From Birds to Drug-Resistant Cancer cells, a Novel \textit{in situ} Methodology to Explore Divergent Genome Evolution

A thesis submitted to the University of Kent for the degree of

DOCTOR OF PHILOSOPHY

In the Faculty of Sciences

2019

Jacob Ward

School of Biosciences
Declaration

No part of this thesis has been submitted in support of an application for any degree or qualification of the University of Kent or any other University or institute of learning

Jacob Ward
Acknowledgements

Firstly it was with great honour to work under supervision of Prof. Darren Griffin these past few years, whose friendship, guidance and generosity has made this entire work possible and his teachings will stay with me throughout my future career.

My thanks also extends to Dr Ellis, whose support and infectious enthusiasm has been invaluable during time spent throughout the PhD, alongside Dr Skinner, where both were foolish enough to listen to my preposterous ramblings and hypotheses regarding the interphase plotting presented in this thesis and aid in its creation. I am also grateful for the friendship shown by Dr Mansfield who I admire and aspire to be as good of a teacher as she is one day.

My thanks extent to current and past members of the Griffin and Ellis Lab, who have been of great support and teaching throughout my time as a member of the laboratory. Whether it is through friendship, compassion or the acknowledgment of my previously undiscovered in-humane strength to break multiple pieces of equipment; I shall take memories made with them forward and cherish them fondly.

I am grateful for the support Michael Ellis (Digital Scientific UK) has provided, from his aid in using his image capturing software to enabling the creation of the new method and for his perspective into possible avenues of improving current technologies. I wish to extend my thanks for the cell lines kindly provided by Prof. Michaelis and Dr Lowndes, on which many sections of this thesis use extensively and I hope my work is of somewhat a benefit to their future research.

I also extend a very special thanks to members of the UMC and associated friends, to Dr Munn for exceptional phlebotomy skills, to Mr Jackson for his excellent down to earth chats, to the work of Prof. Hale, Dr Babiker and Mr Tomlinson and lastly a very special thanks to Mrs Shrimpton whose widely admired ability to write a stern letter has been of great help and I am eternally grateful for her dedication to aid in my time spent here.

The friends I have made and friends who have stayed with me, for keeping me in line and sane I am thankful for and I wish many more years of friendship towards.

Last but not least, my better-half and partner Jessie Norbury for being by my side throughout my journey these last few years supporting me through what life challenges us with. Nothing would be possible without you.
Dedicated to
My Parents, Julie and Simon
Along with
My Grandparents, Doreen and Brian
# Table of Contents

Declaration .................................................................................................................. I  
Acknowledgements ..................................................................................................... II  
Table of Contents ......................................................................................................... IV  
List of figures ................................................................................................................ IX  
List of tables .................................................................................................................. XII  
List of abbreviations .................................................................................................... XIII  
Abstract ......................................................................................................................... XV  

1. Introduction - An overview of cytogenetics ......................................................... 1  

1.1 The Chromosome .................................................................................................... 1  
  1.1.1 A brief history of the chromosome ................................................................. 1  
  1.1.2 Eukaryote chromosome structure ................................................................. 1  
  1.1.3 The cell cycle and cell division ...................................................................... 3  
    1.1.3.1 Interphase ............................................................................................. 3  
    1.1.3.2 Mitosis ................................................................................................. 4  
  1.1.4 Chromosomal abnormalities and aberrations ............................................. 6  
    1.1.4.1 Aneuploidy .......................................................................................... 6  
    1.1.4.2 Deletions .............................................................................................. 6  
    1.1.4.3 Duplications ......................................................................................... 7  
    1.1.4.4 Translocations ..................................................................................... 8  
    1.1.4.5 Inversions ........................................................................................... 10  
    1.1.4.6 Rings .................................................................................................. 11  
    1.1.4.7 Isochromosomes ................................................................................ 11  
  1.1.5 Cytogenetic methods of past and present ..................................................... 13  
    1.1.5.1 Karyotyping ....................................................................................... 13  
    1.1.5.2 Whole chromosome banding ................................................................. 15  
    1.1.5.3 In situ hybridisation ............................................................................ 16  
    1.1.5.4 Digital/Virtual Karyotyping ................................................................. 21  
    1.1.5.5 Fluorescence microscopy ..................................................................... 24  
  1.2 Comparative Genomics ....................................................................................... 25  
    1.2.1 Evolutionary genomics .............................................................................. 27  
      1.2.1.1 Chromosomal mechanisms of evolution and speciation .................. 28  
    1.2.2 Animal genomics ....................................................................................... 29  
    1.2.3 Avian Genomics ......................................................................................... 31  
      1.2.3.1 The Galliformes ............................................................................... 32
1.3 The Biology of Cancer .................................................................................................................. 34
1.3.1 A brief overview of cancer .......................................................................................................... 34
1.3.2 Causes of Tumorigenesis ............................................................................................................ 38
  1.3.2.1 Environmental Carcinogens ................................................................................................. 38
  1.3.2.2 Infectious agents .................................................................................................................... 41
  1.3.2.3 Genetic contributions .............................................................................................................. 43
    1.3.2.3.1 Oncogenes ....................................................................................................................... 45
    1.3.2.3.2 Tumour suppressor genes ............................................................................................... 47
    1.3.2.3.3 Epigenetics and microRNAs ............................................................................................ 49
  1.3.3 The treatment of cancer ............................................................................................................ 50
  1.3.4 Cancer Genomics ...................................................................................................................... 54
    1.3.4.1 Heterogeneity of cancer ....................................................................................................... 56
      1.3.4.1.1 Clonal evolution/ Somatic evolution ................................................................................. 58
      1.3.4.1.2 Cancer stem cells ............................................................................................................ 59
    1.3.4.2 Chromoanagenesis, Chromoplexy and chromothripsis ...................................................... 61
  1.3.5 Non human models for cancer .................................................................................................. 66
1.4 Thesis rationale ............................................................................................................................... 68
1.5 Thesis Aims ..................................................................................................................................... 70

2. Materials and methods .................................................................................................................... 71
  2.1 Materials ....................................................................................................................................... 71
    2.1.1 Chromosome preparation ........................................................................................................ 71
      2.1.1.1 Chromosome preparation from culture ................................................................................. 71
      2.1.1.2 Chromosome preparation from Human blood ......................................................................... 71
    2.1.2 Cell cultures ............................................................................................................................ 72
      2.1.2.1 Avian cells .......................................................................................................................... 72
      2.1.2.2 Cattle cells .......................................................................................................................... 72
      2.1.2.3 DT40 .................................................................................................................................... 72
      2.1.2.4 Human Cancer lines .......................................................................................................... 73
    2.1.3 Fluorescent Probes ................................................................................................................. 73
  2.2 Methods ....................................................................................................................................... 74
    2.2.1 Fluorescence in situ hybridisation (FISH) ................................................................................ 74
      2.2.1.1 Standard FISH ................................................................................................................... 74
      2.2.1.2 IQ FAST Fluorescence in situ hybridisation ....................................................................... 75
      2.2.1.3 Multilayer FISH ............................................................................................................... 75
    2.2.2 Fluorimeter measurements ....................................................................................................... 76
      2.2.2.1 Manual measurement ......................................................................................................... 76
2.2.2.2 Automatic measurement .......................................................... 76
2.2.3 Chromosome Interphase Plotter (CIP) ............................................. 76
2.2.4 Imaging software and hardware ..................................................... 77
2.2.4.1 Merging of Images .................................................................... 77

3. Improving FISH technology to facilitate more rapid, cost-effective multiplex results (specific aim 1) .................................................................................... 78
3.1 The formalities of FISH hybridisation ................................................. 78
3.2 Exploring the option of different hybridisation buffers ....................... 82
  3.2.1 The recipe for good FISH ............................................................... 82
3.3 Comparison of commercial buffers for FISH analysis ....................... 83
  3.3.1 Same species comparison of hybridisation times .......................... 83
  3.3.2 Zoo-FISH comparison of hybridisation times ............................... 87
3.4 Development of the Multilayer FISH method .................................. 90
  3.4.1 Methodology of Multilayer FISH ................................................ 91
  3.4.1.1 Removal of previously hybridised probes ............................... 91
  3.4.1.2 The Iterative Cycle of Multilayer FISH ................................. 92
  3.4.2 The effects of Multilayer FISH on metaphase preparations and probes ...... 94
3.5 Limitations of the Multilayer Method ................................................. 97
3.6 Discussion ......................................................................................... 100
  3.6.1 Exploration into alternative buffers ........................................... 100
  3.6.2 Comparison of commercial buffers .......................................... 100
  3.6.3 The novel Methodology of Multilayer FISH ............................. 101
    3.6.3.1 The effects of Multilayer FISH protocol .......................... 103
3.7 Conclusion ....................................................................................... 104

4. Using the Multilayer FISH Strategy to Improve Our Understanding of Galliform evolution (specific aim 2) ............................................................................. 106
4.1 Cytogenetics of the aves ................................................................. 106
4.2 Galliform chromosome rearrangements ......................................... 109
  4.2.1 Measuring FLpter: script vs. Manual measurements .................. 109
  4.2.2 Genome organisation in Galliform chromosomes 1-5 ............ 111
    4.2.2.1 Chromosome 1 ................................................................. 111
    4.2.2.2. Chromosome 2 ........................................................... 114
    4.2.2.3 Chromosome 3 ............................................................. 117
    4.2.2.4 Chromosome 4 ............................................................. 120
    4.2.2.5 Chromosome 5 ............................................................. 123
  4.2.6 FLpter results ............................................................................. 126
4.3 Development of a Chromosome Interphase Plotter for chromosomal nuclear arrangement ................................................................. 128
4.3.1 Interphase specific Multilayer protocol ................................................................. 128
4.3.2 Mechanics of the Path-Finding Tool ...................................................................... 130
4.3.3 Interphase chromosome analysis ........................................................................... 133
4.3.4 Intricacies of macrochromosome folding ............................................................. 135
4.3.4.1 Normalisation of chromosomes ........................................................................ 135
4.3.4.2 Folding arrangements of G.gallus chromosome 3, 4 and 5 ............................... 136
4.4 Discussion .................................................................................................................. 138
4.4.1 Comparison of measuring techniques ................................................................. 138
4.4.2 Detection of chromosomal rearrangements in Galliform ................................. 138
4.4.3 The Chromosome Interphase Plotter ................................................................. 140
4.4.3.1 Mechanics of the Path-Finding Tool ............................................................... 140
4.4.3.2 Folding arrangements of chromosomes ......................................................... 141
4.4.3.3 The means to predict an unknown order ......................................................... 141
4.4.3.4 Limitations of Interphase studies ................................................................. 142
4.5 Conclusion ................................................................................................................. 142

5. Rapid analysis of the karyotype of DT40 (Specific aim) .......................................... 144
5.1 The characteristics of DT40 ...................................................................................... 145
5.2 The Multiprobe Chicken device plan ....................................................................... 147
5.3 DT40 FISH results .................................................................................................. 148
5.3.1 Chromosome 11 and 28 ....................................................................................... 149
5.3.2 Chromosome 15 and 24 ...................................................................................... 150
5.3.3 Chromosome 2, 5, 8 and 20 ................................................................................ 150
5.3.4 Chromosome 6, 7, 9 and 19 ................................................................................ 151
5.4 DT40 karyotype results ........................................................................................... 152
5.4.1 The state of metaphases ...................................................................................... 152
5.4.2 The state of the karyotype ................................................................................... 153
5.4.3 The State of heterogeneity and microchromosomes ........................................... 156
5.4.4 Final cytogenetic assignment .............................................................................. 156
5.5 Discussion ................................................................................................................. 157
5.6 Conclusion ................................................................................................................. 159

6. Cytogenetic assignment of resistant neuroblastomas by chromosomal aberration detection (Specific aim 4) ................................................................. 161
6.1 Cancer cytogenetic studies thus far ........................................................................... 161
6.1.1 Cancer aberrations .............................................................................................. 161
6.1.2 Neuroblastoma ................................................................................................... 162
6.1.3 Cancer cytogenetics of past and present ......................................................... 163
6.1.4 Method of Multilayer probe groups ................................................................. 165
6.2 Metaphase analysis ................................................................................................. 168
List of figures

Figure 1.1  The cell cycle for eukaryotic cells
Figure 1.2  Examples of human cells in stages of mitosis
Figure 1.3  Schematic representations of chromosomal aberrations.
Figure 1.4  Normal Human male karyotype with ideogram
Figure 1.5  Fluorescence hybridisation of human chromosomes
Figure 1.6  FISH principle and procedure
Figure 1.7  SKY (spectral karyotyping) karyotype of a healthy normal human female, 46<2n>,XX
Figure 1.8  Overview of Array CGH methodology and principles
Figure 1.9  A global analysis technique vs. a single cell approach
Figure 1.10  A simplistic representation of a fluorescent microscope and parallel atomic events
Figure 1.11  Cancer hallmarks and appropriate therapeutic targeting.
Figure 1.12  Intracellular signalling networks of cancer cell regulation.
Figure 1.13  Process of metastasis in typical cancer to colonize new sites
Figure 1.14  Theories of establishing progressive tumour heterogeneity
Figure 1.15  Comparisons of Chromothripsis and Chromoplexy
Figure 1.16  Publications using either bulk sequencing or single cell sequencing techniques
Figure 3.1  Metaphase frequency of successful hybridisation using Hyb I at 60 minutes and under time-points.
Figure 3.2  Metaphase frequency of successful hybridisation using FAST at 60 minutes and under time-points.
Figure 3.3  Metaphase frequency of signal identified at various forced exposure time points upon B.taurus using same species probes.
Figure 3.4  Metaphase frequency of successful hybridisation over 2 days using Hyb I on S.camelus using G.gallus probes
Figure 3.5  Metaphase frequencies of successful hybridisation using FAST over 1 day and 2 day hybridisations
Figure 3.6  Metaphase frequency of signal identified at various forced exposure time points upon Struthio camelus (common ostrich) using G.gallus probes
Figure 3.7  The Multilayer FISH iterative protocol shown in Flowchart form
Figure 3.8  Multilayer FISH does not cause probes to incorrectly position after additional layers
Figure 3.9  An exemplar schematic of a three layered FISH image
Figure 3.10  The maximum layer count achieved using current methods.
Figure 3.11  Five layered chicken chromosome 1 in more detail
Figure 4.1  Phylogenetic Tree of Galliformes studied in this thesis with notable inter-chromosomal changes
Figure 4.2  Probe position placements for G.gallus chromosome 2 measured via different methods
Figure 4.3  Merged metaphase images of five galliformes using selected G.gallus chromosome 1 BAC’s
Figure 4.4  Probe point plot for galliform chromosome 1 for five species, and corresponding chromosome schematic
Figure 4.5  Merged metaphase images of five galliformes using selected G.gallus chromosome 2 BAC’s
Figure 4.6  Probe point plot for galliform chromosome 2 for five species, and corresponding chromosome schematic
Figure 4.7  Merged metaphase images of five galliformes using selected G.gallus chromosome 3 BAC’s
Figure 4.8  Probe point plot for galliform chromosome 3 for five species, and corresponding chromosome schematic
Figure 4.9  Merged metaphase images of five galliformes using selected G.gallus chromosome 4 BACs
Figure 4.10  Probe point plot for galliform chromosome 4 for five species, and corresponding chromosome schematic
Figure 4.11  Merged metaphase images of five galliformes using selected G.gallus chromosome 5 BAC’s
Figure 4.12  Probe point plot for galliform chromosome 5 for five species, and corresponding chromosome schematic
Figure 4.13  Multilayer G.gallus chromosome 3 interphase nuclei with schematic representation of chromosome folding pattern.
Figure 4.14  Graphical outlines of three different permutations of the interphase chromosome path-finding tool of five probes.
Figure 4.15  Excel screenshot of working example of the interphase chromosome path-plotting tool using a G.gallus chromosome 3.
Figure 4.16  Multiple multilayer G.gallus interphase nuclei with schematic representation of chromosome folding pattern.
Figure 4.17  Overview of final schematic interphase nuclei with overlay of data marks.
Figure 4.18  Graphic example of how to normalise chromosome data for analysis
Figure 4.19  G.gallus chromosome 3 interphase common folding pattern
Figure 4.20  G.gallus chromosome 4 interphase common folding pattern
Figure 4.21  G.gallus chromosome 5 interphase common folding pattern
Figure 5.1  Organisation and false colouration of the multiprobe device and glass slide orientation used in this study
Figure 5.2  Chicken chromosome 11 and 28 FISH results on a DT40 metaphase
Figure 5.3  Chicken chromosome 15 and 24 FISH results on a DT40 metaphase
Figure 5.4  Chicken chromosome 2, 5, 8 and 20 FISH results on a DT40 metaphase
Figure 5.5  Chicken chromosome 6, 7, 9 and 19 FISH results on a DT40 metaphase
Figure 5.6  An exemplar DT40 metaphase observed
Figure 5.7  Full chromosome count and frequency for karyotyped DT40 metaphases
Figure 6.1  Metaphase spreads for all the NB3 Cell lines used
Figure 6.2  Probe point plot showing derivative chromosome 1 for NB3 parental/rCis/rVin FLpter scores and corresponding chromosome schematic
Figure 6.3  Merged multilayer FISH images of Human and three NB3 cell lines from panel 1
Figure 6.4  Hypothesised Phylogenetic tree as per results from FISH panel 1
Figure 6.5  Merged multilayer FISH images of Human and three NB3 cell lines from panel 2
Figure 6.6  Hypothesised Phylogenetic tree as per results from FISH panel 2
Figure 6.7  Merged multilayer FISH images of Human and three NB3 cell lines from panel 3
Figure 6.8  Hypothesised Phylogenetic tree as per results from FISH panel 3
Figure 6.9  Average aberrations away from the modal cell subpopulation in each panel tested.
Figure 6.10  Examples of the most mutated subpopulation metaphases in respect to the ‘modal’ subpopulation
List of tables

Table 2.1  
List of Human derived cancer cell lines used in this study

Table 3.1  
Hansen’s Solubility parameters for various organic solvents

Table 3.2  
Differences in methodology within multilayer FISH

Table 3.3  
BAC information and ordering for *G.gallus* chromosome 1 for multiple Multilayer FISH rounds

Table 3.4  
Limitations of traditional FISH with resolutions found in the multilayer method

Table 4.1  
Probes used for metaphase chromosome analysis for Galliform chromosome 1

Table 4.2  
Probes used for metaphase chromosome analysis for Galliform chromosome 2

Table 4.3  
Probes used for metaphase chromosome analysis for Galliform chromosome 3

Table 4.4  
Probes used for metaphase chromosome analysis for Galliform chromosome 4

Table 4.5  
Probes used for metaphase chromosome analysis for Galliform chromosome 5

Table 4.6  
All tables for every chromosome with probe’s FLpter score

Table 4.7  
Minor hypotheses tested within the interphase study

Table 5.1  
Summary of chromosomal aberrations identified via FISH in DT40

Table 6.1  
Probes selected for group 1 panel analysis for the NB3 lines

Table 6.2  
Probes selected for group 2 panel analysis for the NB3 lines

Table 6.3  
Probes selected for group 3 panel analysis for the NB3 lines

Table 6.4  
Probe panel 1 aberration frequency across all cell lines.

Table 6.5  
FLpter scores for derivative chromosome 1 within; NB3 parental/ rCisplatin / rVincristine, with relative distances between signals.

Table 6.6  
Probe panel 2 aberration frequency across all cell lines.

Table 6.7  
Probe panel 3 aberration frequency across all cell lines.

Table 6.8  
Results from chromosome 17 variant analysis
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>aCGH</td>
<td>array comparative genomic hybridisation</td>
</tr>
<tr>
<td>ALK</td>
<td>Anaplastic lymphoma kinase</td>
</tr>
<tr>
<td>ALL</td>
<td>Acute lymphoblastic leukaemia</td>
</tr>
<tr>
<td>ALV</td>
<td>Avian Leukosis virus</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid Leukaemia</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BACs</td>
<td>Bacterial Artificial Chromosomes</td>
</tr>
<tr>
<td>BRAF</td>
<td>B-Raf proto-oncogene</td>
</tr>
<tr>
<td>BRCA 1/2</td>
<td>Breast Cancer type 1/2 susceptibility protein</td>
</tr>
<tr>
<td>CDR1/2</td>
<td>Cerebellar degeneration-related protein 1/2</td>
</tr>
<tr>
<td>CGH</td>
<td>Comparative genomic hybridisation</td>
</tr>
<tr>
<td>Cis</td>
<td>Cisplatin</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic myeloid leukaemia</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>ddH2O</td>
<td>Double Distilled water</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ERBs</td>
<td>Evolutionary breakpoint regions</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FISH</td>
<td>fluorescence in situ hybridisation</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FLpter</td>
<td>Fraction of length from p terminus</td>
</tr>
<tr>
<td>GIMP</td>
<td>GNU Image Manipulation Program</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent protein</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks' Balanced Salt Solution</td>
</tr>
<tr>
<td>HGP</td>
<td>Human Genome Project</td>
</tr>
<tr>
<td>hiPS</td>
<td>human induce pluripotent cells</td>
</tr>
<tr>
<td>HSBs</td>
<td>Homologous synteny blocks</td>
</tr>
<tr>
<td>ISH</td>
<td>In situ hybridisation</td>
</tr>
<tr>
<td>LINE</td>
<td>long interspersed regions</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>M-FISH</td>
<td>Multiplex fluorescence in situ hybridisation</td>
</tr>
<tr>
<td>Mb</td>
<td>mega base pairs</td>
</tr>
<tr>
<td>MDS</td>
<td>Myelodysplastic syndrome</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimal Essential Media</td>
</tr>
<tr>
<td>mFISH</td>
<td>Multicolour fluorescence in situ hybridisation</td>
</tr>
<tr>
<td>MFN2</td>
<td>Mitofusin-2</td>
</tr>
<tr>
<td>NGS</td>
<td>next generation sequencing</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non-homologous end joining</td>
</tr>
<tr>
<td>mlFISH</td>
<td>Multilayer fluorescence in situ hybridisation</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PMP22</td>
<td>Peripheral myelin protein 22</td>
</tr>
<tr>
<td>REEP3</td>
<td>Receptor Accessory Protein 3</td>
</tr>
<tr>
<td>S.E.M</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SHH-MB</td>
<td>Sonic-hedgehog medulloblastoma</td>
</tr>
<tr>
<td>SINE</td>
<td>short interspersed sequences</td>
</tr>
<tr>
<td>SKY</td>
<td>Spectral Karyotyping</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SRY</td>
<td>Sex Determining region Y</td>
</tr>
<tr>
<td>SSC</td>
<td>Saline-Sodium Citrate buffer</td>
</tr>
<tr>
<td>TP53/p53</td>
<td>Tumour protein 53</td>
</tr>
<tr>
<td>USSR</td>
<td>Union of Soviet Socialist Republics</td>
</tr>
<tr>
<td>UKF-NB3</td>
<td>A Neuroblastoma cell line</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>V(D)J recombination</td>
<td>Variable, Diversity and Joining gene recombination</td>
</tr>
<tr>
<td>Vin</td>
<td>Vincristine</td>
</tr>
<tr>
<td>WHIM syndrome</td>
<td>Warts, Hypogammaglobulinemia, Immunodeficiency and Myelokathexis</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organisation</td>
</tr>
</tbody>
</table>
Abstract

Fluorescent hybridisation methodologies have not changed in principles over the past 30 years, with the increase of computational sequencing technologies causing the replacement of in situ hybridisations. Fluorescence *in situ* hybridisation (FISH) is in need of a refresh to be a worthwhile tool in a modern day cytogenetic laboratory to overcome short comings of these new methods. The creation of the novel multilayer FISH protocol has effectively eliminated many negative aspects of classic FISH based experiments, such as a large reduction in cost and is no longer as limited by fluorophore availability. Here presented within this thesis is the creation of this methodology and application to a wide variety of cytogenetic hypotheses.

Key species from the Galliform order were investigated in order to detect previously missed intrachromosomal rearrangements within their macrochromosomes, a premise formerly overlooked. Rearrangements were found within chromosomes of the galliforme species used such as *E.chinensis* which displays a intrachromosomal inversion on the p-arm of chromosome 2. Furthermore, the creation of an interphase state folding prediction tool has been used to assess the arrangement of macrochromosomes during cellular growth stages within *G.gallus*. Here it is noted that there are particular arrangements identified which are similar across chromosomes studied.

The chicken lymphoma cell line DT40 is of great importance in B-cell receptor studies along with gene disruption experiments. Presented here is an updated karyotype for the cell line. Here shows contrasting and more in-depth evidence of aberrations to further develops our understanding of the genomic arrangement of this useful cell line.

The level of tumour heterogeneity in a cancer is a diagnostic tool allowing clinicians to comment on therapeutic choices and prognosis of the disease. Found to be dominant in recurrent cancers, cytotoxic resistant tumour cell populations may indeed exist within initial primary tumours at low frequency to be positively selected during chemotherapy. Within a neuroblastoma cell line, and cyto-toxic resistant derivatives lines, there has been identified a level of genomic heterogeneity which may give clues towards the generation of drug resistance mechanisms.
1. Introduction - An overview of cytogenetics

1.1 The Chromosome

1.1.1 A brief history of the chromosome

Thought to be first observed by von Nägeli in 1848 (Nägeli, 1884) the naming of the chromosome, and more precisely chromatin, was coined by von Waldeyer-Hartz (Waldeyer, 1888) alongside W. Flemming who proposed the idea of a heritable element within the nucleus came from a parental nucleus (Flemming, 1878). It was not until the early 1900’s until the karyotype was defined as the phenotypic representation of the genetic content found within cells in the form of somatic chromosomes. (Levitsky, 1924; Levitsky, 1931) and identifying that it was the chromosome which was the means of inheritance via the Boveri-Sutton theory (Sutton, 1902; Sutton, 1903; Wilson 1925). This work made the Mendelian theory popular and led to the famous Drosophila mutations experiments by Thomas Hunt Morgan showing patterns of inheritance of phenotypic changes from one generation to the next, alongside the proposal of genetic linkage and crossing over (Morgan, 1910). The number of chromosomes present within humans was debated initially, from a XX/XO sex determination system resulting in 48 chromosomes (Winiwarter, 1912), down to 46 (Painter, 1922) but later re-established as 48 with the discovery of the correct XX/XY sex determination mechanism (Painter, 1923). It was not until 1956 when a new methodology of isolating chromosomes was created where the count of 46 was confirmed (Tjio & Levan, 1956). Improvements in cytogenetic methods rapidly improved the study of chromosomes till the end of the century where a predictive computational approach rose in popularity as with most research in the modernising digital age.

1.1.2 Eukaryote chromosome structure

Eukaryotic cells localise their DNA within the nucleus of the cell; with the interaction of scaffold proteins, the DNA-protein structures (chromatin) can be manipulated further as a means of gene switching and packing efficiency. The association of DNA to histone octamers makes up the nucleosome, which in-turn best represents the 10nm ‘beads on a string’ model that allows genes to be actively transcribed from (Olins & Olins, 2003). With regards to the structure conformed by the nucleosome-DNA interactions, there are
additional associations to linker histones causes further condensing of the structure, found commonly in regions of less active genes (Hammond et al., 2017; Wanner & Formanek, 2000). During cellular replicative periods, scaffold proteins associate with the DNA-histone complex to again condense these vast structures (completely inhibiting transcription) into the commonly recognised chromosome shape that is essential for correct cellular division (Fukui, 2009). Following DNA replication, each length of DNA – a single chromatid – now exists as two identical copies (in regards to somatic cells), in which these two identical ‘sister chromatids’ are connected by the centromere complex and cohesin protein rings (Gligoris & Löwe, 2016; Naumova et al., 2013).

Each chromosome has two ‘arms’, which resemble the length of chromatin either side of the chromosome; the ‘p’ (petit) arm being the shorter and the ‘q’ arm the longer. Chromosomes are further categorised by the relative ratio of p to q arm size; a chromosome of equal arm size are termed metacentric, slight offsets of arm size from centromere are referred to as submetacentric chromosomes, when q arms are much longer than p arms places them into the acrocentric description and chromosomes with near non-visible p arms i.e. centromere is at the ‘top’ are termed telocentric (Levan, Fredga & Sandberg, 1964). Throughout each chromosome are lengths of relatively de-condensed (euchromatin) and more condensed (heterochromatin) regions. Within protein coding/associated regions of the chromosome, there are multiple elements that may be found such as enhancer regions, promoter regions, the transcribed and eventually translated exons and introns which are spaced between exons not to be translated. Typical of higher eukaryotes, repeat sequences are abundant throughout the genome, such as satellites and retrotransposons. Satellites are repeat sequences found at specific locations across the chromosomes and classified by the length of repeat, minisatellites for short nucleotide repeats and microsatellites for very small nucleotide repeats, such as di- and trinucleotide repeats. Centromeres can be an example of satellite regions – sequences that are commonly repeated. In humans this can be identified as α-satellites and can be used as targeted markers for chromosomal analysis (Mehta et al., 2010). Retrotransposons, or interspersed sequences are repetitive sequences that are found all over the genome as opposed to one location. Similar to satellites, they are further classified by size, short interspersed sequences (SINE), such as Alu elements which make up approximately 10% of the human genome (international Human Genome Sequencing Consortium, 2001; 2004), and long interspersed regions (LINE) of several thousand base pairs in length to make up approximately 21% of the human genome (Pierce, 2012).
As described, the chromosome is essential for cellular health and correct functionality; it is a highly specialised structure that has been the basis of evolutionary drive of eukaryotic organisms over time. However, chromosomal aberrations that cause a disruption in this specialised structure could be lethal or result in abnormal cellular activity, such as cancers or systemic genetic disorders.

1.1.3 The cell cycle and cell division

The cell must be able to replicate a copy of itself for the purpose of the organism’s growth, repair, and reproduction. Within eukaryotic cells, this is classified as the cell division cycle which defines clear phases of cellular activity relating to this division process. (Bell & Dutta, 2002; Bertoli et al., 2013).

1.1.3.1 Interphase

Before the process of mitosis or meiosis, the cell must progress through interphase (Figure 1.1), this phase can be split into three distinct stages, two growth stages G1 and G2 (Pardee, 1989) which flank S-phase, dedicated for chromosome replication (Takeda & Dutta, 2005). Each stage is shown to have highly conserved regulatory mechanisms crucial for the transition between stages (Nurse & Bissett, 1981; Foster et al., 2010). A newly constructed cell from a previous cycle shall enter at the G1 phase, to undergo high energy dependant metabolic activities such as protein synthesis (Pardee, 1989) to either progress further onto the cell cycle, s-phase (Figure 1.1) or leave the cycle to be in a state of rest, known as G0 where no cellular division happens. The cell will enter G0 any time before the restriction point, but once past this checkpoint stage it is committed towards division (Pardee, 1974). S-phase is the dedicated genetic content replication step, where cellular functions focus on the duplication of material adequate for two identical daughter cells (Nurse & Bissett, 1981; Aparicio et al., 1997). The phase after is G2, the second gap phase (Figure 1.1) where cellular machinery for division is developed and recruited (Zhai et al. 1996; Meraldi et al., 1999) and can be a means of controlling cellular size via Cdr2 regulation, as shown in yeast (Moseley et al., 2009). Before entry into division, G2 acts as a checkpoint to detect any errors in the DNA (O’Connor, 1997) to be repaired via homologous recombination (Burgoyne et al., 2007). Failure to so could lead onto malformed daughter cells but typically end up in cell apoptosis (Li et al., 1998); the mechanisms to achieve this are also highly regulated, such as the ability to detect mutative stress factors via p53 (Taylor & Stark, 2001) and the specific signal pathway to arrest the
cell cycle at this stage in order to correctly repair the genetic content of the cell (Xiao et al., 2003; Charrier-Savournin et al., 2004).

1.1.3.2 Mitosis

The majority of human cells are produced as a result of mitotic cellular division, with the exception of sex gametes which are instead produced by meiosis. Mitosis can be subdivided further into separate stages, each with distinctive identifiers as represented in figure 1.1 and seen in live cells in figure 1.2. The first stage of mitosis is prophase, classified by the phosphorylation and therefore breaking down of the nuclear membrane releasing the now condensing chromatin strands (chromosomes) into the cellular interior (Schermelleh et al., 2008). Microtubules from the centrosome shall start to attach to each chromosome at the kinetochore (Chan et al., 2005a); the kinetochore being a protein/microtubule structure that has been synthesised on each centromere during this stage (Chan et al., 2005). When all microtubules are attached to every kinetochore, the cell now enters metaphase. Here the spindles pull the chromosomes towards the approximate midline of the cell, the metaphase plate (Winey et al., 1995). At the metaphase plate, the spindle/metaphase checkpoint ensures correct attachment of kinetochores and proper alignment of chromosomes (Chan & Yen, 2003); mistakes at this stage would cause the daughter cells to have incorrect chromosomal counts (aneuploidy) to arise in diverse phenotypic results (cancer) or cellular death (Santaguida & Amon, 2015). After the metaphase checkpoint, the cell progresses into anaphase, being signalled by the cleaving of cohesin which bind the sister chromatids together (Rao et al., 2001). The mechanics of kinetochore microtubules cause the pulling of the separated sister chromatids to the opposite poles of the cell, paired with alternate microtubules which push against one another and the cell membrane to elongate the cell in parallel to chromatid separation (Civelekoglu-Scholey & Cimini 2014; Asbury, 2017). With separation finished the cell enters telophase, in which the chromosomes start to de-condense as the nuclear envelope reforms around them (Sansregret & Petronczki 2013), leading directly onto cytokinesis and the development of the cleavage furrow (which acts as a contractile ring) at the metaphase plate to pinch off the two cells completing cellular division with two identical daughter cells (Glotzer, 2005).
Figure 1.1. The cell cycle for eukaryotic cells

The cyclic nature of the cell cycle is shown, split up into interphase (thick black arrow), metaphase (green) and cytokinesis (purple). A representative 2n cell is shown at key points to visually describe the replication and separation of chromatids.

Figure 1.2. Examples of human cells in stages of mitosis

Live human induce pluripotent cells (hiPS) captured in increments along the cell cycle. DNA is labelled with NucBlue (Cyan) and cell membranes with CellMask Deep Red (magenta)

Image credit: Allen Institute for Cell Science, a division of the Allen Institute.
1.1.4 Chromosomal abnormalities and aberrations

1.1.4.1 Aneuploidy

Aneuploidy is generally defined as the ‘state’ of the cell’s genome/karyotype as abnormal due to numerical number variations of whole chromosomes, which is not a result of a multiplication of the haploid number; the term was first named by G. Täckholm in 1922 (Täckholm, 1922). Aneuploidy tends to be as result of chromosome mis-segregation during cellular division, which is a tightly regulated system as the spindle assembly checkpoint identifies most errors (Musacchio & Salmon, 2007; Pfau & Amon, 2012). Incorrect spindle fibre attachment and subsequent incorrect separation causes lagging chromosomes (Janssen et al., 2011), or in the event of a weakened mitotic checkpoint, incorrect pairing or lining up on the metaphase plate could cause whole copy number variations in the two daughter cells, known as non-disjunction. Lagging chromosomes can cause abnormalities in a multitude of ways; one example is be caught in the cleavage furrow formed in telophase, causing damage to elicit the DNA repair action of p53, which may cause aberrations such as translocations or deletions (Janssen et al., 2011; Crasta et al., 2012). Micronuclei can also form around lagging chromosomes, which facilitate a harsh environment for typical nuclear reactions causing a non-functional chromosome, and seen as an indicator for chromosomal instability (Crasta et al., 2012; Luzhna et al., 2013); a micronucleus can also stall DNA replication and induce stresses on the replication machinery to cause extensive DNA damage (Xu et al., 2011). Aneuploidy within cells tends to cause a change of gene expression of up to 50% (Stingele et al., 2012; Kahlem et al., 2004), and to detect the significance of the incorrect dosage at this scale requires complex live-cell imaging methods or extensive quantitative single cell genome analysis tools (Santaguida & Amon 2015). Aneuploidy is also noted to be the leading cause of miscarriages in humans, as frequencies of aneuploidy are, spontaneous abortions 35%, still births 4% and newborns 0.3% (Hassold & Hunt, 2001; Hassold et al., 1996; Hartl & Jones, 2005), and of high frequency in cancers >85% (Gordon et al., 2012; Weaver & Cleveland, 2006).

1.1.4.2 Deletions

Chromosome deletions are classified by segments of DNA being lost from the cell’s genome, typically during cellular division/DNA replication. Small deletions, such as single base deletions, could lead to frameshift mutations which would interfere with the reading frames of codons in eukaryotic organisms, yielding incorrect transcription products (which may ultimately formulate as malformed proteins).
The magnitude of disruption deletions cause are somewhat reliant to a disruption to normal cellular processes, for example, single base deletions occur mainly due to single base flipping events or DNA slippage within DNA polymerase in that stage of DNA replication (Banavali, 2013; Manjari et al. 2014). Other large-scale losses can be explained by losses during translocation events during chromosomal inversion events (Figure 1.3), unequal crossing over during meiosis (Comings, 1966; Jelesko, et al. 2004) and DNA breakage without rejoining.

Deletions can cause systemic wide issues for the organism with large loss of gene products, for instance deletions of the 15q11-13 are indicative of Prader-Willi syndrome with loss of small nuclear RNAs, essential for regulation (Cavaillé et al., 2000; de Smith et al., 2009). Deletions, such as multiple micro-deletions along the Q arm of chromosome Y in males are associated with spermatogenic failures (Krausz et al., 1999; Foresta et al., 2001); these deletions show that the Y chromosome has more function than just sex determination (Tiepolo & Zuffardi 1976; Burgoyne, 1998). One of the more heavily studied in respect to deletions arising to tumorigenesis is the deletion of chromosome 17p, home to the p53 gene, TP53 (Isobe et al., 1986; Baker et al., 1989) and somewhat disrupted in at least 50% of all human tumours (Hollstein et al., 1991) indicating that this gene plays a vital role in cancer suppression (Surget et al., 2014).

1.1.4.3 Duplications

The term ‘duplication’ can compromise events such as gene replication, whole chromosome duplication or gene amplification. These events act as a mechanism to increase the genetic content of the organism thus expanding the coding and conding elements within the genome. Likewise with deletions, there are many cellular events that can cause duplications, such as unequal crossing over which can also duplicate a segment due to a possible large homologous region between chromatid pairs, (Comings, 1966; Jelesko, et al. 2004) and replication slippage. Whole chromosome number increases can be classified as duplication events. In the case of aneuploidy via non-disjunction during meiosis can give rise to whole chromosome increases such as in Down syndrome (Oliver et al., 2008) however aneuploidy events occur in approximately 15-20% of clinical pregnancies that result in a spontaneous abortion (Jia et al., 2015). The whole genome can be duplicated in polyploidy events, typically seen in plants as a means of speciation and adaptation (Rieseberg & Willis, 2007), with many examples of lineages expanding in ancient polyploidy events (Meyers & Levin, 2006). These can arise via autoploidy, such as the
potato (3n) (Potato Genome Sequencing Consortium, 2011; Visser et al., 2009) or by fusion between taxa -allopolyplody- such as forms of wheat (6n) (Hancock, 2004). Polyplody is also present in animals where it is typically tied to evolutionary events (Dehal & Boore, 2005) such as hox gene cluster duplications (Soshnikova et al., 2013) but also used for cellular, tissue and organ differentiation (Velicky, 2018).

Diseases that may arise from duplication events are commonly caused by the over expression of specific gene products, typical of cancers such as MYC and cell cycle regulators CCND1 and CDK4 (Vogelstein & Kinzler, 2002). Examples of congenital conditions such as ‘Cat eye syndrome’, where 22p-q11 is in triplicate or quadruplicate (Rosias et al,2001), or Charcot-Marie-tooth disease which is caused by the duplication of 17p12 containing PMP22 or 1p36.22 containing MFN2 (Krajewski et al.,2000; Baloh et al., 2007) both associated with nerve cell damage (Watila & Balarabe 2015).

1.1.4.4 Translocations

The rearrangement of chromosomes, the sharing of chromosome segments can be classified as translocations. Translocations come under different descriptive varieties, the quantity of DNA transferred between chromosomes (balanced or unbalanced) and by the method of translocation (reciprocal or Robertsonian, figure 1.3).

The distinction between balanced and unbalanced is given as either: balanced, where a swap of equal genetic material occurs between chromosomes, typically non-homologous, which generally cause no loss or gain of function, or unbalanced translocations, which results in the exchanging of unequal volumes of DNA between chromosomes which can give rise to imbalances across the chromosomes affected. Reciprocal translocations are characterised by the exchange of material between non-homologous chromosomes somewhat seen as a high occurrence estimated between 1-500 people (Ogilvie, 2002) to 1-600 people (Estop, 1997). The majority of these are typically balanced, asymptomatic, and harmless to the organism yet lead to fertility issues as gametogenesis can be impaired (Stern et al., 1999; Baccetti et al., 2003). XX male syndrome, or De la Chapelle syndrome, (De la Chapelle, 1972) demonstrates that approximately 90% of the cases are caused by a translocation aberration of the SRY gene to the X chromosome. This effectively causes individuals to either have a female karyotype (46,XX) but present phenotypically as males or alternatively a phenotypic female with a male karyotype (46,XY), without the SRY gene present on the Y (Margarit et al., 2000; Anık et al., 2013).
Robertsonian translocations, like to reciprocal translocations, can also be symptomless. However these abnormalities also carry the risk of unbalanced gametes to cause fertility issues (Keymolen et al., 2011). These translocations typically occur with the acrocentric chromosomes (Figure 1.3) as they contain highly similar DNA sequences within the short arms, thought to increase a possibility towards these types of translocations (Kim & Shaffer, 2002). These events yield a large metacentric appearing chromosome and a smaller gene poor chromosome which is typically lost during cellular division (Kim & Shaffer, 2002). Down syndrome is typically due to the trisomy of chromosome 21, however it can occur due to translocation events involving chromosome 21q, such as in the event of a Robertsonian translocations, between t(14;21) and t(21;21) (Polani et al., 1960; Penrose et al., 1960; Jyothy, 2002). This translocation event is somewhat comparable to trisomy 21 as increase dosage of genetic material on the q arm is still present and expressed. However, findings indicate that the translocation causing sufferers express less severe learning difficulties and less severe obesity as compared to trisomy 21 sufferers (Prasher, 1995).

Translocations require two separate double strand breakages to occur. Double strand breakage can occur via pathological means such as during cellular processes like replication errors, destructive action at fragile sites and topoisomerase failures or via external influences such ionising radiation. Opposed to this, regulated non-random physiologic double strand DNA breaks can occur during V(D)J recombination and during class switching (Lieber, 2010). The breakages can either be repaired by homology directed repair during late S and G2 phase as the sister chromatid present act as a reference or donor, however outside of these stages non-homologous end joining (NHEJ) must occur to fix the breakages (Tsai & Lieber 2010). NHEJ usually induces some nucleotide loss and no use of a template to guide any new nucleotide insertion by polymerases (Lieber, 2010). This can induce a translocation by joining arms from breakages from differing chromosomes during the repair step. It has been suggested that proximity of chromosomes involved in translocation events within the nuclei space are important for the aberration to occur (Roukos et al., 2013). As a possible result from poorly mediated NHEJ, unbalanced translocations are typically iconic in formation of a wide variety of genetic diseases. Frequently acrocentric chromosomes are found to be malformed via unbalanced translocations in many cancers, more-so in blood cancers (Lin et al., 2018), thought to be as result of closer special association around the nucleolus (Bolzer et al., 2005).
1.1.4.5 Inversions

Chromosome inversions occurs when a segment of a chromosome ‘flips’ and the gene order is reversed, either being paracentric when the reversed segment does not involve the centromere or pericentric when the centromere is located within the reversed segment. Many inversions do not cause any abnormalities, and are just evolutionary events that cause genetic dissimilarity and isolation between populations/species, creating post-zygotic barriers that prevent fertile heterozygotes (King, 1995) due to the inability to correctly balance chromosomes during crossing over events in gametogenesis due to mismatched homology. In addition to this, suppression of recombination would prevent the creation of an inversion containing heterozygote chromosomes from being dispersed into the population, causing offspring to either have a ‘normal’ or ‘inverted’ chromosome further fuelling speciation and genetic divergence (Dobzhansky, 1970; Trickett & Butlin, 1994).

Evidence of that the presence of inversions and their impact on evolutionary events throughout time has been long predicted (Sturtevant, 1921; Dobzhansky, 1970). Classic cytogenetic techniques predicted 9 inversions between humans and chimps yet with the introduction of genomic sequencing there has been found to be an excess of over 1,500 (Feuk et al., 2005). The process of inversions rely on several molecular mechanisms, such as reliance on key breakpoint regions which are staged along the genome (Ranz et al., 2007), where these inversions can span several megabases in the case of D.melanogaster (Hoffman & Rieseberg, 2008) or small as can be found to be less than 1KB (Feuk et al., 2005).

However, it is unlikely that an inversion would cause harm to the carrier as there is typically no gain or deletion of genetic material, similar to previously described chromosome aberrations. Nevertheless it is still possible if disruption occurs in gene reading frames/coding regions or expression controlling elements. An example of a possible link to autism is a balanced inverted paracentric inversion involving chromosome 10(q11.1;q21.3), where breakpoints disrupt TRIP8 and REEP3 highlighting possible candidate genes for further study (Castermans et al., 2007).
1.1.4.6 Rings

Ring chromosome aberrations arise due to the loss of both ‘ends’ of a chromosome with repair and recombination events connecting them improperly, first seen in 1926 (Morgan, 1926) and has been reported for every human chromosome (Schinzel, 2001). It is possible for rings to form with just lost of subtelomeric or telomeric sequences, and no loss of genetic content to typically yield a normal phenotype (Vermeesch, 2002; Sigurdardottir et al., 1999) but can show a delay in growth of an individual (Yip, 2015).

Severity of conditions arising from ring chromosomes depend on the location of the ring fusion, a shorter ring will resemble a larger loss of genetic material whereas a ‘larger’ ring of same chromosome origin has a lower level of genetic material loss. Rings can be inherited or rarely spontaneously formed, rings are associated with mosaicism usually due to derivative cell lines/populations attempting to stabilise their genetic content after a previous aberration (Shchelochkov et al., 2008). Alongside other chromosomal aberrations, such as tetrasomy and deletions, the presence of ring chromosome 20 has been seen in cases of epilepsy, indicating that epilepsy is polygenic in nature and can be caused by multiple chromosomal malformations (Schinzel & Niedrist, 2001).

Ring chromosomes have been identified within many plant species (Singh, 2016) and have been seen to be somewhat beneficial as they can confer resistance to commonly used glyphosate based herbicides due to the presence of extra EPSPS (Koo et al., 2018).

1.1.4.7 Isochromosomes

‘Equal’-chromosome or isochromosome is an unbalanced chromosomal aberration where chromosome arms are replicas of one another. In other words, it is in appearance that one arm of a chromosome is deleted and replaced with a duplicated version of its remaining arm. These isochromosomes can arise from two different means during cellular division of an individual, or inherited from either parent’s gamete.

One such mechanism is centromere misdivision; this is the process of incorrect disjuncture during metaphase/anaphase causing the chromatids to divide transversely and not longitudinally along the forming daughter cells. This is due to the action around the pericentromere, where homologous sequences are shared between sister chromatids to cause mismatching of separation complexes to form and incorrect separation around the centromere. This would typically form a di-centric chromosome albeit close in distance with possible gains of genetic material (Wolff et al., 1996). Another such mechanism is via
U type strand exchange, instead of incorrect separation, there is a double strand breakage in the pericentromeric regions of the sister chromatids to be repaired incorrectly. Likewise, two centromeres are present on one new ‘chromosome’ and lack of centromere for the other results in chromosomal loss (Rowe et al., 2009). U type strand exchange can also affect homologous chromosomes (similar and sometimes misinterpreted as Robertsonian translocations (Shaffer et al., 1991), if there are similar enough low copy repeats to allow partial folding onto itself to recombine incorrectly between these repeats, such as in the previous example of acrocentric short arms.

The presence of isochromosomes affects the organism’s phenotypes differently, for example *Candida albicans* can gain a resistance to antifungals with the presence of a specific isochromosome (Selmecki, 2006). The 5L chromosome in isochromosome form, incurs a positive growth advantage when exposed to conditions containing the antifungal fluconazole. Unsurprisingly 5L is the location for ERG11, the target of fluconazole (White 1997), and Tac1 which up regulates ATP binding cassette multidrug transporters CDR1 and CDR2 (Coste et al., 2004; Prasad, 2015). With increased genetic copies, it is predictable that with increased copy number due to the isochromosome increases resistance to the effects of the antifungal.

In humans however, isochromosomes are associated with multiple diseases and syndromes. Turner syndrome is classically categorised by an X0 genotype, yet isochromosome Xq is reported in cases of live Turner syndrome births to exhibit milder symptoms than typical Turner syndrome (Akbas, 2012). In retrospect of current Turner syndrome sufferers, between 7-17% tested were found to be the 46,X,i(Xq) karyotype (Sybert & McCauley, 2004; Ćatović & Kendić, 2005) and can be maternally inherited (Cetin et al., 2011). Both isochromosome variants exist with different symptoms of Turner syndrome expressed, i(Xq) (and therefore deleted Xp) have the short stature and congenital malformations whereas i(Xp) (and therefore deleted Xq) typically display gonadal dysfunction (Sönmez, 1997).

As demonstrated, isochromosomes bring about the phenotypical changes due to dose variation, as effectively there is a trisomy dosage in the gained arm and a monosomy in the lost arm (Sidwell et al., 2000). Isochromosomes unsurprisingly relate to cancerous cell development and survivability, where the presence of isochromosome 17q correlates to high levels of neoplasia and poor survival rates in patients (Mendrzyk et al., 2006). The p arm has many low copy repeats localized to the pericentric region, allowing a higher rate of
recombination and U type strand exchange to occur creating the isochromosome 17q (Barbouti et al., 2004), multiple tumour suppressor genes are located on the p arm of chromosome 17 such as p53 so a worse prognosis is understandable in presence of this aberration (see section 1.3.2.3.2 and section 4).

1.1.5 Cytogenetic methods of past and present

1.1.5.1 Karyotyping

The karyotype of an organism is the visual representation of its ordered chromosomes; this definition can also be expanded to be in reference to the ordered chromosomes of a whole species. The use of karyotyping extends from the detection of abnormalities that may cause pathological symptoms, to evolutionary biology where comparisons can be made to comment on chromosomal similarity or shifts between species.

Using a karyotype, observations can be made about characteristics to further distinguish information about an organism (Fisher, 1919), seen in figure 1.4 as a representation of a normal human male karyotype. Features and characteristics can be; sizes of chromosomes, centromere positioning, chromosome count and ploidy number, featuring of satellites and banding pattern. Together this information can be used to characterise the organism in terms of species level, differ between the sexes (if sex is determined by chromosomes (Bull, 1980; Janzen & Phillips, 2006), differences between life stages of an organism (Goday & Esteban, 2001), differences between the gametes compared to normal body cells, and to identify abnormalities in the event of disease (Santaguida & Amon 2015).
The isolation of lymphocyte cultures in the 1960s allowed easier collection of genetic material that could be stimulated to a growing state ushered in an age of clinical cytogenetics (Morrhead et al., 1960; Ferguson-Smith, 2008). This allowed the discovery of whole chromosomal aberrations to link to medical diagnoses, such as translocation Down syndrome (Polani, 1960) and the Philadelphia chromosome (Rowley, 1973). The Philadelphia chromosome was the first discovered cancer chromosomal aberration (predominantly found in chronic myeloid leukaemia (CML)) with a direct link to tumorigenesis, consisting of a translocation to give a short chromosome 22. Furthermore, prenatal diagnosis was an area of interest; especially genetic analysis (Steele & Breg, 1966) yet the methodologies used yielded poor and low relevant information (Schmid, 1963; Ferguson-Smith et al., 1962).

**Figure 1.4. Normal Human male karyotype with ideogram**

A karyotype of a normal healthy human male, 46\( <2n> \), XY. Chromosomes stained and banding highlighted by DAPI, image and assignment produced using SmartType (Digital scientific UK).
1.1.5.2 Whole chromosome banding

The introduction of chromosome banding revolutionised the field of cytogenetics by quinacrine intercalating into DNA to produce varying dark and light bands visible by UV based microscopy (Caspersson et al., 1969). This technique known as Q-banding was used on the principle that the quinacrine binds to rich AT and CG dense regions, yet only the AT-quinacrine complex produces detectable fluorescence when excited by UV light. AT regions, being common in heterochromatin segments of the chromosomes gave a reliable and repeatable means to classify chromosomes and lead to the ability to classify all human chromosomes by this one technique (Caspersson et al., 1970). Previous identified aberrations were able to be explored more, such as the Philadelphia chromosome (Rowley, 1973) highlighting the significance of chromosome banding. More common stains used to give the same banding effect are DAPI (4’,6-diamidino-2-phenylindole) and Hoechst 33258 (Kapuscinski, 1995; Latt et al., 1975).

Alternative banding techniques arose for general banding pattern, such as G banding. G-banding instead uses Giemsa stain after trypsin digestion which binds to phosphate groups rather than insertion into the DNA structure, initially used to detect the malaria causing *Plasmodium* (Giemsa, 1904). Similar to Q-banding, G-banding gives light and dark bands, however it is the CG light euchromatin that resolves as light bands and the AT right heterochromatic that displays as dark bands (Seabright, 1971). An offspring of G-banding is Reverse banding (R-banding), to give a similar banding pattern but in reverse colouration. This is achieved by heat denaturation in a high temperature phosphate buffer followed by Giemsa stain and in conjunction with G-banding it allows comparison to define any possible irregularities with precision (Richer et al., 1983).

The use of stains can be used to also highlight specific features of chromosomes opposed to staining heterochromatin/euchromatin such as the centromere or telomeres. The use of C-banding highlights constitutive heterochromatin which stains darker (Angell & Jacobs, 1975), and is highly concentrated around the pericentromeric region of chromosomes which consists of high copy count tandem repeats classified into microsatellites, minisatellites, and transposons (Saksouk et al., 2015). C-banding is achieved by the use of Giemsa stain, yet has an alkaline denaturation step before staining which only constitutive heterochromatin binds, used typically to clearly visualise centromeres (Sumner et al., 1971). Lastly T banding is again very similar, where Giemsa stain is used with pre-
staining parameters changed, such as high temperature denaturation, to stain the subset of R-bands found at the telomeres of chromosomes (Jack et al., 1986).

1.1.5.3 In situ hybridisation

Pardue and Gall first displayed a technique using previously isolated DNA (ultracentrifugation obtained mouse satellite DNA) to generate centromere specific probes by conjugation with radioactive isotopes (Pardue & Gall, 1970). This was the first time an in situ hybridisation (ISH) had been used in this way to detect features on chromosomes, with this methodology applied later to shown that ribosomal genes are located on the short arms of human acrocentric chromosomes (Henderson et al., 1972). Up to this point, only large, easy to isolate DNA material was able to be used to form useable probes. With the introduction of recombinant DNA techniques, the cloning of DNA fragments of interest was enabled to be high enough in yield to be labelled and provide sufficient signal radioactively. This allowed smaller features to be identified, such as the location of beta globin genes to be confirmed to the short arm of chromosome 11 (Malcolm et al., 1981). Obviously, use of radioactively labelled probes had plenty of safety issues regarding probe generation and imaging. The imaging process would take a multitude of days to accomplish a sufficient signal. With the drawbacks of isotope labelled probes, the replacement using fluorophore-labelling and UV microscopy became the standard methodology for gene location studies and chromosome analysis, known as fluorescence in situ hybridisation (FISH) (Pinkel et al., 1986; Lichter et al., 1990). Initially, biotin was the primary label used in conjugation with probe DNA (Hofmann & Kiso, 1976; Bayer et al., 1985). After hybridisation, adi vin (which has high affinity towards biotin) labelled with fluorescein was used to produce an observable signal. An example of usage was interspecies translocations between human and hamster in human-hamster hybrid cell lines. This method also allowed the creation of a Y specific chromosome probe (Figure 1.5) and human 28S ribosomal RNA gene probes (Pinkel et al., 1986).
Figure 1.5. Fluorescence hybridisation of human chromosomes
The introduction of more colours from differing fluorophores further allowed specific gene mapping questions to be answered. More fluorophores allow for direction or gene order to be questioned and the establishment of gene loci along the chromosome (Trask et al., 1989).

Flow cytometry allows the isolation of fluorescently labelled chromosomes based upon their size (Gray et al., 1975) with enough genetic material collection, each chromosome (or pool of chromosomes- see section 1.3 and section 4.1) can be amplified and labelled with a fluorophore. This effectively creates a whole chromosome probe, known as a chromosome ‘paint’ which will fluoresces with successful hybridisation to its complimentary sequence (Young et al., 1981). Chromosomes previously had been used to make chromosome specific libraries, but now paints could be used to answer biological questions from clinical uses to ecological assignments (Reid et al., 1998). Now previous phylogenetic assignment could be questioned by direct chromosome comparison, looking for common homologous regions.

**Figure 1.6. FISH principle and procedure**

A schematic representation of the FISH technique. The probe DNA is isolated and labeled either directly with a fluorescent tag (as seen here) or indirectly (e.g. Biotin). The sample of target DNA is denatured alongside the probe DNA and allowed to hybridize with its specific target area. Upon analysis with a fluorescent microscope, a clear signal should be apparent on the targeted position of the chromosome.
across species and taxa to redefine our understanding of evolutionary lineages, or direct karyotype evolution by cross species painting. Complex chromosome aberrations could now be classified and understood at a deeper level as human and mouse paints had been created enabling cancer researchers to draw conclusions easier as compared to previous banding pattern analysis which was technically challenging and gave questionable results (Ferguson-Smith, 1997).

There was also a large relevance for the use of paints diagnostically in IVF, such as the detection of chromosome 21 copy number variants in uncultured amnionocytes using a highly specific contig, unique to human chromosome 21 (Zheng et al., 1992). Sex chromosome paints and probes were used to correctly sex embryos before implantation (Griffin et al., 1992) and link dysmorphism events to specific chromosomal abnormalities with links to prevalence of these abnormalities to maternal age (Munné & Cohen, 1998). This was important for the identification of X linked diseases (Griffin et al., 1993), or associated aneuploidy/mosaicism sex chromosome linked diseases where clinical use of paints resulted in confirmed healthy pregnancy of a selected embryo after screening out affected embryos (Delhanty et al., 1993).

The use of bacterial artificial chromosomes (BACs) in FISH has allowed the creation of whole genomic libraries by fragmenting genomic DNA into short 150-350 kbp lengths (O’Connor, 1989). By using specific BAC clones of model species it is possible to use these with fluorescent molecules to act as positional markers along a chromosome. In the example of a specific gene of interest, the locus of said gene can be identified from positional hybridisation of the BAC now probe sequence (Figure 1.6). Furthermore, hybridisation across different species allows indications towards possible regions of homology and gives information towards possible ancestral lineages between the samples. The use of comparative BAC mapping has shown that the platypus sex chromosomes are more avian like than classic mammalian like in their five X chromosomes (Grützner & Graves, 2004; Veyrunes et al., 2008).

Multiplex in situ hybridisation (M-FISH, also known as Multi-Fluor and similar to multicolour, mFISH) uses many differing fluorophores attached to DNA paints hybridised simultaneously to show different successful hybridisations on the same preparation (Liehr et al., 2004). With this method it is possible to detect at a higher level of accuracy, as has been demonstrated that it is possible to detect new novel chromosomal rearrangements not previously detected by established banding techniques (Speicher et al., 1996). As this
technique is limited by fluorophore wavelength emission overlap, ratios of fluorophores can instead be used and computationally analysed to assign correct pseudo colouration, these can be detected by a typical set of varied fluorescence filters or by the use of an interferometer in the case of spectral karyotyping (SKY).

As of 1996, using the differentiation of different spectra (SKY technique, figure 1.7) allowed the simultaneous identification of all human chromosomes and colourisation in pseudo colours (Schröck et al., 1996) SKY has great practicality when used to detect chromosomal malformations in context of unpredictable karyotype (Guo et al., 2014). One of the first studies on haematological malignancies, acute myeloid leukaemia (AML) and myelodysplastic syndrome (MDS), used SKY to confirm results seen from G-banding yet also indicated that SKY detected more cryptic aberrations that were impossible to detect via G-banding (Veldman et al., 1997). Even more recently, SKY still has applications to improve banding based assignments with more accurate results and to highlight unseen malformations. For example, in acute lymphoblastic leukaemia (ALL) to improve R-banding karyotype (Guo et al., 2014) and a discovery of a novel 5 way translocation event within chronic myeloid leukaemia (CML) via SKY, G-Banding and FISH (Vaidya, 2013). SKY can be also used to further classify and categorise patients of AML into specific aberration groupings when G-banding had failed to identify precise mutations (Mrózek et al., 2002). SKY’s advantages are clear in that it allows the detection of large aberrations which would simply appear as a multitude of differing colours on a derivative chromosome yet it does not come without its drawbacks. There are aberrations that would not directly cause a colour change, such as small deletions, duplications, reciprocal translocations and in particular intrachromosomal events such as inversions (Fan et al., 2000; Imataka & Arisaka, 2012). The financial costs to set up and maintain reagents and equipment for SKY are high as well as skill of the user able to interpret and use the information (Padilla-Nash et al., 2006). SKY loses its usefulness without being paired with another analysis methodology, typically G-banding or Q-banding is usually performed alongside SKY (again requiring a skilled cytogeneticist) and usually requires FISH experimentation to some degree to verify claims such as small reciprocal translocations (Fan et al., 2000) and detection of clone variants (Saito, 2010). SKY can only detect down to an alteration of 1000-2000bp, and fails to detect aberrations well close to telomeres (Fan et al., 2000), this causes what is known as the ‘sandwich effect’. The sandwich effect comes to fruition when the fluorophores from multiple contributing chromosomes (in context of an unknown derivative chromosome) slightly overlap and cannot be distinguished from one another.
often leaving a small region from a third chromosome unseen (Padilla-Nash et al., 2006). In the event of mass malformation or a chromothripsis event (see section 1.3.4.2), SKY cannot possibly be reliable enough to sufficiently characterise a cancer line (Zhang et al., 2013).

1.1.5.4 Digital/Virtual Karyotyping

Digital/virtual karyotyping is a methodology used to identify DNA via copy number on a whole genome scale. To explain further, short sequences from samples of DNA are tagged from previously specified loci across the genome that consistently overlap. These sequences are then processed and compared to a previously established reference genome to identify deletions, amplifications, foreign DNA and copy number variants (Wang, et al., 2002). Contemporary methods to achieve a computational derived karyotype are comparative genomic hybridisations (CGH), more specifically arrayCGH (Figure 1.8) and via single nucleotide polymorphism arrays (SNP).

Figure 1.7. SKY (spectral karyotyping) karyotype of a healthy normal human female, 46\(<n>\),XX
These methodologies have changed the field of cytogenetics as it is able to merge the field of traditional chromosome analysis and specific molecular diagnostics. Constitutional disorders can be easily detected and resolved using these methodologies, such as copy number variant issues (Lee et al., 2007), or abnormalities that can be identified to link towards developmental delays in humans (Shaffer & Bejjani, 2006; Edelmann & Hirschhorn, 2009). These methodologies require the use of disrupted DNA (DNA in which is different from the reference) to detect aberrations, which is also limited by the probes used within the array itself such as the ‘density’ of the probe (size) and the depth used in each sample.

The most basic of disadvantages is a lack of the ability to detect balanced translocations or inversions (Gebhart, 2004), and there are issues regarding the computational analysis of sequenced samples (Roy & Motsinger-Reif, 2012). Studies show that there are variations when comparing results from replicates using various platforms (Baumbusch et al., 2008; Curtis et al., 2009) and even showing that variation of algorithms will provide different results on the same sample provided (Lai et al., 2005; Pinto et al., 2011).

**Figure 1.8. Overview of Array CGH methodology and principles**

Simplistic representative of the methodology used for array-comparative hybridization. Test DNA (green) and reference DNA (red) are mixed and be simultaneously hybridized on ‘pits’ containing complementary DNA to the reference genome. Competition between the differently labeled strands will yield yellow fluorescent results, yet a bias towards red or green signifies a lack of test DNA (red) or an abundance of test DNA (green) signifying a gain or loss of DNA in the test organism.

As previously described, virtual karyotype systems will compare any disrupted DNA found to a known reference, typically a normal diploid genome. In the case that there has been an elevation of genomic material due to an increase of ploidy, this will incur incorrect results as a ‘multi-ploidy’ sample will be condensed into reference of a diploid reference, within context of this example. This can only be rectified by conventional techniques, such as traditional karyotyping or FISH to reassess the sample if this issue is detected. Sub clones will be missed within the circumstance of a clonally diverse tumour and would be seen as low level background interference when averaged against the dominate clone present, which will impact the outcome of the treatment (Landau et al., 2013). There are associations between greater number of detectable subclonal populations and poorer clinical results (Pereira et al., 2016; Morris et al., 2016), and increased diversity can indicate increased resistance to selective pressures such as drug resistance (Zhao et al., 2016). In the context of these points, only detectable subpopulations are taken into account of current treatment and clinical diagnostics which is typically deduced by an ‘average’ population analysis (Figure 1.9). Currently undetectable subpopulations may give rise to recurrent tumours that may be missed by current methodologies at initial treatment stage.

**Figure 1.9. A global analysis technique vs. a single cell approach**

A single cell analysis approach can identify individual cells as part of subpopulations. A) Clear offshoots can be identified over time which may possess different intrinsic characteristics. B) a possible result of an averaged ‘global’ analysis not taking into consideration possible subpopulations present. Adapted from Matson & Cook, 2017.
1.1.5.5 Fluorescence microscopy

The dogma of fluorescence microscopy is that a molecule of fluorescent properties will chemically bind or somewhat help in the detection of a known biomolecule of interest (Sahoo, 2012). Fluorescence can be described as the emission of light at a lower wavelength/energy by a substance after absorption of a higher wavelength/energy light or other electromagnetic radiation. In comparison to radioactive labelling, fluorescence is much safer to use and multiple fluorescent molecules can be used simultaneously.

As described, fluorescent molecules require high energy wavelengths of light to be absorbed and then emit a lower energy wavelength of light, with the use of different spectral emission filters (for different wavelengths of light to be emitted to the sample) and a dichroic mirror/beamsplitter paired with an emission filter (to match the excitation wavelength from the fluorescent molecule) multiple fluorophores can be used of differing absorption and emission to separately identify different targets (Spring & Davidson, 2004)(Figure 1.10).

![Fluorescence Microscope Diagram](image)

Figure 1.10. A simplistic representation of a fluorescent microscope and parallel atomic events

Shown, a basic overview of the rudimentary principles on which fluorescent microscopy requires. Left, a ‘slice-through’ representation of a fluorescent microscope and the path light travels to and from the specimen as different wavelengths of light. Right: a nuclear model showing a) a high wavelength of light exciting an electron to b) jump to a higher energy state to eventually, c) lose the energy and return to its ground state by, d) emission of light at a lower wavelength/energy.
Fluorescent labels such as ethidium bromide and GFP (green fluorescent protein) are used commonly, but there are multiple ways to generate unique targeted labelling options. To create a specific tag, typically a fluorophore will be attached to a unique molecule with affinity towards the target, examples of which include enzymatic labelling (Richter et al., 2002), chemical labelling as an alternative to GFP (Wombacher & Cornish, 2011; Jung, 2013), protein labelling such as histidine tags, and finally genetic labelling such as FISH allowing specific loci or sites along a chromosome to be identified.

1.2 Comparative Genomics

Within the field of genomics, comparisons of genomic features between many numbers of organisms can be studied to determine comparable biological features and evolutionary relationships. Since the work of Fisher in 1918 (Fisher, 1918), by formulating the idea that Mendelian inheritance was to account for the phenotypic variation in nature, gave birth to the research environment of genomics. It was not until 1986 with gene specific DNA sequences for the herpes simplex virus was studied to better understand its gene coding glycoproteins, that the Epstein Barr virus and Varicella zoster virus was also compared alongside to surprisingly discover over 100 shared gene homologs between these pathogenic viral agents (McGeoch & Davidson, 1986). This direct comparison between these viral groups and the identification of a multitude of genetic similarities that would relate to possible shared biological features, the field of comparative genomics was born. The genomic features of interest that can be used to compare species is not just limited to gene sequences. Genomic features analysed can encompass DNA base sequence, gene order, features of regulatory regions, homologous synteny blocks (HSBs), evolutionary breakpoint regions (ERBs), proteins/protein function, and all variants of RNA (Larkin et al., 2009). All of these described genomic features can be used to detect evolutionary relationships between organisms, a closer relationship determined by; more commonality in sequences, alignment of regions, orthologs in sequences, and homologs in genes. The observation of evolutionary relationships will allow insight into possible positive or negative evolutionary changes that may have occurred, and allow a differing perspective of the phenotype observed of said species, such as the viral example above in relation to the pathogenic nature of viral components.

The first genome of a cellular organism fully sequenced was detailed in 1995, *Haemophilus influenzae*, a bacterium thought to be the causative agent of influenza in 1892 (Fleischmann et al., 1995). This was shortly followed by a parasitic bacterium, *Mycoplasma*
genitalium, in the same year (Fraser et al., 1995). A year later, the first eukaryotic organism was fully sequenced Saccharomyces cerevisiae (Goffeau et al., 1996), followed two years later by multicellular organism Caenorhabditis elegans (C. elegans Sequencing Consortium, 1998). The release of the iconic model species Drosophila melanogaster genome in 2000 (Adams et al., 2000) allowed the first major study of all genomes released thus far. This study showed that approximately 60% of genes from D. melanogaster to Human were conserved, and two thirds of genes that were then implicated in human cancer have counterparts in these fruit flies (Rubin et al., 2000). The first human genome draft was released in 2001 (International Human Genome Sequencing Consortium, 2001; McPherson, 2001; Venter et al., 2001), to be completed in the following years (Collins, 2003; International Human Genome Sequencing Consortium, 2004). Interestingly this found around 22,000 protein coding genes in humans, similar to other mammals (Pertea & Salzberg, 2010) and contained large volumes of repetitive identical segments throughout which was a surprising find (International Human Genome Sequencing Consortium, 2004). Following human, a multitude of higher multicellular organisms were fully sequenced, such as the chicken, Gallus gallus (International Chicken Genome sequencing Consortium, 2004), the chimpanzee, Pan troglodytes (The Chimpanzee Sequencing and analysis consortium, 2005), the European honeybee, Apis mellifera (Honeybee Genome Sequencing Consortium, 2006) and even sponges, Amphimedon queenslandica (Sirvastava et al, 2010).

The use of sequenced genomes spans further than just evolutionary comparisons, in terms of pathogen genomics, the advent of whole genome sequencing has changed the way any microbial organism is now researched. This increase of throughput allows easier comparisons between pathogenic strains and from pathogenic to non-pathogenic organism (Hu et al., 2011). The study of microbial genomics has allowed the development of vaccines, the exploration of the microbiome of the human digestive tract and to detect virulence/spread of disease (Kaper et al., 2004). This has allowed the research into ‘metagenomics’- the study of communities of organisms, such as in the previous example of human micro flora reservoirs (Sommer et al., 2009). The tracking of great ape social groups paired with genetic profiling has allowed research into more social/conservation focuses areas, such as the fact that set populations exhibit large levels of inbreeding which will aid conservationists in restoring population numbers (Prado-Martinez et al., 2013). Agriculture is a great receiver from the work of genomic studies, a possible increase of yield in animal products or plant products are only going to be beneficial. For example plants can be selected for higher yield, lower cost, disease resistance and environmental resistances,
essential for an ever growing population under the changes of global climate change (Huang et al., 2010; Morrell et al., 2012).

To best summarise the use of genomics is in the aftermath of the human genome project, where it was set out in light of results to understand all functional parts of the genome, to then apply this to the improvement of human life. To quote architects of the project (Collins et al., 2003a);

“If we, like bold architects, can design and build this unprecedented and noble structure, resting on the firm bedrock foundation of the HGP [human genome project] then the true promise of genomics research for benefiting humankind can be realized.”

1.2.1 Evolutionary genomics

The use of comparative genomics allows the detection of genomic features which are similar (or contrasting) of organisms within the same species and organisms of varying species, this allows a phylogenetic and evolutionary pathway to be predicted. Over evolutionary time, DNA will differ as reflected by the process of natural selection to drive the progression of species and fill the diversity of life we see today (Ellegren, 2008; Diekmann & Pereira-Leal, 2015; Currat et al., 2015). The use of new high throughput techniques of recent years allows better coverage of the individual’s genome and the collective genome to resemble the population, with aid from computational tools, data can be quickly analysed with visualisation to increase efficiency of research (Bergman, 2007; Shendure & Ji, 2008). In reference of human evolution, genomic studies have underpinned the origin of lactose tolerance, originally presumed to be of European north west origin due to high lactase concentration, however modern computational modules suggest that it was in fact near east in origination 8000 years ago (Itan et al., 2009; Gerbault et al., 2011). Genome exploration into individual human populations has also revealed interesting allelic variants to deal with adaptation of environmental conditions, such as a high-altitude positive selection loci and resistance adaptability of select pathogens (Bigham et al., 2009; Toprak et al., 2012; Casanova et al., 2014; Eldholm et al., 2014).
1.2.1.1 Chromosomal mechanisms of evolution and speciation

Implementation of gross chromosomal changes over time can drive the formation of new species via the process of speciation (Livingstone & Rieseberg, 2004). The formation of a new species requires reproductive barriers to exist to maintain the genetic/phenotypic differences established, allowing enough diversion to form a true new species (Dobzhansky, 1936; Conyne & Orr, 1989). Reproductive barriers can be caused by different means: in the method of extrinsic reproductive isolation, it shall cause a divergent selection process of populations due to ecological conditions, i.e. geographical features. Alternatively via genetic drift intrinsic reproductive isolation can occur to drive speciation, genomic conflict and genetic incompatibilities within populations form this reproductive barrier splitting the population apart. Examples of causing genomic conflict include; inversions, gene duplication, transpositions, gene loss and underdominance (Rieseberg & Burke, 2001; Conrad & Antonarakis, 2007; Innan & Kondrashov, 2010).

Inversions within chromosomes to drive speciation will be reflected within the karyotype (Kirkpatrick & Barton, 2006; Larkin, 2010). These chromosomal changes, such as inversions, can create post-zygotic barriers to reproduction such as the reduction of the fertility of chromosomal heterozygotes (heterokaryotypes) by suppressing recombination (Sturtevant, 1917; Dobzhansky, 1947; Dobzhansky, 1970; King, 1995). This reduction of recombination of chromosomes within a population will contribute to the division of said population and the formation of a new species (Felsenstein, 1981; Butlin, 2005).

The process of chromoanagenesis (see section 1.3.4.2) in describing the event of a singular ‘macro mutation’/genomic rearrangement event, can drive evolution in a non-pathogenic event as is typically associated with the formation of cancer genomes. A process of chromoanagenesis is via chromothripsis (section 1.3.4.2 and Figure 1.15). Chromothriptic events are found to occur within germline cells or during early embryonic development; within humans, complex but balanced genomic rearrangements, via chromothripsis, can be tolerated to result in perfectly viable live births (Pellestor et al., 2014). The concept of chromoanagenesis fits with proposed models of evolution, opposed phyletic gradualism which relies on small changes over time to arise in new species. Evolutionary models such as the ‘hopeful monster’, summarising the generation of a new species upon birth (Goldschmidt, 1982) or the theory of ‘punctuated equilibrium’ have credibility if chromoanagenesis is to be found throughout evolutionary events (Goul & Eldredge, 1972). Yet the mechanisms and cellular pathways responsible for these events...
are highly conserved throughout life (Pellestor, 2019), with reference to the previously described chromothripsis event, it could be seen as a short-term adaptation mechanism to quickly generate population variants in response to quickly changing selection processes. Further research should be expected to shed light on the possibility of chromothripsis as an evolutionary process (Pellestor & Gatinois, 2019). As explained in the further section of 1.3.4.2, chromoanagenesis events are classically seen in cancers, however with the view that they could arise due to sudden selection pressure presence (aka cytotoxic drugs) a singular event to rapidly alter the genome, despite high cell death, could be beneficial as a survival strategy (Liu et al., 2014; Christine, 2018). These events have been somewhat eluded to in other organisms: within gibbon lineages there has been an insertion of a retrotransposon within gene regions dealing with cellular cycle progression and chromosome segregation (Meyer et al., 2016), similar events as this appear at the origin of high rate chromothripsis like events presumed to cause the accelerated gibbon karyotype observed in different lineages (Carbone et al., 2014). Not just in the great apes, chromothripsis has been observed within marsupials (Deakin, 2018), S. cerevisiae (Anand et al., 2014), Tasmanian devil cancers (Deakin et al., 2012)) and even plants (Tan et al., 2015; Carbonell-Bejerano et al., 2017). As these events are seen somewhat throughout nature, could it be a means to drive natural genetic variation observed? Could be a means to drive natural genetic variation?

1.2.2 Animal genomics

Motile multicellular eukaryotes with separate differentiated tissue such as dedicated nervous tissue, apart from the sponges (Porifera) make up the kingdom of Animalia. Within the early vertebrate lineage more than 450 million years ago, two independent whole chromosome duplication events took place, referred to as the 1R and 2R event (Dehal & Boore, 2005; Nakatani et al., 2007; Putnam et al., 2008). Evidence of these duplicative events is evident within current human genomes as highly conserved genes exist in duplicate, such as glycolytic enzymes (Singh et al., 2015; Steinke et al., 2006). Reconstructive studies have retrospectively analysed multiple extant animal genomes to reconstruct a hypothesised Amniota genome (Sacerdot et al., 2018). This study takes these extant genomes to predict the chromosome number representative of populations in their respective evolutionary lineage. Pre-vertebrate ancestors (tunicates, i.e. sea squirts) were presumed to have 17 chromosomes, to double within the first whole duplication even to 34, seven chromosome fusions were then undertaken before the second whole duplication up to 54 chromosomes. Furthermore, between the jawed vertebrates (Gnathostomata)
and the jawless fish (Cyclostomata) four further chromosome fusions took place to resemble the bony vertebrate (Euteleostomi) genome of 50 chromosomes, with presumed micro-chromosome ancestors. A third ‘fish’ specific genome duplication ‘3R’ event occurred in the bony/ray finned fish lineage (actinopterygii) to explain the large chromosome count of these animals, however during duplication most genes were lost or gained alternate functions which may explain the high dominance of this class within all ‘fish’ shown by their evolutionary success and wide biological diversity (Meyer & Van de Peer, 2005; Near et al., 2012).

Another interesting example of genomic development over the animal lineage is resembled in the Hox family of transcription factors. These factors are thought to be also duplicated in 1R and 2R events (Krumlauf, 1994; Wellik, 2009). Presumed to originate in choanoflagellates, the single cellular ancestors of Porifera (Wenger & Galliot, 2013), and shown as representatives of these transcription factor products in specification of Cnidaria (i.e. jellyfish and anemones) bodily features (Martindale, 2005). In vertebrates, Hox genes are regulatory factors that specify cellular fate in the spacial axis of embryos, ensuring correct anterior/posterior skeletal structure and morphology (Amores et al., 1998). In the event of deletions they shown to be lethal or concur with vast developmental issues (Kmita et al., 2005)). In evolutionary genomics, Hox clusters are seen in a singular copy within the invertebrate chordates opposed to mammals which have four clusters and zebrafish have duplicated up to seven clusters (Kappen & Ruddle, 1993).

The means to detect conserved features or blocks of chromosome homology has traditionally been detected via the use of cross-species chromosomal painting (See section 1.1.5.3). This painting methodology allows direct comparison of ‘like-like’ sequences seen across differing species to understand possible rearrangements and discover new configurations, these events that would give more insight into their evolutionary lineage and explain the diversity of mammals seen in life (Ferguson-Smith & Trifonov, 2007). To identify chromosomal events to be able to identify karyotype evolution lineages across all animals, and in particular mammalian orders, well help bring light upon interesting areas of evolutionary biology such as the evolutionary events underpinning the arrival of sex determination for example.
### 1.2.3 Avian Genomics

Avian genomes are uniquely interesting by their stable karyotype and presence of microchromosomes (Burt, 2002). An important taxonomic group as they are filled with such rich phenotypic diversity and of great importance throughout human society. In respect to usefulness in human society, birds are of great importance for food production (approximately 20% of meat consumption), culturally significant in terms of prized racing/hunting birds and genetically of great interest due to neural/vocal functionality paired with the abundance of flight (Alexander, 2000; Griffin, 2007). Birds are host to multiple model species for research. The chicken is an easy to manipulate amniote used for developmental studies as well as genetic expression studies, the chicken was also the first fully sequenced avian species and as result is used extensively in genomic studies (Burt, 2007). The pigeon, *Columba livia*, is important within cognitive and intelligence studies (Emery, 2005). Whereas the zebra finch, *Taenopygia guttata*, a songbird used for exploration into non-mammalian auditory systems (Zeigler & Marler, 2004), with many members now having fully annotated genomes (International Chicken Genome Sequencing Consortium, 2004; Warren et al., 2010; Zhan et al., 2013; Jarvis et al., 2014).

Among the amniotes the birds have some of the smallest genomes with multiple hypotheses attributed to this phenomenon, such as the adaptability towards flight (Hughes & Hughes, 1995). Somewhat conserved amongst the avian group is the karyotype, obviously with exception of some families such as the hornbills (2n=42) and the Corythaixoides (2n=136-142) (Christidis, 1990). Most birds have diploid numbers in the range of 74 to 86 chromosomes, and for the majority the avian karyotype appears to resemble one another (Griffin 2007). Exceptions to this karyotypic similarity are the grossly rearranged orders of the psittaciformes (parrots), the falconiformes (falcons) and accipitrids (eagles) (de Oliveira et al., 2005; Nanda et al., 2006; Tagliarini et al., 2011; Joseph et al., 2018), with most showing their microchromosomes fused to larger macrochromosomes. Comparative cytogenetics within the avian class with use of comparative paints has allowed easier comparison of chromosome homology and specific DNA markers generated to question microchromosomes (Lithgow et al., 2014; Romanov et al., 2014; Damas et al., 2017). The use of paints does not allow the correct identification of hidden intrachromosomal rearrangements, as any inversions or duplications of chromosome material for example would not display a change of fluorescent pattern.
These hidden rearrangements are predicted to be found within multiple birds that have already been analysed via paint techniques and are an interesting avenue of possible research (Griffin et al., 2008; Skinner et al., 2009; Volker et al., 2010)

**1.2.3.1 The Galliformes**

The galliformes are found worldwide with many well known taxa, such as the pheasianinae (the common pheasants), the quails (both old world and new world quails, Odontophoridae), cracinae (curassows), Meleagridinae (turkeys) and numididaes (guideafowls). See section 4, for more detailed information into the investigation of possible hidden intrachromosomal rearrangements for these birds.

**1.2.3.1.1 The Chicken**

The chicken (*Gallus gallus*) is of key importance within the genomic studies of the avian group. First sequenced in 2004 (International Chicken Genome Sequencing Consortium, 2004) the chicken was the first bird to be sequenced and is typically used as a reference to compare newly sequenced birds to (Burt, 2007). With the chicken as a key research species, it has allowed important questions to be answered, one of which is the function of the bursa and the classification of B-cells (Glick et al., 1956). The induction of the avian leukosis virus (ALV) caused the generation of the DT40 cell line (Baba & Humphries, 1984; Baba, 1985), a useful cell line that can be easily genetically manipulated allowing wide use for a variety of cellular based investigations (Malhotra et al., 2017)(Section 5.1). Use of this cell line has been invaluable in multiple studies (Sonoda et al 1998; Chi 2018) however it has fallen out of favour recently due to advances has in other means of genetic manipulation, (CRISPR). Regardless, the cell line is a useful tool to assess from a genomic point of view to assess chromosomal aberrations that may cause the tumorigenic state seen of the cell line.

**1.2.3.1.1 Nuclear organisation in chicken**

Chromosome territories within interphase state nuclei have been looked at in multiple species, an early investigation was performed in Indian muntjact due to its low chromosome count (2n=6 for females) allowing easy identification of chromosomes within the nucleus (Yang et al., 1995; Ferguson-Smith, 2011). The organisation and structure of genetic material in the 3D state of a working nucleus is essential for correct cellular functionality, with abnormal structure leading to disease phenotypes (Foster & Bridger, 2005). With most studies focusing on mammals, avian interphase studies are not as
abundant (Skinner 2009b). However initial studies using singular BACs of macrochromosomes and microchromosomes have been performed to show an indication towards a nuclear periphery localization for macrochromosomes opposed to the gene dense microchromosomes which on average occupy a more central position (Skinner 2009b). However with the use of single BAC’s, there is a requirement to somewhat introduce a methodology using multiple BAC’s to assess longer chromosomes (the macrochromosome) position and arrangement within a ‘working’ interphase nuclei to further explore the relation between 3D space and phenotypic effect.
1.3 The Biology of Cancer

1.3.1 A brief overview of cancer

Cancer is a disease that spans throughout all of human history (Hajdu, 2011), detailed in ancient Egyptian and Greek periods (Breasted, 1930; Karpozilos & Pavlidis, 2004) and evidence found of specific cancers in archaeological remains (Strouhal, 1976; Binder et al., 2014). Genetic deviations have been thought of as the key mechanism that drives the prolific growth and malignancy of cancers since 1969, as suggested by Heubner and Todaro (Huebner & Todaro, 1969). More recently molecular genetics has progressed to a stage where the presence and gain of chromosomal aberrations, mutations in proto-oncogenes and tumour suppressor genes clearly drive tumour progression (Harris & Hirohashi, 1992; Bishop & Weinberg, 1996; Curtis et al., 2012). The term ‘cancer’ is a generic term used to a collective of diseases which share the property of abnormal and uncontrolled growth of self cells to spread into other areas of the body (WHO Cancer Control Programme, 2019; Cancer.gov, 2019; CancerResearchUK, 2019). Cancers are a separate group of neoplasms, every neoplasm can be seen as a group of cells that present with unregulated growth, such as hyperplasia or dysplasia, but not for the most part from into a ‘true’ cancer (Cancer.gov, 2019). The initiation of cancer shows no symptoms, only during later periods of expansive cellular growths or metastasizes symptoms can arise. These typically nonspecific symptoms are attributed to other issues such as coughs to respiratory infections and changes of bowel habits to diet. These nonspecific symptoms and failure to immediately diagnose has seen cancer been regarded to as medicine’s ‘greatest imitator’ (Segen, 1992; Kufe & Pollock, 2010; Del Paggio et al., 2017).

The formation of cancers are, in no way, caused by a single step or event, it is estimated that each of the ~$10^{13}$ cells of a human can have from tens of thousands of DNA lesions to one million changes to its DNA in a single day (Lodish et al., 2008; Jackson & Bartek, 2009), yet these have to be advantageous towards tumorigenesis and not repaired by normal cellular responses (Tomlinson et al., 1996; Croce, 2008). When looking at the rate of mutation in the human body, it is surprising we do not have more cancer across the population. With the estimate of at least one million mutations in a single cell per day, we would presume these were equally spread across all 23,000 protein coding genes and that some would be missed. With approximately 350 out of these 23,000 proteins coding genes are known to be attributed with cancers, the cancer rate should be tremendously higher.
across our $10^{13}$ cells that what we currently observe (Frank, 2010). Thankfully the cell and body has systems in place, such as a multitude of internal cell checkpoints and a very specific immune system, we are not over encumbered with malignant cells. However when cancers do arise, they must overcome all of these ‘defences’ via formation of what is known as the ‘cancer hallmarks’.

Proposed in 2000, and later reviewed and improved in 2011 (Hanahan & Weinberg, 2000), Hanahan and Weinberg suggested the classification of hallmarks to “constitute an organizing principle for rationalizing the complexities of neoplastic disease” (Hanahan & Weinberg, 2011). The view conceptualizes the multistep process normal cells must undergo and evolve each hallmark successively to survive, become tumorigenic and eventually malignant. There is also the notion that a cancer must not just be understood by the traits shown by the cancer cells themselves, but by the ‘tumour microenvironment’ characteristics and tumour associated interactions (Hanahan & Weinberg, 2011).

As mentioned previously, overruling cellular growth restrictions to provide uncontrolled growth is seen in all cancers and cells must sustain this unending division. Typically normal cells can respond to internal checkpoints to progress through cellular division, or can respond to external stimuli, such as growth factors or suppressors (Ullrich & Schlessinger, 1990). However cancer cells deregulate this widespread signalling pathway to ensure continuous propagation (Hynes & MacDonald, 2009; Lemmon & Schlessinger, 2010). This can be achieved by the cancer cells either self stimulating by producing their own growth factors or via stimulation of normal cells within the tumour microenvironment to supply them with growth factors (Bhowmick et al., 2004; Cheng et al., 2008).

Alterations in growth factor receptors is another possible avenue taken, the alteration to induce constant signalling provides self-sustaining growth (Jiang & Liu, 2009; Davies & Samuels, 2010) and a deregulation of negative feedback systems, such as ras gene with Ras oncoprotein which would typically regulate that an input of growth signal is temporary or cellular senescence is performed (Bardessy & Sharpless, 2006; Collado & Serrano, 2010).

To continue on the theme of growth, the cells must also become resistant to internal and external suppressor signals. Retinoblastoma-associated protein (RB) interprets both internal and external signals to allow the cell to progress through its cellular cycle (Burkhart & Sage, 2008), defects in this pathway is a means to allow cell cycle progression regardless of any suppressant signals.

Inflammation and recruitment of associated inflammatory cells, (mast cells, neutrophils, B/T cells) has been shown to drive growth by release of tumour growth factor
EGF and chemo attractants/cytokines which induce a higher inflammatory state which can induce more tumorigenesis factors (Egebald et al., 2010; DeNardo et al., 2010). Invasive capabilities can be incorporated by use of these inflammation cells, as they typically release extracellular digestive enzymes (Qian & Pollard, 2010). Alongside this, the release of DNA damaging (therefore mutagenic) reactive oxygen species by these cells can increase their genetic instability and possibly inducing more malignant mutations (Grivennikov et al., 2010).

Internal signals from the cell would occasionally drive the cell towards apoptosis, a highly regulated and pre-programmed method to act as a safeguard against ill functioning cells (Adams & Cory, 2007). Stresses to induce apoptosis such as extensive irreparable DNA damage (Roos & Kaina, 2006) and oncoprotein elevated levels can be evaded by mutation of the apoptosis machinery. Examples of this include; the up regulation of anti-apoptotic Bcl-2 family (Reed, 1995; Adams & Cory, 2007), loss of TP53 function (Ozaki & Nakagawara, 2011), increase of survival signal IGF-1/IGF-2 (Kooijman, 2006) and down regulating pro-apoptotic factors BAX/BIM (Campbell & Tait, 2018). Likewise to apoptosis, autophagy also needs to be prevented as it would activate in response to high levels of internal cellular stress. Inactive autophagy pathway caused by mutations are shown to increase the levels of susceptibility to cancer in mice (White & DiPaola, 2009), indicating another barrier that must be overcome to develop a tumour.

Tumour genomes are seen to have an increase in mutation frequencies, termed genomic instability (Lengauer & Kinzler, 1998). Genomic instability within tumours must exist in a manner to provide cancer cells with the means to drive tumour progression, opposed to waiting for natural DNA damage events to happen and be beneficial. Genomic instability is the foundation feature of a cancer cell to gain every other tumour trait required to form into a developed cancer. Therefore it is essential for there to be a disruption to the genome (i.e. oncogenes/tumour suppressors) that can propagate across the ‘population’ successfully. As previously described (section 1.14), cancers are seen in almost all chromosomal aberration types with key specific aberrations at particular sites of the genome indicative of gene areas, which when disrupted, favour tumour progression (Korkola & Gray, 2010).

Other key hallmarks of cancer are shown in figure 1.11, such as promotion of angiogenesis to enable the growing tumour a steady income of resources to grow further and the induction of metastasis by invasive mechanisms activation. These are detailed further in Hanahan & Weinberg 2011 and in figure 1.12.
Personalized medicine in cancer is gaining traction to allow targeted therapies for individuals rather than a one size fit all approach guided by their genetic profile (Jackson & Chester, 2015). Specific patterns of genomic imbalances can be found across tumours of various types, highlighting specific cancer genes yet also allowing clinicians to comment on disease progression and therapeutic choices used (Gebhart, 2004). Via the use of CGH and aCGH, a top down approach can be used to detect these patterns in individuals, such as copy number of particular genes which may be representative of the incidence of targetable oncogenes. However as previously discussed, this methodology can misinterpret results by the global approach used opposed to looking at the individual level which could miss key details, such as subpopulation aberrations (Pinkel & Albertson, 2005). Missing details in cancer diagnostics can be fatal as incorrect treatment, inability to detect subpopulations and presence of resistant tumours can lead to a cancer recurrence at a later date that could be unresponsive to previously used therapies (Landau et al., 2013; Pereira et al, 2016; Morris et al., 2016; Zhao et al, 2016).

Figure 1.11. Cancer hallmarks and appropriate therapeutic targeting.

The acquired capabilities of cancer needed to survive and eventually metastasize; each function can be gained via different biological means but can be summarized via observable phenotypical trait. Surrounding each hallmark is an investigational or current drug to target these specific characteristics. (Taken from Hanahan & Weinberg, 2011).
1.3.2 Causes of Tumorigenesis

Disruption of multiple cellular processes is vital in the establishment towards formation of a malignant tumour, long has there been fascination over the underlying factors or agents responsible for these progressions. With an increasingly industrialised and globalised world, the rates of cancer have increased dramatically thought to be carcinogens released as a by-product of these processes (Vineis & Wild, 2014). New knowledge paired with education about cancer risk factors is thought to be able to reduce incidence of cancer by a third (Vineis & Wild, 2014), and in an ever developing world prevention opposed to cure is vital for a healthy populace. Here shall be a tour from original theories to modern day hypotheses from extrinsic environmental factors, biological agents to culminate with agents within the genome itself.

1.3.2.1 Environmental Carcinogens

Historically, the first theories about external influences or carcinogens arose from the observation that specific occupations of the time had higher incidences of specific cancers. In approximately 1770-80, chimney sweepers were seen to have an increased rate of cancer of the scrotum which was linked with consistent exposure to soot (Pott, 1775).
Additionally around this same time, tobacco snuff was observed to be in association with
the prevalence of nasal cancers (Redmond Jr, 1970). The later discovery of X-rays (which
was a medical breakthrough in itself by the visualisation of bones without pain) (Röntgen,
1896), radiologists would routinely use their own hands to calibrate and test their
equipment to ensure correct working order. This would lead them to develop a new
condition termed ‘radiodermatitis’ in the following days (Pitkin, 1903), which would result
in eventually develop skin cancers in later years (Porter & White, 1907).

The prevailing theories were that cancer could be caused by three different factors,
chronic irritation, displaced embryonic tissue or an infectious agent/parasite (Oberling,
1994). There was a need to experimentally induce a cancer with an ‘agent’ to disprove
these theories. The first successful induction of cancer was performed on the ears of
rabbits (as cancerous tissue was rarely seen here and must have been due to the influence
of external/exogenous factors) (Yamagiwa & Ichikawa, 1918). This lead to eventual
formation of cancerous lesions after years of tar applications with some eventually showing
the presence of metastases (see figure 1.13) (Yamagiwa & Ichikawa, 1918). Following this
research showing that an environmental agent could cause the formation of cancerous
cells, the cancer inducing compound of tar was identified (Kennaway, 1995), and lead
researchers to look into other professions and retrospectively. Subset of workers in the dye
industry presented with high rates of bladder cancer, found to be due to long term
exposure to β-napthylamine (Case et al., 1954), and high rates of lung cancers in miners
was due to exposure of uranium, the very material they were mining (Wagoner et al.,
1965). World war one veterans and Japanese survivors of Hiroshima/Nagasaki atomic
bomb explosions developed high rates of lung cancer and leukaemia in later life allowing
retrospective conclusions about the cancer-causing effects of exposure to mustard gas and
atomic exposure (Case & Lea, 1955; Folley et al., 1952). Exposure to radioactive material
was identified again as a cancer causing agent years after exposure. USSR scientists
identified an increased risk of liver, lung and bone cancers of those involved in
research/exposure to plutonium and in nuclear plants (Koshurnikova et al., 1994;
Koshurnikova et al., 1998), and after the Chernobyl explosion of 1986 to release radioactive
iodine-131 into the atmosphere there was an increase of childhood thyroid cancers in
surrounding regions (Kazakov et al., 1992; Shibata et al., 2001).

Other examples of carcinogens include; diesel fume exposure (Garshick et al., 2004),
ultraviolet radiation exposure (Ward, 1988; D’Orazio et al., 2013), a possible side effect of
hormone replacement therapies (Smith et al., 1975), the use of immunosuppressant azathioprine for organ transplant recipients (Penn et al., 1969), and cigarette/tobacco smoke (Evans, 1962; Doll & Hill, 1999). It is clear that there are large variations of carcinogens present in a modern day environment, with many taking years to come to fruition. In the aftermath of the collapse of the World Trade Centre (Li et al., 2012) many causative agents have been identified and most likely more will follow in time after delayed onset of cancers (Hutchings & Rushton, 2011; Rushton et al., 2012; Health and Safety Executive, 2018), yet there is now a requirement to investigate possible co-operative synergistic effects between multiple agents that could have carcinogenesis effects (Goodson III et al., 2015). However, modern investigations into carcinogens will likely see the reduction of cancers with application of: prevention strategies, identification of early tumours before they become lethal and better treatment options (Yuspa, 2000).

**Figure 1.13. Process of metastasis in typical cancer to colonize new sites**

Normal cells can undergo transformational changes to form a malignant tumour. From an *in-situ* cancer, further mutations can allow cells to escape extracellular confinement (such as the basal lamina). Cells can spread through the lymphatic system or the circulatory system to invade and colonise other tissues to form metastatic tumours. (Based upon Steeg, 2003).
1.3.2.2 Infectious agents

One of the original hypothesis of cancer induction was the action and presence of parasites (Oberling, 1944), thus it is unsurprising that research has been conducted to attempt to find an infectious agent as an underlying cause of cancer.

Bacteria such as *Helicobacter pylori* has been noted to cause stomach cancer (Egi et al., 2007; Peter & Beglinger, 2007) and a subclass of lymphoma (Morgner et al., 2000), however most other cases of bacteria associations with cancer have unclear relationship between the infection and tumorigenesis (Mager, 2006). The main area of research and interest has been on the influence of viral presence and as the causative agent, as it is not uncommon to see a cancer be attributed to viral infection (Carrillo-Infante et al., 2007).

Many non-human viruses were isolated from animals shown to have various cancers such as the avian sarcoma leukemia virus (ASLV) in 1908 (Blackadar, 2016), the mouse leukaemia virus (Gross, 1951), feline lymphoma virus (Jarrett et al., 1964) and retroviruses found in nonhuman primates and cattle (Kawakami et al., 1972; Kettmann et al., 1975). It was only in the 1970’s that dedicated funding and research was aimed towards discovery of human specific oncogenic retroviruses (Gardner, 1994; Voisset et al., 2008). Although not the first oncogenic virus discovered, a retrovirus was discovered within a T cell lymphoma cell line (HLTV-1) (Poiesz et al., 1980) which was observed to integrate into its hosts genome before any expansion of tumour cells (Yoshida et al., 1984; Hinuma et al., 1981) to produce proteins tax and HBZ which induce cellular disruption and genetic alterations (Mesnard et al., 2006).

The oncovirus Hepatitis B has been indicted in the formation of liver cancer (heptocellular carcinomas, HCC) (Szmuness, 1978). A study performed in Taiwan looked for hepatitis B indicators within healthy males, then retrospectively assessed many years later the prevalence of HCC, which was much higher in infected than those non-infected (Beasley et al., 1981). Due to the high levels of hepatitis B, government driven vaccination for this virus was introduced and saw a marked decrease in prevalence of childhood HCC to further give evidence that this is a true oncovirus (Chang et al., 1997; Chein et al., 2006).

Hepatitis B is known to produce oncogenic proteins (Kim et al., 1991) and when integrated into the hosts genome is observed to increase the rate of chromosomal instability (Tokino et al., 1987), and typically seen in cases of HCC the recruitment of self-immune system cells to damage infected and un-infected cells (Chen et al., 2006).
Human papillomavirus (HPV) comes in approximately over 170 ‘types’ (Bzhalava et al., 2013), it is estimated to infect 12% of women worldwide at any single time (World Health Organisation, 2014) and is predicted that over 50% of US sexually active individuals will be infected with a type of HPV at some point in their life (Baseman & Koutsky, 2005). There is a strong association of HPV-16 and HPV-18 in cervix cancers, where these specific types are observed in 97-98% of all cervical cancer biopsies (Dürst et al., 1983; Boshart et al., 1984; Walboomers et al., 1999; Muñoz et al., 2003). Oncoproteins associated with these two have been shown to influence the degradation of p53 (Scheffner et al., 1990) and increase the rate of chromosomal instability (Boyer et al., 1996). Interestingly, the virus does not typically need to integrate into the host genome to replicate, yet has high incidence in cancer cells (Arias-Pulido et al., 2006; Pett & Coleman, 2007) showing an alternative oncogenic viral mechanism. HPV vaccines are clearly shown to reduce the prevalence of cancers (Lehtinen et al., 2012). Long term retrospective population studies in the UK observed routine vaccinations for past 10 years for girls, to shown a reduction of 90% of pre-cancerous cells of the cervix (Palmer et al., 2019).

Another virus (the first oncogenic virus discovered) associated with oncogenic properties is the Epstein-Barr virus (EBV) Epstein-Barr virus, which is reportedly seen in Burkitt’s lymphoma (Burkitt, 1983; Epstein, 1964; Henle et al., 1969), and nasal pharynx cancer (Ji et al., 2007). With EBV the action of microRNA’s on cellular metabolism is considered to be the malignant inducing pathway (Raab-Traub, 2012). Human immunodeficiency viruses (HIV) can be viewed as an indirect oncogenic virus due to high levels of Kaposi’s sarcoma associated with infections (Centers for Disease Control (CDC), 1981; Bovi et al., 1986). Through the action of the HIV tat protein, cellular expression can be altered within immune cells to suppress immune system functions, activate apoptotic genes (which can cause genomic aberrations, see chromoanagenesis) and cause mass death of non-infected immune cells (Johri et al., 2011). With these functions in mind, it is not surprising that carriers of HIV develop cancers and that HIV can be seen as an oncovirus as a key hallmark of immune system survival has been bypassed.

Other oncoviruses include; the Human T-lymptotropic virus, the first discovered human retrovirus (Yoshida, 1982; Gallo, 2005), Hepatitis C (Houghton 1989), the Kaposi’s sarcoma-associated herpes virus (Antman & Chang, 2000) and Merkel cell polyomavirus (Feng et al., 2008). Strong associations exist between presence of oncogenic virus and associated cancer, such as the example of Merkel cell carcinomas with 70-80% carcinomas presumed
to be caused by the Merkel cell polyomavirus (Feng et al., 2008) and near 100% of cervical
cancers attributed to papilloma viruses (Parkin, 2006).

Some human endogenous retroviruses (HERVs) who’s remnants are left over within the
genome over evolutionary time typically make up non-coding regions of the human
genome with minimal or no purpose (Stoye, 2012). However, there are occurrences where
there is increased expression of HERV in serum, melanomas and in particular breast
cancers (Hohn et al., 2013; Schmitt et al., 2013; Wang-Johanning et al., 2014). Whether this
is due to resurgence or reactivation of viral mechanisms or an effect of malformed
expression due to the cancerous nature of malignant cells is still up for debate.

1.3.2.3 Genetic contributions

Having described thus far the mechanisms of cancer induction via external stimuli,
it is vital we explore and understand the endogenous factors of tumorigenesis. The
formation of cancers is known to typically arise from ‘normal’ DNA mutation events
induced by DNA damage (Kastan, 2008), as previously stated, the typical human cell
receives tens of thousands of DNA lesions per day (Lindahl & Barnes, 2000) and if not
correctly repaired they can lead to further mutations to become harmful to the cell or the
organism as a whole. Typical physiological processes such as DNA mismatches during
replication, DNA strand breaks (Hsieh & Zhang, 2017), hydrolytic reactions of the
phosphodiester backbone or DNA bases (Shapiro, 1981; Gates, 2009) and reactive oxygen
compounds from oxidative respiration or from immune cells at inflammation sites
(Kawanishi et al., 2006). Clearly described thus far and a frequently recurring feature, the
cancer genome requires genomic instability (Stratton et al., 2009).

Typically, germline mutations are only seen in 1% of cancers and are the distinctive
causative effect of highly aggressive tumours (Fearon, 1997), compared to the majority of
other cancers which are sporadic in nature or are thought to have a hereditary component
not yet identified (Lichtenstein et al., 2000). In the context of familial contribution to
cancer, the correlation between distinctive cancer patterns and families is seen to be high
(Goldgar et al., 1994; Hemminki et al., 2007). Examples of this include studies of families
which have high incidence of prostate cancer (Zeeegers et al., 2003), breast cancer
(Collaborative Group on Hormonal Factors in Breast cancer, 2001) and colon cancer (Fuchs,
1994). As it is hard to derive a definitive causative agent to link common cancers in family
lineages due to typical shared experiences/exposures in reference to environmental
influences, it is possible to perform a more crude Mendelian approach in search for ‘cancer
genes for high penetrance in cancers which seem to be inherited. Twin studies show that monozygotic twins (identical) have higher rates of developing cancer if their twin developed a cancer in life compared to dizygotic twins, indicating that there is somewhat a genetic disposition or mechanism at play (Page et al., 1997; Lichtenstein et al., 2000).

By use of Mendelian genetics, it is possible to look into these families for specific causative genes yet these only typically make up 5% of inherited cases. The identification of BRCA1 and BRCA2 variations has an association with increased risk of breast cancers and ovarian cancers (Miki et al., 1994; Wooster et al., 1995; Welch & King, 2001). Understandably BRCA1 and BRCA2 are associated with correct DNA repair after double strand breaks events so it is of no surprise these are associated with the incidence of cancer risk. However it is only observed that between 3-8% of breast cancers actually carry a mutation in either BRCA1 or BRCA2 (Brody & Biesecker, 1998), and 18% of ovarian cancers has a mutated BRCA1 where 13% is inherited through the germline and 5% somatic mutations. Li-fraumeni syndrome is a classic example of an autosomal dominant hereditary disorder (Li & Fraumeni, 1969) that is characterized by early onset of a wide variety of cancers and a development of cancers throughout the life time of an individual (Hisada et al., 1998). This syndrome is associated to p53 mutations that can arise through novel mutations but is shown to be highly heritable (Varley, 2003). Typically, a tumour suppressor, expression occurs during cellular stress (DNA damage) to halt cell cycle progression to either direct DNA repair or induce apoptosis (Zilfou & Lowe, 2009). However in mutated forms this checkpoint is made redundant to possibly allow cells to divide uncontrollably. The mechanisms of inherited Li-fraumeni syndrome are heterozygous for p53 mutation with a high majority having missense mutations in the DNA binding domain to cause a decrease in efficiency of p53 to bind to DNA (Malkin, 2009). As this syndrome is autosomal dominant, this can happen due to the p53 mutant expressed is more stable than wild-type p53 and has a degree of interaction between mutant and wild type proteins that can suppress its function (Willis et al., 2004).

An example of an inherited autosomal recessive defect is shown in xeroderma pigmentosum (XP) first described by Kaposi in 1882 (Kaposi, 1882), who also described Karposi’s sarcoma as a cancer of the skin from which its name derives. Not associated with a single mutation, XP typically has defects in nucleotide excision repair enzymes causing the effected individuals more susceptible to UV light (Kraemer & DiGiovanna, 2016). Multiple genes associated with XP represent separate groupings based upon phenotype expressed.
which are derived by the mentioned different cocktail of mutated genes (Schubert et al., 2014; Fassihi et al., 2016). The 13 known genes are DDB2, ERCC1, ERCC2, ERCC3, ERCC4, ERCC5, ERCC6, ERCC8, GTF2H5, C7orf11, POLH, XPA, and XPC (DiGiovanna & Kraemer, 2012). Most of these genes are associated within pathways of nucleotide excision repair, with DDB2 determines initial lesion recognition in non-transcribed DNA (Clement et al., 2010) and ERCC2/ERCC3 aid in unwinding the DNA in the region of damage to be repaired (Boyle et al., 2008). The multitude of genes involved reflects the median age of death with XP is 37 years or 29 years if an individual is a sufferer of particular gene defect combinations to have neurodegeneration (Kraemer & DiGiovanna, 2016). Inherited factors are easy to identify within family with alleles of high penetrance, however this is much harder with common or more sporadic in nature cancers, where only risk loci can be presumed but nothing substantial can be concluded (Cunningham et al., 2003; Chang et al., 2005) with only 5 loci weakly correlated to prostate cancer (Zheng et al., 2008).

It is clear thus far that the feature of genomic instability such as malformations in DNA repair mechanisms or regulatory pathways in cellular division being ignored, it is vital to reflect on what these specific DNA mutations bring about it directly cause a cancerous tumour. Many have been mentioned and mechanisms described so far in respect to specific conditions or aberrations, but here will be highlighted some of the more globally associated cancer genes, examples of oncogenes and the tumour suppressor genes.

1.3.2.3.1 Oncogenes

Oncogenes are typically viewed as a gene that somewhat can promote the development of cancer by increasing cellular growth/division. Precursors to oncogenes ‘proto-oncogenes’ are normal genes that typically allow normal cellular grown/proliferation which are unsurprisingly essential for bodily function. Whence acquiring a means of altering the proto-oncogenes function to induce a tumour, it becomes recognized as an oncogene (Todd & Munger, 2001). This activation can be brought about in three different modes. Firstly by a mutation in DNA sequence within the coding region or regulatory region, by an increase amount of the protein concentration (such as via increased expression, mRNA modifications or gene duplication –see section 1.14-) or by a chromosomal translocation event (either to a new position with higher expression or by interjecting into expression of another gene- again see section 1.1.4-).
One of the first oncogenes discovered was a small GTPase, the Ras family, compromising of H-Ras, N-Ras and K-Ras (Reddy et al., 1982; Bos, 1989). Mutations in the ras genes can eventually lead to the production of Ras proteins in an activated state, these proteins are involved in signal transduction pathways to induce cellular growth and survival genes to activate (Hilgenfeld, 1995; Goodsell, 1999) (Figure 1.12). Even though there are many other members of the Ras subfamily (Wennerberg et al., 2005), these three mentioned Ras proteins are implicated in many types of cancer (Bos, 1989), these are the most commonly found oncogenes in human cancer at 20-25% prevalence and up to 90% prevalence in pancreatic cancers (Downward, 2003).

An aberrant form of the c-myc gene is also found in a large variety of cancers, from lymphomas, leukaemia, breast cancers, blastomas and small cell lung cancers (Felsher & Bishop, 1999). As part of the myc family, c-myc is a regulatory gene that code for transcription factors, some of which are actively involved in cellular division, growth (Rahl & Young, 2014) and DNA replication (Dominguez-Sola et al., 2007; Gearhart et al., 2007). Translocation aberrations of chromosome 8 (location of c-myc) is seen in almost all cases of Burkitt lymphoma (Dalla-Favera et al., 1982; Finver et al., 1988), and general up regulation of this gene is seen in carcinomas across the body due to its high number of downstream targets (Figure 1.12). C-myc is typically over expressed in the majority of human cancers, and is seen to be a direct cause of at least 40% of these tumours (Dang et al., 2009), whereas in other tumours it is seen as a downstream effect of other activated oncogenes up regulating its function to push growth forward (Miller et al., 2012).

Mentioned in passing in previous sections, the Philadelphia chromosome is formed from a translocation event between chromosome 9 and 22, commonly seen in chronic myeloid leukaemia cells (Nowell & Hungerford, 1960; Kang et al., 2016). The proto-oncogene ABL1 on chromosome 9 is translocated to the BCR gene on chromosome 22, the outcome of this translocation is the production of a BCR-ABL1 fusion gene to encode the oncogenic protein BCR-ABL1 (Collins & Groudine, 1983; Hagemeijer, 1987). The exact breakpoint regions is known to be variable (Score et al., 2010), but will involve the required regions of both genes albeit with different variations of the fusion protein which can act as a marker for different forms of leukaemia (Pane et al., 1996; Li et al., 1999). The BCR-ABL1 protein has an elevated tyrosine kinase activity, normally to activate other signal pathways, yet unsurprisingly alike other oncogenes this hybrid protein actively maintains signal pathways for proliferation, resistance to apoptosis and preventing differentiation in cancer
cells (Lugo et al., 1999; Sattlermc & Griffin, 2003). This oncogenic protein also infers increased genomic instability, to encourage formation of double strand breaks by stimulating single strand repair processes unnecessarily (Cramer et al., 2008; Fernandes et al., 2009) and increasing enzymes involved in double strand breaks to ensure survival yet promote this genomic instability cancers rely on (Slupianek et al., 2011).

### 1.3.2.3.2 Tumour suppressor genes

With the view that proto-oncogenes push the development of cellular growth, we observe that there are regulatory systems in place to antagonize this drive to prevent the uncontrolled and ill-timed proliferation. Tumour suppressor genes are genes that somewhat protects the cell from the progression into cancer either by repressing the cell cycle or promotion of correct apoptosis (Sherr, 2004), initially shown by somatic cell fusion experiments indicating an existence of genes that could restrain tumour development (Harris et al., 1969; Stanbridge, 1979). Tumour suppressor genes functions can be classified into several categories where examples of genes may overlap between functions. As stated, the continuation of the cell cycle and promotion of apoptosis are two categories followed by acting as a link between the progression of the cell cycle and DNA damage, repair of DNA damage themselves and lastly cell adhesion detection (contact inhibitors for example) (Hirohashi & Kanai, 2003; Sherr, 2004).

The first classic suppressor gene identified was linked to retinoblastoma typically caused by a mutation in the gene RB or RB1 located on chromosome 13q14 (Noel et al., 1976), to give rise to a malformed retinoblastoma protein (pRb) (Murphree & Benedict, 1984), now understood to be one of the most important centres of cellular signalling (Weinberg, 1995; Du & Pogoriler, 2006). Manifesting in a rare cancer of immature retina cells in children, retinoblastoma was hypothesized to be caused by inherited mutations (Kundson Jr, 1971; Kundson Jr, 1973). Observed in retinoblastoma are two patterns of formation, a bilateral early onset tumour or sporadically later in life and typically only in one eye. Interestingly the bilateral early onset patients typically had a familial pattern of this disease indicating an inheritable element opposed to the unilateral sporadic patients not (Kundson Jr et al., 1976; Francke & Kung, 1976). This method of inheritance allowed formation of the ‘two hit hypothesis’ (Kundson Jr, 1971; Kundson, 2001) which hypothesizes that both alleles of a tumour suppressor gene was required must be knocked out in separate mutative events to cause a complete loss of function. However, there are known exceptions to this rule, such as p53’s self-repressive functionality (Baker et al., 1990)
or genes which require two non-mutated alleles, haploinsufficiency (NF1 as an example in neurofibroma (McLaughlin & Jacks, 2002), and holoprosensephaly, a incorrect formation of the forebrain due to the haploinsufficiency for Sonic Hedgehog (Nanni et al., 1999). The theory of ‘two-hit’ is appropriate for retinoblastoma as individuals who inherit one copy need only sustain one mutation, hence the expression of early life bilateral tumour formation (Kundson Jr, 1971; Kundson, 2001) opposed to two mutations required for sporadic formation. Furthermore, follow-up studies of hereditary and non-hereditary survivors showed that hereditary retinoblastoma acts as a higher risk factor for new cancers over time as compared to sporadic (Kleinerman et al., 2005). pRb restricts the cells ability to leave G1 phase and enter S phase (see section 1.1.3.2) (Goodrich et al., 1991) as it can bind to inhibit transcription factors such as E2Fs, which regulate multiple genes required for DNA replication (Funk et al., 1997; Nevins, 2001; Trimarchi & Lees, 2002), so unsurprisingly in mutated versions of the RB gene this function is suppressed allowing S phase promoting factors to proceed unchecked.

Another key tumour suppressor gene is TP53 which codes for the p53 protein. As already discussed in previous sections this gene is associated with the majority of cancers (Nigro et al., 1989; Ozaki & Nakagawara, 2011), Li-Fraumeni syndrome (Malkin et al., 1990) and multiple chromosome aberrations involving chromosome 17 (Salido et al., 2005; Zedan et al., 2015). The role of p53 is well understood (Levine & Oren, 2009) and is vitally important in tumour development as it was shown to infer a 100% penetrance cancer phenotype in TP53-null mice (Brož & Attardi, 2010). P53 can detect a wide variety of cellular stresses such as DNA damage, hypoxic conditions, cell cycle abnormalities, ribosome dysfunction and other oncogene expression. This serves to limit the division of cells (Kundson, 2001; Vousden & Prives, 2009) at G1 and G2/M phase (Mercer et al., 1990; Diller et al., 1990) by its functionality as transcription factor with its transactivation domain (Zhu et al., 1998) that can bind to specific DNA regions (Kern et al., 1991; Zauberman et al., 1993; Kitayner et al., 2006). The regulation of p53 by protein kinases that detect stress factors, such as MAPK family members which detect externally induced stresses (Wu, 2004) and others which detect genome integrity (Ciccia & Elledge, 2010), increases the half-life of p53 in cells and forces the conformational change to act as a transcription factor by utilizing its transactivation domain to activate expression of multiple genes (Brooks & Gu, 2010). Examples of targets of p53 are as follows; p21 inhibits cyclin dependent kinases to halt cell growth/drive into cellular senescence, G0 (Brown et al., 1997) amongst other cell cycle arresting agents (El-Deiry, 1998), opposed to other targets which induce apoptosis as pro-
apoptotic genes, Bax and PUMA (Green & Kroemer, 2009). More than 50% of human tumours contain either a mutation or deletion in TP53 to therefore be associated with genomic instability (Hollstein et al., 1991; Schmitt et al., 2002). The importance of p53 has been shown in studies that have restored its functionality causing a degree of regression in tumours (Ventura et al., 2007).

1.3.2.3.3 Epigenetics and microRNAs

Structural changes within chromatin subunits influences the ability of genes to be activated or silenced, such as via histone modification or DNA methylation which can allow or prevent transcriptional machinery access to coding regions. Epigenetic modifications are kept during cellular division, and can be inherited by offspring. A common example of epigenetic silencing is the process of a totipotent embryo cell becoming modified to a pluripotent cell then further specialization to specific cell/tissue types, a process that requires permanent silencing of genes (Mitalipov & Wolf, 2009).

In reference to cancer formation, epigenetics has been shown to play a large role in formation and repression of cancers (Jones & Baylin, 2002; Egger et al., 2004; Sharma et al, 2010), with more modern understanding is that cancer cells show global epigenetic abnormalities paired with genetic mutations (Jones & Baylin, 2007). During all stages of cancer progression epigenetic modifications have been shown to play roles to promote the cancerous phenotype (Feinberg & Vogelstein, 1983; Feinberg et al., 2006), and even initiate. Some cancers typically have more silencing markers associated with oncogenes as compared to specific DNA mutations effectively causing a larger gene silencing effect (Vogelstein et al., 2013). As described within colon cancer, more heavily methylated CpG islands coupled with oncogenes were found to not be methylated in adjacent healthy mucosa cells indicating the vast change of epigenetic features comparing cancer cells to their healthy progenitors (Ilingworth et al., 2010; Wei et al., 2016).

microRNA’s are small non coding RNAs that are seen to regulate the expression of genes via post-transcriptional silencing. The specific bases that the microRNAs possess allow it to pair with messenger RNA to induce silencing as double-stranded RNA (dsRNA) is tagged for destruction by nucleases and prevention of integration into the translational machinery of the ribosome (He & Hannon, 2004; Morris & Mattick, 2014), the presence of dsRNA is also an indicator for viral infections so it is not surprising the cell has a dedicated and good response to the presence of dsRNA (Gantier & Williams, 2007). As compared to normal tissue cells, the levels of microRNAs expression in malignant tumour cells show
large changes during this tumorigenic state (Lu et al, 2005). Typically microRNA’s regulate genes associated with transcriptional regulation, proliferation, apoptosis and the cell cycle, so understandably a disruption in the levels of microRNAs expression will give rise to tumour generating effects. An example of a microRNA implicated in tumorigenesis is miR-127. miR-127 targets the production of BCL6 (BCL6 acts as a repressor of transcription via binding directly to DNA to silence genes), and this is shown to be down regulated in prostate and bladder tumours (Saito et al, 2006) preventing the action of BCL6 and increasing expression of its targets. Another microRNA down regulated in cancer cells is miR-127; this inhibits the translation of protein group EZh2. EZh2 is associated with histone methylation which is used to silence genes (Viré et al., 2006) and this protein itself is shown to be linked to high expression in cancers as it inhibits tumour suppressor genes epigenetically (Kim & Roberts, 2016). Unsurprisingly the down regulation of its microRNA suppressor, miR-101, is seen in bladder carcinomas (Friedman et al., 2009) causing a subset of tumour suppressor genes being down regulated as expected.

Alternatively, there is the incidence of oncogenic microRNAs. One such is miR-21. This microRNA regulates the expression of protein PTEN, a tumour suppressor gene within the ATK pathway that ultimately prevents the cell from dividing too rapidly (Chu & Tarnawski, 2004). PTEN is seen to be lost in 70% of prostate cancers (Chen et al., 2006) indicating its tumour suppressor nature. Alongside this within glioblastoma it is observed that miR-21 is instead up regulated (Chan et al., 2005) to decrease the levels of PTEN in the cell and therefore disallow a level of control around cellular division.

1.3.3 The treatment of cancer

The treatment of cancer has undergone many improvements over the past few centuries of medical knowledge and research, cancer treatment depends on the type of cancer, where it is located, at what stage the cancer is and general health of the patient. In some cases a single treatment is required with no follow-up, and some patients require long lasting repetitive treatments typically in a specific personalised combination (Cancer Research UK “Treatment for cancer”, 2019 Cancer.gov “Treatment for Cancer”, 2019).

Here shall be a demonstration of multiple treatment types currently used for various types of cancers and how can be beneficial or disadvantageous.

The Ebers papyrus dating back to 1550 BC is one of the oldest medical ‘textbooks’ in current existence to display the best-preserved record of ancient Egyptian medicine
A 110 page scroll detailing a vast plethora of knowledge of ‘current’ medical treatments of the time, from recognizing the heart is the centre of blood circulation, to expulsion of disease causing demons. This papyrus is the first known recorded text that references the surgical treatment of tumours with accompanied methodologies (Sullivan, 1996). Obviously, surgical techniques over the past few millennia have made somewhat progress yet it still maintains its status as a core treatment methodology for modern day cancers. Surgery may be the singular treatment needed, if the cancer has yet to metastasise and is located to a singular non-essential organ (e.g. testicular, breast, skin) it is likely that surgery would completely remove all cancerous cells from the body in one go. With this in mind, in theory it would be possible to remove all solid cancers from the body via surgery, however realistically speaking this is not an easy process. To minimize the risk of recurrence of the tumour, typically healthy tissue is cut away around the tumour and analysed until no presence of malignant cells are visible. If every cancer cell is not removed there is a high chance that this will simply reform into a secondary tumour.

Another treatment primarily against solid tumours (but also can be used against leukaemia/lymphoma) is radiation therapy. Different cancer types respond differently to radiation damage so can require higher dosage of radiation for effective treatment, such as melanomas are considered rather radio-resistant for treatment yet can have a beneficial effect as a palliative option (Maverakis et al, 2015). The principle of radiation therapy is the use of targeted high energy particles, such as photons, aimed at cancerous cells to induce sufficient breakages across their genetic material to prevent growth and hopefully cellular death. X-rays are commonly used as an external beam aimed at multiple angles towards the tumour through the patient to reduce unnecessary damage to healthy tissues and induce more damage in the cancerous ones. Other particles used may be in the form of protons, neutrons or heavy positive ions as they all have different levels of energy loss across distance travelled through tissue, with the aim of the highest energy loss being localised to the tumour and not to the surrounding tissue, again another means to prevent unnecessary damage. Alternatively, radioactive treatment can be induced from inside of the body by administration of a radioactive source as a liquid or solid with aim to pass near the tumour. An example of this is auger therapy, by the use of low level electrons released in high dosage via the auger effect (a high energy electron falling to a low electron state to release energy either by light or into another electron which is emitted, the auger electron (Meitner, 1922) opposed to the use of high energy particles which has systemic side
effects. With conjugation of an emitting molecule (a heavy atom) to another molecule with specific affinity to cellular compartments, delivery of low energy electrons can be focused on one particular area of the body rather than a systemic effect. This conjugation and affinity is also required as the electrons emitted only have a short range of travel (nanometres).

Once viewed as the pinnacle of treatment for many cancer types, angiogenesis inhibitors are used to prevent the formation of new circulatory network being formed from malignant stimulation from tumours (Folkman, 2004), effectively starving the growing tumour of resources in a hypoxic environment (Hayden, 2009). The use of angiogenesis inhibitors alongside other cancer therapeutics in combination therapy is seen to still reduce cancer morbidity and mortality (Bagri et al., 2010). However the downsides of angiogenesis inhibitors, such as excessive bleeding and increase of blood clots, can be life threatening for cancer patient users (Akl et al., 2017).

The use of systemic cytotoxic drugs to destroy rapidly dividing cells describes the treatment of chemotherapy. The administration of intracellular agents intended to inhibit cellular division and induce irreparable stresses to trigger apoptosis is the main aim for chemotherapy. Understandably more rapidly dividing cells such as cancers, bone marrow or hair follicles are more susceptible to the effects of these drugs. The efficiently of chemotherapy is dependent on the type of cancer present, leukaemia can be cured via chemotherapy (Nastoupil et al., 2012; Freedman 2012), whereas brain cancers do not respond well (Rampling et al., 2004) and some cancers it is inappropriate to use cytotoxic drugs, such as skin cancers. Dosages are required to be calculated correctly, too low of a dose and it will simply be ineffective against the cancer yet comparatively too higher of a dose will increase the cytotoxic effect on normal body cells more to the dislike of the patient coping with side effects (Gurney, 2002; Corrie, 2008). One type of cytotoxic drug is alkylating agents, with some of the oldest examples being mustard gas used in WW1 to the multiple chemotherapy drugs in use today (Corrie, 2008). These drugs cause the addition of alkyl groups to DNA, RNA and proteins within the cell impairing cellular function. In addition, these drugs can covalently bind to a DNA strand multiple times thus preventing cellular machinery from correct DNA replication as it cannot cope with intra-strand linked DNA to abort cell cycle process (Siddik, 2002; Lind, 2008). An example of a direct DNA damaging compound is the cisplatin family of drugs. These direct DNA binding platinum based cytotoxic drugs are used in many different cancers, such as breast, bladder, lung,
brain, neuroblastoma, and ovarian. In particular it has very good success rates improving the survival of prostate cancers (Einhorn, 1990). However, it is noted that patients can relapse with cisplatin resistant tumours following treatment using this drug (see section 1.3.4). Proposed methods of resistance of this drug include an increase of efflux proteins to expel the drug from the cell, the reduction of cellular uptake, detoxification of the drug, or further mutations in the apoptotic or DNA repair pathways to remedy the effects of the drug (Stordal & Davey, 2007).

Another division of cytotoxic drugs are classified as the anti-microtubule agents, which indicative of their name, prevent the action of microtubule proteins α-tubulin and β-tubulin (Rowinsky & Donehower, 1991). Within anti-microtubule agents there are two classes of drugs, the vinca alkaloids which prevent formation of microtubules and the tazanes which prevent disassembly. As we know microtubules are essential for separation of chromatids during mitosis (see section 1.1.3.2) understandably these drugs prevent mitosis completion to arrest the cell and eventually trigger apoptosis (Yue et al., 2010). The vinca alkaloids are an example of a naturally produced plant based product as they are found within the Madagascar periwinkle, Catharanthus roseus (Jacobs et al., 2004). An example of a vinca alkaloid and a natural product of C.roseus is the cytotoxic drug vincristine (Keglevich et al., 2012). Suitable for use in acute lymphoblastic leukaemia, acute myeloid leukaemia, Hodgkin’s disease, neuroblastoma and small cell lung cancers, vincristine binds to the tubulin proteins preventing the formation of microtubules to prevent chromosome separation during metaphase and therefore induce apoptosis (Jordan, 2002). Unfortunately, in a similar fashion to cisplatin, resistance can arise in tumours to cause relapse. One proposed mechanism is the over expression of the p-glycoprotein pump to increase efflux of this drug out of the cell preventing interaction with forming microtubules (Sui et al., 2012; Breier et al., 2013).

Other systemic cytotoxic drugs used are the antimetabolites. This group of drugs disrupt DNA and RNA synthesis as their structure is comparable to a nucleobase (Parker, 2009), the presence of these drugs in the cell can directly block enzymatic reactions required for DNA or incorporate itself into the DNA polymer formed. The anti-cancer action of these drugs arises from the blocking of DNA interactions thus preventing mitosis from occurring, or alternatively if inserted into DNA can induce DNA damage due to structural damage caused to trigger apoptosis of the cancer cell. Lastly, topoisomerase (TOP) inhibitors target TOP1 and TOP2 which typically aid in the unwinding of DNA during
replication by inducing breakages to release tension build-up. Inhibitors can prevent TOP1/2 action in different ways, they can have an inhibitory feature to prevent the unwinding of DNA altogether physically barring exposure of DNA bases to replicative machinery, or encourage the breakage of DNA that TOP1/TOP2 typically regulate and then prevent re-ligation causing widespread DNA breakage (Goodsell, 2002).

Unlike drugs that non-specifically attack all of the host's cells, targeted therapies rely on the use of precise drugs for individual cancer types indicated by their specific markers expressed (Syn et al., 2016). One such classification of a targeted therapy is the drug imatinib, this drug occupies active sites of tyrosine kinases, in particular the oncoprotein BCR-ABL’s active site (see Philadelphia chromosome in section 1.3.2.3.1 and section 6.1.1) (Deininger & Durker, 2003) as well as c-kit and PDGF-R (Iqbal & Iqbal, 2014). The use of monoclonal antibodies also constitutes as a targeted therapy, these antibodies can either stimulate the patient’s immune system into action to target malignant cells by identification of a biomarker or deliver a chemical/radioactive dose to the specific location of the tumour. A good example of a monoclonal antibody is ramucirumab, this antibody binds to the extracellular domain of VEGFR-2 to prevent activation via ligands VEGF-A, VEGF-C and VEGF-D (Wadhwa et al., 2003). By blocking the activation of VEGFR-2, leads to inhibition of VEGF tumour angiogenesis as the VEGF ligands are typically secreted from malignant cells to promote angiogenesis (Vennepureddy et al., 2017).

1.3.4 Cancer Genomics

The association between genome and expressed phenotype was long debated until rediscovery of Mendelian inheritance by demonstrating that the variation and inheritance of alleles are responsible for the phenotypic variation we see across populations (Fisher 1919). As previously discussed, there are plenty of examples of genetic diseases caused by individual or a sub set of alleles, but to determine the underlying genes at fault in common diseases (or common cancers) are very likely to be a multitude of minor gene interactions multiplied by the external environmental factors at play to cause this disease (Guttmacher et al., 2004; Manolio & Collins, 2007). The detection of underlying genes or polymorphisms within cancer cases is possible, such as this blunt approach worked for bladder cancer (García-Closas et al., 2005; García-Closas et al., 2011). Yet for prostate with a known good gene target, the associated androgen receptor highly and frequently expressed in aggressive tumours (Lee, 2003) shows a large variation within this gene within cancer patients and healthy control patients (Schatzl et al., 2002; Lindström et al., 2010),
furthermore, when studying (relatively speaking) isolated ethic-groups displaying the same cancer type, there was still no correlation to be seen between cancer features and displayed genetic variation (Gallagher et al., 2010).

These studies show that cancer traits, and cancer susceptibility, are not controlled by specific polymorphisms or individual point mutations that can be detected across populations, this highlights the need to look across the whole genome to make associations, of course the use of multiple genome wide association studies has shown the detection of cancer-related trends. The region of 8q24 has now been implicated in many types of cancers. Firstly identified within European and African American prostate cancers (Amundadottir et al., 2006; Freedman et al., 2006) with a novel polymorphism detected (Haiman et al., 2011) and to be further implicated in prostate cancer with other independent polymorphisms found at this site (Haiman et al., 2007; Yeager et al., 2009). These other types of cancers show risk markers that have been associated at this same site, including; colorectal cancers (Tomlinson et al., 2007), bladder cancers (Keimeney et al., 2008) and leukaemia (Crowther-Swanepoel et al., 2010). An exciting result from genome wide studies was that >90% of disease associated polymorphisms are found within non-coding regions of the genome, somewhat surprising as the premise was to look for variations within gene coding regions (Hindorff et al., 2009; Hirschhorn, 2009), yet also somewhat expected knowing that most human variation at the DNA level is within non-functional regions (estimates put 3-5% of single polymorphisms as functional (Collins et al., 1998)). However, detection of individual polymorphisms between individuals would be expensive, in-able to detect very rare variations found and most importantly miss structural variations, such as the previously discussed translocations/inversions/deletion etc (Manolio et al., 2008). It is this structural variation that is attributed to the larger number of ‘base pair differences’ and variation amongst populations than single polymorphisms (1000 Genomes Project Consortium, 2005). As explained, the use of whole genome studies has led to the identification of many causes of specific cancers and seen as beneficial to perform, however cancer cells within a tumour display heterogeneity and this must be appreciated when performing wide scale studies.
1.3.4.1 Heterogeneity of cancer

As cancers are known to exhibit genome instability, it would be incorrect to presume that each cycle of division would yield identical products as the first ‘progenitor’ cell (Schmitt et al., 2012). This differentiation event would yield a tumour with distinctively different features, in the phenotype and genotype, of different sub-clonal populations (Fidler & Hart, 1982). Each cancerous event is a unique evolutionary process where multiple heterogeneous malignant cell clone populations arise via mutation events due to deregulation of their tumour suppressor feature. These cells would compete against one another in the tumour microenvironment for resources, space, nutrition, waste management, ability to metastasise and the ability to resist chemotherapy drugs (Nowell, 1976; Merlo et al, 2006; Yachida et al., 2010; Campbell et al., 2010; Greaves, 2010; Ding et al., 2012; Gerlinger et al., 2012; Greaves & Maley, 2012). Unsurprisingly, with this evolutionary theory being applied to the development of cancers, evolutionary methodologies are being explored to best understand this phenomenon (Peinta et al., 2008; Stearns, 2012; Nesse & Williams, 2012). However, it is also hypothesised that the disproportionate sharing of oncogenic products during cellular replication could drive cells phenotypically apart post-mitosis, indicating that heterogeneity seen across tumours may not entirely be describable by genomic alterations alone (Czerniak et al., 1992).

The issue of heterogeneity within cancers as stated is that it allows a higher frequency of varied alleles in the population to make the ‘species’ more resistant to environmental pressure changes. A higher indication of tumour heterogeneity is associated with poorer clinical outcome (Dagogo-Jack & Shaw, 2018). The higher variety of genetic diversity within the tumour environment, can predict this poorer clinical outcome by indicating a faster progression towards malignancy to reduce patient survival time period. The genetic heterogeneity seen is indicative of that ability of new adaptive hallmarks to be generated to drive tumour progression (Mroz et al., 2015; Andor et al., 2016).

Tumours sampled at initial biopsy may not represent the true tumour population (Bedard et al., 2016) and in the event of a recurrence or metastasis it is not routine to perform another biopsy (Bedard et al., 2016). Instead treatment is based upon the biomarkers identified from the initial biopsy (or equivalent) which may not resemble the recurrent cancers cellular makeup (Bedard et al., 2016). In the example of breast cancer, the presence or absence of oestrogen receptor expression determines treatment and prognosis. Lack of oestrogen receptor lowers response to tamoxifen or aromatase
inhibitors (Early Breast Cancer Trialists’ Collaborative Group, 2011) and is associated with worse prognosis (Lindstrom et al., 2012). However, the difference in expression of this receptor between initial and succeeding tumours, that may appear years after primary tumour treatment, is observed in 7-25% of patients (Amir et al., 2012; Thompson et al., 2010). Another example of melanomas, BRAF mutation is used as a biomarker for prognostic and predictive uses as is seen in more than 80% of melanoma cases (Davies, 2002; Bhatia et al., 2015) and associated with a more malignant phenotype has a 4-25% difference in prevalence of initial biopsy result compared to a subsequent tumour results (Colombino et al., 2012). The lack of detection of these ‘evolved’ tumours will cause incorrect treatments to be administered and a decrease of survival.

Clonal heterogeneity as seen by changes of DNA content and chromosomal abnormalities can be seen in initial tumours before metastases formation (Fidler & Hart, 1982; Coons et al., 1995) via FISH, reported in breast cancers (Yoon et al., 2012)) and gastro-oesophageal cancers (Starczynski et al., 2012). Tumours are assessed based upon the median ‘biomarker’ detected (Figure 1.9) to determine treatment strategy, the failure to detect sub-populations may be the cause of the drug resistant tumours seen in secondary tumours (Turner & Reis-Filho, 2012; Rye et al., 2012; Tougeron et al., 2013; Bai et al., 2013). Incorrect assessment of the primary tumour will act as an advantage selective measure for subpopulations that are treatment resistant, as they are free to continue with less competition within their microenvironment, allowing for more growth, more divergence and possibly more malignant features. Multiple studies have confirmed this as they have shown that treatment resistant sub-clones have been present, albeit at a minor frequency, in the primary tumour (Roche-Lestienne et al., 2003; Shah et al., 2009; Diaz., et al 2012; Shah et al., 2012; Morissky et al., 2016). With a better model of cancer diagnosis at the initial tumour it would be possible to characterise the subpopulations within this tumour, eradicate low frequency resistant clones before they become the primary sub-population and monitor the clonal dynamics over time (Aparicio et al., 2013). The use of chemoresistant cell lines, either taken from biopsy or generated in a laboratory setting, is a vital tool to estimate these heterogenic conditions as they must poses a ‘biomarker’ distinguishing them from the non-resistant population.

To better explain the heterogeneity of tumour cells, there are two currently accepted models that most likely act in tandem to different degrees across different tumour types and environments (Shackleton et al., 2009).
1.3.4.1.1 Clonal evolution/ Somatic evolution

The proposal that neoplasms, both pre-malignant and developed malignant cells, evolved by a natural selection means via the clonal evolution model, first being summarised in 1976 (Nowell, 1976; Merlo et al., 2006). From a single malignant cell, single mutations are acquired via ‘tumorigenic’ processes, as described throughout this section. To allow natural selection to take place the malignant or pre-malignant cells require variety in population (via genetic or epigenetic means) and sub-populations to have a heritable selection advantage over others in the specific tumour microenvironment, ultimately swaying the ratio of subpopulation frequencies throughout the tumour (Merlo et al., 2006; Swanton et al., 2012). The constant changing of the tumour microenvironment itself is shown to be tumorigenic (Chen et al., 2015; Wang et al., 2017) such as cells, or cellular populations, must compete for resources such as space, oxygen availability and waste processing. Understandably, a subpopulation that has an advantageous trait that can be inherited will comparably outperform other subpopulation derivates to generate more daughter cells. Clearly seen, the increased genetic diversity of the tumour is critical for overall survival, as seen throughout nature (Merlo et al., Fernandez et al., 2016).

Applying this Darwinian process of selection to tumour cells (Greaves & Maley, 2012), cancer therapies (particularly chemotherapy) are a means of artificial selection via human interaction. As most cancer deaths are caused by clones which are therapeutically resistant (Greaves & Maley, 2012), subpopulations with mutations that can infer a drug/multidrug resistance will propagate to form the representation of the next ‘generation’/tumour in the event of a relapse. The cellular causes of drug resistance will vary greatly between tumours (Gottesman, 2002), and can form in a multitude of tumour types (Azam et al., 2003; Engelman et al., 2007; Murugaesu et al., 2015). The increased mutation rate by the cancer cells innate genome instability further fuels the likelihood that a drug resistant clone will appear in the population, to proliferate with low competition and is shown to lead to faster malignancy development (Axelrod et al., 2006; Worsley et al., 2016; Sun et al., 2016; Fortunato et al., 2017).

Initial tumour evolution in the ‘clonal evolution’ theory is thought to occur by two different methods. In the view of a linear expansion, sequential ordered mutations are accumulated over time stepwise causing the expansion of tumour cells. Observed is a single population collectively acquiring hallmarks and is most likely only seen in initial tumorigenesis as this process is not seen in developed malignant tumours (Gerlinger et al.,
2012). For example, it is more likely that cellular regulation initially is aberrant, such as a benign neoplasm, followed by other features such as genomic instability to give rise to a more varied population. This development into a varied population is representative of branched expansion (Figure 1.14), the second methodology observed. Via generation into multiple subclonal populations through means as described above (Swanton, 2012), the tumour now has the means to drive distinct branches which would eventually yield in the generation of advantages/resistances to align with the concept of natural selection (Swanton, 2012; Gerlinger et al., 2012).

1.3.4.1.2 Cancer stem cells

An alternative view of cancer development brings the notion that the wide spread epigenetic changes seen in cancer cells development is unlikely to occur in a random fashion to then accumulate inside a tumour via selection (Sharma et al., 2010; Greaves, 2010). The cancer stem cell model proposes that epigenetic changes which typically occur in normal stem or progenitor cells are the earliest events in cancer formation (Feinberg et al., 2006). Furthermore, the fact that these epigenetic aberration events are notably seen to be very early events in cancer development (Cui et al., 2003; Matsubayashi et al., 2003; Sakatani et al., 2005; Peters et al., 2007) (Figure 1.14).

The self-immortalisation of stem cells is seen by silencing genes involved in renewal capacity is observed in cancers (Jones & Baylin, 2007), an aberration in silencing of these replication controlling genes gives rise to the infinite renewal capacity proposed in this model. These immortal stem cells, or abnormal precursor cells, can be subject to genetic mutation events to form the basis of tumorigenesis (Baylin & Ohm, 2006), and go on to reflect a high expression of pluripotency typically seen in embryonic stem cells (Widschwendter et al., 2007; Werbowetski-Ogilvie et al., 2009).

This stem cell model is able to describe the heterogeneity found within tumours. Initial formation creates a small population of pre-malignant cells able to maintain their stem cell properties indefinitely to give rise to a malignant population when influenced by additional genetic mutations at any time (Jones & Baylin, 2007). These cells are hypothesised to exist within the tumours as a distinctively different population in a low proportion of the tumour, typical therapies used to treat tumours may not be effective against these persistent cells to generate new cells, such as in the case of relapses or metastasises (Reya et al., 2001) (Figure 1.13). The removal of an initial tumour population without removal of the cancer stem cells will simply allow the stem cell to produce a genetically different
malignant cell to grow and propagate into a secondary tumour. In the event of therapy, cancer stem cells are thought to be more resistant to typical cytotoxic drugs used (Zhao, 2016), either by the up regulation of DNA repair proteins or slow division cycles, features that classic adult stem cells are shown to possess. Again, the use of already diverged chemo-resistant cell lines is helpful for use in studies exploring cancer stem cells theories as they may contain sub-populations of increased ‘stemness’ that generate these resistant populations.

There is somewhat of a debate about the existence of cancer stem cells, evidence against cancer stem cells is the fact that some tumour cells display no signs of ‘stem’ like properties across the whole cancer (Gupta et al., 2009). Some query the origination of the stem cell itself, is it a malformation in a true stem cell as current models predict via epigenetic malformations or simply from non-malignant cells gaining the ability to self-renew to give appearance of a stem cell which display a level of phenotypic plasticity (Nouri et al., 2017). However, there is current evidence of cancer stem cells found in leukaemia (Bonnet & Dick, 1997), breast cancer (Al-Hajj et al., 2003), colon cancer (O’Brien 2007), prostate cancer (Lang et al., 2009), melanoma (Civenni et al., 2011) and many more (Shackleton et al., 2009). The presence of multiple cell types found in heterogeneous tumours can currently only be explained by the presence of stem cells (Bonnet & Dick, 1997), unless multiple tumours from multiple body sites that have all formed malignant metastasising properties are in play.

However, it is most likely that tumour heterogeneity is best explained by a combination of models, an incremental ‘random’ mutation pattern and the cancer stem cell idea (Wang et al., 2014) as both examples have to be shown in tumour heterogeneity, more research must be conducted that can highlight key genomic and epigenetic changes during tumour development.
1.3.4.2 Chromoanagenesis, Chromoplexy and chromothripsis

The advent of next generation sequencing from tumours and diseases arising from complex chromosomal rearrangements has identified complex rearrangements to occur within chromosomes in a single cellular event (Holland & Cleveland, 2012). As previously described, cancer is typically driven by single mutation events and single chromosomal rearrangements to give individual functions that collect over time to form a true cancer (Hanahan & Weinberg, 2011; Vogelstein et al., 2013). However recent studies show that a single ‘phenomenon’ can occur whereby tens to hundreds of genomic rearrangements can occur simultaneously (Stephens et al., 2011) in many different types of cancers (Kloosterman et al., 2011; Rausch et al., 2012; Molenaar et al., 2012). Interestingly, the frequency of chromoanagenesis is higher, as compared to all types of cancers, in specific types of tumours, such as neuroblastomas (Molenaar et al., 2012), colorectal cancers...
(Kloosterman et al., 2011), bone cancers (Stephens et al., 2011), AMLs and Sonic-hedgehog medulloblastoma (SHH-MB) (Rausch et al., 2012). Unsurprisingly, the presence of chromoanagenesis in tumours typically yields poor survival rates due to correlation with more aggressive tumours (Hirsch et al., 2013; Notta et al., 2016; Rücker et al., 2018), however presence of complex chromosomal events can be used as a biomarker for prognosis and patient outcome (Forero-Castro et al., 2016; Luijtenet al., 2018).

Chromothripsis is the term given to describe ‘chromosome shattering’, a methodology that could explain the highly aberrant chromosomes formed during chromoanagenesis in one single event (Stephens et al., 2011) (see Figure 1.15). Expected during chromothripsis, the chromosome should shatter i.e. multiple double strand breaks, to be immediately followed by aberrant DNA repair (Stephens et al., 2011). The presence of whole chromosome micronuclei are correlated with large levels of DNA damage leading to multiple chromosome breaks and incorrect rejoining (Hatch et al., 2013). Micronuclei are thought to occur when a whole chromosome is lagging during anaphase/telophase due to previous errors in the mitotic process, and not included within the main nucleus when its membrane reforms (Fenech et al., 2011). Experimentally, micronuclei can be used to induce double strand breaks in specific chromosomes (Cveticanin et al., 2009), and the cell can survive with the presence of micronuclei and it is possible for contained DNA to be replicated during the next mitotic cycle (Terradas et al., 2010). However this replication is thought to be defective and lagging due to a lack of correct nuclear conditions/ enzymes, forcing a disrupted replication and multiple double strands breaks to be formed. This DNA damage is not detected, due to the lagging nature so misses cellular checkpoints (Giunta et al., 2010), causing more stress and fragmentation to the chromosome. The resulting fragments can be rejoined, in a most likely erroneous way (Figure 1.15) and these chromosomes could be reincorporated into the main nucleus of the cell to survive multiple generations (Crasta et al., 2012). Where chromothripsis concerns the shattering of only one chromosome, the thought that this process happens in a micronucleus where a singular chromosome can be present and undergo stress pairs well (Forment et al., 2012). The telomeres during mitosis can become vulnerable due to the loss of their protective terminal regions, to cause fusion and formation of a di-centric chromosome. The di-centric chromosome during mitosis can have multiple spindle fibres attached and tension pulling it to opposing daughter cells, to create a chromatin bridge between cells. When decondensing paired with nuclear membrane reformation, incorrect membrane formation will cause exonucleases within the cytoplasm to sporadically cut the chromosome into
single strands to resolve the stress event, effectively causing the fragmentation event (Maciejowski et al., 2015). Alternative theories about the methodology of chromothripsis occurring are possibly, the presence of ionizing radiation (Maher & Wilson, 2012) or the result of aborted apoptosis (Tubio & Estivill, 2011). Another structural aberration typically found in chromothripsis malformed chromosomes is the presence of vast clusters of point mutations around breakpoints, termed Kataegis, seen as a “thunderstorm” event as which its name derives from (Nik-Zainal et al., 2012; Maciejowski & de Lange, 2017).

In normal cells, this level of mass genomic damage would typically undergo apoptosis (Holland & Cleveland, 2012). However as unsurprising as it is, in cancers where chromothripsis is thought to have occurred the regions of the genome affected are associated with cell cycle control, DNA damage, proliferation and apoptosis regulators (Cai et al., 2014; Notta et al., 2016; Rücker et al., 2018). It is quite possible that chromothripsis events outside of these genomic regions may happen, yet it is understandably hard to observe as the cells would not survive to be able to be observed. However, cells that do survive can show the ability to generate multiple hallmarks in one genomic mutative event.

**Figure 1.15. Comparisons of Chromothripsis and Chromoplexy**

Shown is a representation of chromosomal rearrangements created by chromoanagenesis. A) Chromothripsis, showing a single event to shatter the chromosome, followed by reorganization, deletions, inversions and duplications when repaired. **Right**) Chromoplexy, showing chromosomes undergoing a single reorganization event to create multiple malformed chromosomes; also during this event has the deletion of chromosome segments. Based upon: Shen, 2013; Luijten et al., 2018.

In normal cells, this level of mass genomic damage would typically undergo apoptosis (Holland & Cleveland, 2012). However as unsurprising as it is, in cancers where chromothripsis is thought to have occurred the regions of the genome affected are associated with cell cycle control, DNA damage, proliferation and apoptosis regulators (Cai et al., 2014; Notta et al., 2016; Rücker et al., 2018). It is quite possible that chromothripsis events outside of these genomic regions may happen, yet it is understandably hard to observe as the cells would not survive to be able to be observed. However, cells that do survive can show the ability to generate multiple hallmarks in one genomic mutative event.
Typically, it is thought to be seen in early tumorigenesis to be a large initial driver of malignancy, however studies have shown that the chromothripsis event can be late and insignificant in the cancer’s phenotype (Kovtun et al., 2015), or it is a marker shown in late tumour aggressiveness (Bassaganyas et al., 2013).

The presence of chromothripsis has been detected in a wide variety of cancers including; medulloblastoma (Northcott et al., 2012), acute myeloid leukaemia (Rausch et al., 2012; Bochtler et al., 2017), bladder cancer (Morrison et al., 2014), and in many more examples (Luijten et al., 2018). The rate of chromothripsis events is somewhat of a discussion point, recently it was seen to only occur in up to 4% of all cancers (Cai et al., 2014), yet it reported much higher for specific cancer types such as neuroblastoma and bone cancer (Stephens et al., 2011). The variation seen in reports fuels the discussion about frequency of these events, yet reasoning might be that a concise consensus on how to categorise chromothripsis events hasn’t been ratified at the time of these studies (Rode et al., 2016).

The incidence of chromothripsis is highly associated with the inactivation of p53 (Rausch et al., 2011; Cai et al., 2014; Fernandez-Banet et al., 2014; Bochtler et al., 2017). As previously described, the action of p53 is vital for the cellular response for DNA damage to induce cellular cycle arrest, DNA repair and/or apoptosis (Lane, 1992), those with germline TP53 mutations consistently shown massive genomic aberrations with appearance of a chromothripsis event (Rausch et al., 2012), especially in childhood cancers (Gröbner et al., 2018). Using neuroblastoma as an example, consistent chromothripsis has been observed (Molennar et al., 2012) and typically is associated with poor prognosis and aggressive tumours, such as with the deletion of FANC (Molennar et al., 2012). However, the association with chromothriptic events may not be fully penetrant in regards to TP53 mutation, as cells with wild-type TP53 have been observed with the presence of chromothripsis (Cohen et al., 2015) and conversely not all tumours with incorrect TP53 shown chromothripsis (Fernandez-Banet et al., 2014).

Outside of cancers, chromothripsis has been reported within the germline of patients that display a delay in development or dysmorphic features (Chiang et al., 2012; Plaisancié et al., 2014; Gamba et al., 2015; Anderson et al., 2016), and even events can be beneficial as shown as a spontaneous cure WHIM syndrome by deletion of the disease causing double negative allele (McDermott et al., 2015). Chromothriptic events are not a human phenomenon, it has also been reported in cancers within Tasmanian devils (Deakin et al.,
2012) and natural events within plant genomes (Tan et al., 2015; Carbonell-Bejerano et al., 2017), relating back to a possible beneficial evolutionary aspect of this mechanism of mass genome restructuring.

Alternatively to chromothripsis, chromoplexy (Figure 1.15) is understood as another mechanism of mass chromosomal rearrangement that occurs as a single event in cancers. Chromoplexy is defined as a build up of linked translocation events involving multiple chromosomes in a singular event resulting in chromosomal rearrangements that show little in the way of copy number variation (Baca et al., 2013; Shen et al., 2013). Chromoplexy was first described in the study of prostate cancers with an indication of presence in up to 40% of cases (Baca et al., 2013), as indicated by previous research that prostate cancer was typically shown to have frequent large scale translocations resulting in oncogenic fusion proteins (Tomlins et al., 2005). Furthermore, within these prostate cancers, point mutations were seen to be low and chromosomal rearrangements high (Taylor et al., 2010; Barbieri et al., 2012), concluding that the presence of genome rearrangements being the main driver of tumour development.

The mechanisms behind the formation of chromoplexy is less well understood as compared to that of chromothripsis, associations between common oncogenic fusions noted within prostate cancer cells which are caused by double strand breaks (Haffner et al., 2010) is somewhat consistent with the idea that chromoplexy is thought to be a result from inconsistent deletion/rejoining mechanisms (Fukami et al., 2017) and association with open chromatin configurations during times of transcriptional DNA-damaging processes (Baca et al., 2013; Lin et al., 2009).

The presence of chromoanagenesis challenges the view that tumour establishment via gradual mutation events are required and instead a single catastrophic event could be enough to initiate a cell to progress to a malignant state. The presence of chromoplexy and chromothripsis indicate that tumour progression may not be linear and have large bouts of sudden alteration that could explain the heterogeneity seen across cancer cells (Cai et al., 2014). Baca et al., 2013 concludes in their paper which terms ‘chromoplexy’, that “The characterization of clonal progression and chromoplexy in emerging large panels of cancer genomes may provide insights into tumor initiation and progression that impact cancer detection, prevention, and therapy.”, and indicates this level of cancer development would be missed by gene/exome level sequencing to call for a wider whole genome approach instead.
1.3.5 Non human models for cancer

There is somewhat of a tradition in using the mouse or rat as universal model organisms for biological research, and likewise explicit use within cancer research. Being very short lived animals have a rapid reproductive rate and are very prone to cancer, a great choice for a model species. However, for studies exploring the mechanisms behind innate cancer resistance they offer very little use. Other animal models are more beneficial towards this aim of exploring resistance mechanisms. We can look towards long-lived mammalian species instead which may provide new insights towards prevention strategies rather than treatments, which are plentiful but have serious side effects to the organism (Cleeland, 2012.). It would be expected that longer lived mammals have evolved strategies which allows a reduction in observed cancer rates, for example mice have a high incidence of cancer, +50% (Lipman, 2004) where as several other species are known to be resistant to cancer, such as the naked mole rat, elephant and bowhead whale (Seluanov, 2018).

Different species require different ‘hits’ for malignant cells to arise as compared to humans, (Gonzalez, 2010) and possess varying levels of telomerase to solve issues arising from incomplete replication of chromosome ends by DNA polymerases (de Lange, 2009) leading to incorrect expression and reactivation to immortalize tumours (Kim, 1994). To summarise, the larger and long lived the animal, more mechanisms exist (and therefore more ‘hits’ required) for tumours to be formed (Tian, 2018). The naked mole rat (Heterocephalus glaber) is a small bodied yet long living rodent with a maximum lifespan of 32 years in captivity (Buffenstein, 2002) is found to be highly resistant to cancer and therefore of high interest within research (Buffenstein, 2008; Liang, 2010). A phenomenon that occurs within naked mole rat cellular cultures is very early contact inhibition, slowing their growth rate in culture (Seluanov, 2009), an example of a possible mechanism towards cancer resistance as cellular proliferation is arrested at earlier stages. Unfortunately, this will make the use of naked mole rat as a model organisms harder to generate material for research use.

Blind mole rats (Spalax ehrenbergi) again are another example of a long lived rodent that shows resistances to cancers (Gorbunova, 2012). A resistance mechanism arises through concerted cell death, where after a set number of population doubling a culture will die through necrotic and apoptotic processes by a large rise of INFβ (Gorbunova, 2012). Clearly this is a useful mechanism for the organism, but not for culturing cells.
Other non-human examples include the elephants, which have expanded their copy number of TP53 genes as pseudogenes to confer some anti-cancer properties (Abegglen, 2015; Sulak, 2016) and long lived whale species which show examples of positive selected DNA repair genes such as ERCC1 and UCP1 (Keane, 2015). However the process of obtaining primary cultures, maintaining adequate growth will incur high resource input and costs due to the bespoke nature of the cells themselves and the conservational status of some of these animals. By understanding the mechanisms behind these different species and retroactively applying them to human specific medications or prevention strategies could lead to new methods of cancer treatment or prevention, a worthwhile field of research but high costs may impair this (Xia & Chen, 2011).
1.4 Thesis rationale

The techniques of cytogenetics have evolved over the past century to allow us as researchers and medical professionals to perform in-depth analysis on specific genomes, to study diagnostics, comparative genomics and cancer progression amongst other things. From evolutionary studies to medical diagnoses, the use of fluorescent techniques has understandably been a key driver within these fields, as it allows the user to visually interpret the data. A new methodology that can aid in these fields would allow researchers an alternative to expensive next generation sequencing technologies that can visually and quickly provide results. A successful new fluorescent methodology should be able to perform in these areas: be a cheaper/more efficient alternative to current FISH technologies, demonstrate an increase of throughput/reliability of results, be applicable for use in evolutionary studies and be worthwhile to use in ‘non-typical (i.e. malformed cell lines -cancer) studies. The work within this thesis aims to firstly provide an exploration into optimisation of the FISH protocol and establishment of a new methodology, alongside distinctive studies across these mentioned research areas that can use this protocol to discern previously unknown chromosomal features.

Figure 1.16. Publications using either bulk sequencing or single cell sequencing techniques

Transcriptome data from bulk analysis of cells derived from tumour tissues fail to accurately detect the tumour heterogeneity and subpopulations present. The graph indicates the variation in the number of publications reported using whole exome techniques VS single-cell-sequencing based techniques. Adapted from: Shi et al., 2018.

As described in 1.1.5 methods, cytogenetic techniques have advanced over time with a large emphasis on a computational based approach within recent years (Figure 1.16). This has effectively left traditional in situ hybridisation methodologies out of focus without much improvement, bar industry improvements to reagents. With the focus towards a ‘bottom-up’ visualisation of the genome via NGS’s short read approach opposed to the
‘top-down’ visualisation of chromosomal structure via traditional techniques, possible incorrect conclusions could be drawn. As shown in multiple studies throughout section 1, the use of FISH is required alongside NGS to confirm results. If the throughput of FISH was increased paired with a decrease of cost, the ratio of NGS methods to FISH methods may balance out with FISH being a new cost-effective option.

Within section 1.2.3.1, the Galliform order has great importance within research due to G.gallus being the avian model organism of choice for multiple reasons. Within the field of comparative genomics however, the rest of the galliform order has been somewhat overlooked as with research concluding with the fully sequenced turkey (M.gallopavo) and chromosomal paint studies indicating a rather uneventful progression over evolutionary time for other members. Here calls for a study into the intrachromosome rearrangements which paint based studies can miss, to detect any further rearrangement hidden to previous knowledge. As also described (1.2.3.1.1), microchromosomes have been indicated to reside centrally in the nucleus with macro-chromosomes residing in the periphery. This hypothesis can be further explored alongside chromosomal studies with the generation of a new FISH methodology.

With chicken being a useful model organism (1.2.3.1) to derive useable cell lines from, there is somewhat of an imprecise karyotypic description of the useful DT40 cell line. With use of DT40 has diminished with the advent of CRISPR systems, it is still a useful model cell line to explore the genomic structural changes when inducted into a malignant state. As per section 1.3.2.2, the research into oncogenesis via oncoviruses is a worthwhile area to explore; with the DT40 cell line being induced by AVL genomic rearrangements may be found. As per section 1.1.5, the need for speed and efficiency in FISH methodologies is one of the major requirements and drawbacks from matching current NGS technologies. Here also shall be demonstrated how a new FISH methodology can be used to improve current technologies created to improve efficiency.

As per section 1.3.4.1, current opinion of cancer heterogeneity highlights the need to develop newer methodologies that can assess the heterogeneity of cancer populations. Currently, methods can lack the specificity to detect small sub-populations that derive from the ‘modal’ population. This modal population is used to assess the details of the tumour and may not be an accurate representative. Methods such as M-FISH and SKY are possible avenues, but due to the high cost of equipment and reagents paired with the questionable results provided they are not suitable for sustained use in a modern cytogenetics
laboratory. By using currently established neuroblastoma cell lines (UKF-NB3), and derivative cytotoxic lines (rVincristine/rCisplatin), it is possible to observe these at a cytogenetic single cell level to; assess heterogeneity within the population, detect key aberrations that could infer resistance or act as molecular markers and provide an up-to-date cytogenetic assignment.

1.5 Thesis Aims

With reference to the rationale set about from the previous section, the aims of the thesis are thus:

**Aim 1**: To generate a new FISH based methodology that can decrease current costs and improve throughput which is applicable for all FISH research areas, but particularly to apply to other aims in this study.

**Aim 2**: To use the above methodology (or adaptations of it) a) to detect intrachromosomal rearrangements within the macrochromosomes of the Galliform order in five species and b) to formulate a methodology to determine folding patterns and location within interphase state nuclei for *Gallus gallus* macrochromosomes.

**Aim 3**: To analyse the karyotype of the DT40 cell line, to assess heterogeneity and detect any novel chromosomal aberrations using adaptations of the new FISH methodology developed.

**Aim 4**: To analyse the human cancer cell line UKF-NB3 (neuroblastoma) alongside its derived cytotoxic resistant cell lines, UKF-NB3-rCisplatin/UKF-NB3-rVincristing to detect novel aberrations, assess the level of heterogeneity and provide a cytogenetic assignment.
2. Materials and methods

2.1 Materials

2.1.1 Chromosome preparation

2.1.1.1 Chromosome preparation from culture

Cells were harvested at 70-90% confluence and mitotic doublets seen and abundant before harvesting was carried out of culture flasks. To each individual flask colcemid (Gibco) was introduced at 1ug/ml and incubated at their previous environments, 37°C for mammalian and 40°C for avian, for one hour. Medium was aspirated and rinsed with HBSS(Fisher) to then be followed by each cell culture subjected to a brief trypsin-EDTA(Fisher) exposure (37°C for 2-3 minutes) to detach cells, and use of HBSS to rinse and neutralise the effect of trypsin.

This was then followed by a hypotonic treatment of 75mM KCL for 15 minutes at 37°C, and have repeated washes of a 3:1 mixture of glacial methanol:acetic acid with 150 G centrifugations to aspirate supernatant and re-suspend cell pellet formed in fresh methanol:acetic acid mixture until the sample is cleaned of debris.

Sample quality and metaphase index was observed by fixation onto a glass slide with 3:1 methanol:acetic acid and stained with DAPI in VECTASHEILD antifade medium (Vector Laboratories)

2.1.1.2 Chromosome preparation from Human blood

Sample was taken from a consenting adult human male via typical phlebotomy means into a standard sodium-heparin coated tube. The sample was then cultured in PB-MAX karyotyping medium (invitrogen) at 37°C 5%CO₂ for 72hours, to have cell division arrested with the introduction of colcemid (Gibco) at 1ug/ml for 35 minutes before a hypotonic solution of 75mM KCL was introduced. Repeated washes were performed with a glacial 3:1 methanol: acetic acid until sample was sufficiently clean of debris and cytoplasmic elements. Sample quality and metaphase index was observed by fixation onto a glass slide with 3:1 methanol:acetic acid and stained with DAPI in VECTASHEILD antifade medium (Vector Laboratories)
2.1.2 Cell cultures

2.1.2.1 Avian cells

All avian samples used within this thesis were from egg derived cultured fibroblasts (5-7 day old embryos) or from skin biopsies. Cells were subject to collagenase treatment to be cultured at 40°C, 5% CO$_2$ in alpha MEM (Fisher) with 10% (FBS) fetal bovine serum (Gibco) and 1% L-Glutamine-Penicillin-Steptomycin solution (10mg/mL) (Sigma).

2.1.2.2 Cattle cells

Cattle ovaries, of mixed age and breed, were delivered in phosphate buffered saline (PBS) at 38°C within 6 to 8 hours of culling from a local abattoir (Charring meats, Charing, UK). A biopsy was taken from the surface of the ovary tissue and subjected to a collagenase treatment for 4-6 hours. The sample was then to be cultured in 20% FBS (Gibco) alpha MEM (Fisher) with 1% L-Glutamine-Penicillin-Steptomycin solution (10mg/mL) (Sigma) at 37°C 5% CO$_2$ until 70/80% confluent to then be harvested (2.1.1.1.).

2.1.2.3 DT40

Cell line was cultured, donated and received from the laboratory of Noel Lowndes (Queen’s University, Belfast), in the form of a frozen cell culture delivered overnight by courier. Re-suspended in alpha MEM (Fisher), supplemented with 10% fetal bovine serum (Gibco), 1% L-Glutamine-Penicillin-Steptomycin solution (10mg/mL) (Sigma) at 37°C and 5% CO$_2$. Cells were harvested in the same way as previously mentioned cell cultures.
2.1.2.4 Human Cancer lines

<table>
<thead>
<tr>
<th>Cell line name</th>
<th>Accession</th>
<th>Disease</th>
<th>Resistance</th>
<th>Species / Tissue</th>
<th>Thesis Abb.</th>
</tr>
</thead>
<tbody>
<tr>
<td>UKF-NB-3</td>
<td>CVCL_9904</td>
<td>Neuroblastoma</td>
<td>-</td>
<td>Homo sapiens Bone marrow</td>
<td>NB-3 Par</td>
</tr>
<tr>
<td>UKF-NB-3rVCR10</td>
<td>CVCL_9905</td>
<td>Neuroblastoma</td>
<td>Vincristine</td>
<td>Homo sapiens Bone marrow</td>
<td>NB-3 rVin</td>
</tr>
<tr>
<td>UKF-NB-3rCDDP1000</td>
<td>CVCL_M478</td>
<td>Neuroblastoma</td>
<td>Cisplatin</td>
<td>Homo sapiens Bone marrow</td>
<td>NB-3 rCis</td>
</tr>
</tbody>
</table>

Table 2.1. List of Human derived cancer cell lines used in this study
Details of the human cell lines used within this study, UKF-NB-3 will be referred to as the ‘parental’ cell line as UKF-NB3rVCR10 and UKF-NB3rCDDP1000 are children cell lines derived from this cancer line. More information can be found in Kotchetkov et al., 2005.

All human cancer cell lines were grown in Iscove’s modified Dulbecco’s medium (Fisher) supplemented with 10% fetal bovine serum (Gibco), 100 IU/mL penicillin - 100 mg/mL streptomycin (Gibco) at 37°C 5% CO₂ until harvest and kindly donated by the Michaelis Lab, further information can be found in Kotchetkov et al, 2005.

2.1.3 Fluorescent Probes

G. gallus probes were selected from BAC clones of the CHORI-261 library (ranging in sizes from 150,000 kb to 200,000 kb) and B. taurus in similar fashion from the CHORI-240 library, inserted into E. coli stocks to be subject to collection via mini prep (Qiagen) which was then directly labelled via standard nick translation with either FITC-fluorese-in-12-UTPP (Roche) or Texas Red-12-UDP (Invitrogen) prior to purification using a nucleotide removal kit (Qiagen).

Alternatively, probes were purchased from Cytocell in pre-mixed solutions (probe and hybridisation buffer) and used as per manufacturer’s instructions, a table of which can be seen in section 9 (appendix) and a chromoprobe multiprobe chicken device (Cytocell) which can be seen in detail in section 5.2 and figure 5.1.
2.2 Methods

2.2.1 Fluorescence in situ hybridisation (FISH)

2.2.1.1 Standard FISH

Metaphase preparations from cell culture or blood samples were re-suspended to be fixed onto clean condensation covered microscope slides using fresh 3:1 methanol:acetic acid solution and allowed to dry. Slides were then dehydrated through an ethanol series at room temperature (2 minutes for each wash, 2XSSC (saline-sodium citrate buffer), 70% ethanol, 85% ethanol and 100% ethanol).

Probes were then diluted with a formamide based hybridisation buffer HybI (Cytocell) and respective species hyblock (Insight Biotech), chicken for avian experiments, cattle for cattle specific experiments. Human specific repetitive sequence blocker was already mixed in the pre-mixed purchased probe mixtures from Cytocell. These probes were then applied to the dried metaphase slides on a 37°C hotplate in order to denature both DNA strands of both target and probe (Figure 1.6) before sealing with cover slip and rubber cement. This now probe-sample mixture was denatured simultaneously at 75°C for 2 minutes to then be moved into a humid hybridisation chamber at 37°C for their required hybridisation time (typically an ‘overnight’ hybridisation time of 12-16 hours). Hybridisation time is detailed in the specific experimental sections as investigated in section 3.

Post hybridisation, coverslips and rubber cement was removed and washed for 2 minutes at 72°C in 0.4XSSC, and then had a 30 second wash in 2xSSc with 0.05% Tween 20 at room temperature, only ‘same species’ i.e. identical species as probe DNA origin required the first 2 minute stringency wash. Alternatively, when Zoo-FISH was performed the first wash was omitted and instead the 30 second wash was undertaken in place. Slides were then counterstained using VECTASHEILD anti-fade medium with DAPI (Vector labs) and allowed to resolve in the dark for 10 minutes.

In experiments that used the ‘Cytocell chromoprobe multiprobe chicken device’, metaphase preparations were instead dropped and fixed onto the 24 chamber slide provided by the kit and probes were rehydrated on the 24 slot device using HybI, to both be merged before denaturation aligned to their corresponding boxes and configuration. The device required 5 minute denaturation at 75°C and hybridisation in a 37°C water-bath.
in their provided hybridisation chamber. Post hybridisation steps are the same as standard FISH as seen above.

### 2.2.1.2 IQ FAST Fluorescence in situ hybridisation

Use of the fast buffer was near identical to the standard FISH protocol, albeit with a few minor adjustments. At the stage of denaturation, the sample was denatured at 90°C for five minutes and transferred to a hybridisation chamber at 45°C for 60-90 minutes.

Post hybridisation, the first wash of using 0.4xSSC at 63°C for 10 minutes is followed by a 37°C dH2O wash for one minute, at each stage the slide was vigorously agitated by movement up and down inside the jar. Before counter stain can be applied, the slides went through another ethanol series (70%, 80% and 90% ethanol all for 2 minutes) and allowed to dry. Counterstaining and visualisation is identical to other FISH procedures.

### 2.2.1.3 Multilayer FISH

The multilayer FISH (mlFISH) methodology can be seen in more depth in section 3.4 and in flowchart form in figure 3.7.

The multilayer slides were identical to the standard FISH protocol seen above yet requires additional post analysis steps to continue the iterative method. When visualising, absolute positions on the slide were saved and imported each time the experiment was required to be imaged, via usage of an automated stage and compatible image software, Smartcapture 3 (Digital scientific UK).

After final collection of images from the slide, a pre-wash of 2xSSC with 0.05% Tween 20 for approximately 30 seconds was performed to remove the coverslip and residual DAPI from the slide, this was followed by immersion into 72°C ddH2O for a minute. Upon removal agitation the slide by removing the slide in and out of the 72°C ddH2O jar a few times, to be fully submerged and re-emerged at each step.

At this stage, slides were then prepared to re-enter the ‘standard’ FISH method into the ethanol series step.
2.2.2 FLpter measurements

The means to measure FLpter is discussed in section 4.2.1 where their usefulness is compared for this study.

2.2.2.1 Manual measurement

FLpter scores (first used as a standard means to measure chromosome position, (Lichter et al., 1990) were manually measured using the segmented line feature within ImageJ (version 1.50g, W.Rasband, National institutes of Health, USA, https://imagej.nih.gov/ij/), points were selected upon start of the chromosome, at each probe location and a final measurement upon reaching the end of the chromosome to give =2+n (n being number of unique probes) number of points as distances from the first point. Each chromosome in each metaphase was measured individually and given an unique identifier.

2.2.2.2 Automatic measurement

Using an automated FLpter measuring script (In house, B.Skinner), it was possible to insert a merged 3 colour FISH image and have the script to give an output measured FLpter values after the user has clicked the path of the chromosome needed to be analysed. This script is currently limited to 5 points and works within ImageJ in a similar fashion to the segmented line tool, yet splits the image according to RGB value and measures where the ‘white’ position is found upon the users selected path.

2.2.3 Chromosome Interphase Plotter (CIP)

The chromosome Interphase Plotter was designed within Microsoft office Excel 2007 (Microsoft) using user generated data retrieved from ImageJ.

Images, chromosomes and probes were given set identifiers upon post-merging. Merged metaphase images were taken into ImageJ and points was selected upon probe locations observed, maximum of 16 probes (2n, 8 each) and given set X and Y co-ordinates. These coordinates were then inputted into the set excel tables and chromosomes were calculated and plotted automatically in text short hand.

By using ImageJ, the centre of mass was detected by use of an inbuilt tool as well as area/diameter recorded for each individual metaphase.

Lines connecting points were then drawn on manually using Design 4 (Microsoft) as governed by the output of the plotter.
2.2.4 Imaging software and hardware

Images captured were done so by using an Olympus BX61 epifluorescence microscope with cooled CCD camera and relevant filters for probes used in this study.

Software used for all experiments requiring microscopy imaging was SmartCapture (Version 3, Digital Scientific, Cambridge, UK).

2.2.4.1 Merging of Images

Images were compiled and given false colouration using GIMP (GNU Image Manipulation Program, 2.82, 2018, S.Kimball P.Mattis. GIMP development team) manually using raw channel outputs from SmartCapture 3.

DAPI images were inserted and colour levels were then curved to eliminate low level background DAPI fluorescence and each probe layer was inserted sequentially aligned to its paired DAPI channel. Each probe layer was automatically applied to a threshold using GIMP’s inbuilt tool to give each signal to appear as ‘white’ on a ‘black’ background, these ‘white’ sections were easily selected using GIMP’s selection tools and assigned a false colour and the black layer removed to see the probe location on the chromosomes. Each DAPI layer after this was then layered on top of the original DAPI aligning chromosomes perfectly and new probe layers were added on at this stage until the image was full of all required layers. The intermediate DAPI layers were then hidden to give a single DAPI layer with all probes seen in correct positions. See section 3.4.2 and section 3.5 for examples.
3. Improving FISH technology to facilitate more rapid, cost-effective multiplex results (specific aim 1)

3.1 The formalities of FISH hybridisation

As pointed out in the main introduction (section 1.1.5), while FISH technology has advanced in many areas, the composition of the hybridization buffer has hardly changed. The ability to multiplex experiments is somewhat limited by the expense of dyes (other than the standard red and green), and time-consuming steps and the complexities of multiple hybridizations and dye mixing (Ridolfi et al., 2000; Yaziji et al., 2004).

While using formamide as the basis for a hybridization buffer has proved highly successful, the “if it isn’t broken, don’t fix it” approach may well be impeding the use and adoption of alternatives. From a chemical point of view, there may be better alternatives to formamide an organic solvent. It has been well-documented the effect organic solvents have on biomolecules (Cordone, et al., 1980), oligonucleotides require a relatively non-aqueous media to function, essential for intracellular concentrations and typically created by high presence of macromolecules and osmolyte compounds.

Organic solvents solvated by water reduce the amount of free water, charge the dielectric permittivity (degree of electrical polarization) (Garlid, 1999; Luby-Phelps et al., 1999; Cuervo et al., 2014) and are observed to interrupt the level of hydrogen bonding, polarity and hydrophobicity of DNA, dependant on organic solvent used. These adjustments bring about conformational changes in DNA stability and the kinetics of hybridization. The introduction of an organic solvent will generally destabilise nucleic acid base paring and interruption of hydrophobic stacking interactions between bases and thus bring down the temperature of DNA’s melting point (Yakovchuk et al., 2006; Šponer et al., 2013). The addition of an organic solvent to polynucleotides shall result in precipitation dependant on the ratio of organic solvent to water, length of polynucleotide and properties of the organic solvent itself, again this area is well researched (Mel’nikov et al., 1999; Bonner & Kilbanoc et al., 2000; Stanlis 2003).

Typically, formamide is the organic solvent of choice to lower the melting point of DNA in standard ISH experiments (McConaughy et al., 1969; Blake et al., 1996), however within the context of cross species hybridization (zoo-FISH), and whole genome hybridization, a longer hybridization step is required (Kallionieme et al., 1992). The current theory is that
organic solvents destabilize the hydrophobic stacking of bases (as mentioned previously) thus reducing temperature of denaturation, opposed to interfering with the hydrogen bonding of complementary strands. It is imperative to look into alternative organic solvents that possess the same base de-stacking potential as formamide but a reduction of hydrogen bonding potential to propel the denaturation reaction forward and not interrupt with the annealing of probe DNA. By conferring to parameters devised to predict if one material will dissolve into another to form a solution, the Hansen solubility parameters (Hansen, 1967) assign three parameters to molecules to describe different energy in various intermolecular bonds; dispersion forces, dipolar intermolecular forces and hydrogen bonds between forces. The closer these parameters are together, the more likely they form a solution (Hansen, 1967) (Table 3.1).

Indeed, other molecules and organic solvents have been tested as alternatives to formamide for DNA denaturation, such as; urea (Sinigaglia et al., 2018), trehalose (Mathlouthi, 2013), propylene carbonate (Matthiesen & Hansen, 2012), DMSO (Markarian et al., 2006), dimethylformamide and acetonitrile (Tateishi-Karimata & Sugimoto, 2014). Some alternative organic solvents have yielded successes when applied to in situ experiments, in particular ethylene carbonate (Matthiesen & Hansen, 2012; Golczyk, 2019). By reflection of each organic solvents Hansen’s solubility factors (Table 3.1) it would be possible to theoretically predict an alternative organic solvent that has similar properties as formamide, similar van-der-Walls and dipolar intermolecular forces, yet with a reduction in hydrogen bonding potential. With the reduction of hydrogen bonding, it is hypothesized that it shall reduce hybridisation time to fit in with the hypothesis that the time requirement of this step is constrained by the annealing of the base DNA to the newly added probe DNA.
Table 3.1. Hansen’s Solubility parameters for various organic solvents

Reported organic solvents that could be a replacement for formamide in FISH based hybridisation buffers, where $\delta D$ = energy from Van-der-Waals forces between molecules, $\delta p$ represents energy from dipolar intermolecular force between molecules and $\delta H$ represents energy from hydrogen bonds between molecules. The key aspect is to note similar $\delta D$ and $\delta p$ as to formamide yet not $\delta H$ as this is viewed as the ‘lagging’ component of hybridisation of probe to DNA target.

<table>
<thead>
<tr>
<th>Organic Solvent</th>
<th>$\delta D$</th>
<th>$\delta p$</th>
<th>$\delta H$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethylene Carbonate</td>
<td>19.4</td>
<td>21.7</td>
<td>5.4</td>
</tr>
<tr>
<td>Formamide</td>
<td>17.2</td>
<td>26.2</td>
<td>19.0</td>
</tr>
<tr>
<td>DMSO</td>
<td>9.0</td>
<td>8.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Acetonitrile (methyl cyanide)</td>
<td>7.5</td>
<td>8.8</td>
<td>3.0</td>
</tr>
<tr>
<td>Dimethyl Formamide</td>
<td>17.4</td>
<td>13.7</td>
<td>11.3</td>
</tr>
<tr>
<td>Urea</td>
<td>20.9</td>
<td>18.7</td>
<td>26.4</td>
</tr>
<tr>
<td>Methanol</td>
<td>15.1</td>
<td>12.3</td>
<td>22.3</td>
</tr>
<tr>
<td>Ethanol</td>
<td>15.8</td>
<td>8.8</td>
<td>19.4</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>15.8</td>
<td>6.1</td>
<td>16.4</td>
</tr>
<tr>
<td>Ethylene Glycol</td>
<td>17.0</td>
<td>11.0</td>
<td>26.0</td>
</tr>
<tr>
<td>Glycerol</td>
<td>17.4</td>
<td>12.1</td>
<td>29.3</td>
</tr>
</tbody>
</table>
In regards to thesis aim 1, a new organic solvent that can reduce the time taken for hybridization experiments should increase throughput of FISH, in same species and cross species experiments. With this in mind, the first aim of this chapter was to test the hypothesis that there are alternatives to formamide based buffers that can significantly shorten hybridization times. Secondly, changes in methodology of FISH may be viable in terms of effectively of experiments and this shall be explored via the use of two commercial buffers in typical FISH experiments and Zoo FISH experiments, culminating in devising a new low-cost high throughput FISH methodology.

This chapter is thusly divided into 2 sections

a. Exploring the option of different hybridization buffers to test the hypothesis that there are better alternatives to formamide based buffers. This section sub divides into two areas;
   a. FISH testing of buffers made in the lab
   b. Direct comparison of formamide vs. non-formamide based commercial buffers

b. Development of a low-cost, multiple probe strategy. This section subdivides into three sections
   a. A newly developed multilayer FISH method
   b. The effect on how the multilayer method affects probes and preparations
   c. An assessment of how many layers can be practicably hybridised
3.2 Exploring the option of different hybridisation buffers

3.2.1 The recipe for good FISH

The first part of the exercise was to establish whether the various solvents were capable of producing a solution that, by necessity, contains dextran sulphate to be a fit for purpose hybridisation buffer. Multiple organic solvents were selected from table 3.1 based upon their Hansen’s’ solubility factors being, ethylene carbonate, DMSO, acetonitrile and dimethylformamide, alongside the typical hybridisation buffer solvent formamide. These solvents were tested in a variety of conditions and concentrations (of NaCl and dextran sulphate). Initial results indicated that dimethylformamide, acetonitrile and DMSO disrupt the dissolution of dextran sulphate in the presence of a high salt concentration. Their counterparts of formamide and ethylene carbonate can effectively form a working solution at any concentration, and are the key components of commercially available buffers.

In house synthesised buffers were tested in a variety of conditions and concentrations however presented with poor levels of hybridisation throughout with even the ‘home made’ formamide and ethylene carbonate buffer failing to produce viable/useable signals, whereas commercial buffers - HybI and FAST- worked alongside in identical conditions consistently(>95%). With the inability to conveniently reproduce commercially available buffers, the possibility of forming a newer cost effective methodology with primary focus on the buffer chemistry drew to an end with focus now being applied to changes of methodology.
3.3 Comparison of commercial buffers for FISH analysis

Following on from the attempts to make alternative organic solvent-based buffers, attention was then turned to commercially made buffers, one formamide based (HybI, Cytocell) and one ethylene carbonate based (FAST, DAKO). Typically, all FISH hybridisation buffers are formamide based so a selection of a reputedly high formamide based buffer, HybI was selected to be tested alongside relatively new yet expensive ethylene carbonate based buffer from DAKO which reportedly can work within the hour as compared to the ‘overnight’ requirement of typical formamide based buffers.

As the time of hybridisation is the key difference with the protocol of these buffers, it was decided to query the formamide buffers ability to successfully hybridise using reduced time for the hybridisation stage, then to compare to the FAST buffer.

As described, two key usages of cytogenetics are for medical diagnostics in ‘same species’ hybridisations and for chromosome mapping of ‘like-species’ across evolutionary time and distance. A means to test these two buffers in light of cytogenetic usages was conducted in order to justify a change of protocol for further work.

3.3.1 Same species comparison of hybridisation times

Initial experiments to compare the buffers has indicated that HybI can successfully hybridise probes with high efficiency (>= 95%) as shown in figure 3.1, with indication that there is a moderate amount of successful hybridisation at 40 minutes and even less success at 20 minutes hybridisation with no evidence seen at 10 minutes or 5 minutes.

With the initial results thus far on HybI indicating similar decreased times for hybridisation, the emphasis will now shift and focus on the ability of these two commercial buffers in tailored experiments which would be typical of a cytogenetics lab and influence subsequent experiments contained within this thesis.
Figure 3.1. Metaphase frequency of successful hybridisation using HybI at 60 minutes and under time-points.

A), *Bos taurus* metaphase with successful probe hybridisation (chromosome 2, P-terminus Green, Q-terminus Red) using standard FISH procedure with hybridisation time shortened to 60 minutes. B), The frequency of successful hybridisation (characterised by two chromosomes with two coloured probes on each) on aforementioned metaphases at the greatly lowered hybridisation time points of 60 minutes and under, using HybI with its specific methodology.

Repeating the experiment using this time the FAST buffer and its methodology, yet changing the hybridisation time points to be comparable to the HybI results seen in figure 3.2, we can indentify that the FAST buffer does not exhibit the drop off of successful hybridisations that HybI expresses. With all time points tested having a successful hybridisation rate of above 90%, (60 minutes 100%, 40 minutes 95%, and 20 minutes 95%, total n=60).

Results thus far show that the variation between the two buffers for same species hybridisation at greatly reduced hybridisation times is considerable, yet a more quantifiable scheme of measuring hybridisation quality is required to further pinpoint buffer quality for this genre of experiments, especially when discussing and experimenting on cross species evolutionary work.
Many means of semi-quantifying data was trialled, such as grading scales and recording software defined automatic capture times yet the most fruitful measurement of scale was found to be forcing set exposure times on metaphases known to have successful signals. This method effectively lowers the overall level of light entering the camera, theorizing that if the signal can be seen with a low level of light then the signal must be stronger therefore hybridisation is of higher quality. The amount of light captured by a lens (camera in this case) is directly proportional to the area of the aperture, something here which cannot be easily changed, yet the exposure time which governs how long the aperture mechanism is open can be directly controlled by the software, enabling a means to control the light levels entering the camera.

It was noted that the higher limit for FITC and Texas Red filters for this particular hardware set up (Figure 3.3) was deemed to be an exposure time of 5 seconds, an ideal higher limit cut-off, where if signal is not identifiable within the 5 second exposure time it is deemed to not have successfully hybridised. Set time points were then selected limited by pre-set levels via the software (SmartCapture 3, Digital Scientific UK) in near equal decreasing increments of roughly half the previous at each step (5 seconds, 2 seconds, 1 second, 0.5 seconds, 0.1 seconds and 0.05 seconds).
With level of exposure time being the precedent for quality of hybridisation, a more direct comparison between different experimental procedures can be observed. Figure 3.3 shows the variation of required exposure times for this same species section of buffer comparison and stress testing (n=88). Here we see that although both buffers have comparable success rates for 1 hour hybridisations (Figure 3.1 and Figure 3.2), we can see a large shift of exposure time between the two samples, the FAST buffer manages to achieve the majority of their metaphase samples in the lowest time frame of 0.05 seconds exposure (55%) whereas HybI at this time point failed to achieve this higher standard with its modal exposure time being 1 second (57%), $X^2 = 21.6 > p(11.070)$, indicating a significant difference between methods at this time frame. The decreasing success rate of HybI (Figure 3.1) somewhat follows suit with the frequency pattern observed; where the modal values drop from 1 second, 1 hour hybridisation down to 2 seconds for 40 minutes and 20 minutes. Statistical analysis follows suit in showing that there is no significant difference between the methodologies used at these time points. For 40 minutes $X^2 = 4.01 > p(7.815)$ and 20 minutes $X^2 = 1.38 > p(7.815)$ showing no significant difference between buffer image quality.

Figure 3.3. Metaphase frequency of signal identified at various forced exposure time points upon B. taurus using same species probes. Histograms showing the percentage frequency of metaphases which show identifiable signal at the lowest exposure time point. Top, using the FAST buffer at incremental hybridisation time points, bottom, identical experiments yet using HybI as buffer at the same time increments. Lower exposure time (0.05 seconds) is noted to correlate with stronger signal therefore better hybridisation quality.
3.3.2 Zoo-FISH comparison of hybridisation times

As discussed, cytogenetic research uses FISH as a means of identifying and validating cross species genomic mapping from species to species. Within this experiment *Struthio camelus* (common ostrich) metaphase preparations are used with *G.gallus* BACs conjoined with fluorophores as probes to again test these two commercial buffers for their effectiveness to hybridise said probes in shortened time frames (n=52). BAC clones were selected from a universal probe set which derived from multigenome alinment computational algorithms intended to act as a means to rapidly anchor PCFs (predicted chromosome fragments) to chromosomes as described in Damas, 2017. Zoo FISH hybridisation is typically performed over three days yet here we shorten this to two days and one day hybridisation, similar to same species FISH we would predict a high percentage of successful hybridisation for the ‘standard’ timeframe (>=95%). The connotation ‘day’ is referred to an overnight hybridisation i.e. initiated on one day and stopped the next working day would be ‘1 day hybridisation’.

![Figure 3.4. Metaphase frequency of successful hybridisation over 2 days using HybI on S.camelus using G.gallus probes](image)

A) *S.camelus* metaphase with probe hybridisation (chromosome 2, P arm, assignment 172N3 Green, Q arm assignment 44D16 Red) using standard FISH procedure with hybridisation time changed to two day hybridisation. B) The frequency of successful hybridisation of *S.camelus* metaphases at the lowered hybridisation time points using HybI buffer and its methodology for zoo-FISH. Note, BAC 172N3 is assigned to the distal portion of the P arm in *G.gallus* and in *S.camelus*, the green signal seen in the image is a repetitive region (centromere) being falsely indicated and overshadowing the correct signal. Upon examination of images the correct signal can be seen via thresholding and repeats of the experiment under normal conditions.
Results from variation of HybI on a cross species hybridisation does not vary to the degree same species FISH varies, with 1 day having a success rate of 93% and 2 days having a success rate of 86% as seen in figure 3.4. A somewhat similar outcome to FAST buffer within the same experimental parameters, 1 day hybridisation had a success rate of 87% and 2 days had 80% success.

Likewise, the use of set exposure times can further delve into the effectiveness of these buffers across shortened typical hybridisation periods. A similar pattern across the 1 day hybridisations can be observed, as both buffers perform similar across the set exposure ranges averaging at 1.9 seconds +/− 0.51 S.E.M for HybI and 2.0 seconds +/− 0.5 S.E.M for FAST. Yet results across the 2 day hybridisations (Figure 3.5) yield differing quality. It can be noted that HybI has a higher count for lower exposure time cut offs where FAST averages out much lower, 0.8 seconds +/− 0.37 S.E.M and 1.7 seconds +/− 0.47 S.E.M respectively, indicating that even with similar percentage success rates, HybI can produce better hybridising conditions at this two-day hybridisation time as compared to the FAST buffer.
Figure 3.6. Metaphase frequency of signal identified at various forced exposure time points upon *Struthio camelus* (common ostrich) using *G. gallus* probes

Shown, histograms representing four different hybridising environments; two selected different buffers (HybI, Cytocell, Top and FAST, DAKO, Bottom), alongside two different hybridisation times (1 day hybridisation, left and 2 day hybridisation, right). Exposure time for the microscope camera was forced at the time points shown and selected metaphases imaged at each point; metaphases are categorized based upon the lowest exposure time image where there is clear identifiable probe signal in the correct position, guided by an automatic exposure image. Lower exposure time is theorized to be indicative of more probe signal therefore better probe hybridisation in said condition.

With results being fairly comparable for both same species and zoo-FISH in terms of overall success and neither being statistically significant from one another. For 1 Day $X^2 = 0.29 > p(7.815)$ and 20 minutes $X^2 = 5.93 > p(9.488)$

It is important to note other differences in the actuality of both buffers. At time of testing the FAST buffer was a new and expensive product which equalled out to ~£40 per reaction whereas HybI in comparison costs ~£0.50 per reaction.

Given that work contained in later chapters uses pre-mixed HybI probes, devices intended and designed specifically to use the formamide based buffer and cross species comparative genomic work, coupled with the fact that the producer of HybI, Cytocell, collaborates with this and likewise projects, the cost of buffer solution is near zero. Therefore all subsequent experiments were using HybI as the hybridisation buffer for all FISH experiments.
3.4 Development of the Multilayer FISH method

As pointed out in the introduction, there have been many attempts to produce multicoloured FISH. The advantages of this approach are self-evident. Visualization of multiple target on the same preparation can be very useful e.g. if there are few cells available for analysis, if each cell in a population of potentially karyotypically different or if multiple targets need to be analysed on the same chromosome e.g. for BAC ordering purposes. As outlined in section 1.1.5, beyond red and green dyes, fluorochromes can be prohibitively expensive, single hybridizations of multiple probes can be more temperamental than one or two colour (often requiring much higher concentrations of probe, which increases the cost), and the mixing of dyes (e.g. red and green to produce yellow) can yield inconsistent results.

In order to address this, this study involved the creation of a relatively new methodology of FISH (in reality a variant and combination of previously tried strategies). The intention was for it to be relatively cheap, high throughput, rapid, and allow for visualization of multiple targets. Understandibly, the current FISH methodology is the rate-limiting step in creating chromosome level genome assemblies and it was with this in mind that the following protocol was developed.
3.4.1 Methodology of Multilayer FISH

A reappraisal of current FISH technology led us to the conclusion that, with modern microscope hardware and software improvements, it is entirely feasible to return to the same cell saved on any individual slide and re-observe it. This paired with the evidence that probe DNA held in place by hydrogen bond pairing can easily be removed and new DNA probes hybridised to the same preparation, I pursued the strategy of a multilayered FISH (mlFISH) approach, involving two dyes at a time in three or four rounds of hybridization as follows.

3.4.1.1 Removal of previously hybridised probes

In creating the protocol the following considerations were taken into account: Probes must be sufficiently removed to not display residual signals in later hybridization rounds. Each individual layer must have their own unique signals from the fluorophore(s) chosen for that ‘layer’ and should not have any interference from previous signals that may be the same fluorophore yet at a different position on the metaphase. It is also essential that the stripping process does not interfere with the metaphase preparation so they can easily be located and imaged for the next iteration of the multilayer cycle.

After some experimentation, it was established that complete removal of signal caused by residual hybridized probe DNA could be achieved with a prewash of 2xSSC and 0.05% Tween 20 for 2 minutes to allow gentle removal of immersion oil, cover slip and counter stain (typically DAPI). Following this a stringency wash, aka ‘strip wash’ (100% ddH₂O, at 72°C) for 30 seconds to 60 seconds with agitation upon removal effectively removes all fluorophore signals from the sample with no observed damage to the chromosomes in the preparation. These changes to the protocol are outlined further in table 3.2 and the resulting imagery is illustrated in figure 3.8.
TABLE 3.2. Differences in methodology within multilayer FISH

Here described, the multilayer methodology steps which are different to classical FISH experimental procedure paired with their resulting change to the sample as rationale behind said methodology.

<table>
<thead>
<tr>
<th>Method Step</th>
<th>Resulting change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clean slide with 2xSSC + tween (Pre Wash)</td>
<td>Removes immersion oil and counter stain (DAPI) from previous imaging round, allows the cover slip to be floated off the slide with minimal disturbance to the metaphase preparation</td>
</tr>
<tr>
<td>High temperature ddH₂O wash</td>
<td>A high stringency wash (no ionic molecules present, i.e. salt) to remove all strongly bound probes from the chromosome preparation, and rehydrates the slide</td>
</tr>
<tr>
<td>Agitation when removing the slide</td>
<td>Flushes any probes that may still attached by mechanical action</td>
</tr>
<tr>
<td>Rinse in increasing concentration of ethanol</td>
<td>With the washes taking place, the slides are hydrated and need to be dehydrated for effective hybridisation to occur for the next round of FISH</td>
</tr>
</tbody>
</table>

3.4.1.2 The Iterative Cycle of Multilayer FISH

A standard model of mFISH was produced visually in flowchart form as shown in figure 3.7. The method follows the similar standard FISH methodology, yet has the iterative cycle element after microscopy where the user can remove probes as previously mentioned and insert the slide into the pre-ethanol dehydration stage as if they were freshly prepared samples. It was observed that there is mild deterioration in chromosome morphology, increasing within each cycle, creating a limit on how many times it is practicable to go through the process.
Figure 3.7. The Multilayer FISH iterative protocol shown in Flowchart form

A flowchart following standard shape meanings describing the process a user must go through to perform multilayer FISH. Note that the process is similar to standard FISH allowing experienced users to use this technique if it would serve fit for purpose in their experiments. Estimated time is displayed next to each step in the process to show the gained time from subsequent rounds being quicker to image and no need to prepare new metaphases.
3.4.2 The effects of Multilayer FISH on metaphase preparations and probes

The process of mlFISH is not the most gentle on samples as the conditions, such as temperature, are constantly changed. The purpose of this experiment was thus to ask what is the quality of the preparation and the hybridization signal under the same conditions and multiple stripping and re-probing. In order to maintain consistency two commercially available Human centromeric probes were used (Human centromeric 8 and human centromeric 12) in alternate red and green in consecutive hybridization layers.

In layer 1 (green 8, red 12), 100% showed the correct number of signals, in layer 2 (red 8, green 12) 95% of metaphase showed the correct number of signals. A slight deterioration in metaphase quality was noted. In layer 3 (green 8, red 12) 92% of metaphases the correct number of signals. Significant deterioration in metaphase quality was noted however chromosomes were still visible and distinct. Signal quality was also noticeably less bright by visual inspection. On the basis of the above, and after some experimentation, the decision was taken not to routinely include a fourth layer. It was also concluded via further visual inspection and later testing, interphase quality deteriorated the same rate as metaphase quality yet likewise with metaphases, the signals were still highly visible and distinct (see results from section 4., 5., and 6.).

Figure 3.8 shows an example of a *H.sapiens* metaphase describing the above results, albeit with a atypical low level of metaphase distortion across layers. Figure 3.9 shows much more typical distortion of the metaphase across the layers, this time *G.gallus* is the sample. Furthermore, there appeared to be no obvious pixel shift of signals from one preparation to the next, giving me confidence that a three layer, six probe strategy could be developed for BAC mapping on the same chromosome and/or preparation.
Figure 3.8. Multilayer FISH does not cause probes to incorrectly position after additional layers

A Human, *Homo sapiens*, metaphase preparation showing centromere locations for chromosome 8 (FITC 1st+3rd image, Texas red 2nd image) and chromosome 12 (FITC 2nd image, Texas red 1st+3rd) Left to right, images in columns are each layer starting with the first on the left moving right. Top row; combined image from each raw colour channel obtained. Second row; shown is the raw Dapi (blue) channel for each layer showing no major distortion to the metaphase preparation. 3rd row; Raw FITC channel (green) output showing the levels of probe intensity across the metaphase, note that the first and last images occupy the same position on the sample and the second shows no signal at these locations. Last row; similar to the third yet using the red channel (Texas red), again note that the signal is on the same location within the first and third layer yet not on the second layer.
Given that the overall shape and structure of the metaphase was maintained throughout a three layering process (and signal position/brightness was not significantly compromised), the final stage of the process was to develop a protocol for 6 probe rapid hybridization involving two fluorochromes only, Hyb I as a buffer (as established above) and subsequent capture and re-capture of the same metaphase. In order to re-locate the same cell, the automated stage on the microscope hardware was used. In order to differentiate each probe a pseudo-colour function in the image capture software was used.

Figure 3.9. An exemplar schematic of a three layered FISH image
A *G. gallus* metaphase prepared with six differing BAC based probes with three different FISH iterations using identical fluorophores (Texas red (red) and FITC (green)) at different positions along chromosome 3. Top, the three layers of combined three channel images produced via fluorescence microscopy (1000x) moving in increasing layer from left to right. Bottom, the ‘merged’ combined metaphase with ‘false’ colouration to each unique probe, green = First layer FITC, red = Second layer Texas red, Yellow = second layer FITC, Purple = second layer Texas red, aqua = third layer FITC, Orange = third layer Texas red.
3.5 Limitations of the Multilayer Method

While, for most experiments, six probes in three layers were considered sufficient, an obvious question was how many layers could we feasibly apply before no (or a negligible) signal was seen at all.

An assortment of BAC probes were arranged for chromosome 1 of chicken (*G.gallus*) in a way that allowed successive layers to be hybridised and stripped until the experiment failed, in this experiment using dual colour FISH, 9 unique layers were hybridised with the BACs from chicken chromosome 1 indicated in Table 3.3.

<table>
<thead>
<tr>
<th>Position</th>
<th>Start pos.</th>
<th>End pos.</th>
<th>Hex code</th>
<th>RGB</th>
<th>BAC ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>875,622</td>
<td>1,046,980</td>
<td>FF0000</td>
<td>255,0,0</td>
<td>89C18</td>
</tr>
<tr>
<td>2</td>
<td>20,538,145</td>
<td>20,775,062</td>
<td>F54C00</td>
<td>245,76,0</td>
<td>89G23</td>
</tr>
<tr>
<td>3</td>
<td>29,623,402</td>
<td>29,863,423</td>
<td>EB9300</td>
<td>235,147,0</td>
<td>119K2</td>
</tr>
<tr>
<td>4</td>
<td>34,010,417</td>
<td>34,242,656</td>
<td>E2D400</td>
<td>226,212,0</td>
<td>120J2</td>
</tr>
<tr>
<td>5</td>
<td>65,968,509</td>
<td>66,176,072</td>
<td>A2D800</td>
<td>162,216,0</td>
<td>36B5</td>
</tr>
<tr>
<td>6</td>
<td>71,546,463</td>
<td>71,784,301</td>
<td>5ACF00</td>
<td>90,207,0</td>
<td>25P18</td>
</tr>
<tr>
<td>7</td>
<td>77,066,050</td>
<td>77,273,343</td>
<td>1BC500</td>
<td>24,197,0</td>
<td>125F1</td>
</tr>
<tr>
<td>8</td>
<td>98,389,770</td>
<td>98,619,006</td>
<td>00BC23</td>
<td>0,188,35</td>
<td>118M1</td>
</tr>
<tr>
<td>9</td>
<td>110,513,385</td>
<td>110,771,740</td>
<td>00B259</td>
<td>0,178,89</td>
<td>18J16</td>
</tr>
<tr>
<td>10</td>
<td>120,693,003</td>
<td>120,889,688</td>
<td>00A889</td>
<td>0,168,137</td>
<td>29N14</td>
</tr>
<tr>
<td>11</td>
<td>132,642,594</td>
<td>132,847,168</td>
<td>008B9F</td>
<td>0,139,159</td>
<td>9B17</td>
</tr>
<tr>
<td>12</td>
<td>140,904,296</td>
<td>141,166,019</td>
<td>005495</td>
<td>0,84,149</td>
<td>168O17</td>
</tr>
<tr>
<td>13</td>
<td>146,261,866</td>
<td>146,502,123</td>
<td>D0238C</td>
<td>0,35,140</td>
<td>83O13</td>
</tr>
<tr>
<td>14</td>
<td>155,895,248</td>
<td>156,124,183</td>
<td>080082</td>
<td>8,0,130</td>
<td>107E2</td>
</tr>
<tr>
<td>15</td>
<td>166,741,351</td>
<td>166,944,259</td>
<td>2D0079</td>
<td>45,0,121</td>
<td>58K12</td>
</tr>
<tr>
<td>16</td>
<td>172,851,270</td>
<td>173,851,270</td>
<td>4C0D6F</td>
<td>76,0,111</td>
<td>184E5</td>
</tr>
<tr>
<td>17</td>
<td>190,251,863</td>
<td>190,251,539</td>
<td>660066</td>
<td>102,0,102</td>
<td>98G4</td>
</tr>
</tbody>
</table>

Table 3.3. BAC information and ordering for *G.gallus* chromosome 1 for multiple Multilayer FISH rounds

Showing the position along chromosome 1 for *G.gallus* for owned BAC probes, detailing their start position and end position (according to distance from the p-terminus), with corresponding clone ID. The relevant hexadecimal and equivalent RGB value is also displayed to design the colouration of the finished merged experiment.
After the first layer of FISH was performed, metaphases were captured and positions saved and total recorded, and layers were repeatedly added onto the metaphases until an absence of metaphases were seen or no specific signal was observed. The metaphase drop out was as follows; First layer n=50, second layer n=41, third layer=40, third layer n=39, fourth layer n=39 and final fifth layer n=31 (a total of 62% survival of decay). Whereas, specific probe signal was not directly correlated to metaphase survivability as n=11 was useable for merging and analysis, (22% success rate at 5 layers). Further layers provided negligible results with no conclusive probe signals seen and even more decay of metaphases was observed. An example metaphase as described here is shown in figure 3.10 and a more detailed isolated chromosome image is detailed in figure 3.11 with arrows corresponding to the table 3.3 of probes.

**Figure 3.10. The maximum layer count achieved using current methods.**
 Five layers on a single G.gallus metaphase showing positions of 10 different probes, with reference to table 3.3. This also shows the quality / state of the metaphase and probe clarity between layers to fully form the final five layer image on the right. In total 11 channels, 10 false colouration of probe signal and one final DAPI layer for chromosome identification.
Figure 3.11. Five layered chicken chromosome 1 in more detail
Increased resolution of the merged five layered image from figure 3.10. Right, Arrows are used for clarity and visibility with numbering signifying the appropriate probe from table 3.3.
3.6 Discussion

3.6.1 Exploration into alternative buffers

Overall, this study was successful in its objectives to re-examine the FISH procedure and emerge with a novel, fit for purpose protocol. The setback of not being able to generate sufficient signals using homemade buffers was not an unproductive endeavour. The commercial formamide based buffer Hyb I showed a significant improvement and is sufficient enough for most purposes currently faced and we were fortunate to have a ready supply of it. The carbonate-based buffer (Matthiesen & Hansen, 2012) certainly was effective, but not significantly more so than Hyb I, but was prohibitively expensive for routine use (costing £40 for a single assay, at time of experiments). Through attempts to synthesise new buffers the lesson learned was that it was more difficult than one might imagine. Formamide was clearly wisely chosen and even attempts to switch to de-methyl formamide (theoretically a better solvent with near identical energy from van der Waal forces, a greatly reduced energy from hydrogen bonding, critical for the ethylene carbonate buffer, yet lower dipolar energy forces which would an ideal area to query next), did not even progress past the stage of even making a soluble buffer. While it presumably would be possible to synthesise a carbonate buffer like the commercial one used in this study, this proved not to be possible (at least in my hands with available time and resources). The “take home message” was to continue with Hyb I.

The precise composition of Hyb I is a trade secret of Cytocell Ltd however personal communication reveals that it is formamide based and benefits from being rigorously batch tested on an industrial scale with a wide range of probes.

3.6.2 Comparison of commercial buffers

With the formulation of ‘home made’ hybridisation buffers being somewhat of a fruitless endeavour, the question of how well the differing commercial buffers with differing organic solvent basis can increase throughput for common cytogenetic experiments. Outlined in this chapter, it is demonstrated the usefulness of both buffers (Hyb I-formamide, FAST – EC) for same species FISH and Zoo-FISH/cross species FISH. Quite an unexpected outcome was the development of an ‘in-house’ system of testing buffers which could be applied for testing newer buffers should they be developed in the future. Overall results across these experiments does indicate that Hyb I is more reliable in cross-species hybridisation experiments and the EC based buffer unsurprisingly works better at
lower hybridisation times. However, it does show the reliability of a well made commercial buffer where Hyb I can somewhat compete with a newer, albeit more expensive, buffer in regards to quick hybridisation testing (Figure 3.1).

In reflection on the methodology used to assess the two buffers, it would be recommended that a more rigorous grading criteria be developed as this is very hard to achieve with current methods available. The idea of using the software derived ‘auto-capture’ time showed little success where metaphase/image usability would not correlate to this variable. Furthermore the algorithm used to determine the auto-exposure time cut-off/selection is within the capturing software itself and could easily vary based upon current hardware used adding to uncertainty of results. From this a more reliable way of assessing probe strength, i.e. hybridisation success, was to manually restrict the exposure time allowing only set quantities of fluorescent light to be received by the camera, governed by probe availability on the sample. Again this is a questionable means of testing as it poses the question of does the level of fluorescent light received necessarily equate to better probe binding/hybridisation conditions?

In light of results from this section, it was clear that Hyb I was suitable enough to carry forward throughout the rest of the thesis experiments. The use of a cheaper as reliable buffer commonplace in cytogenetic laboratories would gain favour for those wishing to follow the multilayer methodology outlined in the rest of this thesis.

### 3.6.3 The novel Methodology of Multilayer FISH

The creation and refinement of the multilayer methodology of FISH has here demonstrated a means of improving yields in a simple and effective way. Outlined in table 3.4, common limitations of FISH are listed with multilayer FISH justifications as use of an alternative methodology.
Limitation of FISH | mIFISH Counterpoint
---|---
Low number of fluorochrome analysis at any one time | Experiments can be organised to reuse the same colour fluorochrome at different positions
Low number of metaphases present, i.e. valuable cell sample or poor cell harvest/growth | The same metaphases can be reused for different experiments
Metaphases are of poor quality | Few metaphases are required to generate a final merged image
Fluorophore reagents are expensive to generate probes | The number of experiments required to get the same results as typical FISH is far smaller thus using less reagents
Metaphases vary within the sample due to growth and harvest conditions introducing error when comparing metaphases | This error is eliminated as there is no variation as the same metaphase is used for the multitude of layers
Laboratory and analysis time needed to produce a wide set of data | mIFISH can reduce the time taken in the laboratory and on the microscope as less slides are used, yet analysis time is increased due to the merging process

Table 3.4. Limitations of traditional FISH with resolutions found in the multilayer method
Limitations reported as why the ‘classic’ FISH methodology is unequipped for a modern-day laboratory setting and how these points are remedied with the use of the multilayer method.

These simple but worthwhile changes to the traditional FISH protocol can dramatically reduce the cost of FISH, one of the main disadvantages against its use (Huber et al., 2018). This reduction is achieved by reducing the amount of probes required for a large comparative study, for example probe ‘A’ would only have to be used once per metaphase/sample rather than ‘n’ times, with ‘n’ being the number of other probes used. Probe ‘A’ would otherwise have to be paired with probe ‘B’, probe ‘C’, probe ‘D’ and so on in multiple experiments which increases probe usage and experimental time required, another cost saving feature. The idea that a sample may become irreplaceable, such as a transgenic mutant within genetic screening processes for the food industry. The animal would be culled immediately to reduce cost, however from a research standpoint it is a very valuable phenomenon with now no means to gain more material. The use of multilayer FISH somewhat removes this experimental limit by increasing usage of remaining material. Again, the use of only two fluorophores further reduces costs required, yet also lessens pre-experimental planning issues as there is no need to work out tricky combinations of specific colour probes to specific loci which can result in redundant probes only being used a handful of times. The fact that a single metaphase can be used for a
whole experimental question reduces some uncertainties with traditional FISH. Now the metaphase variability between experiments is removed, which could lower ranges of results seen during analysis.

For a worked coated example. As a hypothetical estimation on the monetary and time cost of hybridising six probes to a single human chromosome, by the multilayer method (six probes on one metaphase, ‘3 rounds’) or by traditional FISH (two probes per metaphase, unique combinations with cheapest probe combination, 15 ‘rounds’ total). An approximation of cost per probe, (as outlined in sections 2.1.3 and 2.2) for reagents only (probe manufacturing, hybridisation buffer and species relevant repetitive sequence blocker) is £1.40 for a FITC labelled probe and a £1.65 for a Texas red labelled probe per ‘round’ of FISH. The multilayer methodology requires three of each probe, totalling at £9.15 for a single chromosome study. Opposed to this, the traditional method requires 15 rounds for all six probes to be in unique combination with one another, using the cheapest fluorophore combination (as much FITC as possible) costs approximately £45.75 for the chromosome study. In terms of time, preparing a single slide vs. 15 slides is somewhat troublesome to directly compare. For example, using Figure 3.7 as a guide for timings, the 8 minute ethanol series will not be multiplied 15 times as this can be performed simultaneously in the same time frame and the multilayer method will not gain time here. Where time is saved on is repeated rounds of microscopy. Per slide of traditional FISH would take approximately 20/30 minutes to get enough metaphases of good quality imaged, with 15 slides this will be approximately 6/7 hours of microscopy time. The multilayer method initially takes time but time is saved by reliance on saved positions on a mechanised stage to speed up subsequent microscopy. For this example, the total microscopy time for multilayer FISH would be approximately 2 hours. Post microscopy, analysis time will vary greatly dependant on skill/experience of the user and the computational aids they have access to, this is further discussed in section 4.2.1.

3.6.3.1 The effects of Multilayer FISH protocol

The ‘re-FISH’ process within the multilayer methodology is shown not to distort the cellular preparations. This allows determination of results with confidence as there is little to no detriment to the sample based upon what ‘layer/cycle’ the sample is on. As clearly seen in figure 3.8, the movement of probe is incalculable from the first FISH stage to the last. In traditional FISH it is notable that chromosomal morphology is distorted by the classic ‘fuzzy edge’ feature as caused by rapid denaturation interfering with DNA-histone to
scaffolding protein structure, more so with extra rounds of denaturation (Figure 3.10), somewhat making a limit on the amount of cycles a sample can withstand.

The introduction of more stringent washes was a clear route to take to remove previously bound probes. This process is shown to have no effect upon sample quality, as we would expect in molecules only held on by hydrogen bond interactions. Standard FISH requires a post hybridisation stringency wash, typically 0.4% SSC at 72°C, to remove weakly bound probes and sharpen correct signal. By taking the principle that a high ionic solution, such as in the hybridisation buffer, helps stabilised the negatively charged DNA backbone and also aids in to stabilise the probe DNA- chromosome DNA hybrid, then a lower ionic environment shall destabilise this hybrid and allow disassociation of the probe DNA to occur. Furthermore, an environment lacking relatively any ionic component should cause complete destabilisation of the hybrid compound thus causing removal of the fluorophore attached to the probe into the surrounding solution. These checks allow the confident use of multilayer FISH as the methodology of choice for further biological questioning in the field of cytogenetics.

3.7 Conclusion

The necessity of a good protocol and quality reagents in FISH is commonplace in cytogenetic laboratories. As with many processes however a reappraisal is often required.

In retrospect to the buffer comparison tests, a more rigorous set of control samples would be essential to validate this concept of testing further. As a recommendation, a set of human metaphases with commercially made probes as the golden standard of 'perfect materials' to test either homemade buffers or newer commercial buffers. Likewise, a generation of cross-species panels would be useful if the focus is more on evolutionary genomic studies, metaphases from species that increase in evolutionary distance/increasing diversity using probe targets of known homology. With better predefined criteria to judge metaphase/probe quality paired with controlled blind marking/grading of images would further added to the validity of these tests. Regardless, the creation of a preliminary buffer comparison test has shown good results to influence the methodology of all future chapters.

The multilayer methodology is not without its own downfalls. This approach is technically hard as requires an experienced cytogenetic scientist and understanding of multiple pieces of software (for microscope control and image merging/analysis), alongside
the ‘all eggs in the same basket’ approach where time and reagents can be wasted if later layers fail to hybridise correctly. As seen in the 5th layer of chicken chromosome 1 stress testing, the viability of end metaphases was 22%, comparing to amount useable after initial hybridisation. However, this is exciting to see as it represents a wide pool of very specific BAC’s being used to give lots of information in a short period of time. Compared to traditional methods which would require each probe to be tested individually with one another, represented by this equation with ‘n’ being number of probes, \( n=(n^2)-n \) it would equate to 90 simple experiments against a single more arduous challenging multilayer experiment.

It is a error filled task to correctly predict the exact complete cost of a modern day cytogenetics lab using the traditional method against the multilayer method, as we would have to discern; labour costs, general lab consumable costs, the running costs associated with use of a fluorescent microscope (in regards to bulb wear, fluorescent filter cube costs, immersion oil and maintenance) and possible software licensing costs. But with the example outlined in 3.6.3, we can see that the ‘per experiment’ cost of the multilayer FISH method greatly reduces the overall cost due to the reduction of fluorophore usage, where a single two colour test costs £3.05. For a general formula in an ideal situation (i.e., all probes currently exist in the correct and cheapest fluorophore combination), the cost for multilayer two colour FISH would be £3.05 times the number of layers opposed to in traditional FISH compromising of £3.05 times \((n^2(n-1))/2\), where n is the number of probes required. To put a price on individual metaphases cannot be comprehended, as some may be ‘priceless’ as they could be irreplaceable (such as in the event of novel genomic mutations) or from hard to source organisms (see 1.3.5)

It is clear to be seen that the multilayer FISH method gains in efficiency in throughput the more probes that are needed to question a given sample, with reduction in time and money the user will have higher gratification for hybridisation techniques for their research projects. With this in mind, the multilayer methodology has been applied to other biological questions contained within this thesis to truly show its capabilities.
4. Using the Multilayer FISH Strategy to Improve Our Understanding of Galliform evolution (specific aim 2)

4.1 Cytogenetics of the aves

Gene mapping using cytogenetic techniques was first employed to map individual clones, once isolated, to chromosomes (Lichter et al., 1988). As explained in the main introduction however its main utility for modern genomic efforts in recent years is as either confirmation for the reliability of newly assembled genomes or de-novo mapping to chromosomes of scaffolds generated by next generation sequencing. In either event, the use of multi-colour strategies has been largely under-explored in this context, but would nonetheless potentially find utility for visualization of the order of several probes simultaneously, at a decreased cost to previous cytogenetic methods.

As the avian genome is relatively small as compared to mammals (Schmid, 2000; Smith, 2000; Habermann et al., 2001), hypothesised due to evolutionary requirements of flight as allows a higher degree of energy conservation (Hughes, 1995). Members of the aves have a characteristic stable karyotype pattern (Masabanda et al., 2004; Griffin, 2007) consisting of around 10 macrochromosomes and approximately 30 gene rich microchromosomes (Habermann et al., 2001; Burt, 2002) (Section 1.2.3). These microchromosomes are seen to be relatively gene-dense as compared to macrochromosomes and appear to be highly conserved across the avian lineage (Skinner, 2009a; Griffin 2008). Comprehensive interchromosomal rearrangements are reported in only a small number of groups such as parrots and falcons (Seibold-Torres et al., 2015; Nishida et al., 2008; Nanda et al., 2007; Joseph et al., 2018; Damas et al 2007).

The identification of similar morphologies of the karyotype was first achieved through typical banding pattern techniques, such as G-banding (Takagi & Sasaki, 1974; Ansari, 1986) to be further improved by the creation of fluorescent linked whole chromosome paints which were necessary to light up a whole chromosome (Griffin et al., 1999) which allowed a more rigorous means to facilitate the physical mapping of the avian genome (Schmid et al., 2000). An example is the comparative painting between the chicken, *G.gallus*, and the emu, *D.novaehollandiae*, which identified high homology between the nine largest macrochromosomes (Shetty et al., 1999)
The Galliform order is well studied, as *G.gallus* is a key model species, thought to be most similar to the avian ancestor (Romanov et al., 2014). An early study of homologs between chicken and Japanese quail showed no interchromosomal rearrangements (Kayang, 2006), and it is generally agreed that most of these macrochromosomes 1 to 9 (including Z) have direct homologs in most other bird species (Griffin 2000, first report, Griffin et al 2007; Damas et al., 2018). Notable exceptions among the Galliformes (Figure 4.1) include a fission of the ancestral chromosome 2 to form turkey chromosomes 3 and 6 and a fusion to form a sub-metacentric chromosome present in chicken and at least 2 quail species (Griffin et al., 2008; Shibusawa et al., 2002).

Figure 4.1. Phylogenetic Tree of Galliformes studied in this thesis with notable inter-chromosomal changes
Phylogenetic tree of the galliform species used within this study, arrows depict already categorized gross chromosomal changes over evolutionary time.
Note, *Coturnix chinensis* has recently been re-grouped into the Excalafactoria Genus and can appear as *C.chinensis* or *E.chinensis* in literature or phylogenetic trees/databases.
In interphase nuclei, the organization of the avian chromosomes is well described. The microchromosomes occupy a central position, whereas the macrochromosomes cluster around the nuclear periphery (Haberman et al 2001). This in fact fits both classic models of nuclear organization (size-based and gene-density based) given that the microchromosomes are far more gene-rich. Skinner et al (2009a) provided evidence that the gene-density based model was more likely by analysis of relatively gene poor microchromosomes. The clear organization of the whole chromosome territory however raises the question of whether intra-chromosomally, the organization is similarly conserved i.e. that the chromosome territory organises itself consistently each time. If this were the case, then it would be possible to map the order of clones on interphase nuclei and thus negate the need to prepare metaphases for genome assembly studies. The need for dividing cells to make metaphase preparations is one of the impeding factors

With the above in mind, the purpose of this chapter was twofold and thus split into two sections

- **In regards to aim 2.1**: To develop a multilayer FISH protocol specifically designed to map clone order (and hence interchromosomal and intrachromosomal rearrangements) in Galliform chromosomes 1-5.

- **In regards to aim 2.2**: To use a version of the above and thence develop an interphase chromosomal predictive finding tool to better understand chromosomal folding in interphase nuclei and test the hypothesis that gene order can be mapped interphase nuclei
4.2 Galliform chromosome rearrangements

Using a multilayer strategy of 3 sequential layers, each with 2 colours, results demonstrated that the following questions could be addressed.

1): Are all the signals seen on the same chromosome?

Results showed that, immediately, the user can see if the probes used localise to the same chromosome of choice in the new species of bird. An observation of ‘splitting’ indicating either fission of fusion of an ancestral chromosome can be identified easily by all six probes not co-localising to the same chromosome.

2): Are all the signals in the same order as G.gallus?

As pseudo colours are applied to each unique probe in the panel, results show that it is relatively easy for the user quickly to identify any intra-chromosomal rearrangements such as inversions by visual inspection rather than relying on FLpter measurements.

3): Are the signals the same distance across the chromosome compared to a chicken reference?

This approach allows a more rigorous examination where the FLpter scores are generated to detect any minor difference across the chromosome that may not be apparent with a brief examination of the signals.

4.2.1 Measuring FLpter: script vs. Manual measurements

In the materials and methods (section 2.2) usage of a script for measurement of FLpter values in a mlFISH strategy is described. In order to establish that the method was equivalent to the “standard” manual approach of measurement of FLpter values from individual experiments, the two approaches were compared as shown in figure 4.2.

Identical images for G.gallus chromosome 2 panel, merged multilayer images and unmerged two probe images, were measured manually and automatically (outlined in detail in the method section 2.2.2) to give FLpter scores for the identical probe locations. As seen in figure 4.2, the recorded probe positions do match, yet what is observed also is that there is a much larger inconsistency in the variance of the automated script measured sample. This is depicted as error bars showing the standard deviation and confirmed by a series of Paired two sampled, two tailed T-Tests (Probe A, p=0.0025; Probe B, p=0.0046; Probe C, p=0.0288; Probe D, p=0.003; Probe E, p=0.2371; Probe F, p=0.0316; Length, p=0.0003, See appendix). All measurements must have failed the T-test to show that there
is no difference between the methodologies tested; only probe E had a higher p value than the standard 0.05 significance level. The variance is larger in samples measured ‘last’ (the last layer, which has the most chromosome distortion as previously reported), especially seen in probe assigned ‘C’ as this was a probe used in the 3rd round of multilayer FISH.

As rounds of multilayer FISH increase the script incorrectly interprets the length of the chromosome, generating a more erroneous score associated to the probe at that point on the chromosome. Here we see the scores do average out to their expected value, governed by the manual reading of the six-layer image. However, this level for same species FISH sets a worrying precedent for cross species FISH where the large variance could hide or incorrectly report position changes as compared to the chicken.

The results therefore suggest that using the FLpter script could possibly lose precision for the following experiments, with the increase of time taken to measure each sample (1 image for manual and 3 images if using the script), from this point on manual measurements will be taken to determine the FLpter scores.

---

**Figure 4.2.** Probe position placements for *G. gallus* chromosome 2 measured via different methods

FLpter scores obtained by manual reading (blue) and via an in house automated script (red), on *G. gallus* chromosome 2 using probes outlined in table 4.2. Error bars are given as standard deviation in respective colours. Identical images were used to obtain scores then averaged across all recorded data for chromosome 2 for both measuring methods and plotted against one another above.
4.2.2 Genome organisation in Galliform chromosomes 1-5

In the following diagrams and tables, for each chromosome, the BAC ID and genomic position are given. In the initial layer approx n>50 metaphases for G.gallus, and n>20 metaphases for other species (due to lower mitotic index) were captured. Metaphases washed and then re-layered with the next pairing of probes in the standard multilayer iterative approach to generate a multitude of merged metaphases as seen in figure 3.9 for each species used in this study, 3 metaphases of varying quality selected are subsequently shown from overall pool of merged metaphases.

After merging, metaphases were then analysed manually to give averaged Flpter scores for each point across the chromosome and then cross compared to G.gallus. Using a chromosome plot point, FLpter values can be plotted against one another according to distance and probe to be directly measured against the reference of chicken.

4.2.2.1 Chromosome 1

For chromosome 1 (Table 4.1, Table 4.6), the BAC order remained the same in all 5 species. The relatively different position of probe B (yellow) in Japanese quail is suggestive that there may be a rearrangement in this region beyond the resolution of this system (Figure 4.3, Figure 4.4)

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Start Position</th>
<th>End position</th>
<th>BAC ID (CH261-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A)</td>
<td>875,622</td>
<td>1,046,980</td>
<td>89C18</td>
</tr>
<tr>
<td>(B)</td>
<td>65,968,509</td>
<td>66,176,072</td>
<td>36B5</td>
</tr>
<tr>
<td>(C)</td>
<td>98,389,770</td>
<td>98,619,006</td>
<td>118M1</td>
</tr>
<tr>
<td>(D)</td>
<td>132,642,594</td>
<td>132,847,168</td>
<td>9B17</td>
</tr>
<tr>
<td>(E)</td>
<td>146,261,866</td>
<td>146,502,123</td>
<td>83O13</td>
</tr>
<tr>
<td>(F)</td>
<td>190,251,863</td>
<td>190,251,539</td>
<td>98G4</td>
</tr>
</tbody>
</table>

Table 4.1. Probes used for metaphase chromosome analysis for Galliform chromosome 1

G.gallus BACs from the ‘Chori’ library, selected based upon position upon chromosome one of G.gallus, Probe ID with colour corresponds to the appropriate graph and false colour applied to signals seen in images. Start position and end position display the base number on which the BAC spans along the chromosome (approximately 200,000 bp); and BAC ID is the associated identifier associated with the sequence used as a probe. Six probes in total, A to F.
Figure 4.3. Merged metaphase images of five galliformes using selected *G.gallus* chromosome 1 BACs

15 individual metaphases, 3 per species shown, displayed with hybridised *G.gallus* chromosome 1 probes outlined in table 4.1 with corresponding false colouration of probe signal seen. *G.gallus* (first row) is shown as a control to display expected position on that species with possible variances seen in other species, with descending rows being differing species. Images were merged according to the protocol outlined in section 2.2.4.1. Scale bar, 10uM, all images are to the same scale.
Figure 4.4. Probe point plot for galliform chromosome 1 for five species, and corresponding chromosome schematic

A) Probe position measured as FLpter values and averaged across all metaphases obtained using probes outlined in table 4.1. Each individual species has a corresponding colour seen, and colours assigned to each probe correspond to false colours applied to related metaphase images. Error bars are given as S.E.M.

B) Generated schematic chromosomes for each species by using metaphase FLpter data generated in this study, all relative to G. gallus chromosome 1. Position of coloured band corresponds directly to FLpter scores generated and colour corresponding to assigned probe.
4.2.2.2. Chromosome 2

For chromosome 2 (Table 4.2, Table 4.6), the system identified an inversion of probes B and C present in Chinese quail only as well as the well described centric fission in turkey to form 2 chromosomes (Figure 4.5, Figure 4.6)

<table>
<thead>
<tr>
<th>Experiment ID</th>
<th>Start Position</th>
<th>End position</th>
<th>BAC ID (CH261-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A)</td>
<td>5,936,192</td>
<td>6,131,770</td>
<td>169N6</td>
</tr>
<tr>
<td>(B)</td>
<td>29,361,223</td>
<td>29,588,465</td>
<td>186J5</td>
</tr>
<tr>
<td>(C)</td>
<td>43,177,303</td>
<td>43,412,434</td>
<td>50C15</td>
</tr>
<tr>
<td>(D)</td>
<td>103,580,339</td>
<td>103,809,599</td>
<td>169E4</td>
</tr>
<tr>
<td>(E)</td>
<td>128,029,292</td>
<td>128,240,032</td>
<td>44H14</td>
</tr>
<tr>
<td>(F)</td>
<td>143,308,662</td>
<td>143,489,517</td>
<td>17J16</td>
</tr>
</tbody>
</table>

Table 4.2. Probes used for metaphase chromosome analysis for Galliform chromosome 2

G.gallus BACs from the ‘Chori’ library, selected based upon position upon chromosome two of G.gallus, Probe ID with colour corresponds to the appropriate graph and false colour applied to signals seen in images. Start position and end position display the base number on which the BAC spans along the chromosome (approximately 200,000 bp); and BAC ID is the associated identifier associated with the sequence used as a probe. Six probes in total, A to F.
Figure 4.5. Merged metaphase images of five galliformes using selected *G.gallus* chromosome 2 BACs

15 individual metaphases, 3 per species shown, displayed with hybridised *G.gallus* chromosome 2 probes outlined in table 4.2 with corresponding false colouration of probe signal seen. *G.gallus* (first row) is shown as a control to display expected position on that species with possible variances seen in other species, with descending rows being differing species. Images were merged according to the protocol outlined in section 2.2.4.1. Scale bar, 10μM, all images are to the same scale.
Figure 4.6. Probe point plot for galliform chromosome 2 for five species, and corresponding chromosome schematic

A) Probe position measured as FLpter values, obtained using probes outlined in table 4.2. Each species has a corresponding colour. Error bars are given as S.E.M.

A clear inversion can be seen for probe B (purple) and C (orange) for *E.chinensis* (green line) as compared to the other species.

B) Generated schematic chromosomes for each species by using metaphase FLpter data generated in this study, all relative to *G.gallus* chromosome 2. Position of coloured band corresponds directly to FLpter scores generated and colour corresponding to assigned probe. Labels on ‘Turkey’ represent corresponding chromosomes assignment in that species.
4.2.2.3 Chromosome 3

For chromosome 3 (Table 4.3, Table 4.6), the system identified no rearrangements in all 5 species (Figure 4.7, Figure 4.8)

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Start Position</th>
<th>End position</th>
<th>BAC ID (CH261-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A)</td>
<td>106,508</td>
<td>289,139</td>
<td>115J5</td>
</tr>
<tr>
<td>(B)</td>
<td>25,940,915</td>
<td>26,171,148</td>
<td>160I6</td>
</tr>
<tr>
<td>(C)</td>
<td>40,872,930</td>
<td>41,111,215</td>
<td>97P20</td>
</tr>
<tr>
<td>(D)</td>
<td>65,843,346</td>
<td>66,066,979</td>
<td>17B14</td>
</tr>
<tr>
<td>(E)</td>
<td>95,661,126</td>
<td>95,801,606</td>
<td>169K18</td>
</tr>
<tr>
<td>(F)</td>
<td>108,067,425</td>
<td>108,248,097</td>
<td>120H23</td>
</tr>
</tbody>
</table>

Table 4.3. Probes used for metaphase chromosome analysis for Galliform chromosome 3

G.gallus BACs from the ‘Chori’ library, selected based upon position upon chromosome three of G.gallus, Probe ID with colour corresponds to the appropriate graph and false colour applied to signals seen in images. Start position and end position display the base number on which the BAC spans along the chromosome (approximately 200,000 bp); and BAC ID is the associated identifier associated with the sequence used as a probe. Six probes in total, A to F.
Figure 4.7. Merged metaphase images of five galliformes using selected *G.gallus* chromosome 3 BACs

15 individual metaphases, 3 per species shown, displayed with hybridised *G.gallus* chromosome 3 probes outlined in table 4.3 with corresponding false colouration of probe signal seen. *G.gallus* (first row) is shown as a control to display expected position on that species with possible variances seen in other species, with descending rows being differing species. Images were merged according to the protocol outlined in section 2.2.4.1. Scale bar, 10uM, all images are to the same scale.
Figure 4.8. Probe point plot for galliform chromosome 3 for five species, and corresponding chromosome schematic

A)- Probe position measured as FLpter values and averaged across all metaphases obtained using probes outlined in table 4.3. Each individual species has a corresponding colour seen, and colours assigned to each probe correspond to false colours applied to related metaphase images. Error bars are given as S.E.M.

B)- Generated schematic chromosomes for each species by using metaphase FLpter data generated in this study, all relative to G. gallus chromosome 3. Position of coloured band corresponds directly to FLpter scores generated and colour corresponding to assigned probe.
4.2.2.4 Chromosome 4

For chromosome 4 (Table 4.4, Table 4.6), interchromosomal rearrangements were identified with probes A and B appearing on a different chromosome to the others in chicken and the two quails (Figure 4.9, Figure 4.10). This is the well described fusion that appears convergent during avian evolution. The relatively lower position of these two probes in Japanese quail suggests a further rearrangement although the nature of this cannot be determined using this system. Intrachromosomally, the probe order remains unchanged in all five species.

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Start Position</th>
<th>End position</th>
<th>BAC ID (CH261-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A)</td>
<td>4,024,168</td>
<td>4,195,906</td>
<td>83E1</td>
</tr>
<tr>
<td>(B)</td>
<td>15,369,304</td>
<td>15,591,613</td>
<td>111A15</td>
</tr>
<tr>
<td>(C)</td>
<td>30,671,456</td>
<td>30,924,202</td>
<td>18C6</td>
</tr>
<tr>
<td>(D)</td>
<td>43,256,005</td>
<td>43,521,139</td>
<td>185L11</td>
</tr>
<tr>
<td>(E)</td>
<td>65,099,056</td>
<td>65,344,197</td>
<td>85H10</td>
</tr>
<tr>
<td>(F)</td>
<td>74,051,622</td>
<td>74,193,313</td>
<td>89P6</td>
</tr>
</tbody>
</table>

Table 4.4. Probes used for metaphase chromosome analysis for Galliform chromosome 4

\( G.gallus \) BACs from the ‘Chori’ library, selected based upon position upon chromosome four of \( G.gallus \), Probe ID with colour corresponds to the appropriate graph and false colour applied to signals seen in images. Start position and end position display the base number on which the BAC spans along the chromosome (approximately 200,000 bp); and BAC ID is the associated identifier associated with the sequence used as a probe. Six probes in total, A to F.
Figure 4.9. Merged metaphase images of five galliforms using selected *G.gallus* chromosome 4 BACs

15 individual metaphases, 3 per species shown, displayed with hybridised *G.gallus* chromosome 4 probes outlined in table 4.4 with corresponding false colouration of probe signal seen. *G.gallus* (first row) is shown as a control to display expected position on that species with possible variances seen in other species, with descending rows being differing species. Images were merged according to the protocol outlined in section 2.2.4.1. Scale bar, 10μM, all images are to the same scale.
Figure 4.10. Probe point plot for galliform chromosome 4 for five species, and corresponding chromosome schematic

A) Probe position measured as FLpter values and averaged across all metaphases obtained using probes outlined in table 4.4. Each individual species has a corresponding colour seen, and colours assigned to each probe correspond to false colours applied to related metaphase images. Error bars are given as S.E.M.

B) Generated schematic chromosomes for each species by using metaphase FLpter data generated in this study, all relative to \textit{G.gallus} chromosome 4. Position of coloured band corresponds directly to FLpter scores generated and colour corresponding to assigned probe. Labels correspond to equivalent assigned chromosomes in respective species.
4.2.2.5 Chromosome 5

Chromosome 5 (Table 4.5, Table 4.6) appears to be near identical across all species examined (Figure 4.11, Figure 4.12)

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Start Position</th>
<th>End position</th>
<th>BAC ID (CH261-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A)</td>
<td>5,387,924</td>
<td>5,581,233</td>
<td>49B22</td>
</tr>
<tr>
<td>(B)</td>
<td>10,219,524</td>
<td>10,389,786</td>
<td>122F8</td>
</tr>
<tr>
<td>(C)</td>
<td>25,301,609</td>
<td>25,488,411</td>
<td>78F13</td>
</tr>
<tr>
<td>(D)</td>
<td>26,734,106</td>
<td>26,902,004</td>
<td>2I23</td>
</tr>
<tr>
<td>(E)</td>
<td>57,382,304</td>
<td>57,541,027</td>
<td>161B22</td>
</tr>
</tbody>
</table>

Table 4.5. Probes used for metaphase chromosome analysis for Galliform chromosome 5

_G.gallus_ BACs from the ‘Chori’ library, selected based upon position upon chromosome five of _G.gallus_, Probe ID with colour corresponds to the appropriate graph and false colour applied to signals seen in images. Start position and end position display the base number on which the BAC spans along the chromosome (approximately 200,000 bp); and BAC ID is the associated identifier associated with the sequence used as a probe. Five probes in total, A to E.
Figure 4.11. Merged metaphase images of five galliformes using selected *G.gallus* chromosome 5 BACs

15 individual metaphases, 3 per species shown, displayed with hybridised *G.gallus* chromosome 5 probes outlined in table 4.5 with corresponding false colouration of probe signal seen. *G.gallus* (first row) is shown as a control to display expected position on that species with possible variances seen in other species, with descending rows being differing species. Images were merged according to the protocol outlined in section 2.2.4.1. Scale bar, 10μM, all images are to the same scale.
Figure 4.12. Probe point plot for galliform chromosome 5 for five species, and corresponding chromosome schematic

A)- Probe position measured as FLpter values and averaged across all metaphases obtained using probes outlined in table 4.5. Each individual species has a corresponding colour seen, and colours assigned to each probe correspond to false colours applied to related metaphase images. Error bars are given as S.E.M.

B)- Generated schematic chromosomes for each species by using metaphase FLpter data generated in this study, all relative to *G. gallus* chromosome 5. Position of coloured band corresponds directly to FLpter scores generated and colour corresponding to assigned probe.
### 4.2.2.6 FLpter results

#### Chromosome 1

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Chicken</th>
<th>Turkey</th>
<th>Chinese Quail</th>
<th>Japanese Quail</th>
<th>Guinea Fowl</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(A)</strong></td>
<td>0.03576±0.0040</td>
<td>0.04114±0.0040</td>
<td>0.03889±0.0068</td>
<td>0.04094±0.0038</td>
<td>0.03367±0.0035</td>
<td>All similar to chicken</td>
</tr>
<tr>
<td><strong>(B)</strong></td>
<td>0.32422±0.0069</td>
<td>0.3401±0.0086</td>
<td>0.29169±0.0188</td>
<td>0.40515±0.0075</td>
<td>0.33968±0.0086</td>
<td>Small increase in J.quail. Rest similar</td>
</tr>
<tr>
<td><strong>(C)</strong></td>
<td>0.54114±0.0062</td>
<td>0.53495±0.0056</td>
<td>0.52683±0.0146</td>
<td>0.54982±0.0058</td>
<td>0.54540±0.0111</td>
<td>All similar to chicken</td>
</tr>
<tr>
<td><strong>(D)</strong></td>
<td>0.69616±0.0054</td>
<td>0.68255±0.0086</td>
<td>0.69542±0.0087</td>
<td>0.71659±0.0045</td>
<td>0.70557±0.0133</td>
<td>All similar to chicken</td>
</tr>
<tr>
<td><strong>(E)</strong></td>
<td>0.79122±0.0050</td>
<td>0.76985±0.0102</td>
<td>0.78807±0.01015</td>
<td>0.79669±0.0048</td>
<td>0.79168±0.0182</td>
<td>All similar to chicken</td>
</tr>
<tr>
<td><strong>(F)</strong></td>
<td>0.97149±0.0036</td>
<td>0.95458±0.0063</td>
<td>0.95538±0.00885</td>
<td>0.97107±0.0031</td>
<td>0.96386±0.0051</td>
<td>All similar to chicken</td>
</tr>
</tbody>
</table>

#### Chromosome 2

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Chicken</th>
<th>Turkey (merged)</th>
<th>Chinese Quail</th>
<th>Japanese Quail</th>
<th>Guinea Fowl</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(A)</strong></td>
<td>0.071217±0.0045</td>
<td>0.074961±0.0037</td>
<td>0.062752±0.0027</td>
<td>0.064314±0.0039</td>
<td>0.051563±0.0034</td>
<td>All similar to chicken</td>
</tr>
<tr>
<td><strong>(B)</strong></td>
<td>0.217778±0.0063</td>
<td>0.237357±0.0329</td>
<td>0.263951±0.009</td>
<td>0.208003±0.0092</td>
<td>0.171193±0.0173</td>
<td>Reduction in G.fowl Increase in C.Quail</td>
</tr>
<tr>
<td><strong>(C)</strong></td>
<td>0.302730±0.0190</td>
<td>0.297389±0.0080</td>
<td>0.159543±0.0088</td>
<td>0.301784±0.0102</td>
<td>0.247032±0.0272</td>
<td>Reduction in G.fowl, Large reduction in C.Quail, inversion in C.quail</td>
</tr>
<tr>
<td><strong>(D)</strong></td>
<td>0.71019±0.0098</td>
<td>0.698639±0.0128</td>
<td>0.691089±0.0139</td>
<td>0.713300±0.0072</td>
<td>0.689708±0.012</td>
<td>All similar to chicken</td>
</tr>
<tr>
<td><strong>(E)</strong></td>
<td>0.840772±0.0050</td>
<td>0.840519±0.0073</td>
<td>0.850802±0.0091</td>
<td>0.848246±0.0055</td>
<td>0.854927±0.0107</td>
<td>All similar to chicken</td>
</tr>
<tr>
<td><strong>(F)</strong></td>
<td>0.933419±0.0063</td>
<td>0.948405±0.0031</td>
<td>0.945349±0.0050</td>
<td>0.95080±0.0035</td>
<td>0.945320±0.0082</td>
<td>All similar to chicken</td>
</tr>
</tbody>
</table>

#### Chromosome 3

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Chicken</th>
<th>Turkey</th>
<th>Chinese Quail</th>
<th>Japanese Quail</th>
<th>Guinea Fowl</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(A)</strong></td>
<td>0.077477±0.0043</td>
<td>0.096464±0.0037</td>
<td>0.090693±0.0059</td>
<td>0.100627±0.0073</td>
<td>0.078408±0.0044</td>
<td>All similar to chicken</td>
</tr>
<tr>
<td><strong>(B)</strong></td>
<td>0.292917±0.0063</td>
<td>0.345963±0.0212</td>
<td>0.236132±0.009</td>
<td>0.262916±0.0091</td>
<td>0.259185±0.0126</td>
<td>Turkey has small increase</td>
</tr>
<tr>
<td><strong>(C)</strong></td>
<td>0.430784±0.0086</td>
<td>0.430989±0.0111</td>
<td>0.374026±0.0174</td>
<td>0.414425±0.0142</td>
<td>0.408507±0.0115</td>
<td>All similar to chicken</td>
</tr>
<tr>
<td><strong>(D)</strong></td>
<td>0.624298±0.0057</td>
<td>0.619202±0.0133</td>
<td>0.583256±0.0204</td>
<td>0.594358±0.0122</td>
<td>0.611436±0.0102</td>
<td>All similar to chicken</td>
</tr>
<tr>
<td><strong>(E)</strong></td>
<td>0.848472±0.0091</td>
<td>0.810436±0.0208</td>
<td>0.812473±0.0222</td>
<td>0.834502±0.0121</td>
<td>0.853728±0.0103</td>
<td>All similar to chicken</td>
</tr>
<tr>
<td><strong>(F)</strong></td>
<td>0.946176±0.0031</td>
<td>0.917545±0.0114</td>
<td>0.940903±0.0041</td>
<td>0.927622±0.0055</td>
<td>0.942590±0.0056</td>
<td>All similar to chicken</td>
</tr>
</tbody>
</table>
### Chromosome 4

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Chicken (merged)</th>
<th>Turkey (merged)</th>
<th>Chinese Quail</th>
<th>Japanese Quail</th>
<th>Guinea Fowl (merged)</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A)</td>
<td>0.09128±0.0041</td>
<td>0.079414±0.0071</td>
<td>0.099145±0.0052</td>
<td>0.194463±0.0084</td>
<td>0.080248±0.0073</td>
<td>Increase in J.Quail</td>
</tr>
<tr>
<td>(B)</td>
<td>0.204958±0.0094</td>
<td>0.20763±0.0104</td>
<td>0.21712±0.0097</td>
<td>0.313284±0.0087</td>
<td>0.202805±0.0149</td>
<td>Increase in J.Quail</td>
</tr>
<tr>
<td>(C)</td>
<td>0.409365±0.0099</td>
<td>0.422356±0.0312</td>
<td>0.340093±0.0139</td>
<td>0.422849±0.009</td>
<td>0.499626±0.0404</td>
<td>Decrease in C.Quail Increase in G.fowl</td>
</tr>
<tr>
<td>(D)</td>
<td>0.527074±0.0100</td>
<td>0.56061±0.0289</td>
<td>0.453212±0.0164</td>
<td>0.541592±0.0085</td>
<td>0.643777±0.0305</td>
<td>Decrease in C.Quail Increase in G.fowl</td>
</tr>
<tr>
<td>(E)</td>
<td>0.682414±0.0092</td>
<td>0.687141±0.0264</td>
<td>0.610872±0.01423</td>
<td>0.703868±0.0070</td>
<td>0.777363±0.0150</td>
<td>Decrease in C.Quail Increase in G.fowl</td>
</tr>
<tr>
<td>(F)</td>
<td>0.803423±0.0073</td>
<td>0.798217±0.0202</td>
<td>0.761814±0.0099</td>
<td>0.810826±0.0054</td>
<td>0.868288±0.0068</td>
<td>Decrease in C.Quail Increase in G.fowl</td>
</tr>
</tbody>
</table>

### Chromosome 5

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Chicken (merged)</th>
<th>Turkey (merged)</th>
<th>Chinese Quail</th>
<th>Japanese Quail</th>
<th>Guinea Fowl (merged)</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A)</td>
<td>0.109038±0.0043</td>
<td>0.129676±0.0257</td>
<td>0.122423±0.0060</td>
<td>0.117247±0.0068</td>
<td>0.102716±0.0080</td>
<td>All similar to chicken</td>
</tr>
<tr>
<td>(B)</td>
<td>0.238225±0.0073</td>
<td>0.241229±0.0126</td>
<td>0.273694±0.0106</td>
<td>0.25774±0.0115</td>
<td>0.222562±0.0153</td>
<td>All similar to chicken</td>
</tr>
<tr>
<td>(C)</td>
<td>0.397523±0.0085</td>
<td>0.431651±0.0120</td>
<td>0.410412±0.0121</td>
<td>0.422116±0.0129</td>
<td>0.415747±0.0176</td>
<td>All similar to chicken</td>
</tr>
<tr>
<td>(D)</td>
<td>0.506113±0.0087</td>
<td>0.543204±0.0155</td>
<td>0.506192±0.0139</td>
<td>0.522311±0.0126</td>
<td>0.506414±0.0159</td>
<td>All similar to chicken</td>
</tr>
<tr>
<td>(E)</td>
<td>0.8984±0.0052</td>
<td>0.886032±0.0082</td>
<td>0.860873±0.0160</td>
<td>0.901113±0.0053</td>
<td>0.903014±0.0100</td>
<td>All similar to chicken</td>
</tr>
</tbody>
</table>

**Table 4.6. All tables for every chromosome with probe’s FLpter score**

All tables shown correlate to probes used outlined in the previous section, each cell contains FLpter score for the probe on respective species with standard error of the mean displayed alongside. In the case of chromosome fissions (Turkey 2 and 4, Guinea fowl 4) merged scores have been displayed as if the chromosomes are ‘stacked’ as if they are fused to align to the reference of G.gallus.
4.3 Development of a Chromosome Interphase Plotter for chromosomal nuclear arrangement

Previous work on paints gives general region/territory within the nuclei yet no means of direction or specific region identification. However there is a need to improve on this to encapsulate the chromosome territory indication direction and multiple bands on the chromosome in de-condensed state via an ‘automatic’ measuring system.

Using the multi-layered approach, it is possible to detect, give direction and order to chromosomes within the nuclei more specifically as compared with previous exploration using whole chromosome paints (Griffin et al., 1999). It is then possible to ask multiple questions, such as is there a specific direction/orientation of the chromosome in question, or do the chromosomes express a linear direction or are they overlapping themselves? From this we can ask, what happens when things go wrong within the cell, for example partial chromosomal translocations to affect fertility or mass chromosomal deformity in the event of a cancerous cell.

As shown, it is currently possible to plot BACs along the chromosome within a metaphase preparation, to give direction, orientation and distance, and this can easily be applied to work within interphase nuclei as well.

4.3.1 Interphase specific Multilayer protocol

FISH protocol was identical to previous section outlined in section 4.2.2, using identical probes with respective ordering and false colouration. Final merged images were imported into Microsoft Design4 (Microsoft) in original state with no resizing staying true to original pixel state as captured during microscopy. Here imposed coloured circles were placed centrally over signals seen in each interphase cell and bounding nuclear drawn over the perimeter seen. As seen in figure 4.13, not all interphase cells are taken into this stage as they do not exhibit the full count of signals required, in the case of chromosome 3 shown here, requires 2 of each individual 6 probes up to a total of 12.

From this importing into ImageJ with no re-sizing to then calculate the exact position of each probe as exact X Y co-ordinates. Other data can be retrieved such as centre of mass, a built-in function contained within ImageJ, and then diameter of the nuclei, taken in multiples across the nuclei going through the centre of mass.
After the chromosome path finding tool (explained shortly) had been run, black arrows, again seen in figure 4.13, were inserted within Microsoft Design4 to show final chromosome orientation and direction between probes used for each chromosome for user visualisation. Post path finding, analysis was carried out on a selected population of chromosomes/interphase nuclei. From n>50 images each containing from 2~5≤ individual nuclei approximately 100≤ nuclei (therefore 200≤ individual chromosomes) were brought forward to path finding stage. From this medial length was calculated and inter-quartile ranges used as a corrected range of normal nuclei as to account for those nuclei which were of incorrect orientation of plain of view, which would yield a dramatically adverse total length and morphology. Chromosomes studied that had at least n=50 at this point were taken onto further analysis as shown below.

![Figure 4.13. Multilayer G.gallus chromosome 3 interphase nuclei with schematic representation of chromosome folding pattern. A) final six layer merged image of typical G.gallus interphase stage nuclei using aforementioned probes in table 4.3 in corresponding colours. B) schematic representation of signals and nuclear boundary as observed from the final six layer merged image, only nuclei are brought to this stage if they exhibit all required signals (12, 6x2 chromosomes) C) schematic representation of the nuclear position and direction of G.gallus chromosome three in this example after being processed through the nuclear predictive path-finding tool.](image)
4.3.2 Mechanics of the Path-Finding Tool

There was a requirement of a means to automatically predict the correct path of two chromosomes using all probe signals seen in the interphase nuclei, as manual human prediction gave conflicting and debatable results for chromosomes that were localised near to one another. Here shown are the underlying mathematical principles used to calculate and assign probes to their correct chromosome and therefore path.

In order to calculate the total number of chromosome pairs that will needed to be calculated (where N to be number of chromosomes, and R to be number of probes along a single chromosome) firstly the total number of permutations the two chromosomes might have using all probes needs to be calculated, to then be divided by number of chromosomes to return the number of chromosome pairs required.

\[
\frac{n^r}{n}
\]

The total length of a chromosome can be represented as the distance from each point to the next, starting from the known initial point represented as ‘A’ up to a final point, i.e. ‘E’ if the experiment uses 5 probes.

\[
\overline{TOTAL} = \overline{AB} + \overline{BC} + \overline{CD} + \overline{DE}
\]

As data exists as positional (X,Y) co-ordinates, the distance between the first point along the chromosome ‘A’ and the second point ‘B’ can be found using this formula; to be repeated for each point pairing.

\[
\overline{AB} = \sqrt{(X_B - X_A)^2 + (Y_B - Y_A)^2}
\]

However, with each nucleus possessing two chromosomes the total distance needs to be calculated twice, using unique probes and their co-ordinates.

\[
\overline{TOTAL. ChrA} = \overline{A_aB_a} + \overline{B_aC_a} + \overline{C_aD_a} + \overline{D_aE_a}
\]

\[
\overline{TOTAL. ChrB} = \overline{A_bB_b} + \overline{B_bC_b} + \overline{C_bD_b} + \overline{D_bE_b}
\]

\[
\overline{TOTAL Combined} = \overline{TOTAL. ChrA} + \overline{TOTAL. ChrB}
\]
This ‘total combined’ must be calculated multiple times according to the total permutation number first generated, each using a different combination of probes as chromosomes cannot share signals. Figure 4.14 explains this graphically.

Finally, each ‘total combined’ shall be sorted in order of size and the shortest combination selected as the true path of the two chromosomes presented within the nucleus, to best represent the path of ‘least resistance’ with two chromosomes of similar size.

Currently, the path-finding tool exists as sheets within Microsoft excel (Microsoft), see figure 4.15, where the X Y co-ordinates retrieved from ImageJ can be inserted into respective tables and each permutation can be calculated and compared to one another to then output two text strings of the correct ordering of probes for the two respective chromosomes.
The path-finding tool is currently set up to accept chromosomes with probe count =5 (32 permutations 16 pairs), =6 (64 permutations and 32 pairs) and although not used =8 (256 permutations and 128 pairs).

Figure 4.15. Excel screenshot of working example of the interphase chromosome path-plotting tool using a G. gallus chromosome 3.

Here shown, a working example of previously discussed theory of chromosome path-plotting in an interphase nuclei with six probes. With six probes used a total of 64 permutations (32 pairs) are generated and calculated from inputted co-ordinates (ImageJ) and a text string of the correct outcome is displayed, here path 'aaaaaa' and 'bbbbbb' is correct.
4.3.3 Interphase chromosome analysis

Once interphase chromosomes were correctly associated and given direction (Figure 4.16), it was then possible to perform analysis to detect any trends in patterns of nuclear organisation. Using mathematical and trigonometric means, a high amount of data can be retrieved from a rather simplistic methodology from probe positions and little data from the interphase nuclei itself, such as diameter and centre of mass; this is demonstrated in figure 4.17.

<table>
<thead>
<tr>
<th>Post-merged</th>
<th>Intermediate schematic</th>
<th>Final path chromosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken Chr 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chicken Chr 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chicken Chr 5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.16. Multiple multilayer G.gallus interphase nuclei with schematic representation of chromosome folding pattern.

From top to bottom displays a different chromosome (3, 4 and 5) on G.gallus using aforementioned probes in previous sections. From left to right, separations show a representation of each different stage in the path finding process. Initially displaying the initial merged image to a schematic representative then finals a plotted chromosome schematic drawing. Scale bar, 10uM, all images are to the same scale.
Whilst data generated is fruitful, patterns and trends relating to nuclear organisation remain unclear even using high chromosome count for analysis ($n \geq 50$), few trends could be concluded. One clear conclusion is that these macrochromosomes generally occupy the outer periphery of the nuclei and stay there, consistent with previous reports (Haberman et al 2001). Somewhat interestingly, chromosomes examined do show a trend in their folding pattern, when chromosome angle is corrected for, which is shown in the next section in more detail.

Figure 4.17. Overview of final schematic interphase nuclei with overlay of data marks.

A fictitious representative example of a single chromosome within an interphase nucleus, indicated by five probes (A to E), with regions that data can be obtained from. Distance from each probe to the next can be obtained by measuring the distance between points (black), Centre of mass can be obtained (yellow), and diameter (black dotted) to yield area/perimeter. Probe distance from centre/periphery for territories can be measured again by distance of line (light green dotted). Chromosome angles between probes and angles around centre of mass can also be obtained (orange).
4.3.4 Intricacies of macrochromosome folding.

4.3.4.1 Normalisation of chromosomes

In order to process the data generated from fully ‘pathed’ interphase chromosomes, it was necessary to normalise the chromosome orientation to the same plane/direction rather than being determined by random assortment of where they were on the slide when captured, demonstrated in figure 4.16 and figure 4.18. Each chromosome undergoes a transformation and rotation, to have identical start positions and identical ‘direction’ for first to second probe, then to be ‘flipped’ so that each chromosome path starts ‘forward’ along the +X axis and flipped again if required if the third probe resides along the -Y axis so that all chromosomes fold ‘left’. These new transformed normalised co-ordinates can now be processed for further analysis (Figure 4.19).

Figure 4.18. Graphic example of how to normalise chromosome data for analysis

A possible chromosome configuration with known order of probes (=5) has unique X,Y co-ordinates according to data retrieved from ImageJ. (1) Black, is the initial chromosome before translation from original position to new position anchored by first point at 0,0. (2) Blue, translated chromosome in same orientation with the first point anchored at 0,0 with angle from second point(red circle) to X axis expressed as Θ (theta). (3) Green, rotated chromosome about point 0,0 (first probe point) for every probe point according to previously discovered Θ to give a final normalised chromosome. (4) Not shown for clarity sake, the chromosome may be flipped if its second point (red circle) has ‘-x’ coordinate so all second points are along a 1D plane. (5) Again not shown, the chromosome can be flipped again if the third point (purple) has ‘-y’ so that all chromosomes fold ‘left’.
4.3.4.2 Folding arrangements of G. gallus chromosome 3, 4 and 5

After normalisation, the most common folding pattern of each chromosome can be identified, at least n≥50 individual chromosomes were used and plotted on graphs using averaged X,Y co-ordinates across the selected population of chromosomes.

**Figure 4.19. G. gallus chromosome 3 interphase common folding pattern**
Averaged positions of chromosome probes (table 4.3, 6 probes) for chicken chromosome 3 within the nucleus normalised according to figure 4.18. Data points shown as mean ±S.E.M.

**Figure 4.20. G. gallus chromosome 4 interphase common folding pattern**
Averaged positions of chromosome probes (table 4.4, 6 probes) for chicken chromosome 4 within the nucleus normalised according to figure 4.18. Data points shown as mean ±S.E.M.
With these selections of chromosomes shown, it appears that interphase stage nuclei arrange/fold their chromosomes in particular ways, albeit with a large boundary of variation (Figure 4.19, Figure 4.20, and Figure 4.21). With a degree of a defined folding pattern, it is interesting to spot possible ‘shapes’ or motifs within each individual chromosome studied.

Both chromosome 3 and chromosome 5 display a ‘horseshoe’ folding pattern with little degree of ‘overlapping’ itself. This resolves in the final probe, the most distal of the q arm, on average being a close position as compared to the first probe used here as the anchor at 0,0 (Figure 4.18, 4.20). Oppositely so, chromosome four does not show as a distinctive pattern to fit the ‘horseshoe’ folding motif in respect to closely plotting final probe, however it does fit into the narrative consistent with other chromosomes where they all fold ‘backwards’ towards the initial on average.

More analysis has been made to give motions towards some degree of higher organisation yet unfortunately lacks the precision to make indications of the respective de-condensed chromosome interphase structure.

Figure 4.21. *G. gallus* chromosome 5 interphase common folding pattern
Averaged positions of chromosome probes (table 4.5, 6 probes) for chicken chromosome 5 within the nucleus normalised according to figure 4.18. Data points shown as mean ±S.E.M.
4.4 Discussion

The multilayer FISH protocol has here been shown to perform concurrent high-quality FISH experiments for questioning of cytogenetic evolutionary theories. It is possible to use a selection of BAC based probes to analyse and quantify changes and properties of set macro-chromosomes within a metaphase stage cell and an interphase stage cell. Use of *G. gallus* and related Galliform species as models allows robust scrutiny into the methodology used as they are reliable well-researched samples opposed to possibly more interesting branches of avian life such as previously mentioned Psittaciformes or Falconiformes.

4.4.1 Comparison of measuring techniques

The method of choice to quantify final merged image data derives from the standardised form of FLpter scores (Lichter et al., 1990), the data generated by the FLpter script is reliable in other standard FISH but is shown here to introduce a wider variation in scores obtained. A somewhat downside to multilayer FISH, this introduces a larger analysis time as compared to previous standard methodology to generate the FLpter scores used for BAC positioning.

The issues identified could be attributed to the variation in the metaphase/DAPI layers of the images post first hybridisation arising to a small degree of altered metaphase morphology as discussed in the previous chapter, yielding incorrect scores. Whilst long and repetitive, manual scoring of the probe positioning is shown here to be accurate with an increase of probe count does not cause a dramatic increase in time taken to measure, i.e. 5 probes is 7 mouse clicks opposed to 6 probes being 8 mouse clicks.

4.4.2 Detection of chromosomal rearrangements in Galliform

Results here show that there are oversights, not mistakes, in Galliform macrochromosomes studies. Progressing on from an older paint methodology to a newer more precise means of BAC based probes, allowed a more in detail analysis of these macrochromosomes with predicted intrachromosomal rearrangements. It is easy to see in context of the avian ancestral chromosome 2 (Romanov et al., 2014; Griffin et al., 2007) that this multilayered FISH methodology yields complimentary results when presented in this way, it is immediately obvious to see that turkey’s ancestral chromosome has undergone a ‘clean fission’ to give rise to its chromosome 3 and 6.
Other large possible rearrangements can be identified by simply looking at the colour order of probes used, such as the single example found in this study being the rearrangement within chromosome 2 of Chinese quail, by the reversal of probes D, CH261-50C15 and, CH261-186J5. This clear rearrangement is not shared with any other member of the galliform group tested, which is interesting in context of both Japanese and Chinese quail showing that there are further detailed rearrangements within the old world quails, possibly adding evidence into the reclassification of Chinese quail into the *Excalfactoria* genus away from the general grouping of *Coturnix* (Crowe et al., 2006, Christidis & Boles, 2008)

This method also shows smaller changes across the chromosomes tested, such as across chromosome 4, Japanese quail expresses an increase of FLPpter scoring for the first two probes used, a possible shift of DNA around the P-arm of this chromosome, yet returns to match up with the reference *G.gallus*. Again, Chinese quail seemingly differs from its closest relation in this study as this quail instead has identical first two probe positions as chicken, yet the later four signals score much lower FLPter values possibly indicating a shift of material to the Q terminus of the chromosome as the ratio between signals is consistent across species.

Results demonstrated here show that there is much more going on within the macrochromosomes of the Galliformes and should not be neglected for study in favour of the ‘more interesting’ microchromosomes.

It is plausible to relate this methodology of chromosome positioning in the nucleus to chromosome conformation capture technologies such as Hi-C. These capturing technologies quantify the interactions between elements of chromatin that are nearby in the 3-D space of the nuclei yet may be a vast distances apart on the actual linear genome (Dostie and Bickmore, 2012). Hi-C can be used to detect balanced and unbalanced chromosome rearrangements, copy number variants and does not require the expense of whole genome sequencing to detect these rearrangements at the whole chromosome level arguably more useful than at a breakpoint level (Harewood, 2017). It would not be correct to suggest that either method should replace the other. Hi-C methodologies are typically verified and reinforced by FISH studies (Harewood, 2017). Hi-C methods require extensive library preparation to perform along with the computational knowledge required to perform and interpret. With the now reduction of cost and increased through put of FISH via multilayer FISH we could suggest that the 3D arrangement of a chromosome of interest
could be determined by both FISH and Hi-C methodologies rather than FISH used for verification. This would allow costs to be saved for an already established cytogenetics lab and allow them to get a foothold into the realm of nuclear 3D mapping and cellular specialisations.

4.4.3 The Chromosome Interphase Plotter

Here is a demonstration that is possible to adapt the multilayer FISH methodology to give an insight towards the structure and positioning of chromosomes in their de-condensed interphase state. As shown, it is now possible to use this methodology and attached post-merging analysis to correctly path chromosomes within an interphase nucleus to then yield vast amounts of information about the condition of said chromosomes in its interphase state.

4.4.3.1 Mechanics of the Path-Finding Tool

The computational analysis of the path finding tool is currently in the form of vast excel sheets and formulas which is currently limited to 5 to 8 probes, and only two chromosomes. The mathematical formulas used seemingly provide an accurate means to calculate and therefore represent the two chromosomes within the nuclei using all probe signals after being given co-ordinate details via the user which has been retrieved from ImageJ. A lengthy task indeed, considering each nucleus has approximately 10/12 probe signals representing two chromosomes, and nucleus count can exceed the 100’s, a possible justification towards an automated means of performing this task. To apply this further for other questions, in the event of aneuploidy, or to be more precise polysomy, an increase in the ploidy of the sample would cause the underlying mathematics to change, currently not possible in this version of the path finding tool. As an example, a ploidy of 3n for the chromosome of examination would increase the number of signals from 12 to 18 using a six probe series, using the initial permutation equation \(n!\) we would have to compute 729 individual permutations, to be separated into 243 trios. To lower this task with fewer probes would reduce accuracy and information given to the user so it would become essential that an automated process to path chromosomes be created.
4.4.3.2 Folding arrangements of chromosomes

Here it is demonstrated that there is an observable pattern of chicken macro-chromosome arrangement of which this level is not yet reported. Data aligns with the proposal that macro-chromosomes exhibit a peripheral territory bias (Haberman, 2001) yet here this method reinforces that by indicating that the whole chromosome stays in the external region yet some terminus located probes tend to either occupy a more central or more peripheral location as compared to the rest of their chromosome indicating a ‘bending’ or a horseshoe shape back towards the initial probe point, i.e. one telomere ‘curves’ backwards towards the telomere at the end of the opposing arm. Yet these findings require data to reinforce these trends, as currently there is only a poor means to calculate the direction of bending involved to determine if there is a tendency to bend inwards or outwards in respect to nuclear location. A methodology that requires more mathematical and possibly probability prediction means to see if there is any significant patterns of this observable trend. More hypothesises have been tested using data obtained in this study from nuclear positioning (hypothesises tested in table 4.7). However due to the lack of consistent data only weak assumptions can be made but nonetheless are possible avenues for future work.

4.4.3.3 The means to predict an unknown order

Although not tested explicitly, it is possible to give an interpretation on how this method may be applied to a sample with unknown metaphase/karyotype and merely rely on FISH signals shown within an interphase preparation. This changes the underlying mathematical principles where going from an ordered list of objects to an unordered list, the formula changes to;

\[ \text{Permutations} = r! \times 2^r = \text{No probes!} \times 2^{\text{No probes}} \]

From this equation, repeating above experiments would yield, for 5 probes 3840 permutations compared to 32 and for 6 probes 46,080 permutations as compared to 64 and up to 8 (being the current limit) is 10,321,920 permutations as compared to 256, all far beyond the capacity of a spreadsheet.

To work in reverse, it would be possible to compare signals seen to determine whether the chromosome in question is of same properties and therefore order to that of a reference, in this case chicken. This will negate the need to perform mathematical calculations as you would apply the known set order of chicken to the unknown sample.
species and statistically determine if the patterns are the same, i.e. Length of chromosome or possibly folding pattern, if there were dramatic changes we could presume the order of the unknown sample’s chromosome in question is different to that of the reference.

Regardless, this will require a substantial level of detail and further work beyond the scope that this thesis provides.

4.4.3.4 Limitations of Interphase studies

The first issue revolves around the fact that interphase nuclei are 3D structures, flattened down by the process of FISH. This requires many images to be taken to get a fair representation of the structure of the chromosome inside of the cell. Also there is the issue of the time of fixing of the cell, as all the DNA is in its de-condensed form, there will be variation between cells of transcriptional activity at the DNA level causing a possible shift of physical arrangement as areas are unwrapped/re-condensed as the cell requires to allow this mentioned transcriptional level of activity on the DNA for multiple cellular functions. These points direct towards variability within the nuclei, and where there is uncontrollable variability comes questionable conclusions that require changes to methodology to rectify. Understanding that this is a 2D study to represent a 3D space ultimately reduces the usefulness of the trends found within this study, yet acts as a good initial insight into further way to understand delicate highly organised chromatin structure. Again in reference to previous, Hi-C methods could be used along side FISH interphase studies to verify results.

4.5 Conclusion

The multilayer methodology has enabled the easy comparison of intrachromosomal rearrangements between multiple closely related species showing previously undiscovered rearrangements that previous studies have failed to find via other methodologies. Shown within the Galliform study, the stark similarity between member species of this order is represented within their conserved macrochromosomes. However with more specific fluorescent methodologies (the combination of the multilayer method paired with the probe point plots) shows that previously overlooked and somewhat ‘presumably conserved’ chromosomes are worthwhile to investigate. The selection of the Galliform order for this study was somewhat chosen as a ‘safe’ order for the multilayer methodology to be tested upon, whilst answering previously overlooked arrangements. However as the success of results shown here, there is plausibility that this approach be applied to other
species, avian or not, for other unexplored intrachromosomal rearrangements. As seen in the previous chapter, it is plausible that an increase of layers/probes could be applied to high quality prepared samples. This would allow more in-depth studies into specific chromosomes, or a panel of microchromosomes, for more precise re-arrangements to further our understanding of phylogenetic divergence as consequence of chromosomal changes.

The successful adaptation of the multilayer methodology to form an interphase chromosome point plotter for chromosomal nuclear arrangement has been an unexpected outcome of mlFISH. With creation of multiple extensive excel sheets it was possible to create a predictive means of assigning probes to chromosomes, then the easy analysis of said chromosomes within ImageJ/Excel to detect folding arrangements. Due to the nature of interphase mlFISH, there is an observed higher dropout of individual nuclei as compared to metaphases (personally found/presumed to be cytoplasmic connective remnants from the hypotonic isolation of genetic material linking nuclei together more so than metaphase chromosomes). However as there is an overabundance of interphase nuclei in samples as compared to metaphases this may not be too drastic of a drawback.

Recommendations for further work in light of results generated within this chapter are as follows. Currently all ‘software’ elements of interphase prediction exist within excel sheets as formulas with input from ImageJ. With a more experienced user of ImageJ or a proficient user of a computer language (I would recommend Java due to cross-capabilities with ImageJ and SmartCapture) the generation of a succinct script/plug-in would allow the ease of use for further studies (such as hypothesised in table 4.7).

With adaptation into a more computational automated system, more advanced algorithms can be generated that can improve current limitations, such as; increasing the number of signals upon a single chromosome, an increase in ploidy which would be useful for plant or cancer studies and the aforementioned prediction of an unknown gene order where many permutations will exist. Applying these principles towards research in a 3D nuclei opposed to a randomly positioned flattened 2D nuclei would extraordinarily increase the potential use of this methodology. With an extra dimension brings extra co-ordinates, and more mathematical functions. However this would allow a reduced number of interphase to be imaged due to orientation being less of an issue which typically eliminates many chromosomes for study. The multilayer methodology as outlined in the previous
chapter should be adaptable to a 3D system in terms of multiple layers, but this would require extensive testing and refinement as 3D imaging hardware is expensive.

<table>
<thead>
<tr>
<th>Hypothesis</th>
<th>Data used</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Does length of metaphase chromosome correlate to interphase length?</td>
<td>Distances from each individual probe point to the next – total length</td>
<td>Very weak correlation after correction for not true terminus locations i.e. interphase shows true chromosome end where ‘First’ probe point does not equal start of chromosome</td>
</tr>
<tr>
<td>Are the distances between each point in the same ratio?</td>
<td>Individual probe locations, therefore distances</td>
<td>Minimal trend, probes do tend to have similar distances between them, but requires more data such as angles to ratify</td>
</tr>
<tr>
<td>Is there a consistent intra-chromosomal angle seen between set probes?</td>
<td>Generation and measurement of angles around ‘mid’ probes, angle count = probe count - 2</td>
<td>Angles seemingly do not follow a pattern unless correlated with their length and orientation, to reflect solely on angles will generate inconsistent conclusions.</td>
</tr>
<tr>
<td>Are chromosomes in a set ‘angle’ within the nuclei?</td>
<td>Generation of angle from centre of mass to probe, using a false periphery point as reference to draw angle from.</td>
<td>With no way to pre-orientate nuclei, chromosomes seemingly occupy a random territory when testing this hypothesis.</td>
</tr>
<tr>
<td>Does nuclei size affect chromosome length?</td>
<td>Total chromosome length, with area/perimeter derived from diameter measurements</td>
<td>Nuclei tend to be a set range of sizes and chromosomes tend to be a set range of sizes, a very weak positive trend can be seen but with very low R² scores, 0.037 for chromosome 5</td>
</tr>
<tr>
<td>Does the chromosome occupy a peripheral territory?</td>
<td>Distance from centre of mass to each point along the chromosome. Diameter of nuclei as a scale of 0 to 1.</td>
<td>A rather wide territory region, yet it is relatively more external than internal, occupies outer 50% of the area of the nuclei, e.g. chromosome 4 averages from 60% ~ 75% ±20% S.E.M</td>
</tr>
<tr>
<td>Are there any particular points of the chromosome that occupy a more peripheral or internal region?</td>
<td>Distance from centre of mass to each point along the chromosome.</td>
<td>Weak indications towards some points preferring a variation caused by a ‘bending’ of chromosome as previously discussed. Requires means to quantify level of bend and direction of bend to further determine favourable regions.</td>
</tr>
</tbody>
</table>

**Table 4.7. Minor hypotheses tested within the interphase study**

Various questions that the multilayer methodology could provide data for in exploration of chromosome arrangement within interphase nuclei. Each hypothesis indicates what data is used to answer the question gained from the same sample, and the relevant outcome that the data sourced within this study resolves as.
5.1 The characteristics of DT40

Identifying chromosome aberrations is an essential step in tackling questions regarding genome stability which in turn allows the ability to question other fundamental essential cellular steps such as correct cellular division, chromosome inheritance, cell death or inability to initiate cellular death. As well established, fluorescent probes are vital for the characterization of the chromosomally erroneous. From humans to farm stock and onto other species of interest, it is important to characterise chromosomes in the event of individuals’ fertility or alternatively in the event of an aberrant cell line.

The use of the DT40 cell line with its ease of genetic manipulability allows it to be used in a large variety of cellular questions, examples of which include structural analysis of the BRCA2 gene (Warren et al., 2002), sister chromatid exchange mediation (Sonoda et al., 1998), infectious viral disease binding (Chi, 2018) and B-cell receptor signalling (Yasuda & Yamamoto, 2004).

The DT40 cell line is a chicken bursal lymphoma induced by the avian leukemia virus (Baba & Humphries, 1984; Baba, 1985), cells carry IgM on their surface (Baba, 1985) and have a disruption in the c-myc locus typical of avian leukemia viral infection. DT40 has integrated the viral long terminal repeat upstream of the myc-c gene (Hayward, 1981; Westaway et al., 1984) and is in the same orientation of c-myc thus causing an increase of transcription levels (Linial, 1985). The myc-c locus is found upon chicken chromosome 2. In the context of human cancer, the myc-c proto-oncogene is persistently expressed in many cancers and imperative in the formation of Burkitt lymphoma as a common chromosomal translocation between chromosome 8 (location of myc-c) and chromosome 14 causing an over expression (Finver, 1988), drawing parallels to the up regulation seen in DT40.

Initially, the DT40 cell line has been known to display a stable karyotype with a trisomy of chromosome 2 an extra unidentified microchromosome to give a karyotype of 2n=80 (Sonoda et al., 1998) although further work has identified variation to this previous karyotype and a degree of cytogenetic mosaicism (Chang & Delany, 2004). Cheng and Delany argue that there is variation seen to chromosome number and structure based upon source of cells and means of growth environment. They agree with the trisomy of chromosome 2 they identify a monosomy for chromosome 4 and a ‘4p’ variant and insist that this cytogenetic instability has the probable outcome for inaccurate results and interpretations using this cell line for investigations. More recently (Molnár et al., 2014)
proposes instead that DT40 has a relatively intact genome and suited as a model cell line for DNA based investigations attributed to different culture conditions or a stabilisation of the karyotype.

Chang and Delany use a karyotype approach opposed to Molnár who uses whole genome shotgun sequencing and single nucleotide polymorphism array hybridisation, neither using FISH as a means of verification of their findings. As previously discussed (1.3.4) and in light of the multiple examples of human cancers characterised by chromosomal aberrations and translocations, it is not sufficient to simply indicate that there is a loss or gain of genetic material without identifying their location and structure especially as the uniqueness of human cancers are revealed to us and the need for a more personalised approach. An example of this is the generation of resistant cell lines which shall be introduced in the next chapter. This chapter aims to better characterise the cell line DT40 with precise cytogenetic information and the verification of previous claims via the multilayer FISH technique.

Thus, as per the aim 3 set out in section 1.5, questions aimed to be answered in this chapter are as follows;

1. How well can we characterise the DT40 cell line genome, using a pre-set FISH based device improved with the multilayer method, to detect heterogeneity and novel chromosome aberrations
2. In light of the multilayer method, how well can we compare to old genomic data and generate a new karyotype for the DT40 cell line
5.2 The Multiprobe Chicken device plan

The chromoprobe multiprobe device (Cytocell) with pre-dried probes in either FITC, Texas Red or Aqua spectra allows the simultaneous hybridisation of near every chromosome of the chicken genome, barring the not yet fully described D group. In this section, the multilayer methodology was implemented by first hybridising and capturing metaphases using the multiprobe device as outlined by the manufacturer, then adding the second layer by inversion of the device onto the same slide and therefore metaphase preparations. Figure 5.1 outlines the position and coloration of probes used.

Figure 5.1. Organisation and false colouration of the multiprobe device and glass slide orientation used in this study

A representation of the glass cover slip of 24 ‘boxes’ used with corresponding pre-dried on probe in either FITC (green/Yellow) Texas red (red/purple) or Aqua spectra(aqua). The boxes containing aqua are all whole chromosome paints (Chr. 1-9 +Z) and the rest are subtelomeric probes. The top left number in each box represents the first layer, coloured in the previously used way of green/red; and in the bottom right, is the chromosome probed in the second layer when the device has been inverted falsely coloured yellow and purple. Note that pairings repeat after 12 boxes to give the same pair yet with inverted probe fluorochrome, in this event the false colouration has been applied to the colours shown.
5.3 DT40 FISH results

After successful FISH, images were merged and assigned into 12 pairs, due to the symmetrical nature of the device (Figure 5.1), and assigned false coloration to be then analysed. Analysis includes the identification of chromosome abnormalities, such as translocations, and chromosome copy numbers.

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Result</th>
<th>Chromosome</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal</td>
<td>16</td>
<td>Normal</td>
</tr>
<tr>
<td>2</td>
<td>3 Copies</td>
<td>17</td>
<td>Normal</td>
</tr>
<tr>
<td>3</td>
<td>Normal</td>
<td>18</td>
<td>Normal</td>
</tr>
<tr>
<td>4</td>
<td>Normal</td>
<td>19</td>
<td>Normal</td>
</tr>
<tr>
<td>5</td>
<td>Normal</td>
<td>20</td>
<td>Robertsonian translocation</td>
</tr>
<tr>
<td>6</td>
<td>Normal</td>
<td>21</td>
<td>Normal</td>
</tr>
<tr>
<td>7</td>
<td>Normal;ins/dup(2)(?pter)</td>
<td>22</td>
<td>Normal</td>
</tr>
<tr>
<td>8</td>
<td>Normal</td>
<td>23</td>
<td>Normal</td>
</tr>
<tr>
<td>9</td>
<td>Normal</td>
<td>24</td>
<td>4 Copies</td>
</tr>
<tr>
<td>10</td>
<td>Normal</td>
<td>25</td>
<td>Normal</td>
</tr>
<tr>
<td>11</td>
<td>Normal</td>
<td>26</td>
<td>Normal</td>
</tr>
<tr>
<td>12</td>
<td>Normal</td>
<td>27</td>
<td>Normal</td>
</tr>
<tr>
<td>13</td>
<td>Normal</td>
<td>28</td>
<td>-1; ins(28p)(?macro); ins(28q)(?micro)</td>
</tr>
<tr>
<td>14</td>
<td>Normal</td>
<td>Z</td>
<td>Normal</td>
</tr>
<tr>
<td>15</td>
<td>Normal</td>
<td>W</td>
<td>Normal</td>
</tr>
</tbody>
</table>

Table 5.1. Summary of chromosomal aberrations identified via FISH in DT40

Chromosome and associated result from the multilayer FISH strategy, Chromosomes post 28 have been omitted due to lack of probes currently available.

FISH results conclude that there is a trisomy of chicken chromosome 2, the same as reported in multiple previous studies (Figure 5.4). Chromosome 7 appears to be normal, yet there is a noticeable portion of signal (red) present on another single macrochromosome (possibly Zq) indicating a possible translocation or duplication of a short region (Figure 5.5).
Chromosome 20 expresses a single normal copy and a duplicated inverted conjoined chromosome, possibly representing a Robertsonian fusion on the same chromosome, which would effectively resemble a trisomy of 20 (Figure 5.4). Chromosome 24 was seen to have a tetrasomy in all metaphases viewed (Figure 5.3). Chromosome 28 has seemingly lost a single copy and the other has undergone a fission event to split and fuse the P-terminus and presumed arm to a macrochromosome and the q-terminus and presumed associated arm has fused to a large undistinguishable microchromosome (Figure 5.2).

In reference to the sex chromosomes, the sample was previously reported as a female expressing a ZW genotype with reports of a subset of ZZW cells present in some cell lines (Wang and Leung 2002). Here whole chromosome paints reveal a universal ZW genotype with a fully intact W in every metaphase analysed. Image results follow in respect to the order they were retrieved on the device post FISH analysis (Figure 5.1).

5.3.1 Chromosome 11 and 28

![Figure 5.2. Chicken chromosome 11 and 28 FISH results on a DT40 metaphase](image)

Chicken subtelomeric probes hybridised onto a DT40 metaphase; Green 11p, Red 11Q, Yellow 28p, Purple 28q. Chromosome 11 presents normally. There has been a whole chromosome deletion for 28 and the remaining chromatid has split to fuse its p and q terminuses onto distal ends of macrochromosomes.
5.3.2 Chromosome 15 and 24

Figure 5.3. Chicken chromosome 15 and 24 FISH results on a DT40 metaphase
Chicken subtelomeric probes hybridised onto a DT40 metaphase; Green 15, Red 15q, Yellow 24p, Purple 24q. Chromosome 15 is shown to be normal whereas chromosome 24 shows four typical chromosome signals indicating that there are four copies of this chromosome.

5.3.3 Chromosome 2, 5, 8 and 20

Figure 5.4. Chicken chromosomes 2, 5, 8 and 20 FISH results on a DT40 metaphase
Chicken subtelomeric probes and paints hybridised onto a DT40 metaphase; Green 2 whole chromosome paint, Red 5 whole chromosome paint, Yellow 20p, Purple 20q and Aqua, 8 whole chromosome paint. Chromosome 2 presents as a trisomy, and one copy of chromosome 20 exists as a Robertsonian translocation.
5.3.4 Chromosome 6, 7, 9 and 19

Figure 5.5. Chicken chromosomes 6, 7, 9 and 19 FISH results on a DT40 metaphase
Chicken subtelomeric probes and paints hybridised onto a DT40 metaphase; Green 6 whole chromosome paint, Red 7 whole chromosome paint, Yellow 19p, Purple 19q and aqua 9 whole chromosome paint. Chromosomes all appear normal except for an insert from chromosome 7 on a single macrochromosome.
5.4 DT40 karyotype results

5.4.1 The state of metaphases

To karyotype avian metaphases are a difficult but albeit possible task with the use of DAPI and Propidium iodide for complete stain and coverage of all macro and microchromosomes. Demonstrated here, with the aid of FISH results, multiple metaphases of good quality were imaged and analysed via SmartType (Digital Scientific UK).

Full chromosome counts were established with all macrochromosomes correctly identified and the microchromosomes, lacking defining specific features to characterise, assigned as a collective count. Chromosome count thus far has been totalled to average at 79.76 (n=30) with the modal count being 78 at 30% of the total population, as represented in figure 5.6 and 5.7.
5.4.2 The state of the karyotype

As previously stated, the average chromosome count of the DT40 cell line was calculated at 2n=79.76 with modal count at 2n=78 representing 30% of the population, Figure 5.7 shows this breakdown of counts in more detail. Only three metaphases were above previously reported count of 2n=80 (2n=81, 124 and 131), and the rest under 80, there were no metaphases observed with a count of exactly 80 chromosomes. Consistently, in light of and complementary FISH results, observed were three chromosome 2s, four copies of microchromosome 24s and a monosomy of chromosome 18. In terms of macrochromosomes (1-8), all samples karyotyped and observed via FISH had consistent macrochromosome counts and features. However analysis of the post chromosome 28 group was not possible by FISH, this group was determined by size of chromosomes within a karyotype (Figure 5.6, Figure 5.7 and Figure 5.8).

From karyotype analysis, no clear sub-populations were able to be distinguished. Clear divisions of metaphase’s were seen with comparable total chromosome count (again 30% of all metaphases analysed had 2n=78, and other chromosome counts had 10%-15% of the population, Figure 5.6 and Figure 5.7), however due to the nature of microchromosomes being hard to karyotype it is impossible to identify a shared genome / clonal nature to these populations.
Figure 5.7. Full chromosome count and frequency for karyotyped DT40 metaphases

DT40 metaphases karyotyped via staining with DAPI and Propidium Iodide using SmartType (Digital Scientific) software for analysis.

A) Chromosome counts for individual metaphases. Normal *G. gallus N*=78 is highlighted in the black horizontal dotted line with individual metaphase chromosome counts from the DT40 cell line being shown

B) Frequency of total metaphases grouped by chromosome count of aforementioned karyotyped DT40 metaphases.
Figure 5.8. Representative DT40 karyotype
Example of a karyotyped DT40 cell with chromosome count of $2n=78$. Stained with Propidium iodide and DAPI, assignments have been made according to standard chicken ideograms and karyotypes. Macrochromosomes are large enough for distinctive assignment; however microchromosomes are of insufficient size to correctly distinguish from one another (highlighting the importance of FISH testing). Regardless, the unclassified microchromosomes should represent the assignment seen in 5.4.4.
5.4.3 The State of heterogeneity and microchromosomes

Within the population of DT40 cells investigated, there are somewhat conflicting results in terms of heterogeneity. In light of FISH results, there was no evidence of alternating chromosome state/morphology on a cell to cell basis; all signals seen were consistent with one another across all metaphases. The multilayer approach allowed singular cells to be investigated further with use of the chromoprobe being inverted to gain a better observational depth on a single cell level which identified no chromosomal deviations. However, within the analysis of metaphases via karyotyping there is an observable range of chromosomal counts present throughout the population, thus indicating some plausible degree of heterogeneity. With the macrochromosomes being consistent in number and features, the heterogeneity displayed must resolve in the microchromosomes.

The microchromosomes vary in number from sample to sample, however during FISH analysis there was no observed variation of microchromosomes probed. In respect to both methodologies, the heterogeneity seen within the DT40 cell line must be concluded to arise from variation within the smallest of the microchromosomes, 29-35. To predict the behaviour and possible aberration features these chromosomes possess is not within capabilities of this study, as currently consistent BAC probes for these chromosomes are not available for this style of testing.

5.4.4 Final cytogenetic assignment

\[2n = 78, ZW, +2, +24, +24, -28, rob(20;20)(p;p), ins(7, ?macro), ins(28p, ?macro), ins(28q, ?macro)\]
5.5 Discussion

Using the multilayer methodology with a high quality chromoprobe device has allowed the formulation of a new karyotype and further classification of previously identified mutations (Sonoda et al., 1998, Chang & Delany 2004, Molnár et al., 2014). These previous reports have correctly identified the majority of aberrations seen within this study, such as the trisomy of chromosome 2. However these reports lack depth and precise description of these malformations seen as a result of their methodologies.

Results here are consistent with the above-mentioned chromosome 2 trisomy and the tetrasomy in chromosome 24. Molnár correctly identifies the effective trisomy 20 present within the cell line, yet FISH results further details this by indication that this is due to one normal 20 and a Robertsonian translocation involving two other chromosome 20’s, rob(20;20)(p;p). This immediately stands out as a downfall of virtual genotyping where important chromosomal aberrations such as this are missed which may have been of importance of the characteristics of the DT40 cell line. By presuming a trisomy of chromosome 20, it would have also inferred an incorrect assignment of microchromosomes to then not be reflected in the total chromosome number seen.

Furthermore, in reference to previous studies, multiple previously reported aberrations were not observed. Chromosome aberrations not seen within this study were the trisomy of chromosome 14, the ZZW sex chromosome variant or a mosaicism of an ‘alternative 4p’.

With very little heterogeneity previously reported, barring the ZZW/4p variants, somewhat interestingly this study has eluded that there may be an underlying variation as highlighted by karyotype results within the microchromosomes. With this inconsistency observed in total chromosome count, with constant macrochromosome count/assignment, the smallest microchromosomes must be culprit for this phenomenon as mentioned in section 5.4.3.

Results obtained may be specific to the DT40 cell line studied here, it is plausible that other laboratories have had various derivative clones used instead, which may possess the chromosomal variants. This has been well documented as seen in karyotypic heterogeneity (Worton 1978) and functional heterogeneity (Davies, 2013) in CHO cells to give rise to useful functional variants. However, variants may not give rise to useful sub clones and instead cause mass variation in cellular cultures used across laboratories to affect transcriptome, proteome and phenotypic responses of cells. This has been found within
HeLa cells taken from 13 international laboratories implicating proving evidence towards this reproducibility issue (Liu, 2019). With heterogeneity brings variation and therefore an inability to reliably replicate results, this causes questioning into the findings obtained from cultured cells. It would be expected if DT40 cell lines that have undergone many successive passages from other laboratories or stocks to have somewhat of a heterogenic element, but how this implicates upon expression of the ‘usefulness’ of the cell line needs to be explored via multi-omic studies.

Karyotyping microchromosomes is an arduous process in a typical avian organism, let alone with an aberrated cell line; so this must be taken into consideration when referencing karyotypic conclusions. The complete deletion of the microchromosomes is somewhat hard to use as a valid conclusion for results seen due to the high gene density observed within these microchromosomes (Burt, 2005; Smith, 2000). It is entirely plausible that these microchromosomes have instead of being lost in deletion events, have undergone fusion/translocation events with the larger macrochromosomes which would not be detected within the confines of this study. One must reflect on methodology used and if results presented here are in fact artefacts caused by the karyotyping method and user error. Henceforth are alternatives to the methodology that could be used in future studies when other obstacles have been overcome.

As mentioned the lack of reliable probes specific for the ‘D’ group subtelomeric regions prevents usage in the typical multilayer FISH way of incremental probes on chromosomes of interest. With access to these probes, it would be entirely possible to step-wise probe along the macrochromosomes, in a similar fashion to section 4.2.2, to question the hypothesis of microchromosomes fused to alternative larger chromosomes. The use of the chromoprobe device clearly aided in speed and reliability of results when testing this large quantity of chromosomes yet it also acts somewhat as a limiting factor. Indeed, the reversal of the device does cause a repetition of probe pairings across the device, however this is somewhat beneficial as it effectively doubles up the quantity of metaphases able to be analysed which relates to the study of heterogeneity. The use of custom devices would understandably increase the usefulness of the multilayer technique here as it could be designed with more layers in mind to deepen the resolution in regards to the cell by cell approach this method currently provides. However, this is entirely possible if the user has access to a large panel of probes that detail every locus within the chicken genome that the
device becomes redundant and even wasteful if only specific chromosomes are required for investigation.

On the oncogenic properties of aberrations detailed, it is understood that the DT40 cell line has metastatic properties, as is able to form distal tumours in immunosuppressed chicks, so there must be a degree of tumorigenic mutation or deregulation within ontogenetic promoting/suppressing factors regarding cell regulation, prevention of apoptosis and metastatic capabilities. The proto-oncogene myc-c is found on chromosome 2 of the chicken, with similar functionality as human of the regulation of approximately 15% of all genes (Gearheart et al., 2007), and unsurprisingly the DT40 cell line expresses chromosome 2 in triplicate, possibly increasing the dosage of this regulator to have tumorigenic properties. Commonly in ALV induced tumours, there is an association with the hybrid oncogenic protein gag-myc (Watson et al., 1983; Chesters et al., 2001; Law et al., 2001). To correctly determine genome aberration as the causative reason for gag-myc, such as translocation/viral insertion onto the myc-c reading frame, within the three copies of chromosome 2 an incremental BAC approach along the typical myc-c region could be performed to detect malformations.

5.6 Conclusion

As current reagents and equipment are limiting factors to provide a concise valid final cytogenetic assignment of the DT40 cell line; ultimately, the refinement and identification of aberrations, along with a modern interpretation of its karyotype via the multilayer technique has improved the current understanding of this cell line. Unfortunately the ‘D’ microchromosome group (29-38) still eludes us and such this study is limited in its scope of a complete characterisation of DT40, yet this can easily be incorporated into the multilayer methodology at a later time. Nevertheless, this study has shown that DT40 is a rather reliable stable model cell line and should be of further use to the scientific community not only for its capability for easy genetic manipulation of DNA integration ratio but for oncogenic studies as well.

One interesting avenue would be the possible oncogenic properties of aberrations identified, such as the tetrasomy of chromosome 24 and the effects of that dosage level on cellular function, or the differences a trisomy compared to a structural trisomy as seen within this cell line in regards to chromosome 20 where dosages of gene products might be near identical. Overall, this methodology has given new insights into the cytogenetic
makeup of the DT40 cell line and new questions can be asked about how these specific aberrations attribute to its oncogenicity.

Use of the multilayer methodology has allowed easy analysis of this cell line and the generation of a new updated karyotype. Furthermore, this methodology has clear capabilities to classify cancer cell lines which may exhibit a wider variety of intra-tumour heterogeneity, such as in the example of cytotoxic drug resistances which is a topic of substantial research in human cancers.
6. Cytogenetic assignment of resistant neuroblastomas by chromosomal aberration detection (Specific aim 4)

6.1 Cancer cytogenetic studies thus far

6.1.1 Cancer aberrations

It has been well known and discussed, that tumour cells exhibit chromosomal aberrations (Solomon et al., 1991). Error such as aneuploidy or an increase in ploidy number has the consequences of an imbalanced dosage of genes, such as a gain of oncogenes or a decrease/loss of tumour suppressor genes (Savage, 1999). See section 1.1.4 and 1.3.2.3 for more information.

Chromosomal aberrations increase the tendency to form particular types of malignancies, as they could cause the deregulation of genes to act as the basis of the progression of a normal cell into a cancerous cell. This theme has been greatly explored (Mitelman, 2005; Van Gent et al., 2001). As an example, chromosomal aberrations events could give rise to a repositioning of an oncogene within the regulatory parameters of another gene, such as in the case of the famous Philadelphia chromosome. The Philadelphia chromosome, a translocation between chromosome 9 and 22, t(9;22)(q34;q11), results in the fusion protein BCR-ABL to cause the uncontrolled replication of the cell and its genome leading onto a cancerous cell, found in chronic myeloid leukaemia, (Shteper & Ben-Yehuda, 2001) and imatinib resistant leukaemia’s (Talpaz et al., 2006). Other examples include large scale deletions, as in a typical cancer 25% of the genome is thought to be affected by arm-level deletion events (Beroukhim et al., 2010). The deletion of large segments of chromosomes will cause a loss of hetrozygosity; the genes contained in the lost segments are deleted hemizygously causing a shift in cellular product dosage (Dong, 2001; Solimini et al., 2012). This loss of hetrozygosity may not directly infer an oncogenesis event, yet may contribute to the malignant phenotype of an already cancerous cell (Cox et al., 2005; Bignell et al., 2010), an example of which can be described by deletion of 8p. The short arm of chromosome 8 is a common aberration found within cancers originating from epithelial tissue such as breast cancers and is linked to poor prognosis (Yarernko et al., 1995; Lebok et al., 2015). The loss of hetrozygosity disrupts the dosage of genes that negatively regulate cell growth (possible tumour suppressor genes), examples of which are MTUS1 and TUCS3, and also essential
genes that promote cell growth, CLU and PTK2B (Tabares-Seisdedos & Rubenstein, 2009; Solimini et al., 2012). With the loss of positive and negative genes associated with cell proliferation is presumed to balance one another out, no change in tumorigenic transformation. However, the loss of hetrozygosity and therefore the allelic loss of other 8p genes give rise to a shift in lipid metabolism to increase metastatic potential and resistant to hypoxic conditions, further promoting the survival of these cancerous cells (Cai et al., 2016).

6.1.2 Neuroblastoma

Neuroblastoma is classified as a type of cancer that originates from immature nervous tissue, neuroblasts, typically in the adrenal glands but can also be found in the neck chest or spinal cord. Neuroblastoma is the most common cancer in newborns and the third most prevalent in children (Maris et al., 2007). The direct cause of neuroblastoma is viewed to be caused by genetic mutations inherited or present during development and birth, compared to causes such as environmental tumorigenic events. Key gene mutations and aberrations have been identified thus far as possible causes or contributing factors towards the development of neuroblastoma, such as in anaplastic lymphoma kinase (ALK) (Mossé et al., 2008), KIF1B, amplification of the MYCN oncogene (Brodeur et al., 1984), duplicated segments of the LMO1 gene (Wang et al., 2011) and copy number variation of the NBPF10 gene (Diskin, 2009).

Via array-based virtual karyotyping (section 1.1.5.4), neuroblastoma tumours of differing lethality- governed by survival rates- could be assessed (Janoueix-Lerosey et al., 2009). Tumours which show whole chromosome copy number variants are correlated with high survival rates, a segmented chromosome copy number changes decrease survival and the worst survival rates correlate with key aberrations such as MYCN amplification and specific chromosomal arm deletions (Michels et al., 2006).

Cancerous cell lines have been experimentally used to generate resistances to currently used cytotoxic drugs in order to identify mechanisms of resistance, sensitivity to other drugs and biomarkers specific to the acquired drug resistance (Domingo-Domenech et al., 2012; Zahreddine et al., 2014; Göllner et al., 2017). Examples of findings specific to neuroblastoma include the generations of sub-lines which have gained resistance to MDM2 inhibitors, found to be results of p53 mutations (Michaelis et al., 2011; Michaelis et al., 2012a). More so, mechanisms behind varied drug resistances for the UKF-NB-3 cell line
(section 2.1.2.3) has been documented alongside key chromosome aberrations found via karyotyping and SKY (Kotchetkov., 2005; Michaelis et al., 2012b) with more information to resistance mechanisms and aberrations as compared to the closely related UKF-NB-2 and UKF-NB-4 cell lines (Kotchetkov, 2005; Bedrnicek et al., 2005).

6.1.3 Cancer cytogenetics of past and present

The use of G or R banding techniques was soon sequestered by the rise of fluorescence-based hybridisation techniques such as FISH, m-FISH (Jentsch et al., 2001), spectral karyotyping (SKY) (Liyanage et al., 1996), comparative genomic hybridisation (CGH) (Kallioniemi et al., 1992) and array-CGH (see section 1.1.5 for details on these methods). The arrival of high throughput methods, commonly known as next generation sequencing, allowed whole genomes to be sequenced in a quick, inexpensive and automated process in vast parallel quantities (Grada & Weinbrecht, 2013). However, over the past 20 years that these techniques have been available, they have not gained popularity due to their extensive labour requirements, high running costs and somewhat vague results produced. Focuses turned to the ‘functional’ aspects of the genome, typically the exome, as interests were in finding answers to medical problems due to the mutations within genes, such as inherited disease conditions (Lai-Cheong & McGrath, 2011). Initially, exome studies could only be carried out on few samples due constraints of the quantity of data that could be obtained (T. Sjöblo, et al., 2006) yet from this specific gene candidates were proposed to focus on based upon identified cancer mechanisms and possible targets for therapy (Greenman et al., 2007). Improvements accumulated over time via increased knowledge, computational power increases and falling costs, to allow high numbers of cancer genomes to be sequenced to genome level, expression of genes -transcriptome level- (Maher et al., 2009) and exome level (Ley et al., 2008). As of current it is possible via ultra-high-throughput sequencing that hundreds of thousands of samples can be run in parallel (Tucker et al., 2009) and has allowed the sequencing of an entire human genome within a day (Straiton et al., 2019).

However, even with technological advances, it has become more apparent that the data obtained from the analysis of the tumour as a unanimous population, can only draw limited conclusions due to the intra-tumour genetic heterogeneity (Navin et al. 2011). An example of which, in order to define copy number variants of a cancer line, it is made difficult by the unknown level of ploidy caused by probable multiple numerical and structural chromosome abnormalities present (Storchova. & Pellman, 2004; Storchova &
Alongside this difficulty, the heterogeneous population could cause a shift towards an ongoing subclonal ‘speciation’ event, the generation of sub-populations that could become phenotypically distinct from the original tumour (Navin et al., 2010; Navin et al. 2011) (see section 1.3.4). The current methods used to combat this problem are array CGH (Lyng et al., 2008) or via single cell sequencing (Navin et al. 2011), are not as useful of a strategy as once thought to be in order to fully characterise the cancer genome (International Cancer Genome Consortium, 2010). With NGS, the analysis is needed to be at a great depth in order to correctly identify translocations or alternatively by using other techniques to pre-identify translocations (Allen et al., 2014), currently FISH remains the best candidate in terms of cost and ease to identify the overall chromosomal divergence/structure and to correctly assess the level of heterogeneity within the sample. FISH will always be limited in its resolution and inability to detect single point mutations or polymorphisms between samples where NGS will always outperform in this regard. The use of either method must be trialed against the primary research target, for a now quick relatively inexpensive cytogenetic analysis on the state of the chromosomes multilayer FISH shall exceed. Opposed to a potentially highly sensitive methodology that allows identification of base level aberrations.

With the increase of data obtained via NGS coupled with falling costs, it is an attractive model for researchers as it could be argued that the technical expertise and cost of FISH based genomics is off-putting. However by increasing throughput of FISH and lowering the overall cost it could be seen as a useful tool to counter the shortcomings of NGS methods mentioned previously. The use of specific BAC’s via FISH can enable the identification of chromosome number, rearrangements, and complex aberrations such as multiple or balanced translocations or centromere abnormalities. The current downside to a FISH based approach is the limited analysis few fluorophores can provide in one single experiment (Hasty & Montagna, 2014). Modern FISH methodologies have been tried successfully, such as the use of alternative fluorophore ratios tied to specific BACs to increase the number of alternative colours seen, a strategy borrowed from M-FISH, to detect aneuploidy in large quantities. These experiments called for a strategy to detect the full 24 chromosomes via FISH in order to aid clinical diagnosis in a cost effective and reliable manner (Ioannou et al., 2011). As described in this thesis these issues of cost, labour intensively and accuracy of results can be overcome by the multilayer methodology allowing FISH to be a worthwhile and comparable methodology compared to NGS used commonplace today.
We are presented with two main problems that multilayer FISH may be a useful methodology to explore, the possibility of detecting the level of heterogeneity in cancer cell lines, and detecting unknown chromosomal aberrations that may be hidden by traditional sequencing techniques which may bring about new oncogenic properties or resistances to specific therapeutic agents. The neuroblastoma cell line is an ideal model to use; as already established derivative resistant populations exist (Table 2.1) with chromosome aberration identification performed using classical hybridisation based techniques.

The objectives aimed to be answered in this chapter are as follows and to achieve thesis aim 4:

Via use of the multilayer FISH strategy

- Assess the heterogeneity and inheritance of neuroblastoma cell line (the UKF-NB-3) and derivative resistant lines
- Highlight key chromosomal aberrations that could possibly infer a means to detect or cause resistance in subpopulations
- Assign the neuroblastoma cell lines a cytogenetic notation in reference of all results seen

### 6.1.4 Method of Multilayer probe groups

To cover all human chromosomes with at least one probe and to have as little ‘panels’ or ‘groups’ as possible to increase the chance of detecting suspected subpopulation genetic variants, an proficient methodology was required. The resources used in this chapter were composed of a varied selection of commercially available probes, in an assortment of fluorophores and classifications (9. Appendix) (Cytocell) arranged in a way to not overlap in fluorophore signal to be consistent within the multilayer process.

To summarise the overall methodology requirements, in order of importance;

1. To follow the standard 2 colour FISH with sequential layers, the protocol established in section 3.4 of this thesis
2. To have minimal panels/groups
3. To have the ability to identify a wide spread of, if not all, chromosomes as experimentally possible
With being limited to six unique layers for a single round of mLISH, the requirement would be at least 4 differing panels to encompass at least a single probe on each unique chromosome. This was reduced by the use of commercial probes that consist of multiple targets that could be analysed during the merging process to identify chromosomes. An example of this is the 1/5/19 alpha satellite probe (Table 6.1) which consists of three FITC probes at differing concentrations, thus allowing the identification based upon probe intensity post-FISH. Likewise it is possible to combine colours in layers such as in the example of chromosome 3, 6 and 14 in panel 2 (Table 6.2). Chromosome 3 can be identified using the chromosome paint in FITC, alongside the IGH / CCND3 Translocation Dual fusion probe set which identifies 14q32.33 in Texas Red and 6p21 in FITC. One would predict a normal result to be easily to differentiate between a paint signal and a specific location signal in the same colour therefore able to differentiate the chromosomes. An inability to do this would signify there is a degree of chromosomal abnormality, a significant finding and the aim of these experiments.

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Identifier</th>
<th>False Colour</th>
<th>Label + Loci</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1/5/19 α. Satellite</td>
<td>00FF00</td>
<td>FITC 1p11.1-q11.1</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>FFFF00</td>
<td>FITC 5p11.1-q11.1</td>
</tr>
<tr>
<td>19</td>
<td></td>
<td>00EEFF</td>
<td>FITC 19p11.1-q11.1</td>
</tr>
<tr>
<td>2</td>
<td>ALK Breakapart</td>
<td>CC00CC</td>
<td>FITC 2p23.2-p23.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FF00FF</td>
<td>TX 2p23.2-p23.1</td>
</tr>
<tr>
<td>8</td>
<td>α. Satellite</td>
<td>CC6600</td>
<td>TX 8p11.1-q11.1</td>
</tr>
<tr>
<td>10</td>
<td>α. Satellite</td>
<td>FF0A00</td>
<td>FITC 10p11.1-q11.1</td>
</tr>
<tr>
<td>12</td>
<td>α. Satellite</td>
<td>FFFFFF</td>
<td>FITC 12p11.1-q11.1</td>
</tr>
<tr>
<td>18</td>
<td>α. Satellite</td>
<td>FF0000</td>
<td>TX 18p11.1-q11.1</td>
</tr>
</tbody>
</table>

**Table 6.1. Probes selected for group 1 panel analysis for the UKF-NB-3 lines**

*H. sapiens* probes are shown here with chromosome assignment and specific associated loci. Cytocell product identifier is shown to give an indication towards the intended purpose of these products; each probe has been assigned a false colour used in the multilayer process.

The order of FISH was as follows; 1) 1/5/9 α-Satellite + 18 α-Satellite 2) ALK Break-apart + 10 α-Satellite 3) 8 α-Satellite + 12 α-Satellite.
<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Identifier</th>
<th>False Colour</th>
<th>Label + Loci</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Paint</td>
<td>00FF00</td>
<td>FITC Paint</td>
</tr>
<tr>
<td>4</td>
<td>α. Satellite</td>
<td>FFFF00</td>
<td>TX p11.1-q11.1</td>
</tr>
<tr>
<td>6</td>
<td>IGH / CCND3 Translocation Dual fusion</td>
<td>FF0000</td>
<td>FITC 14q32.33</td>
</tr>
<tr>
<td>14</td>
<td>α. Satellite.</td>
<td>00EEFF</td>
<td>TX 6p21</td>
</tr>
<tr>
<td>11</td>
<td>α. Satellite.</td>
<td>FFFFFF</td>
<td>FITC 11p11.1-q11.1</td>
</tr>
<tr>
<td>16</td>
<td>CBFβ (CBFB)/MYH11</td>
<td>CC66CC</td>
<td>FITC 16p13.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FFAA00</td>
<td>Tx 16q22</td>
</tr>
<tr>
<td>22</td>
<td>IGL Breakapart</td>
<td>CC00CC</td>
<td>FITC 22q11.23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FF00FF</td>
<td>Tx 22q11.21</td>
</tr>
</tbody>
</table>

**Table 6.2. Probes selected for group 2 panel analysis for the UKF-NB-3 lines**

*H. sapiens* probes are shown here with chromosome assignment and specific associated loci. Cytocell product identifier is shown to give an indication towards the intended purpose of these products; each probe has been assigned a false colour used in the multilayer process.

The order of FISH was as follows; 1) 3 Paint + IGH/CCND3 translocation dual fusion 2) 4 α-Satellite + IGL Breakapart 3) CBFB/MYH11 + 11 α Satellite.

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Identifier</th>
<th>False Colour</th>
<th>Label + Loci</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>α. Satellite</td>
<td>00FF00</td>
<td>FITC 9p11.1 9q11.1</td>
</tr>
<tr>
<td>13</td>
<td>D13S25 Deletion</td>
<td>CC00CC</td>
<td>FITC 13q34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FF00FF</td>
<td>Tx 13q14.3</td>
</tr>
<tr>
<td>15</td>
<td>α. Satellite</td>
<td>FFFFF00</td>
<td>FITC 15p11.1-q11.1</td>
</tr>
<tr>
<td>17</td>
<td>Classical Sat.</td>
<td>00EEFF</td>
<td>Blue 17p11.1-q11.1</td>
</tr>
<tr>
<td>21</td>
<td>TMPRSS2/ERG Deletion/ Breakpoint</td>
<td>CC6600</td>
<td>FITC 21Q22.2-22.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FFAA00</td>
<td>TX 21Q22.2-22.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FFFFFF</td>
<td>AQUA 21Q22.13-q22.2</td>
</tr>
<tr>
<td>X</td>
<td>α. Satellite</td>
<td>FF0000</td>
<td>TX Xp11.1-q11.1</td>
</tr>
</tbody>
</table>

**Table 6.3. Probes selected for group 3 panel analysis for the UKF-NB-3 lines**

*H. sapiens* probes are shown here with chromosome assignment and specific associated loci. Cytocell product identifier is shown to give an indication towards the intended purpose of these products; each probe has been assigned a false colour used in the multilayer process.

The order of FISH is as follows; 1) 9 α-Satellite + 17 Classical Sat 2) 15 α.Satellite + D13S25 Deletion, 3) TMPRSS2/ERG Deletion/Breakpoint + X α.Satellite.
6.2 Metaphase analysis

Attempts to consistently fully karyotype the UKF-NB-3 cell line were unsuccessful (n≥60), partial karyotypes were possible and it was clear that there was an abundance of chromosomal aberrations and a degree of instability between individual nuclei of the same sample/cell line. The UKF-NB-3 Parental cell line (Parental) and the UKF-NB-3 rVincristine (rVincristine) line both had near normal chromosome counts in respect to total chromosome count, parental average range 48±4 (n=22), rVincristine average range 48±4 (n=24), whereas UKF-NB-3 rCisplatin (rCisplatin) did not show any commonality or consistency with results on average counts from 50 to 120≤ (n=35).

![Image of metaphase spreads](image_url)

**Figure 6.1. Metaphase spreads for all the UKF-NB-3 Cell lines used**

A) Normal *H.sapiens* male 46<2n>XY used as a control in this study. B) Parental UKF-NB-3 cell line. C) UKF-NB-3 rCisplatin metaphase. D) UKF-NB-3 rVincristine metaphase. All metaphases were stained with DAPI (white), and immediately imaged; the scale bar in each image is representative of 10μm.
6.3 Multilayer FISH results

Multilayered FISH was performed on the three neuroblastoma cell lines alongside a control healthy human metaphase preparation by the standard multilayer methodology, using the probes detailed previously in tables 6.1, 6.2 and 6.3. After imaging, layers were merged and probe signals given false colouration to allow identification of abnormalities on individual metaphases across the four populations. Results of these are detailed in the following sections as tables of frequency of aberrations, metaphase representatives of cells from each cell line along side normal human metaphases and a hypothesised phylogenetic tree. As the control sample displayed no abnormalities, it has been excluded from some result figures.

6.3.1 Probe panel one results

<table>
<thead>
<tr>
<th>Aberration</th>
<th>Parental Frequency (%)</th>
<th>rCisplatin Frequency (%)</th>
<th>rVincristine Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+1</td>
<td>-</td>
<td>16.67</td>
<td>-</td>
</tr>
<tr>
<td>+1,+1</td>
<td>-</td>
<td>11.11</td>
<td>-</td>
</tr>
<tr>
<td>-1</td>
<td>89.29</td>
<td>16.67</td>
<td>-</td>
</tr>
<tr>
<td>+der 1, 1/2/18</td>
<td>100.00</td>
<td>-</td>
<td>100.00</td>
</tr>
<tr>
<td>+der 1, 1/2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+der 1, 1/18</td>
<td>-</td>
<td>94.44</td>
<td>-</td>
</tr>
<tr>
<td>+2</td>
<td>-</td>
<td>38.89</td>
<td>-</td>
</tr>
<tr>
<td>+2,+2</td>
<td>-</td>
<td>27.78</td>
<td>-</td>
</tr>
<tr>
<td>+2,+2,+2,+2,+2,+2</td>
<td>-</td>
<td>5.56</td>
<td>-</td>
</tr>
<tr>
<td>-2</td>
<td>3.57</td>
<td>5.56</td>
<td>15.38</td>
</tr>
<tr>
<td>+5</td>
<td>-</td>
<td>-</td>
<td>7.69</td>
</tr>
<tr>
<td>-5</td>
<td>14.29</td>
<td>44.44</td>
<td>7.69</td>
</tr>
<tr>
<td>+8</td>
<td>3.57</td>
<td>45.45</td>
<td>-</td>
</tr>
<tr>
<td>+8,+8</td>
<td>-</td>
<td>18.18</td>
<td>-</td>
</tr>
<tr>
<td>-8</td>
<td>32.14</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+10</td>
<td>-</td>
<td>41.18</td>
<td>-</td>
</tr>
<tr>
<td>-10</td>
<td>10.71</td>
<td>11.76</td>
<td>-</td>
</tr>
<tr>
<td>+12</td>
<td>53.57</td>
<td>36.36</td>
<td>-</td>
</tr>
<tr>
<td>-12</td>
<td>7.14</td>
<td>18.18</td>
<td>-</td>
</tr>
<tr>
<td>-18</td>
<td>100.00</td>
<td>94.44</td>
<td>100.00</td>
</tr>
<tr>
<td>-18,-18</td>
<td>-</td>
<td>5.56</td>
<td>-</td>
</tr>
<tr>
<td>+19</td>
<td>-</td>
<td>16.67</td>
<td>-</td>
</tr>
<tr>
<td>-19</td>
<td>28.57</td>
<td>33.33</td>
<td>23.08</td>
</tr>
<tr>
<td>-19,-19</td>
<td>-</td>
<td>5.56</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 6.4. Probe panel 1 aberration frequency across all cell lines.
Displayed is the level of individuals of the given cell line population that expresses a particular aberration as compared to the total number of nuclei examined for that cell line in this probe panel. Aberrations are identified and classified as if ranked against a normal 46<2n>XY genotype in light of FISH results. A ‘-’ notates that this particular abnormality was not found within this cell line.
It is possible to identify similar and dissimilar 'individuals' with classification of expressed abnormalities, and therefore generate a list of subpopulations that exist within the sample population as a whole. Results thus far indicate that there is a clear mixture of subpopulations within each sample indicating a heterogeneous nature of each cell line. From this, modal (or most frequent) genotypes of these subpopulations were chosen to represent the state of the individual cell lines and are as follows.

6.3.1.1 Modal Parental

Within the parental cell line in context of results from panel 1, the most abundant genotype (and will be identified as Par.1A) was as follows (32% of sample population);

\[ Par.1A = -1, +\text{der}(1)\text{dup}(1;2)(\text{pter};\text{p}23.1\text{-}23.2)t(1;18)(p?;p11.1), +12,-18 \]

6.3.1.2 Modal rVincristine

The rVincristine cell line, the most abundant genotype (rVin.1A) was as follows (62% of sample population);

\[ r\text{Vin.1A} = +\text{der}(1)\text{dup}(1;2)(\text{pter};\text{p}23.1\text{-}23.2),-18 \]

6.3.1.3 Average rCisplatin

rCisplatin did not show any evidence for a modal genotype, as all metaphases analysed had a unique aberration profile, i.e. no two metaphases were the same. In light of this, the most abundant aberration (modal aberration) for individual chromosomes is selected to represent the 'modal' (rCis.1\(\overline{X}\)) as follows;

\[ r\text{Cis.1}\overline{X} = +\text{der}(1)t(1;18)(p?;p11.1),+2,+8\text{-}18 \]

6.3.1.4 Derivative chromosome 1

A noticeable conserved chromosome aberration is a presumed chromosome 1 with a double p-arm translocation with 18p11.1 (red) and chromosome 2p23.1-23.2 (purple/pink) in the parental cell line (Figure 6.3, top right/ 100% of sample metaphases). Surprisingly, both resistant lines seemingly express a similar derivative chromosome 1 yet with only one of the translocations seen as in the parental line (Figure 6.3 bottom left, bottom right. FLpter analysis was performed to investigate the similarities or differences between these chromosomes to assess a possible means of inheritance or spontaneous mutation in each cell line (Figure 6.2).
Figure 6.2. Probe point plot showing derivative chromosome 1 for NB3 parental/rCisplatin/rVincristine FLpter scores and corresponding chromosome schematic

A) Probe position measured as FLpter values and averaged across all metaphases obtained using probes from panel 1 (table 6.1).

B) Generated schematic chromosomes for each cell line by using metaphase FLpter data generated. Position of coloured band corresponds directly to FLpter scores generated and colour corresponding to assigned probe. Not a true representative of actual length of chromosome, only relative distance along the chromosome from the p-terminus.
Figure 6.2 indicates that the parental and rVincristine have identical FLpter scores for their similar probe locations, whereas rCisplatin has a reduction in both centromere location and its paired probe location in reference to the parental. Alternatively, assessing the relative chromosomal distance between the centromere location and the 18p signal, an identical distance is noted between the parental and rCisplatin (Table 6.5), this is also shown by equal gradients on the probe point plot (Figure 6.2) generated from FISH results as seen in Figure 6.3.

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Parental (FLpter)</th>
<th>rCisplatin (FLpter)</th>
<th>rVincristine (FLpter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1p11.1-q11.1</td>
<td>0.45958±0.0085</td>
<td>0.52774±0.0197</td>
<td>0.48424±0.00997</td>
</tr>
<tr>
<td>TX 18p11.1-q11.1</td>
<td>0.68195±0.0073</td>
<td>0.75913±0.0192</td>
<td>-</td>
</tr>
<tr>
<td>2p23.2-p23.1</td>
<td>0.89553±0.0057</td>
<td>-</td>
<td>0.87177±0.0175</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Distance between signals (% of total length)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 to 18</td>
</tr>
<tr>
<td>1 to 2</td>
</tr>
</tbody>
</table>

Table 6.5. FLpter scores for derivative chromosome 1 within; NB3 parental/ rCisplatin / rVincristine, with relative distances between signals.

Rows 2-4, the associated FLpter scores for the position of the indicated probes found on the derivative chromosome 1 conserved in each cell line. Error is given as S.E.M.

Rows 5-6, the distance between the probes as a fraction of total chromosome length recorded. Error is given as S.E.M. `-'annotates that this was not observed for the signified cell line at that specific probe location.
6.3.1.5 Proposed Phylogenetic Principles of classification.

It is possible to use the results of the FISH panels to deduce a feasible hypothesis of phylogeny within the cell line populations between sub-populations, and if sufficient, detect possible divergence positions for the resistant cell lines. In order to speculate a ‘best fit model’ of phylogeny, principles must be rationalised in order to fit this differing model of evolution. The rules that will be obeyed to generate the phylogenetic trees (Figure 6.4, Figure 6.6 and Figure 6.8) in this study are as follows;

I. Assume there is no change in selection pressure since initial cell line divergence
   i. Therefore, subpopulation abundance is in proportion to its ‘age’
      i. New subpopulations with lower abundance are products of more recent mutative events.
      ii. New genotypes should not out-compete old genotypes as there is no new ecological niche to fill.

II. All deviations from the ‘progenitor’ should exist within the population, yet in smaller abundance.

III. The most appropriate phylogenetic pathway should have the least sum of ‘missing genotypes’ to fill.
    i. Assume single chromosome malformation events would cause these to be filled and not be removed from the population as per initial assumption.

IV. Those cells which generate non-viable chromosomal aberrations shall not survive to metaphase stage, and therefore will not be able to generate any further diverged subpopulations. These shall not be incorporated into the best fit model.

These principles can allow a mixed population of varying genotypes to comprise subpopulations that can be linked by single chromosomal mutation events, with reference to subpopulation abundance.
Figure 6.3. Merged multilayer FISH images of Human and three NB3 cell lines from panel 1
A) Normal *H. sapiens* male 46<2n>XY B) Parental NB3 (Par.1A). C) NB3 rCisplatin (Cis.1X). D) NB3 rVincristine (rVin.1A). All metaphases were stained with DAPI (Blue); the scale bar in each image is representative of 10μm. Arrows with numbers annotate the determined chromosome seen as represented by the false colour signal (Table 6.1) size of signal is not indicative of strength of signal.
Figure 6.4. Hypothesised Phylogenetic tree as per results from FISH panel 1

Individual subpopulations of all three cell lines arranged by predicted lineage based upon commonality between aberrations. The first 3 letters classify the cell line, the number represents the FISH panel and the last letter represents the subpopulation. Par.1A and Vin.1A is the ‘modal’ cell population found, representing the hypothesized ‘ancestral’ subpopulation. Where a modal subpopulation is not found, the ‘average aberrations’ is given, denoted by $\bar{X}$. Parental is Black, rVincristine is Purple and rCisplatin is green. The dotted line represents the original hypothesised progenitor.
### Table 6.6. Probe panel 2 aberration frequency across all cell lines.

Displayed is the level of individuals of the given cell line population that expresses a particular aberration as compared to the total number of nuclei examined for that cell line in probe panel 2. Aberrations are identified and classified as if ranked against a normal 46<2n>XY genotype as a result of FISH results. A ‘-’ notates that this particular abnormality was not found within this cell line.
FISH results from panel two (Table 6.6, Figure 6.5), likewise to the first panel, indicated towards a presence of subpopulations defined by chromosome features with varying levels of abundances. Genotypes of these subpopulations were chosen to represent the state of the individual cell lines and are as follows.

**6.3.2.1 Modal Parental**

Within the parental cell line in context of results from panel 2, the most abundant genotype (and will be identified as Par.2A) was as follows (53% of sample population);

\[ Par.2A = t(16;macro?)(q22;p?), -11 \]

**6.3.2.2 Modal rVincristine**

The rVincristine cell line, the most abundant genotype (rVin.2A) was as follows (27% of sample population);

\[ rVin.2A = +3 \]

**6.3.2.3 Average rCisplatin**

Similar to before, the rCisplatin sample did not show any non-unique metaphases to derive a modal genotype. Likewise to the previous panel, the modal aberration for individual chromosomes was selected to represent the ‘modal’ (rCis.2\( \overline{X} \)) as follows;

\[ rCis.2\( \overline{X} \) = +3, t(3;?)(3qArm;?), +4, +11, dup(11q), +der(11)t(11p;6q)(?;32.33), t(16;macro?)(q22;p?), -22, t(22;?)(11.21;?), t(22;?)(11.21;?), dir(22)t(22;16)(qter;q22) \]
Figure 6.5. Merged multilayer FISH images of Human and three NB3 cell lines from panel 2

A) Normal *H.sapiens* male 46<2n>XY. B) Parental NB3 (Par.2A). C) NB3 rCisplatin (Cis.2X). D) NB3 rVincristine (rVin.2A). All metaphases were stained with DAPI (Blue); the scale bar in each image is representative of 10μm. Arrows with numbers annotate the determined chromosome seen as represented by the false colour signal (Table 6.2) size of signal is not indicative of strength of signal.
Figure 6.6. Hypothesised Phylogenetic tree as per results from FISH panel 2

Individual subpopulations of all three cell lines arranged by predicted lineage based upon commonality between aberrations. The first 3 letters classify the cell line, the number represents the FISH panel and the last letter represents the subpopulation. Par.2A and rVin.2A are the ‘modal’ cell population found, representing the hypothesized ‘ancestral’ subpopulation. Where a modal subpopulation is not found, the ‘average aberrations’ is given, denoted by $\overline{X}$. Parental is Black, rVincristine is Purple and rCisplatin is green. The dotted line represents the original hypothesised progenitor. Note that rVin.2D appears twice to highlight the similarity between subpopulations in each cell line.
**Table 6.7. Probe panel 3 aberration frequency across all cell lines.**

The quantity of individuals of the three differing cell line populations that shows the given aberration as compared to the total number of nuclei examined for that cell line in this probe panel. Aberrations are identified and classified as if ranked against a normal 46<2n>XY genotype in light of FISH results. A ‘-’ notates that this particular abnormality was not found within this cell line.

The final panel contained less variation of chromosomes due to the fluorophore required for individual probes, for example chromosome 21 required three coloured fluorophore severely limiting what it could be paired with. Regardless, even with a reduced number of targeted chromosomes, the panel and FISH results provided enough data to comment on the heterozygosity of the three samples (Table 6.7, Figure 6.7), keeping with the consistency of previous panels. Populations were analysed and subpopulations identified with differing genotypes, again characterised by identifiable chromosome aberrations (Figure 6.8).
6.3.3.1 Modal Parental

The parental line was found to have a most abundant genotype (Par.3A, 33% of sample) and is as follows;

\[ Par.3A = -17, \text{dic}(17) \]

6.3.3.2 Modal rVincristine

rVincristine, with the most abundant genotype (rVin.3A) was as follows (44% of sample population);

\[ rVin.3A = +13, +21 \]

6.3.3.3 Average rCisplatin

With no homologous metaphases identified, the modal aberrations are selected to represent the ‘modal’ (rCis.3X) as follows;

\[ rCis.3X = +9, t(13;macro?)(q14.3;?), +15, t(16;macro?)(q22;p?), -17, \text{iso}17(q), \text{dic}(17), +\text{der}(17)\text{dup}(17q)t(17;15-\text{or}-13)(p;p11.1-\text{or}-q34), +21 \]

6.3.3.4 Variants of Chromosome 17

Chromosome 17 within this panel has highlighted very different abnormalities, from a suspected isochromosome, a di-centric chromosome and a possibly highly duplicated q arm. As seen in table 6.7, these variants are somewhat found in the parental and rCisplatin yet absent in rVincristine. As the probe for chromosome 17 used is a centromeric probe, it is possible to predict the lengths of the p and q arms via FLpter scores to further quantify and classify these seen variants.

As both cell lines possessed a normal 17, individual metaphases were measured (section 2.2.2.1) for all chromosome 17 variants to be directly compared to its own chromosome 17 to account for the differing levels of chromatin condensation seen across metaphases within the same sample. Each ‘normal’ 17’s length is represented by ‘p+q=1’ where p and q can be derived from the FLpter scores to give a ratio of length of arms with associated physical length. These lengths can be directly compared to the variants within the same metaphase measured for their own respective FLpter scores in order to identify characteristics about them; data is presented in table 6.8.
Parental p=0.2778±0.0133
q=0.7222±0.0133

ST->FS= 0.206±0.011
FS->SS= 0.700±0.150
SS->EN= 0.093±0.008
1.73±0.084

rCisplatin p=0.2833±0.0125
q=0.7167±0.0125

ST->FS= 0.308±0.081
FS->SS= 0.604±0.01
SS->EN= 0.087±0.008
2.79±0.113

Table 6.8. Results from chromosome 17 variant analysis

FLpter analysis of chromosome 17 and its derivatives presumed to be malformations of chromosome 17 in all cell lines. Use of ‘normal’ 17 FLpter scores within singular metaphases as compared to the malformed 17 present in said metaphase.

ST=Start of chromosome. FS= first probe signal. SS=second probe signal. En=end of chromosome.

Each segment of the di-centric chromosomes were split up into; Start to first signal (ST->FS), First signal to second signal (FS->SS) and lastly second signal to end (SS->EN) and compared to their metaphase counterpart to identify possible p and q segments. Analysis signified that the length of individual segments from the di-centric chromosome did not match either p or q value of the normal 17 previously obtained.

Distances between segments could be represented as a ratio between parts and therefore compared across cell lines to see if they were homologous. The data obtained (table 6.8) indicated that these are in fact differing di-centric chromosomes. Total size relative to the normal 17 was also obtained and is also displayed in table 6.8. With low abundance, the parental isochromosome could not be adequately measured, yet this was not the case of rCisplatin. A ‘p’vs’q’ length was obtained and presented in table 6.8. The presumed duplicated q arm 17 found in the rCisplatin line was measured in a similar fashion as previous examples, measurement of FLpter scores and the proportional representative as length in respect to its ‘normal 17’ metaphase pair. Data is presented in table 6.8 to determine the makeup of this chromosome.
Figure 6.7. Merged multilayer FISH images of Human and three NB3 cell lines from panel 3
A) Normal *H. sapiens* male 46<2n>XY. B) Parental NB3 (Par.3A). C) NB3 rCisplatin (Cis.3X). D) NB3 rVincristine (rVin.3A). All metaphases were stained with DAPI (Blue); the scale bar in each image is representative of 10μm. Arrows with numbers annotate the determined chromosome seen as represented by the false colour signal (Table 6.3) size of signal is not indicative of strength of signal.
Figure 6.8. Hypothesised Phylogenetic tree as per results from FISH panel 3

Individual subpopulations of all three cell lines arranged by predicted lineage based upon commonality between aberrations. The first 3 letters classify the cell line, the number represents the FISH panel and the last letter represents the subpopulation.

Par 3A and rVin3A are the ‘modal’ cell population found, representing the hypothesized ‘ancestral’ subpopulation. Where a modal subpopulation is not found, the ‘average aberrations’ is given, denoted by $\overline{\text{X}}$. Parental is Black, rVincristine is Purple and rCisplatin is green. The dotted line represents the original hypothesised progenitor.
6.3.4 Predicted modal cell genotypes

Whilst somewhat incorrect to assume a subpopulation from one probe panel is identical to the same subpopulation shown in another probe panel without a complete probe panel, a more probable expected genotype can be hypothesised of the most abundant genotypes seen. Below, in context of the FISH probe panels, predicts would be the most common subpopulation genotype seen in each individual cell line at this stage of ‘tumour’ progression. (Lines are presumed of male origin due to only one X being seen consistently, if not then X0 for all cell lines).

**Parental** = <2n> XY?, -1, +der(1) dup(1;2)(pter;23.1-23.2);t(1;18)(p?;p11.1), -11,
+12, t(16;macro?)(q22;p?), -17, dic17, -18

**rVincristine** = <2n> XY?, +der(1) dup(1;2)(pter;23.1-23.2), +13, -18, +21

**rCisplatin** = <2n> XY?, +der(1)t(1;18)(p?;p11.1), +3, t(3;?)(3qarm?;?), +4, +8,
+9, +11, dup(11q), t(13;macro?)(q14.3;?), +14, +15, t(16;macro?)(q22;p?), -17,
iso17(q), dic17, +der(17)dup(17q)t(17;15or13)(p;p11.1 or q34). -18 +21, -22,
t(22;?)(11.21;?), t(22;?)(11.21;?), dir(22)t(22;16)(qter;q22)
6.4 Scale of aberrations identified within cell lines

Aberrations away from the modal (Parental and rVincristine) or average (rCisplatin) were calculated to assist the prediction of phylogenetic trees. Each lines population’s average aberrations away from the modal subpopulation can be calculated across probe panels to highlight the deviation across these populations, a means to highlight the level of homo or heterozygosity. As seen in figure 6.9, parental and rVincristine lines exhibit on average between 1 to 3 aberrations from the mean with minimal deviation, opposed to rCisplatin which has a much higher level of average aberrations and variation across all panels. Examples displaying the most abnormal cell lines discovered within the sample over each cell line with each probe panel are shown in figure 6.10.

Figure 6.9. Average aberrations away from the modal cell subpopulation in each panel tested.

A count of differing chromosome aberrations individual cells are away from the ‘modal cell’ of that population. Each value is given as an average of the altered nuclei chromosome aberration count (not including modal cell populations) with Standard error of the mean shown as error bars. Each group of three represents the three different cell lines used in this study, with each grouping from left to right, panel 1 (p1), panel 2 (p2) and finally panel 3 (p3).
Figure 6.10. Examples of the most mutated subpopulation metaphases in respect to the ‘modal’ subpopulation

Subpopulation merged metaphase examples from each cell line (rows) from each probe panel (columns, left to right, 1, 2, 3). Colours refer to the specific false colour attributed to each probe as seen in the tables in section 6.1.4. Each metaphase has the highest aberration count seen as compared to the modal or average mutation as reported; to represent some of the most phylogenetically distant subpopulations identified.
6.5 Discussion

The use of multilayer FISH has allowed the identification of novel chromosomal aberrations alongside identification of sub-populations indicating a level of heterogeneity in all cell lines studied. The presence of unique aberrations and the level of heterogeneity within both resistant neuroblastoma cell lines could be indicative of a mechanism for said resistance or a means to identify as a ‘marker molecule’. Thus in light of these results, we can say it has been worthwhile to apply the multilayer methodology to improve current understanding in retrospect to just using NGS techniques.

Detected throughout all three panels were clear divisions of genotype across sub-populations, from which presumed phylogenetic assignment can be hypothesised as seen in figures 6.4, 6.6 and 6.8. Whilst hypothetical lineages have been presumed, in no way should results seen in this section be treated as indicative of the true phylogenetic assignment of these cell lines as evident by results presented. Results from this study should instead be used as an introductory insight into the level of heterogeneity and level of chromosomal variety within these cell lines to highlight the need for more in-depth multilayer FISH testing. Nevertheless there is somewhat evidence that shows the derivative nature of both resistant cell lines from the parental, indicated by shared chromosomal aberrations.

6.5.1 Oncogenic properties of aberrations

Shown throughout all FISH results panels are the conservation of specific chromosomal aberrations across the cell lines. An interesting aberration is that of chromosome 1, seen in figure 6.2 and figure 6.3 where seemingly the distal p arm has been lost, most likely through deletion events as common in advanced neuroblastomas (Maris et al., 2001), and insertions of chromosome 18 and 2p.23.1-23.2 (the loci of ALK, which is frequently involved in translocations that lead to malignant gene fusions (Van Slooten et al., 1998). Fascinatingly the parental derive 1 has both of the 18 and 2 insertion yet each resistant line has lost one of these aberrations rCisplatin the ALK from chromosome 2 and rVincristine has lost the 18 insertion. Loss of either insertion is clearly not lethal however the presumed importance would be from the presumed loss of 1p36.3 location of a tumour suppressor gene (Munirajan et al., 2008). The loss of a whole chromosome 18 is near universal across all cell lines, bar the possible insertion into chromosome 1, is the loss of BCL-2 a regulator of apoptosis implicated in aggressive tumours with proliferative activity (Van Slooten et al., 1998; Van Goethem et al., 2017).
The abundant copy number amplification of chromosome 2 in rCisplatin could resolve in the increased dosage of MYCN, associated with many tumours but in particular neuroblastomas (Durbin et al., 2018; Hald et al., 2019). The translocation of chromosome 6p21 onto a chromosome 11 in 85% of rCisplatin cells examined indicates the translocation of the CCND3 gene. CCND3 is within the conserved cyclin family to regulate progression through the cell cycle (section 1.1.3) and seen in many cancer cases either as a translocation (Wlodarska et al., 2008; Beltran et al., 2013) or observed to undergo mutations (Cancer Genome Atlas Network, 2012; Arneja & Gujar, 2015; Rohde et al., 2017).

Chromosome 11 loss and gain across subpopulations of rCisplatin, absence of NCAM at 11q23.2 is seen as an unfavourable prognostic phenotype for advanced stage neuroblastoma as seen in 33% of cells identified in this study (Valentiner et al., 2011). Alternatively the apparent duplication of chromosome 11q in 79% of rCisplatin cells could induce over expression of cyclin D3, another cell regulatory gene that has association in some cancers (Lopez-Beltran et al, 2010).

There is an apparent duplication and insertion of 16q22 in the parental line and rCisplatin, this probe is specific for CBFβ which is of significance in cancer development such as leukaemia (The Cancer Genome Atlas Network, 2012). Translocation events cause impaired functionality of CBFβ, and form oncogenic fusion proteins further contributing to the oncogenesis factors of these cell lines (Park et al., 2010; Noort et al., 2018). Within rCisplatin observed with near exclusively, bar a small sub-clonal population within rVincristine, there are multiple aberrations regarding chromosome 22. With either whole chromosome loss or apparent translocations with many other chromosomes (16 and other unidentified) indicates a possible generation of oncogenic fusion proteins. The BCR gene located on 22q (section 1.3.2.3.1) has been implicated in many fusion proteins and could relate to the progressive tumour features of this resistant cell line.

6.5.2 State of heterogeneity and comparison to previous studies

The level of heterogeneity within all three cell lines was larger than was originally anticipated. Previous work involving the generation of these neuroblastoma cell lines relied on SKY to assign their respective karyotypes (Kotchetkov, 2005). Detailed here are abnormalities observed within this study, such as the derivative chromosome 1 with translocations, the whole chromosome loss of 18, an extra 21 and 13 in rVincristine, only one chromosome X, and more. However, rVincristine was found to have the derivative 1
t(1;18) which was not observed in a single instance within this study, and instead exclusively found within rCisplatin. In comparison of results, whilst the ‘average’ cytogenetic assignment is comparable in both studies, lower frequency aberrations are seen within sub-populations which are not detailed in the report. Especially within the parental line, the loss of chromosome 11 is not seen alongside the large aberrations regarding chromosome 17 (detailed below).

These findings could be summarized by two different events, either as individual or most probable to be a combination of both. Firstly, the SKY methodology used incurred erroneous assignments due to the previously described fluorochrome ‘sandwich effect’ (section 1.1.5.3) hiding translocations or causing false positives. With the use of SKY comes the question of number of singular cells examined with the post-analysis of deciding which aberrations are non-representative of given karyotype. Secondly, there could be an element of continuous evolution of said cell lines over time; a new sub-clonal population arises to out-compete the previous dominant with new chromosomal aberrations. This factor is relevant more so to the specific tumour microenvironment (section 1.3.4.1) within a person, yet the possible progression seen could comment on the microenvironment of ‘cell culturing’ and the effect that has on the genome of cancer cells. Far beyond reach and aims of this thesis, but it is plausible to be of focus in further studies.

With regards to the heterogeneity seen in the parental and rVincristine somewhat displays what to be expected with a ‘progenitor’ being the initial population that has been selected for to give rise to a genetically diverse population. Here observed is a ‘core’ high frequency common genotype population with smaller populations branching off in regards to further low number chromosomal mutations (figure 6.9). This process would generate populations that would slowly drift from the ‘progenitor’ but fail to establish large subpopulations due to a lack of selective pressure. Quite the opposite story is that of rCisplatin, in none of the panels tested here was there a single cell that existed as a ‘clone’ of another. In other words, every singular cell examined was unique at the chromosomal level; true there were commonalities with chromosomal aberrations shared amongst individuals, but a high level of genomic instability must exist to have caused this large variation (also represented by large unstable chromosomal count (section 6.2).

The suggestion is that the level of heterogeneity itself can be used as a bio-marker for treatment/progression as a more heterogeneous tumour may be more likely to contain a resistant sub-population (Marusyk et al., 2012). This could be represented with the
parental/rVincristine direct overlap observed in panel 2, exact aberrations detected in cells of parental and rVincristine samples are the same. rVin.2D is found within both samples and more interestingly Par.2E (in low frequency) exhibits the same chromosomal features as rVin.2A the most abundant population within rVincristine. This somewhat gives some evidence that there may be resistant populations residing as low frequency populations within the ‘parental tumour’ that could be selected again with introduction to more selective pressures.

6.5.3. On the chromothripsis of chromosome 17

Chromosome 17 has mass aberrations within the parental and rCisplatin cell line (and surprisingly not observed in rVincristine), location of p53. Within neuroblastoma, p53 mutations are rare at first diagnosis/testing yet seen after a selective pressure, such as chemotherapy, to be seen as a potential role in acquiring of drug resistance (Xue et al., 2007). Obviously there may be genetic manipulations within the gene that the study presented here cannot detect, but the vast aberration of chromosome 17 within the parental and rCisplatin is something of great interest. The suspected isochromosome 17(q), as determined from (table 6.8), would cause a lower dosage of P53 (as is located on the p arm) regardless of its ‘coding-gene-mutation-state’ to infer a higher oncogenic state of the cell. Isochromosome 17(q) specifically within neuroblastoma is seen as one of the more frequent isochromosome aberration seen within neoplasia and also used as a marker for poor patient survival (Gilbert et al., 1984; Barbouti et al., 2004; Mendrzyk et al., 2006). The presence of a di-centric chromosome seen consistently in both lines indicates an inheritance from the parental to rCisplatin at time of ‘branching’, not to presume it has been the selective initial cytotoxic drug resistant causing aberration. However, there is variation within this di-centric chromosome across the two lines; the rCisplatin’s being almost twice the length of that in the parental (table 6.8). The ratio between ‘segments’, described within the table are seemingly near equal between the cell lines, indicating a post-branching duplicative event across the whole di-centric chromosome within rCisplatin. The derivative chromosome 17 only seen within the rCisplatin can be somewhat explained via this study. The ‘p’ arm has the same relative ‘length’ as a normal 17q, although some with translocations from chromosome 13 or 15 yet this is presumed to be balanced. The new ‘q’ arm for this longer 17 is presumed to be three duplications of the original 17q or an amalgamation from something else entirely.
Regardless of specific state/location of p53, both parental and rCisplatin have in-depth malformations of their chromosome 17 and derivatives. A plausible explanation is the event of a single catastrophic process within the parental that catalyzed the ability for large swathes of genomic instability seen explicitly within rCisplatin. A ‘possible’ singular chromothriptic event regarding chromosome 17 within a sub-population of the parental cell line allowed the onset of a possible rCisplatin precursor with higher levels of genomic instability due to a lower dosage of p53. This event must have happened after the branching of rVincristine due to two apparently normal copies of chromosome 17 observed in all cells and vastly less noted genomic instability indicated by lower aberration counts and lower levels of heterogeneity. Somewhat interesting as previous reports a loss of WT p53 in these cells (Kotchekov, 2005). Vincristine is thought to induce cellular death by p53-independent-pathways as is a microtubule binding drug to prevent the expansion of mitotic spindles thus cellular division. By vincristine resistant cells circumventing this requirement the resistant cells must have alternative mechanisms to prevent the cytotoxic action of vincristine (Hientz, 2017). The resistant cells therefore can maintain a relatively stable (as compared to rCisplatin) genome as their mechanism(s) of resistance has little interaction with direct DNA mutative events to cause instability and instead allows the correct formation of spindle fibers for cellular replication. Cisplatin resistance can arise through increase in DNA repair mechanisms (Lin & Howell, 2006; Heintz, 2017), this somewhat explains the large heterogeneity and large chromosome count/vairability (Rao, 1998) observed in the resistant cell line. With increased DNA repair, any breakages or replicative errors (caused through the direct DNA damaging action cisplatin or alternative means) can be successfully repaired and not drive apoptopic factors to cause cellular death. With a population of resistant cisplatin cells via the action of upregulated repair mechanisms (TP53 linked or not) would be subjected to mutations on the DNA at different locations across the genome to be repaired independently of one another and not become ‘unselected’ and therefore push this high level of growth and heterogeneity observed. The incidence of chromothripsis is seen to be correlated with the lack of p53 ((Rausch et al., 2012; Cai et al., 2014; Fernandez-Banet et al., 2014; Bochtler et al., 2017) where the lack of activity to induce cell cycle arrest can explain the faster growth of rCisplatin observed from previous personal observations of culture maintenance. Furthermore these chromoanagenesis events are seen consistently in neuroblastoma and other childhood cancers paired with poor prognosis most likely caused by the cytotoxic drug resistance (Molenaar et al., 2012; Luijten & Ting, 2018).
One can presume that the addition of the cytotoxic drug cisplatin during selective growth for rCisplatin cells had indeed induced a ‘chaotic’ survival event with more chromoanagenesis like aberrations throughout its genome spawning many derivatives, as represented by its heterogenic state, which are resistant to the drug. The lack of heterogeneity seen in the parental line, as compared to rCisplatin, can be explained by the lack of a selective pressure to cause a resistant sub-population to become dominant. Furthermore this pairs with the fact that rVincristine has much lower levels of genome reorganization and chromoanagenesis like events by the lower aberration and heterogeneity level seen.

6.6 Conclusion

Analysis of results provided by the use of the multilayer method within this study it is notable that there has been a new perspective of already established cancer cell lines and their cytotoxic drug resistant derivatives. This suggests that opposed to a ‘bottom-up’ approach, whereby prospective oncogenic genes are analysed via specific base pair sequences for mutations and copy number, a ‘top-down’ view may be considered alongside to look for aberrations first then investigate what it may specifically cause. It is plausible to say that both methodologies are worthwhile into the investigation of cancer lines for causative effects, treatment options, detection of heterogeneity, identification of biomarkers and possible evolutionary pathways taken. One must also be aware that genomic aberrations may not be indicative of phylogenetic attributes seen. In the example of the rCisplatin cell line, a vast diversity in the genome across the population may not have a reflection upon the characteristics of the cancer.

With reference to patient diagnostics and treatment options, the current means is dependent on specific tumour drivers presented, such as ALK, MYCN and TrkB. The current focus is placed more so on therapeutic developments such as immunotherapy and targeted delivery of drugs opposed to specific treatments for specific ‘genomes’ (Pastor & Mousa, 2019). Whilst it is true that initial biopsies of tumours yield invaluable genomic information to refine treatments and prognosis, it is clearly evident that genomic evolutionary models and modes of resistance be developed to fully grasp the idea of a personalised treatment program.
What this study has indicated is that there is an overabundance of future work that can be proposed. One such would be the idea of a probe specific to each human locus along a chromosome or being tailored to specific genes/aberrations of interest (e.g. MYCN). Here within this study, it was only possible to use a ‘one probe to many chromosome’ approach rather than a ‘many probes to single chromosome’ demonstrated within previous chapters due to reagent constraints (hence why not every chromosome was assessed due to lack of specific probe). However even with a limited pool of specific FISH probes it has demonstrated the usefulness of the multilayer approach by efficiently and cheaply providing a worthwhile insight into cancer genomics.
7. General Discussion

7.1 Summary of accomplishments

In regards to the aims and themes set out within the opening chapter, I believe that this thesis has been successful in demonstrating how each of these aims was accomplished.

1. The analysis of the ‘state of play’ for current in situ hybridisation, with the testing of new reagents and current commercially available buffers, has allowed an apt view in regards to the use of FISH to solve comparative genomic questions. The supposed high throughput ‘fast’ hybridisation buffer performed marginally better than the current ‘standard’ formamide based buffer and would reliably reduce hybridisation times for experiments. However, with regard to a comparative genomics lab (opposed to a time sensitive diagnostics lab) the gain of output is vastly insignificant to the costs attained for this alternative faster acting buffer. In light of this, a new means of increasing effective output vs. cost was required and the refined multilayer methodology was devised. Further refinements and augmentation of this methodology can only be of benefit to reliably use more layers (as demonstrated up to 10) for further research.

2.

a. Detection of intrachromosomal rearrangements within the Galliform order has been presented within this thesis; hypothesised to exist yet overlooked due to the nature of this order and methodologies needed. The identification of novel rearrangements within the ‘conserved’ macro-chromosomes of these species has brought light onto the fact that more may yet exist within this order and are worthy of attention to further influence phylogenetic assignments. One rearrangement within this order is to be of note and relevant to recent re-assignment of E.chinensis from Coturnix to Excalafactoria indicating a clear chromosomal event which may exist within other members of both species to refine their assignments between these two genera.

b. Although only three of the five chromosomes studied were suitable enough to analyse within an intra-nuclei arrangement, the multilayer method was sufficient at generating data to allow a preliminary insight into folding
arrangements as a de-condensed state. The generation of a ‘skeleton’ system to computationally organise and path/fold has been of great benefit as it has shown that there is somewhat of a distinctive conserved folding pattern of the macrochromosomes studied indicated by a ‘horseshoe’ shape. Further studies are obviously required to validate these findings and see if the same principle occurs for other chromosomes in other species or an alternate arrangement is found. This work also highlights that spatial modelling in a 2D environment is fundamentally flawed in design, yet with some conclusions being drawn from this study it shows that it may be worthwhile to adapt the multilayer method into a 3D methodology. Furthermore, adaptation into 3D nuclei arrangement would pair well with Hi-C methodologies of current as a two angle approach to determining arrangement of chromosomes, especially in species not commonly researched upon. Multilayer FISH could be performed rapidly and fairly inexpensively on any species of choice, even those hard to source and grow as only interphase stage cells are required whereas bespoke libraries for Hi-C will have to be created.

3. Adapting an existing multiprobe device it was possible to use within context of the multilayer method to refine and put forth an updated karyotypic representation of the DT40 cell line. With comparison to previous research using next generation sequencing techniques, it has been highlighted that previously identified chromosomal aberrations exist in a different conformation that previously thought or do not exist at all. Use of the two combined methods allowed the quick classification of this cell line to inform researchers of genomic changers underpinning the features which make DT40 a useful research tool. Understandably, it is clear that this could be adapted further for other aberrated cell lines to assess the genomic state and level of heterogeneity.

4. Applying a ‘one probe to many chromosomes’ approach via multiple layers has allowed an easier system in order to classify vastly rearranged cancer lines and their derivative cytotoxic daughter lines. As shown within chapter 6, there are observable chromosomal differences between each cell line which may be indicative of a means of cytotoxic resistance or a biomarker used for other
neuroblastomas. Interestingly it is also seen that the level of heterogeneity within these cell lines vary considerably, with the parental and rVincristine having a key ‘modal’ sub-population with lesser frequent alternative sub-populations with minor genomic structural changes, rCisplatin expresses the opposite where there is no modal sub-population seen with every cell possessing an unique cytogenetic assignment. Further work is required to assess the nature of other chromosomes within these cell lines, and the possibility of sequential probes across chromosomes of interest (such as the possible chromothripsis of 17) to understand the mechanisms of this cancer better.

To summarise, this thesis has shown that the technique of FISH is suitable for modern genomic studies across different fields via use of the multilayer methodology enabling extensive data to be produced at a much low cost compared to previous FISH methodologies (section 3.6.3). Rightly so, the technique of FISH has been effectively ‘refreshed’ for use in a modern day laboratory. Also shown, new methods that can apply to cytogenetic studies are; a means of testing new commercial or homemade buffers and the creation of algorithms to determine chromosome probe order and assignment in interphase nuclei. These techniques alongside the multilayer method have demonstrated usefulness in a wide range of cytogenetic areas, such as the identification of rearrangements that exist within previously studied species, an updated assignment of a useful research cell line, and the exploration into human neuroblastoma cell lines which have gained resistance to modern day cytotoxic drugs. These summarise a small area within cytogenetics where the multilayer method could be a valuable tool for and many more questions can be aided furthermore in respect to the work outlined in this thesis.

7.2 Impact of the Multilayer Fluorescent in Situ Hybridisation method

As previously stated the use of the multilayer method has enabled a variety of areas to be investigated relating to multiple areas of genetics. The number of studies that exist whereby the use of whole exome sequencing as compared to a single cell approach is vastly increasing (Figure 1.16, Shi, 2018), it has been reported and seen in many studies/cases that this wider approach can miss small yet significant events relating to a multitude of issues, the key one being the development of resistant cancers (Baca, 2013; Navin et al., 2011; Storchova & Pellman, 2004; Storchova & Kuffer, 2008; International
The use of multilayer FISH can increase the usefulness of the cytogenetic research opposed to sole reliance on NGS techniques to somewhat balance the scales between the two overarching approaches. The reduction of cost as demonstrated in chapter 2 (approximately four times the difference when performing large studies, using the example from 3.6.3, applied to 23 chromosomes of a human we would expect a cost of £210 for the multilayer method as compared to £1,189 for traditional FISH) should be an important factor in use of this methodology as costs of multiple fluorophores and a limitation of number of fluorophores used at any one time is a major off-putting reason to abstain from use of FISH techniques (Eastmond et al., 1995; Hasty & Montagna, 2014). Now having established a technique that has been shown to display useful results across multiple areas of study, one should hope that its application has a positive effect across multiple genomic fields and allow the appreciation of FISH as a suitable 21st century technique.

7.3 Future studies in light of this thesis

Presented throughout this thesis are many examples of areas of research that could be further explored in light of results presented here, however here I wish to highlight some key areas that are worthy of being singled out.

1. Re-investigating intrachromosomal rearrangements within species of similar order to reinforce phylogenetic assignment. As seen in chapter 4, there are hidden intrachromosomal modifications which have been overlooked by traditional painting methods to be detected using multilayer FISH. These may be of significant use in the classification of debatable existing assignment for any organism with established sequences along their chromosomes to generate probes. This would be a substantial aid, to be paired alongside NGS techniques, for evolutionary cytogenetic areas of questionable assignment.

2. The generation of a standalone script or plug-in to adapt methods represented in chapter 4 to correctly assign and interpret signals within an interphase nuclei. Far beyond the reach of this thesis, the means to correctly identify folding patterns of chromosomes within nuclei would be a tremendous factor to better understand gene expression and control in any cell. In particular, finding differences between a ‘normal’ and aberrated chromosome, such as in the event of a cancer within an interphase state can only be of benefit to make
links towards how a mutative event in the genome can induce observable phenotypic changes across a cell. Furthermore, future work to follow on from the expansion of the current chromosome folding software would be to adapt its usage for a 3D environment, such as with a confocal microscope, which would only benefit users further.

3. More work should be conducted on the NB3 cell line and its resistant derivatives to close links between the phylogenetic trees proposed within chapter 6. As limited by probe material in this study, the generation of varied panels of probes for every chromosome can fully assess the genome at a single cell level and reflect upon the heterogeneity across the cell line population. It would be of great reward to synthesise a system of ‘a BAC at every loci’ to be applied to future cell lines of interest (possibly in a similar fashion to the chromoprobe device used in chapter 5) to assess the genome of a cancerous cell line from a ‘top down’ perspective.

4. The excessive heterogeneity identified within chapter 6 can be used to comment on the genomic instability cancers embrace, especially those with poor prognosis and limited clinical choices. It is also reflective of the state of research cell lines used over many years, as they may have mutated to become non-comparable as compared to when they were first isolated; is this a disadvantage of laboratory-based research? I would propose that (with aid of expert clinicians) when a primary tumour is identified within a patient the genomic arrangement be tracked at this stage and at further biopsies during the course of treatment. Here possible clinical events such as remission, reoccurrence and resistance could be identified throughout treatment rather than a singular ‘snapshot’ from a single biopsy (which could be at any stage of cancer progression) that may not be representative of the cancer at these key events leading to false avenues being followed.
7.5 Concluding remarks

The journey that this PhD and culmination into a thesis is one that will leave a lasting effect upon me, which I shall look fondly upon in the future. The opportunity to be involved in a field of vast progression and groundbreaking research where I have somewhat contributed towards is an achievement I can be proud of. With the synthesis of the multilayer method, I like to entertain the possibility that this is a means of giving back to the scientific community; to further aid research in a multitude of genetic fields for the selflessness means to benefit society as a whole. I will treasure the fact that I have been able to isolate myself in such a precise niche of scientific research by solving the problem of modernising FISH to apply to many different yet interesting areas of biology where in truthfulness each deserve a whole PhD project of their own to fully appreciate.

With such a deeper understanding of genomics at this time we shall soon expect vast improvements within many aspects of everyday life. Even now there are many products that allow a level of personalised genomic information to be revealed such as ancestry and a drive within the National Health Service to fully sequence multiple thousands of healthy patients for medical focused research. The ‘genomic age’ is soon to be upon us with extensive personalised medicine and tailored products we cannot even start to guess may look like. To comment on the state of society and it’s capability for these upcoming changes is far too in-depth for me to comment on, however though understanding of biology and genetics shall enable a prepared generation to exceed and flourish in this new age. This is but a single reason of many to direct my own future endeavours; by pursuing a career in education, to divulge the wonders of life (governed by genetics) to future generations we can be proud of.
8. References


202


Hancock, J. F. (2012). Plant evolution and the origin of crop species. CABI.


association loci for human diseases and traits. Proceedings of the National Academy of Sciences, 106(23), 9362-9367.


Lindström, S., Ma, J., Altshuler, D., Giovannucci, E., Riboli, E., Albanes, D. & Chanock, S. J. (2010). A large study of androgen receptor germline variants and their relation to sex hormone levels and prostate cancer risk. Results from the National Cancer Institute Breast and Prostate Cancer Cohort Consortium. The Journal of Clinical Endocrinology & Metabolism, 95(9), E121-E127.


Molnár, J., Póti, Á., Pipek, O., Krzystanek, M., Kanu, N., Swanton, C., ... & Szüts, D. (2014). The genome of the chicken DT40 bursal lymphoma cell line. G3: Genes, Genomes, Genetics, 4(11), 2231-2240.


chromosomes of platypus imply recent origin of mammal sex chromosomes. Genome research, 18(6), pp.965-973.


Welcsh, P. L. & King, M. C. (2001). BRCA1 and BRCA2 and the genetics of breast and ovarian cancer. Human molecular genetics, 10(7), 705-713.


## 9. Appendix

<table>
<thead>
<tr>
<th>Chromosome number</th>
<th>Identifier</th>
<th>Colour</th>
<th>Position</th>
<th>Loci</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 /5 /19</td>
<td>1/5/9 Alp.Sat</td>
<td>FITC</td>
<td>Centromere</td>
<td>FITC 1p11.1-q11.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>FITC 5p11.1-q11.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>FITC 19p11.1-q11.1</td>
</tr>
<tr>
<td>1</td>
<td>ALK Breakapart</td>
<td>FITC + TX</td>
<td>2P</td>
<td>FITC 2p23.2q23.1 TX 2p23.2-p23.1</td>
</tr>
<tr>
<td>3</td>
<td>Paint</td>
<td>FITC</td>
<td>Paint</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Alp.Sat.</td>
<td>TX</td>
<td>Centromere</td>
<td>TX p11.1-q11.1</td>
</tr>
<tr>
<td>6/14</td>
<td>IGH / CCND3</td>
<td>FITC + TX</td>
<td>6P + 14Q</td>
<td>FITC 14q32.33 TX 6p21</td>
</tr>
<tr>
<td></td>
<td>Translocation, Dual</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>fusion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Alp.Sat.</td>
<td>TX</td>
<td>Centromere</td>
<td>TX 8p11.1-q11.1</td>
</tr>
<tr>
<td>9</td>
<td>Alp.sat</td>
<td>FITC</td>
<td>Centromere</td>
<td>FITC 9q12</td>
</tr>
<tr>
<td>10</td>
<td>Alp.Sat.</td>
<td>FITC</td>
<td>Centromere</td>
<td>FITC 10p11.1-q11.1</td>
</tr>
<tr>
<td>11</td>
<td>Alp.Sat.</td>
<td>FITC</td>
<td>Centromere</td>
<td>FITC 11p11.1-q11.1</td>
</tr>
<tr>
<td>12</td>
<td>Alp.Sat.</td>
<td>FITC</td>
<td>Centromere</td>
<td>FITC 12p11.1-q11.1</td>
</tr>
<tr>
<td>13</td>
<td>D13S25 Deletion</td>
<td>FITC+TX</td>
<td>13Q</td>
<td>FITC 13q34 / Tx 13q14.3</td>
</tr>
<tr>
<td>15</td>
<td>Alp.Sat.</td>
<td>FITC</td>
<td>Centromere</td>
<td>FITC 15p11.1-q11.1</td>
</tr>
<tr>
<td>16</td>
<td>CBFβ (CBFB)/MYH11</td>
<td>FITC+TX</td>
<td>16P + 16Q</td>
<td>FITC 16p13.1/ Tx 16q22</td>
</tr>
<tr>
<td>17</td>
<td>Classical Sat.</td>
<td>Blue</td>
<td>Centromere</td>
<td>Blue 17p11.1-q11.1</td>
</tr>
<tr>
<td>18</td>
<td>Alp.Sat.</td>
<td>TX</td>
<td>Centromere</td>
<td>TX 18p11.1-q11.1</td>
</tr>
<tr>
<td>21</td>
<td>TMPRSS2/ERG Deletion/Breakpoint</td>
<td>FITC + TX + AQUA</td>
<td>21Q</td>
<td>FITC 21Q22.2.2-22.3 TX 21Q22.2.2-23.3 AQ 21Q22.13-q22.2</td>
</tr>
<tr>
<td>22</td>
<td>IGL Breakapart</td>
<td>TX+FITC</td>
<td>22Q</td>
<td>FITC 22q11.23 TX 22q11.21</td>
</tr>
<tr>
<td>X</td>
<td>Alp.Sat.</td>
<td>TX</td>
<td>Centromere</td>
<td>TX Xp11.1-q11.1</td>
</tr>
</tbody>
</table>
## Measuring FLPter script VS Manual measurement, Paired T-test results 4.2.1

<table>
<thead>
<tr>
<th></th>
<th>Script score</th>
<th>Manual score</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Probe A</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>0.04702</td>
<td>0.071992</td>
</tr>
<tr>
<td>Variance</td>
<td>0.001561</td>
<td>0.000505</td>
</tr>
<tr>
<td>Observations</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Pearson Correlation</td>
<td>0.58294</td>
<td></td>
</tr>
<tr>
<td>Hypothesized Mean Difference</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>df</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>t Stat</td>
<td>-3.47802</td>
<td></td>
</tr>
<tr>
<td>P(T&lt;=t) one-tail</td>
<td>0.001259</td>
<td></td>
</tr>
<tr>
<td>t Critical one-tail</td>
<td>1.729133</td>
<td></td>
</tr>
<tr>
<td>P(T&lt;=t) two-tail</td>
<td>0.002518</td>
<td></td>
</tr>
<tr>
<td>t Critical two-tail</td>
<td>2.093024</td>
<td></td>
</tr>
</tbody>
</table>

| **Probe B** |              |              |
| Mean   | 0.193485     | 0.224616     |
| Variance | 0.00168      | 0.000762     |
| Observations | 20           | 20           |
| Pearson Correlation | 0.250599    |              |
| Hypothesized Mean Difference | 0            |              |
| df     | 19           |              |
| t Stat | -3.21545     |              |
| P(T<=t) one-tail | 0.002277   |              |
| t Critical one-tail | 1.729133   |              |
| P(T<=t) two-tail | 0.004554    |              |
| t Critical two-tail | 2.093024   |              |

| **Probe C** |              |              |
| Mean   | 0.282626     | 0.306693     |
| Variance | 0.002182     | 0.001979     |
| Observations | 19           | 19           |
| Pearson Correlation | 0.532197    |              |
| Hypothesized Mean Difference | 0            |              |
| df     | 18           |              |
| t Stat | -2.37605     |              |
| P(T<=t) one-tail | 0.014403   |              |
| t Critical one-tail | 1.734064   |              |
| P(T<=t) two-tail | 0.028807    |              |
| t Critical two-tail | 2.100922   |              |

| **Probe D** |              |              |
| Mean   | 0.768715     | 0.707258     |
| Variance | 0.007119     | 0.001612     |
| Observations | 20           | 20           |
| Pearson Correlation | 0.32406     |              |
| Hypothesized Mean Difference | 0            |              |
| df     | 19           |              |
| t Stat | 3.399726     |              |
| P(T<=t) one-tail | 0.001503   |              |
| t Critical one-tail | 1.729133   |              |
| P(T<=t) two-tail | 0.003006    |              |
| t Critical two-tail | 2.093024   |              |

<p>| <strong>Probe E</strong> |              |              |
| Mean   | 0.82814      | 0.839619     |
| Variance | 0.001868     | 0.000427     |
| Observations | 20           | 20           |
| Pearson Correlation | 0.294843    |              |
| Hypothesized Mean Difference | 0            |              |
| df     | 19           |              |
| t Stat | -1.22068     |              |
| P(T&lt;=t) one-tail | 0.11857    |              |
| t Critical one-tail | 1.729133   |              |
| P(T&lt;=t) two-tail | 0.237141    |              |</p>
<table>
<thead>
<tr>
<th></th>
<th>Probe F</th>
<th>Total Length of chromosome</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>t Critical two-tail</strong></td>
<td>2.093024</td>
<td>2.093024</td>
</tr>
<tr>
<td>Mean</td>
<td>0.91841</td>
<td>104.9502</td>
</tr>
<tr>
<td>Variance</td>
<td>0.001354</td>
<td>747.042</td>
</tr>
<tr>
<td>Observations</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Pearson Correlation</td>
<td>0.538261</td>
<td>0.973931</td>
</tr>
<tr>
<td>Hypothesized Mean Difference</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>df</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td><strong>t Stat</strong></td>
<td>-2.32048</td>
<td>-4.46056</td>
</tr>
<tr>
<td><strong>P(T&lt;=t) one-tail</strong></td>
<td>0.015798</td>
<td>0.000134</td>
</tr>
<tr>
<td><strong>t Critical one-tail</strong></td>
<td>1.729133</td>
<td>1.729133</td>
</tr>
<tr>
<td><strong>P(T&lt;=t) two-tail</strong></td>
<td>0.031596</td>
<td>0.000268</td>
</tr>
<tr>
<td><strong>t Critical two-tail</strong></td>
<td>2.093024</td>
<td>2.093024</td>
</tr>
</tbody>
</table>