2-3 hours before or during nuclear blebbing [216, 217]. It has been reported that active blebbing during apoptosis lasts only approximately 60 minutes [215, 218], although these studies were performed on cells attached to tissue culture flasks, and the relatively short duration of the process may simply be a reflection of the
loss of adherence of apoptotic cells. The apoptotic cells which were misclassified as normal were typically larger than average. While there was a definite decrease in nuclear area in these cells, and nuclear fragmentation characteristic of apoptosis, there was an atypically large cytoplasm area. Furthermore, the DNA content of these cells was near tetraploid. It is possible that these cells represented apoptosis induced in late-G2 phase or during mitosis, and were "true" misclassifications. The only other significant misclassifications were between debris and apoptotic cells. The majority of debris misclassified as apoptotic were those cells which demonstrated apoptotic morphology but were of less than 15% diploid DNA content. This is not unexpected, as the DNA content dependent features used in DSF1-2b exhibited much less discriminatory power compared to other shape, size and texture-related features used in the classification functions.

In each case, the characteristics of misclassified cells appeared qualitatively similar in both the test set and the learning set. The resulting discriminant functions effectively introduced a hierarchical structure to the classification scheme, where those features which had the greatest discriminatory power could over-ride some conditions for classification. While in absolute terms this is not a truly desirable quality, the consistency in classification afforded by this procedure is a useful trait for comparative measures of apoptotic frequencies.

**4.1.2 Learning Set Characteristics for Future Studies**

The use of discriminant functions as a method of providing consistent classification of apoptotic cells performs well. However, it should be noted that due to visible differences in texture between cells of different origin (for instance, human cell
Section 4 - Discussion

lines), the classification functions should be reconstructed for each cell line studied. In such a case, using information from the present study, how much data should be gathered for future learning sets? In the present case, the learning set was based on the sampling of 1000 cells from each of 80 different slides, resulting in less than 500 apoptotic cells. For CHO cells, the overall classification accuracy of the method was 98.9% (i.e. the percentage of all learning set cells correctly classified). In contrast, the percentage of apoptotic cells correctly classified correctly was 76.5% for the learning set and 81.7% for the test set. Because one would not normally expect a greater accuracy in the test data set this indicates the characteristics of the apoptotic cell feature distributions were slightly different between the two groups. This is most likely a result of the lower number of cells comprising the test set (approximately one-third of the learning set) and the influence of random errors. In future cases the size of the learning set might be decreased by building discriminant functions from cells irradiated with a dose which induces a greater level of apoptosis. The use of prior probabilities in the discriminant functions, which reflect the expected levels of the four classifications, could then be used to shift the constant term in the classification functions. This approach assumes that the features of the cell classifications are invariant with dose. This was indeed the case for the apoptotic cell population by t-tests on the features comprising the discriminant functions, from randomly chosen apoptotic cell images collected at 5 Gy and those collected at all other doses (N=300, p<0.05). However, normal cells irradiated at high doses exhibit a marked visible difference in cell area compared to unirradiated or low dose-irradiated cells. For example, from a single experiment, normal cells irradiated with 5 Gy had a mean area of 2900 ± 750 compared to an area of 2450 ± 600 for normals from control slides (although there is a large variance in area for each population, this
result is highly reproducible). A dose dependent size increase following irradiation has been noted by other investigators [139, 219]. Therefore, in the absence of a method of decreasing the learning set size necessary for accurate discrimination, the considerable work involved in compiling new learning sets for each cell line studied remains a significant drawback to this technique, and therefore its use should be restricted only to those studies such as the present one, where a large sample size is necessary to investigate the question under study.

A further note should be added that at this time, it is doubtful that this technique could have applications for clinical material. The considerable work required to construct a learning set of images for the varied nuclear textures of each tumour sites is a significant drawback. Furthermore, cells of varied origins and a large amount of debris are typically contained in clinical material and therefore would add several extra classifications to the discriminant analysis, which would effectively lower the overall accuracy of the technique.

4.2 APOPTOSIS FOLLOWING HIGH VERSUS LOW DOSE IRRADIATION

4.2.1 APOPTOSIS FOLLOWING 5 GY IS A POST-MITOTIC EVENT IN CHO CELLS

The time-course for apoptosis in irradiated CHO cells was shown in Figure 3.2. The only published data regarding radiation-induced apoptosis in the CHO cell lines is a single dose point at 15 Gy, evaluated at 24 hours post-irradiation and measured by the comet assay [201]. The value reported was 3%, roughly equivalent to the value obtained using the automated microscopic assay for cells irradiated at 5 Gy, which was 3.8% ± 1.3 after 24 hours. The 3-fold greater dose used in the comet assay study would be
expected to produce a larger apoptotic fraction. This may in part be a reflection in the end-point used to classify a cell as apoptotic. Extreme fragmentation of nuclear DNA, which migrates within the agarose gel when an electric field is applied, is used as the classification criterion for apoptotic cells in the comet assay. As DNA fragmentation is an early feature of apoptotic cells compared to the nuclear disintegration and blebbing, one might expect differences in the time-dependence of apoptosis as measured by the two methods. Although no other published data are available for this cell line, the development of apoptosis after 5 Gy of x-rays follows a pattern consistent with that found by other investigators, in other cell lines: following a specific time after irradiation, a rapid increase in the apoptotic fraction followed by a comparatively slower decrease. A peak apoptotic frequency of approximately 12% of cells was detected 42 hours post-irradiation. The colony-forming assay performed on CHO cells irradiated with 5 Gy results in a surviving fraction of approximately 6% (i.e. 94% of cells died) [220, 221]. It is difficult to estimate the fraction of this cell loss resulting of apoptotic death. While the cellular shrinkage and active blebbing of apoptotic cells have been observed in fibroblasts to have a duration of less than an hour [215], the length of time in which this morphology persists is unknown for CHO cells grown in suspension culture. Furthermore, the significant effects of cell cycle perturbations (i.e. G2-block, either reversible or irreversible) must be taken into account. However, ignoring any effects of cell doubling and persistent apoptotic morphology, the summation of all apoptosis measurements was 50% (cf. colony-forming assayed 94% death). The dependence of apoptosis on passage through at least one mitosis indicates that apoptotic cell death directly accounts for only a small fraction of cell death in CHO cells, a fact which concurs with the general belief that fibroblast cell lines generally die through non-apoptotic
mechanisms [222]. Although the necrotic fraction was not measured in these experiments, this most likely represents the primary form of cell death in CHO cells.

The initial appearance of apoptotic cells following radiation occurred somewhere between 12 and 18 hours. This coincides with the time when approximately half of the cells blocked in the G2 phase of the cell cycle were released. This finding is in agreement with the pattern observed in several other cell lines [201, 223, 224]. This in fact, may offer a possible explanation for the discrepancy between the comet assay result and those obtained here. The duration of the G2 delay is known to increase with x-ray dose [86, 225] and therefore measurement of the apoptotic fraction at 24 hours following 15 Gy may have been performed at a time when the majority of cells were still blocked within G2.

The increase of apoptosis which occurs immediately following the G2 release provides evidence that the primary form of apoptosis observed in CHO cells is dependent upon passage through mitosis. With the initial appearance of apoptosis occurring approximately 12 hours following initial subsiding of the G2 block, it can be inferred that the cells have most likely progressed through a single mitosis before undergoing apoptosis (this would also depend on the time required to develop the apoptotic morphology). In general, the appearance of post-mitotic apoptosis is believed to be related to abortive mitoses or chromosome aberrations, although more studies are needed [226]. It is unclear at this time whether the observed apoptosis occurred in the actual parent cells in which cytokinesis had not yet occurred, or whether it was observed in the daughter cells. Casual analysis of the DNA content of apoptotic cells showed that they varied widely, from sub-diploidy to near tetraploidy. Should apoptosis occur in the daughter cells only, the DNA content would be expected to be exclusively sub-diploid. An
incomplete description of the length of time required for DNA-staining loss in apoptotic cells confounds this problem, as sub-diploid apoptotic cells could actually be tetraploid cells which have lost the ability to bind the DNA stain in the time between the onset of apoptosis and its measurement by morphological appearance. In time-lapse studies of apoptosis in rat embryo cells, apoptosis caused by higher doses of radiation (9.5 Gy) occurred in subsequent generations, following one to four mitoses [215, 227].

4.2.2 Low Doses of X-Rays Result in “Delayed” Apoptotic Cell Death

Peak apoptosis levels for low dose irradiation (0.25 and 0.5 Gy) occurred at a similar time to the high dose (5 Gy) irradiated cells. The findings here would indicate that there is no temporal difference in the appearance of peak apoptotic levels following irradiation with high or low doses (both peaked at ~42 hours). Dose dependent kinetics in radiation-induced apoptosis have been reported in F9 teratocarcinoma cells [228], but an ovarian carcinoma irradiated in vivo failed to show such an effect [229]. Two separate studies in HL-60 cells have alternately reported both the presence [230] and absence [231] of a strictly temporal dose dependence, and each reported the appearance of a peak in apoptosis at different times. The kinetics of apoptosis-induction following irradiation is of special importance for the in vivo case, where cells are rapidly phagocytosed, and therefore more information regarding this feature is needed before the clinical implications of radiation-induced apoptosis may be understood.

While the peak apoptotic levels occurred at a similar time following low and high dose irradiation, the shapes of the curves were markedly different. Whereas the results of high dose irradiation described here, and described by others using different cell lines report a single peak followed by a decline in apoptotic frequency, the low dose data suggest the appearance of two peaks within the 72 hours studied (Figure 3.1 and 3.4).
Furthermore, no G2-block was detected at each dose up to 1.0 Gy, in contrast to the apparent dependence of the 5 Gy-induced apoptosis on the passage of the cells through the G2/M checkpoint. Considering the lack of cell cycle delay as noted in Appendix A, it is reasonable to assume that there was near-normal cell cycling times of 12 hours for the low dose irradiated populations. The appearance of apoptotic cells above control levels 36-42 hours post-irradiation suggests that the cells had progressed through the cell cycle approximately 3 times, and for the second observed peak, 5 times. The appearance of apoptosis following low doses of radiation is therefore observed in cells several generations removed from the irradiated parent cell. This is not unprecedented, as a "second wave" of apoptosis has been noted in irradiated HL-60 cells at significantly longer times [230].

4.2.3 Apoptosis in the Progeny of Irradiated Cells

The appearance of an apoptotic peak in high-dose irradiated cells after fewer mitoses than the similar peak observed at low doses indicates there may be a dose dependence in the mechanisms of apoptosis-induction. A dose-dependence for the number of mitoses prior to apoptosis has not been previously reported. There are however, reports of such differences between cells of varied origin. In particular, there are reports of both pre-mitotic [232, 233] and post-mitotic [224, 234] forms of apoptosis following irradiation: the former is a rapid interphase death, which does not require passage of the cell through mitosis, the latter is apoptosis occurring in the progeny of irradiated cells. Pre-mitotic apoptosis appears to have a rapid onset and occurs most

† During each experiment, this was checked by counting cells (Coulter counting) at each time point, however due to very large uncertainties from frequent dilutions, these measurements are unreliable and are thus are not included in the "Results" section.
prominently in those cells which are very radiosensitive [235], particularly cell lines of haematopoietic origin [222]. Rapid interphase death has been postulated to be a result of increased sensitivity of these lines to DNA double-strand breaks [222, 236] although there is other evidence that this form of death is induced through membrane damage alone [237, 238]. Lines of fibroblastoid origin appear to apoptose primarily by post-mitotic methods [235]. Vidair et al. have studied the post-mitotic form of apoptosis and observed a marked variation in the number of cell divisions which occur prior to the onset of apoptosis in fibroblasts, the majority of cells entering apoptosis 20-60 hours post-irradiation with 9.5 Gy, or following 1-4 mitoses [215]. Remarkably, both the time and number of mitoses before apoptosis varied widely even between sister cells (originating from the same stem cell). While this may be a surprising result in view of the general acceptance of apoptosis as a tightly regulated process, it has been widely accepted for many years that radiation-induced cytotoxicity in general may be delayed for several generations [2, 239], and that the mode and timing of death occurring in progeny is highly unpredictable [240, 241]. In fact, delayed effects in progeny of irradiated cells have been described in several different manifestations including variability in colony size, persistent reductions in plating efficiency, clonal heterogeneity and delayed chromosomal instability (see review by Morgan et al. [242]). In particular, Weissenborn and Streffer have shown that new chromosome aberrations may be expressed two to three mitoses following irradiation [243, 244]. The appearance of chromosome aberrations following one or more mitoses is known to be correlated with the subsequent death of the cells [245]. Following the hypothesis put forward by Vidair et al. [215], it is therefore possible that apoptosis induced following one or more mitoses is triggered by the appearance of chromosome aberrations. Clonal heterogeneity caused by low doses
Section 4 - Discussion

of radiation [240] would therefore explain the observed differences in post-mitotic apoptosis between low and high dose irradiation. It should be emphasised, however, that this apoptosis which occurs as a post-mitotic event still accounts only for a fraction of the observed cell death, and that the majority of cell death at either dose appears to be non-apoptotic.

4.3 NON-LINEAR APOPTOTIC RESPONSE AT LOW DOSES

In the original hypothesis put forth in this study, it was postulated that an induced repair mechanism which operates following low dose irradiation might be reflected in an inverse dose dependence for apoptosis. That is, at doses spanning the IRR range of the cell survival curve, a decrease in the apoptotic frequency would be observed. Despite the preceding discussion, examination of figure 3.4 reveals that this may indeed be the case: for each time point studied, the apoptotic fraction in cells irradiated with 0.25 Gy was greater than that observed in cells irradiated with 0.5 Gy. The cumulative levels of apoptosis displayed in Figure 3.5 illustrate this in a much more dramatic fashion. Over the 72 hours post-irradiation time period studied the 0.25 Gy irradiated cells produced approximately twice the amount of apoptosis as the cells irradiated with 0.5 Gy.

4.3.1 Is it Real?

The presentation of the "cumulative" apoptosis levels at various post-irradiation times (Figure 3.5) dramatically illustrates the greater apoptosis levels following 0.25 Gy compared 0.5 Gy. It may be noted however, from Figure 3.4, that only at 42 hours post-irradiation does the apoptotic fraction in the 0.25 Gy differ from that of the 0.5 Gy population by a large amount. This difference at a single time point is largely responsible for the high cumulative difference measured at 72 hours. However, the cumulative data
do indicate that the 0.25 Gy response is consistently greater over time, as a higher cumulative value is found immediately within the range of times included in the analysis (24 hrs. +). This is further demonstrated by the fact that for every time studied, the 0.25 Gy irradiated population showed a greater mean apoptotic fraction over three (6-42 hours) or two (48-72 hrs.) experiments (Figure 3.4).

T-tests performed on the experimental values of the apoptotic fractions for all doses at each time point, however indicated that no significant differences between any of the doses existed, save for a few exceptions. To illustrate this, data from individual experiments have been re-plotted in Figure 4.1 so that an idea of the overlap of the data might be obtained. Inspection of this plot reveals that the only time where all 0.25 Gy data are greater than the corresponding 0.5 Gy data is the point at 42 hours. Again these differences were not found to be statistically significant. The t-tests applied to this data were calculated in a pairwise fashion, grouping the individual dose data by the day of the experiment. This in essence assumes that the most significant source of variance between samples would arise due to experimental considerations. Because care was taken during each experiment to ensure all flasks (irradiated at different dose levels) were treated in exactly the same manner (i.e. temperature, pH of medium, general handling, sampling time, etc.) it is a valid assumption that these samples would not be completely independent†.

The fact that statistical analysis suggests that the differences are not significant is not surprising. Because a maximum of only three data points were used for each dose

† In fact, when the t-tests were applied in an independent manner, statistical significance in the differences in apoptotic fraction between 0.25 and 0.5 Gy and between 0.5 and 1.0 Gy was found. Generally, this test is more likely to generate significant results. However, it is difficult to justify the use of the independent test as a result of the above considerations.
Figure 4.1: All low dose apoptotic fraction data plotted from each of three experiments. Each point represents the mean of 3-4 slides sampled from the same cell population: 0.25 Gy (●); 0.5 Gy (▲); 1.0 Gy (□). At 42 hours, all 0.25 Gy measurements (●) were greater than the 0.5 Gy (▲).

point, the statistical tests were actually performed with very few degrees of freedom and therefore the validity of these tests is questionable. It is widely accepted that t-tests performed where \( n < 10 \) are considered suspect. This would indicate that for conclusive statistical validation of the difference between the 0.25 and 0.5 Gy cases, at least seven further experiments would be necessary. However, it is possible to forecast the number of additional measurements necessary before a significant difference between the two populations would be discernible. A more complete treatment of the assumptions and methods used for this approximation are provided in Appendix B.
As expected, due to the observed variations in both the differences between means and in the variances observed between 0.25 and 0.5 Gy responses at each time point, there were large differences in the predicted number of experiments, ranging from 4 to 760. Because the 42 hour time point represented the time which contributed most to the cumulative apoptosis data, this point may be of the greatest interest. With the data as presented, statistical analysis predicts that four experiments would be necessary to achieve a statistically significant difference, assuming the means of the two populations did not vary. However, considering only three experiments were performed, the addition of a fourth could affect these means by a considerable amount. Therefore, the calculations were performed again with using the lower limit of the 95% confidence interval for the 0.25 Gy mean apoptotic fraction (0.78%). In this case, there would be approximately 6 experiments needed to show a statistically significant difference. In light of the unexpected results of the priming experiments, where 0.25 Gy yielded less apoptosis than even the unirradiated controls (Section 3.3), even more experiments may be necessary before sufficient data is collected to obtain an accurate description of the low dose apoptotic response. As each experiment thus far has involved the preparation and scanning of approximately 250 slides, the effort remaining to satisfy these statistical requirements is considerable (at present performance of the system approximately 2 years).

4.3.2 RELATIONSHIP OF APOPTOSIS TO CELL SURVIVAL AT LOW DOSES

The original hypothesis set forth in Section 1.2.5 suggested the possibility that the induced repair mechanism postulated by Marples and Joiner [15] was coupled to apoptosis in such a way that the apoptotic mechanism would be down-regulated...
following a sufficient dose of radiation in order to allow the opportunity for damage repair. With the appearance of a greater level of apoptosis following 0.25 Gy than 0.5 Gy, it may follow that there is indeed a role for apoptosis in the development of increased radioresistance. Comparison of the apoptosis data with the low dose CHO response confirms that the decrease in apoptosis observed between 0.25 and 0.5 Gy occurs at similar doses to the appearance of IRR [220, 221]. Furthermore, the recovery of the apoptotic response following 1.0 Gy appears to agree with the overall decrease in the clonogenic survival found at this dose. Is apoptosis therefore at least partly responsible for the appearance of HRS/IRR?

In Section 4.2.2 it was noted that apoptosis in CHO cells in response to low dose irradiation appears to be a delayed response, occurring several generations removed from the initial irradiated cell population. While there is the possibility that a very few cells are irreversibly suspended within the cell cycle prior to apoptosis, it is evident from the 6-18 hour points in Figure 3.5, that there is little, if any, rapidly induced apoptosis that would be characteristic of the pre-mitotic form. The delayed appearance of apoptosis as reported here confuses the relationship between cell death by apoptosis and the clonogenic survival. If the apoptosis observed here were pre-mitotic, it could be directly related to the loss in clonogenicity as measured by the survival assay: that is to say that the reproductive failure of the cell could be directly attributed to the apoptotic mechanism. Perhaps unfortunately for this project, the majority of apoptosis observed seems to occur following several mitoses, where its impact on the colony-forming ability of the cell is uncertain. Therefore, an appropriate question regarding the consequences of apoptosis with respect to HRS/IRR may be: what proportion of the cells undergo
apoptosis before mitosis? And subsequently, can this account for the observed
differences in clonogenicity in the HRS/IRR region?

Another confounding factor of the delayed apoptotic response is the lack of
predictability of the time and rate at which daughter cells undergo apoptosis [215]. The
heterogeneity of the response between sister cells makes prediction of the impact upon
the survival assay difficult. For instance, in the typical application of the DMIPS assay for
cell survival, individual cells are allowed to grow for 3 days, which is equivalent to 6
progressions through the cell cycle. Normally proliferating colonies formed from
undamaged cells would therefore contain $2^6$ or 64 cells, greater than the 50 cell
minimum to be scored as a "survivor". Consider however, the scenario where apoptosis
claims a single cell following the second mitosis: the resulting colony would contain $2^4$
fewer, or 48 cells. In contrast, if 3 cells were to die following the fourth mitosis, the
impact on the colony size would be much less, such that $3 \times 2^2$ cells would be lost such
that 52 cells would form the colony at the time of counting, and therefore would be
scored as a survivor. Therefore, there is some potential for delayed apoptosis to affect
the results of the clonogenic assay performed on the irradiated cell population.

In fact, the dose-dependence of colony size has been investigated in V79
Chinese hamster cells using the DMIPS device [246]. Figure 4.2 shows the effect of low
dose irradiation on the size of colonies classified as survivors. In manner consistent with
the measured survival, the dose response exhibits a concave region at very low doses,
followed by a region of recovery mimicking the IRR, followed by a gradual decrease as
the applied dose escalates. While the valley and peak in colony size occur at different
doses for this particular cell line, this emphasises the possibility that the presence of
delayed apoptosis may indeed be reflected in the colony size of surviving cells. In the present study, the cells were followed for a similar duration as the incubation period for the DMIPS variant of the colony forming assay (72 hrs.), offering a possible explanation for the observed decrease in survival. The enhanced cell death in the progeny of cells noted at 0.25 Gy would result in a decreased colony size and therefore fewer colonies scored as survivors.

However, the HRS/IRR phenomenon is observed regardless of the method used for colony scoring (automated versus manual) [15, 21, 246] and incubation for longer periods of time has little impact [247]. As well, survival assessed in human cell lines by the FACS assay is determined following 2 weeks of incubation, thus allowing several more divisions to take place before colony counting [26, 27]. These results suggest another question which should be addressed, namely: what proportion of each cell’s progeny undergo apoptosis? Indeed, it should also be determined when these cells undergo apoptosis, as the kinetics of this response may have an impact on the size of the resulting colony.
4.3.3 Non-linearity of the Apoptotic Response to Low Doses

While there is insufficient information to judge the impact of the low dose apoptotic response with respect to clonogenic survival, the presence of an increased level of apoptosis at the 0.25 Gy level warrants an explanation. This response could be adequately explained if apoptosis occurred later in cells irradiated at 0.5 Gy than at 0.25 Gy. There is in fact a basis for this argument from studies of cell proliferation following very low doses of x-rays. There are reports that low dose irradiation in vivo can have a stimulatory effect on cell proliferation [248, 249]. In vitro, Korystov et al. recently demonstrated that the growth of Chinese hamster cells is stimulated by x-ray doses in the region of 0-0.5 Gy, with a peak stimulation occurring at 0.25 Gy [250]. This effect was found to give rise to an increase in both cell number and in the size of colonies as a result of a shortened lag phase of cell growth. Beyond this dose range, cell proliferation was inhibited due to cell cycle perturbations. In effect, they found that low-dose irradiated cells re-enter the cell cycle faster following attachment, when transferred from spinner culture. The apoptosis measurements performed here were carried out exclusively in suspension culture. However, irradiation was performed at room temperature before the cultures were returned to optimum growth conditions at 37°C and a similar lag phase could be expected. The effect of differences in the lag phase of growth are difficult to predict, but could result in the scenario whereby peak apoptotic rates are observed sooner at 0.25 Gy than at 0.5 Gy. Higher doses could result in slower proliferation, thereby masking this effect. While there is little indication as to the probability of this explanation from the data obtained (Figure 3.5), it suggests subsequent experiments should involve irradiation at 37°C to control for this effect.
The inverse dose response observed between 0 and 0.5 Gy could, in fact, reflect a greater propensity for apoptosis in cells irradiated with 0.25 Gy than those irradiated with 0.5 Gy. As there is an almost complete lack of information regarding the molecular responses to radiation doses in this range, explanations for this can only be hypothesised. While there are numerous reports of gene products induced or stabilised by ionising radiation which have been shown to be actively involved in the apoptotic process, organisation of these into a possible regulatory pathway for low-dose stimulation of apoptosis is difficult. Many of these activities are dependent upon the cell type used, and further dependent upon the particular clone which has been studied. And despite speculation, there appears to be little commonality in the apoptotic pathways between different cell lines. There is as yet no consistent information regarding the regulation of apoptosis related proteins in CHO cells. For instance, some investigators have found that ionising radiation acts to rapidly up-regulate both the apoptosis-promoting protein \textit{bax} [251] and the apoptosis antagonist \textit{bcl-2} [252, 253] in various cell lines. In our hands, no difference was found in either \textit{bcl-2} or \textit{bax} protein levels up to 42 hours following irradiation with 0-5 Gy (data not shown\textsuperscript{†}). Therefore, while it is possible to envision molecular pathways which could result in a non-linear apoptotic response following low dose irradiation, such speculation is premature and further information specific to CHO cells is needed.

\textsuperscript{†} While no discernible difference in \textit{bcl-2} or \textit{bax} levels were found between irradiated and unirradiated cell populations as measured by FACS detection of indirect immunocytochemical staining, a significant variation over time was found in both cell populations. These variations may be a result of several technical factors and thus these data were not included pending further investigation.
4.4 SUGGESTED FUTURE WORK

4.4.1 FURTHER DEFINITION OF THE LOW DOSE RESPONSE

As forecast in Section 4.3.1, a minimum of one to three further experiments would be necessary to show statistically significant differences at the 42 hour point between the 0.25 and 0.5 Gy apoptotic fractions (although it may be argued that at least 10 experiments in total are required for statistical validation). As this point seemed to contribute a significant portion of the difference between cumulative data, further data should be gathered for this time point. While a further characterisation of the complete time course would be desirable, in light of the limited information this technique provides due to the delayed nature of apoptosis in CHO cells, it may be more efficient to isolate this single time (and perhaps ± 6 hours) for further analysis. Furthermore, a more detailed characterisation of the dose response (for instance in 0.1 Gy increments from 0-1.0 Gy) at this time might prove relevant to the HRS/IRR phenomenon, by more precisely indicating the doses where the apoptotic response is down-regulated and where it recovers, in analogy to the IRR phenomenon. Moreover, it may be beneficial to extend the post-irradiation period to determine if any further apoptosis is observed (i.e. whether the 0.5 Gy irradiated cells simply take longer to undergo apoptosis).

It may also be relevant to the initial hypothesis to study the apoptotic response of cell lines known to be deficient in DNA repair processes, or in other cell lines which show no increased radioresistance. The presence of an inverse dose response for apoptosis only in those cell lines which exhibit HRS/IRR and not in others, would strongly support the hypothesis that apoptosis is involved in an induced response and suggest that this relationship should be further investigated, perhaps at a molecular level.
4.4.2 Time-lapse Studies of Apoptotic Response

The delayed nature of the apoptotic response has been noted by others in different cell lines. The use of time-lapse video analysis in some of these studies has allowed a more complete analysis of the number of mitoses prior to a cell's undergoing apoptosis [215]. Such a technique could be quite valuable in this instance to determine the impact of post-mitotic death on the resulting colony size in the cell survival assay. Without such information, the exact relationship between the inverse dose response of apoptosis observed at low doses and the HRS/IRR phenomenon as measured by cell survival cannot be determined.

The DMIPS system which is routinely used for the cell survival assay at low doses would be highly suitable for such purposes. A modified video-based DMIPS has been used to track individual cells over time [254, 255]. A simple modification could be made to adapt this system to phase-contrast microscopy, which enhances interfaces within biological material and would therefore highlight the nuclear and cytoplasmic blebbing of the apoptotic cells [256]. Following individual cells over the course of 72 hours or more would allow direct quantitation of the fraction of cells within individual colonies which undergo apoptosis and direct assessment of the impact this would have on cell survival.

4.4.3 Other Uses for Two-Colour Fluorescence Hardware/Software

The methods developed in the course of this study, while useful, have presented limited information regarding the involvement of apoptosis in the HRS/IRR phenomenon, they may prove significantly more useful for other applications, particularly in the study of micronuclei.
Micronuclei arise from whole chromosomes or acentric chromosome fragments that are not incorporated into the daughter nuclei at mitosis [257]. Thus, they appear as small membrane-enclosed pieces of chromatin separated from the nucleus, but still within the cytoplasmic boundaries of the cell. Since the use of texture features to characterise apoptotic cells has proven useful, the existing imaging software might be applied to the discrimination of cells containing micronuclei. Furthermore, the quantitative nature of the staining methods would allow densitometric analysis of individual micronuclei, which has been shown to provide further information not readily obtainable by conventional means [258].

The micronucleus assay has numerous applications, including biologic radiation dosimetry (assessment of dose received post-irradiation) [259], radiosensitivity prediction [260, 261] and drug and chemical mutagenicity testing [262, 263]. There are conflicting results as to whether the fraction of micronucleated cells following 2 Gy X-rays provides predictive information related to the surviving fraction, or intrinsic radiosensitivity. Interestingly, Abend et al. have shown that combined measurements of apoptotic fraction and micronucleus fraction correlate with the surviving fraction at 2 Gy [231]. An automated assay which could perform both measurements within a single cell population would be highly desirable to further investigate this relationship, and could have some clinical utility owing to the high sample throughput it would allow.
4.5 CONCLUSIONS

Prior to the initiation of this project, little was known regarding the apoptotic response of cell lines of fibroblast origin, and in particular CHO cells. Such cell lines were generally regarded as non-apoptotic and therefore little effort was extended towards characterising this response following irradiation. In fact, the present study has shown that CHO cells x-irradiated with 5 Gy exhibited a peak apoptotic response of 12%, and therefore the apoptotic potential of such cell lines may generally be under-estimated.

The apoptosis in CHO cells occurs following one or more mitoses, as evidenced by the appearance of apoptotic cells only following release of the G2 block in cells irradiated with 5 Gy. This is in agreement with studies involving other non-haematopoetic cells lines. However, the apparent lack of a dose-dependence in the time of appearance of peak apoptotic frequencies could not be predicted based on the conflicting results available in the literature. Furthermore, the delayed nature of apoptosis following low doses of x-rays (following several mitoses after irradiation) suggests that apoptosis in CHO cells is mediated not by initial damage, but as a consequence of tertiary damage caused by aberrant mitoses.

While the significance of apoptosis regarding the HRS/IRR phenomenon is still unclear, the greater level of apoptosis observed following 0.25 Gy irradiation compared to 0.5 Gy offers an explanation for variations in colony size observed following low dose irradiation. This underscores the need for further characterisation of the effects of colony size on the interpretation of results from the clonogenic assay.

A large portion of the work reported in this thesis has involved the development of an automated method for the detection and classification of apoptotic cells. This
technique, based upon the most reliable feature of apoptotic cells, their morphology, provides a standardised method for quantitation of apoptosis in cultured cells which is free of inter-observer variability. It will provide a valuable tool in the screening of other cytotoxic agents for apoptosis-induction and provides a technological basis for automated measurement of other end-points such as micronucleus formation.
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APPENDIX A

In the case of those cells irradiated with 5.0 Gy and followed over time, a distinct cell cycle delay was noted, where cells accumulated in G2 for up to 24 hours post-irradiation. This cell cycle perturbation was not noted in the control population, which remained relatively constant throughout the duration of the experiment. Figure A.1 illustrates the time dependence of the cell cycle parameters for the control population. Values in Figure A.1, as well as all further graphs of cell cycle parameters, represent the mean and standard deviation over three experiments. Variation in the control population over time was minimal, and no statistical difference was found between any of the times studied as tested by ANOVA.

For each of the other doses studied, the mean cell cycle parameters over the course of the experiment are listed in Table A.1. In all cases, there were no significant deviations in time as determined by ANOVA. Interestingly, the mean fraction of cells in G2/M over the course of the experiment showed a small increasing trend with dose, while the mean S-phase fraction showed a similar decrease. While these differences may reflect a small G2 block, no statistical differences were found when t-tests were performed on values from individual experiments. Further experimental data may provide a greater resolving power.

Table A.1: Cell cycle parameters after low doses over 72 hours. (Values are mean ± s.d.)

<table>
<thead>
<tr>
<th>Dose</th>
<th>% G1</th>
<th>% G2/M</th>
<th>%S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>39.3±4.2</td>
<td>14.2±3.6</td>
<td>46.7±5.9</td>
</tr>
<tr>
<td>0.25 Gy</td>
<td>38.7±3.8</td>
<td>15.5±3.1</td>
<td>45.3±4.1</td>
</tr>
<tr>
<td>0.5 Gy</td>
<td>38.4±4.4</td>
<td>17.4±3.0</td>
<td>44.3±4.6</td>
</tr>
<tr>
<td>1.0 Gy</td>
<td>39.0±3.1</td>
<td>19.0±3.5</td>
<td>41.9±4.3</td>
</tr>
</tbody>
</table>
Figure A.1: Variation of cell cycle parameters with time for control cells. Values represent averages of 2 (>42 hours) or 3 (≤42 hours) experiments, and error bars are standard deviation in the mean.
Figure A.2: Variation of cell cycle parameters with time for 0.25 Gy irradiated cells. Values represent averages of 2 (>42 hours) or 3 (<42 hours) experiments, and error bars are standard deviation in the mean.
Figure A.3: Variation of cell cycle parameters with time for 0.5 Gy irradiated cells. Values represent averages of 2 (>42 hours) or 3 (≤42 hours) experiments, and error bars are standard deviation in the mean.
Figure A.4: Variation of cell cycle parameters with time for 1 Gy irradiated cells. Values represent averages of 2 (>42 hours) or 3 (≤42 hours) experiments, and error bars are standard deviation in the mean.
APPENDIX B

The hypothesis we will be trying to test is that the mean apoptotic frequency following 0.25 Gy is greater than that at 0.5 Gy. We first assume that measurements of the apoptotic frequency are normally distributed with true means $\mu_{0.25}$ and $\mu_{0.5}$. Our measured values have sample means of $\overline{X}_{0.25}$ and $\overline{X}_{0.5}$, and sample variances of $S^2_{0.25}$ and $S^2_{0.5}$. In the current case, it will be assumed that the sample variances are in fact representative of the true population variances, $\sigma^2_{0.25}$ and $\sigma^2_{0.5}$.

We will test the null hypothesis,

$$H_0: \mu_{0.25} = \mu_{0.5}$$

and the alternate hypothesis,

$$H_1: \mu_{0.25} > \mu_{0.5}$$

To test the null hypothesis then, the difference between sample means such that,

$$Z_0 = \frac{\overline{X}_{0.25} - \overline{X}_{0.5}}{\sqrt{\frac{\sigma^2_{0.25} + \sigma^2_{0.5}}{n}}} \approx N(0,1),$$

where the $\sigma^2/n$ term represents the variance in the mean $\mu$. At this point, it should be noted that if $\sigma^2_{0.25}$ and $\sigma^2_{0.5}$ are not equal, then $Z_0$ is not necessarily normally distributed. Therefore, this represents a Behrens-Fisher problem, for which approximations exist based on Bayesian inference exist. However, this approximation requires at least 6 degrees of freedom ($n = 7$) and therefore is not applicable to the
problem at hand. Furthermore, it most situations, the deviation of $Z_0$ from normality is negligible.

Given that $H_0$ is true then, the probability of rejecting it is $\alpha$. In terms of the distribution of the difference between measured sample means $\bar{X}_{0.25}$ and $\bar{X}_{0.5}$, then $H_0$ is rejected when

$$\frac{\bar{X}_{0.25} - \bar{X}_{0.5}}{\sqrt{\frac{\sigma^2_{0.25} + \sigma^2_{0.5}}{n}}} > z_{1-\alpha} \quad \text{(Eq. 1)}$$

where $z_{1-\alpha}$ is the quantile of the standard normal distribution such that $P(Z \geq z_{1-\alpha}) = \alpha$.

Similarly, for the alternate hypothesis, $H_1$, where $\beta$ is the defined limit of the type II error, then

$$\frac{\bar{X}_{0.25} - \bar{X}_{0.5} - (\mu_{0.25} - \mu_{0.5})}{\sqrt{\frac{\sigma^2_{0.25} + \sigma^2_{0.5}}{n}}} > z_{\beta} \quad \text{(Eq. 2)}$$

where $z_\beta$ is the point of the standard normal distribution such that $P(Z < z_\beta) = \beta$.

To determine $n$, equations (1) and (2) can be solved for $\bar{X}_{0.25} - \bar{X}_{0.5}$ and equated to give

$$n = \frac{(\sigma^2_{0.25} + \sigma^2_{0.5})(z_{1-\alpha} - z_\beta)^2}{(\mu_{0.25} - \mu_{0.5})^2} \quad \text{(Eq. 3)}$$
which assures the probabilities $\alpha$ and $\beta$ for type I and type II errors, respectively for testing the null and alternate hypotheses.

Application of equation (3) above requires knowledge of the true means and standard deviations of the apoptotic responses for 0.25 and 0.5 Gy. The problem at hand therefore requires the assumption that the measured sample means $\bar{X}_{0.25}$ and $\bar{X}_{0.5}$ are faithful approximations of the populations means $\mu_{0.25}$ and $\mu_{0.5}$. Similarly, assumptions must be made regarding the true variances of the measured populations.

The values of standard error in the mean as reported in Figure 3.5 are the estimates of the biological variation as measured over three experiments. While this may accurately reflect the inter-experiment variance, another significant source of variance is the intra-experimental error resulting from sampling of individual slides, as reported in Table 2.15. In effect, the sampling of the slide to determine the number of apoptotic cells is a counting experiment and therefore follows Poisson statistics. Examination of Table 2.15 reveals that this is approximately true when viewed in terms of the number of cells counted. For instance, in the high dose case, the mean number of apoptotic cells sampled over the 10 slides was 43. The expected standard deviation from Poisson statistics is therefore $\sqrt{43}$ or 6.6. The observed value was 9.7. Similarly, in the low dose case, an average of 1 apoptotic cell was detected per slide, yielding a Poisson error of 1.0, similar in magnitude to the 0.7 observed. It is therefore appropriate to apply Poisson statistics to the intra-slide variance.

The variance introduced by biological sources can be estimated from the spread in experimental means. An estimate of the variance of resulting distribution incorporating
Appendix B - Statistical Analysis

both the slide sampling variance and the biological variance may therefore be obtained by the addition of the variance from each: the Poisson error from intra-slide sources and the calculated variance between experiments for the biological.

In those cases calculated in Section 4.2.1, the power of the test was determined at levels of $\alpha=0.05$ and $\beta=0.10$, for which $z_{1-\alpha}=1.96$ and $z_\beta=-1.28$. Table B.1 lists the variances used in the calculations together with the resulting values of $n$, calculated from Equation (3). As expected, due to variations in the variances between different times, the number of experiments forecast varies considerably. Further tests were done using the upper and lower bounds for the 42 hours point means to determine the "worst case" scenario. At the 42 hours point, there were on average $9.7 \pm 1.9$ apoptotic cells detected per 100 cells. Therefore, the calculations in Table B.1 were performed using a mean $\mu_{0.25} = 9.7 - 1.9 = 7.8$ as the basis for comparison instead. The resulting number of experiments was $n=6$.

Table B.1: Number of experiments required for statistical validation of difference in means. For each time studied, the experimental mean and calculated variances (see text) are listed, together with the number of experiments, as calculated in Equation (3).

<table>
<thead>
<tr>
<th>Time</th>
<th>$\mu_{0.25}$</th>
<th>$\sigma^2_{0.25}$</th>
<th>$\mu_{0.5}$</th>
<th>$\sigma^2_{0.5}$</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>3.17</td>
<td>4.50</td>
<td>1.48</td>
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