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Techniques for Quantifying Structure-Function Relationships of Interstitial Cell of Cajal Networks

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A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

in

Biomedical Engineering

University of Auckland, 2014
Abstract

Interstitial cells of Cajal (ICC) are specialised cells present throughout much of the gastrointestinal (GI) tract. These cells perform various functions to facilitate normal GI motility, with one of the most prominent roles being the electrical pacemaking of the GI tract. ICC loss and injury is now a major research focus as it is recognised as a hallmark of several GI functional motility disorders, but the mechanism relating ICC structure to GI function and dysfunction remain poorly defined. Progress in elucidating this mechanism is limited with experimental techniques alone due to factors including: 1) the absence of methods for quantifying the complex network structures formed by ICC; 2) challenges in associating cellular and tissue level activity across multiple spatial and temporal scales; and 3) the lack of a comprehensive ICC imaging data set encompassing large-scale network structures across a range of network properties. This thesis therefore aims to use a mathematical modelling approach to address these experimental shortcomings and investigate ICC network structure-function relationships.

A set of six numerical metrics were developed to quantify the structural properties of confocal ICC network imaging data: density, thickness, hole size, contact ratio, connectivity and anisotropy. These metrics were applied to discern the effects of various gene knockouts (KO) and postnatal maturation (three-day- versus four-week-old) on murine intestinal ICC network structural properties, allowing for the first detailed automated analyses and unbiased quantitative comparisons of ICC network structures. The analysis revealed a novel remodelling phenomenon occurring during $5-HT_{2B}$ KO (ICC depletion), namely a spatial rearrangement of ICC and the preferential longitudinal alignment of processes, and an apparent pruning of the ICC network occurring during postnatal maturation was identified as well. On the other hand, no changes in the ICC network structure were observed during $Ano1$ or $Spry4$ KO.
ABSTRACT

The feasibility of employing multiscale computational models to relate ICC network structure to its electrical pacemaker activity was then evaluated as the models can be upscaled to span multiple spatial and temporal scales provided sufficient computational resources. First, to demonstrate utility, the established biophysically-based simulation approach was used in conjunction with the developed ICC network structural metrics to investigate the structural and functional changes that occur in postnatal development (from birth to 24-day-old) of murine intestinal ICC networks. Four measures based on the average membrane potential and intracellular calcium concentration over the network ([Ca$^{2+}$]$_i$) were used to quantitatively assess the simulated ICC pacemaker activity: activation rate, peak [Ca$^{2+}$], time to peak [Ca$^{2+}$]$_i$ and half peak [Ca$^{2+}$]$_i$ time ratio. The results identified a pruning-like mechanism occurring during postnatal ICC network development which may facilitate mature digestive function, and elucidated the temporal course of this developmental process. Next, a new cellular automaton model was constructed and used to demonstrate impaired pacemaker activity propagation during ICC depletion. This simulation approach represents a more simplistic but computationally-efficient option in comparison to biophysically-based simulations.

Subsequently, an alternative strategy to experimental imaging for obtaining a comprehensive ICC network imaging data set encompassing large-scale ICC networks across a spectrum of network properties was presented. Both small- (0.225×0.225 mm with a resolution of 362×362 pixels) and large-scale realistic virtual ICC networks (≈25.2×12.4 mm with resolution of 40,500×20,000 pixels) were generated in silico using the stochastic Single Normal Equation Simulation (SNESIM) algorithm. The algorithm was also modified to enable virtual networks with a range of structural and functional properties to be generated. The SNESIM algorithm was then employed in conjunction with cellular automaton modelling to demonstrate the first multiscale computational framework in the GI field spanning from cellular structures to tissue level electrophysiology. The simulation results showed only a minor reduction in propagation velocities over 5-HT$_{2B}$ KO networks (4.3 mm/s) in comparison to WT networks (4.9 mm/s), which is logical as 5-HT$_{2B}$ KO does not affect intestinal transit times.

In total, the work presented in this thesis constitutes novel mathematical and computational tools which form a comprehensive framework providing unprecedented analyses and a virtual platform for investigating ICC network structure-function relationships. Further refinement of this framework combined with concur-
rent improvements in computational resources will enable the elucidation of these key structure-function relationships, leading to a more extensive understanding of GI physiology and pathology, as well as holding promise for the clinical utility of ICC network information from patients to transform the diagnosis, prognosis and treatment of GI functional motility disorders.
To my parents and sister, for their unconditional love and support.
Acknowledgements

Foremost, I thank the late Prof. Andrew Pullan for leading me into the fascinating world of research in biomedical engineering. I also thank my supervisors, Assoc. Prof. Leo Cheng and Prof. Rosalind Archer, for their guidance and support throughout this work.

Team and Collaborators: This work succeeded thanks to the assistance of the GI research group at the Auckland Bioengineering Institute. In particular, I thank Dr. Greg O’Grady, who contributed as a supervisor to this work. I am forever grateful for your mentorship, inspiration and encouragement. I also particularly thank Drs. Peng Du, Nira Paskaranandavadivel, Tim Angeli and Rachel Lees-Green for their collegiality and support.

Several others deserve special mention for generously contributing their time and effort to this work. I thank Drs. Simon Gibbons, Gianrico Farrugia, Jean-Marie Vanderwinden, Feng Mei and Juan Han for providing the experimental data which facilitated this research, and for their guidance, support and collaboration.

Funding: Primary scholarship support for this work was funded by the University of Auckland Health Research Doctoral Scholarship. Additional personal funding came from the Freemasons Postgraduate Scholarship from the Freemasons Charity, Freemasons New Zealand; the R. H. T. Bates Postgraduate Scholarship from the Royal Society of New Zealand; and the Young Investigators Award from the International Electrogastrography Society. Group funding came from the Riddet Institute, the New Zealand Health Research Council and the National Institutes of Health (R01 DK64775). Additional travel and research funding was provided through the Riddet Institute, the Japan Student Services Organization, the University of Auckland and the Auckland Bioengineering Institute.

Personal Acknowledgement: Lastly, but most importantly, I thank my loving parents and sister, Alex, Annie and Jennifer, for their endless support.
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# Nomenclature

## Acronyms

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<tr>
<td>AT</td>
<td>Activation times</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>DMP</td>
<td>Deep muscular plexus</td>
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<tr>
<td>DSN</td>
<td>Data search neighbourhood</td>
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<tr>
<td>EENG</td>
<td>Electroenterography</td>
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<td>EGG</td>
<td>Electrogastrography</td>
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<tr>
<td>FSM</td>
<td>Finite-state machine</td>
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<tr>
<td>GI</td>
<td>Gastrointestinal</td>
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<tr>
<td>ICC</td>
<td>Interstitial cells of Cajal</td>
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<tr>
<td>IP$_3$</td>
<td>Inositol 1,4,5-triphosphate</td>
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<td>KO</td>
<td>Knockout</td>
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<td>MENG</td>
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<td>MP</td>
<td>Myenteric plexus</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PDF</td>
<td>Probability density function</td>
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<tr>
<td>SCF</td>
<td>Stem cell factor</td>
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<td>SMC</td>
<td>Smooth muscle cells</td>
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<tr>
<td>SNESIM</td>
<td>Single Normal Equation Simulation</td>
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<tr>
<td>SQUID</td>
<td>Superconducting quantum interference device magnetometer</td>
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<td>WT</td>
<td>Wild-type</td>
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Chapter 1

Introduction

Gastrointestinal (GI) motility encompasses the muscular contractions in the GI tract required for digestion and transportation of contents. Throughout much of the tract, this process is facilitated by specialised cells called interstitial cells of Cajal (ICC) [51]. One of the most prominent roles of ICC is the pacemaking of GI motility patterns, achieved by actively initiating and propagating electrical oscillations that excite and cause contractions in the GI musculature [92, 182].

ICC loss and injury is now a major research focus as it is recognised as a hallmark of several GI functional motility disorders [93], including gastroparesis [77, 141], intestinal pseudo-obstruction [62, 95] and slow-transit constipation [125, 185]. In mice, the development of gastroparesis is directly correlated with ICC loss, and restoration of ICC is associated with reversal of the delayed gastric emptying [27]. Also, in humans the delay in gastric emptying seen in gastroparesis inversely correlates with the number of ICC [76].

ICC loss also occurs throughout the normal ageing process without disrupting normal GI motility, and this loss is to a reduced extent in comparison to disease states [74]. Therefore ICC loss occurs over a spectrum of severities, and GI motility is only impacted after some depletion threshold is exceeded. Younger tissues possess an ICC ‘reserve’ offering more resilience towards ICC damage, and with age the reserve decreases, providing less buffer against damage and hence increasing the susceptibility of the damage manifesting as GI motility disorders (Fig 1.1).
1. INTRODUCTION

Figure 1.1: Changes in (a) ICC body counts and (b) volumes from the human gastric circular muscle layer throughout normal ageing. Although both ICC body counts and volumes decrease with age, ICC depletion occurring throughout normal ageing does not exceed the threshold beyond which GI motility is disrupted. There is, however, a decreased ICC ‘reserve’ at older ages, increasing the susceptibility of ICC damage manifesting as motility disorders. The field size is 0.12 $mm^2$. Each point is the mean ± standard error of the mean, and the regression line (solid) and limits of the 95% confidence interval (dotted) are shown. Reproduced from [74].

1.1 Motivation

Although the importance of ICC in GI health and functional disorders is now well established, the mechanism relating ICC structure to GI function and dysfunction remain poorly defined. However, elucidating this mechanism proves difficult with only experimental techniques. Current methods for assessing ICC depletion are limited to cell body counts and volume computations [74, 173], and these simplistic measures are inadequate to explicitly define the complex network structures formed by ICC (Fig. 1.2). In the recent study by Grover et al. [77] full-thickness gastric biopsies were taken from gastroparetic patients and age-matched controls, and ICC body counts from the circular muscle layer showed a loss of ICC in gastroparesis. However, at the same time gastroparetic patients with normal ICC numbers were also present (Fig. 1.3). This example shows the necessity of more detailed assessment techniques in order to reveal the crucial ICC network structural properties contributing to GI function.

Also, ICC activity and GI function occurs over very different spatial and temporal scales, and hence simultaneously capturing these processes for association is challenging. Currently, the only experimental technique that has successfully captured
1.1. Motivation

Figure 1.2: Example 2D bitmaps of ICC-MP networks from the jejunum of four-week-old (a) WT and (b) \(5-HT_{2B}\) KO (ICC depleted) mice. The simplistic measures of cell body counts and volume computations are inadequate to explicitly define these complex network structures.

Figure 1.3: Comparison of ICC body counts from the gastric circular muscle layer between gastroparetic patients and age-matched controls. On average, there is a decrease in ICC numbers in gastroparetic patients, but patients exhibiting normal ICC numbers are also present. The field size is 0.0367 \(\text{mm}^2\). Dots represent individual patients and the means of each group are shown with a solid line. Reproduced from [77].
spatial propagation of ICC pacemaker activity at the cellular level is \textit{ex vivo} \( \text{Ca}^{2+} \) imaging in mice [144]. However, the spatial and temporal scales at which \( \text{Ca}^{2+} \) imaging operates at is in the order of micrometres and milliseconds respectively, which is several orders of magnitude smaller than that of GI function. Therefore, a holistic view demonstrating how cellular activity scales up to GI function cannot be obtained.

Another major constraint in elucidating the mechanism relating ICC network structure to GI function is the limitations of current ICC imaging data. The imaging of \textit{ex vivo} ICC networks is technically challenging, and the obtained data is still generally limited to small fields of view in the order of a few hundred micrometres to millimetres [72]. Also, most studies on ICC loss compare normal networks against depleted networks at some set depletion severity, such as that induced by a gene knockout (KO) [92, 173] or disease [95, 141]. There is no experimental method to systematically control the network properties so that networks at an intermediate depletion level can be imaged and investigated. A comprehensive imaging data set encompassing large-scale ICC networks across a spectrum of network properties would be of substantial benefit in investigating the pathophysiology of ICC loss.

Mathematical modelling has been an effective strategy in forming integrative systems descriptions of GI function [26, 46], and hence this thesis aims to use a mathematical modelling approach to address the above challenges and investigate the relationship between ICC network structure and GI function. Once defined, this information can potentially make ICC network samples from biopsies of patients with GI motility disorders assist in deciding treatment protocols or become part of routine clinical assessments of GI health and disease.

\section*{1.2 Objectives}

This thesis constructs three separate sets of computational tools for addressing each of the aforementioned experimental challenges, which are jointly used to investigate ICC network structure-function relationships. The development of these tools presents the first integrative multiscale computational framework spanning from ICC network structure at the cellular level to GI electrical activity at the tissue level.

Chapter 2 reviews current knowledge of ICC. The location, identification, roles and modelling of these specialised cells are covered.
1.2. Objectives

Chapter 3 introduces novel numerical metrics for quantifying ICC network structural properties. Such quantification isolates particular features of the ICC network, and thus enables unbiased and consistent analyses.

Chapter 4 describes multiscale computational models for coupling ICC network structure to its pacemaker function. That is, by providing the models with ICC structural information obtained from imaging data, the corresponding electrical pacemaker activity can be simulated. These models are capable of incorporating activity spanning across multiple spatial and temporal scales, and hence offers a feasible method for relating ICC activity to GI function.

Chapter 5 proposes the generation of realistic virtual ICC imaging data in silico to gain access to large-scale ICC network structures across a spectrum of network properties. This is accomplished by employing the stochastic Single Normal Equation Simulation (SNESIM) algorithm, a geostatistical algorithm originally used in the petroleum industry for building models of the geological formations which host oil reservoirs [166, 167].

Chapter 6 summarises the main findings of this thesis and presents directions for future work before concluding.
Chapter 2

Interstitial Cells of Cajal

Interstitial cells of Cajal (ICC) have a pivotal status in gastrointestinal (GI) health and disease. This chapter focuses on these specialised cells and begins with identifying their location within the GI tract, followed by reviewing the history of their discovery. The physiological roles played by ICC are then discussed, as well as detrimental causes and effects of ICC loss. Finally, the efforts made to model these cells and their function are summarised.

2.1 Location of ICC

In order to identify where ICC reside, a thorough understanding of GI anatomy is required. This section uses a top-down approach to first cover the anatomy of major GI organs along the GI tract, followed by dissecting the GI wall, and finally pinpointing the location of ICC.

2.1.1 The GI Tract

The GI tract is a continuous tube approximately nine metres in length in humans from the mouth to the anus (Fig. 2.1), and is comprised of several distinct compartments categorised into separate organs. The integrated activity of these GI organs serve the foremost purpose of providing the body with a continual supply of water,
2. INTERSTITIAL CELLS OF CAJAL

Figure 2.1: The major organs along the GI tract. Food enters the body through the mouth, past the oesophagus and into the stomach, where it is stored, ground and mixed. The processed contents, termed chyme, are then released into the small and large intestines for further digestion and absorption. Any excess waste material is temporarily stored in the rectum before leaving the body through the anus. Reproduced from [153].

electrolytes and nutrients [78]. Here, the two major GI organs on which this thesis focuses are discussed: the stomach and small intestine.

The stomach serves the crucial functions of storage, grinding and mixing of ingested contents, as well as controlling the release of the processed contents, termed chyme, into the small intestine [5]. The longer side of the organ is named the greater curvature, whereas the shorter side is the lesser curvature. The stomach is anatomically divided into the four regions of the fundus, corpus, antrum and
2.1. Location of ICC

Figure 2.2: An illustration of the stomach and its major anatomical landmarks. Reproduced from [6, 64].

The pylorus (Fig. 2.2). The different anatomical zones have different functions. The fundus and corpus act as a reservoir of the ingested contents and are responsible for the emptying of liquids, whereas forceful contractions in the antrum mix and grind the solid contents [109]. The pylorus connects the stomach to the duodenum in the small intestine, regulating the outflow of chyme through the pyloric sphincter muscle [78].

The small intestine is a convoluted conduit connecting the stomach and large intestine, forming the longest section of the GI tract. Digestion and most of the absorption process is completed here [127]. The small intestine is subdivided into the duodenum, jejunum and ileum. The duodenum is the first and shortest section of the small intestine where chyme is further digested with enzymes. The second and third sections, the jejunum and ileum respectively, are responsible for the uptake of water and nutrients before propelling the contents further along the GI tract to the large intestine.
2. INTERSTITIAL CELLS OF CAJAL

Figure 2.3: An illustration of the generic GI wall cross-section, indicating the various tissue layers and their disposition. Note that the oblique muscle layer only exists in the stomach. Reproduced from [35, 63].

2.1.2 The GI Wall

The GI wall consists of multiple different tissue layers, and subtle variations in this tissue composition exist along the GI tract. An illustration of the generic GI wall cross-section is shown in Fig. 2.3. The innermost layer of the GI wall is the mucosa, which comes into contact with luminal contents via its epithelial lining. It is involved in absorbing nutrients and electrolytes, and secreting liquids to aid in digestion. The next layer is the submucosa, formed of loose connective tissue, nerves and blood vessels which supply and support the mucosa. Beyond the submucosa lies the muscularis externa, which is the layer consisting of smooth muscle tissue responsible for the contractile activity of the GI tract. The muscularis externa is split into two layers of smooth muscle tissue – one with muscle fibres aligned in the circular direction, which form rings around the GI tract, and another with muscle fibres aligned in the longitudinal direction along the GI tract. A rich plexus of nerves known as the myenteric plexus (MP) lies in between and innervates these two muscle layers.
The circular smooth muscle layer can also be further divided into inner and outer layers. The inner circular layer contains smaller smooth muscle cells (SMC) that are packed more densely, whereas the outer circular layer is thicker than the inner layer [5]. In the small intestine, another plexus of nerves called the deep muscular plexus (DMP) exists between the inner and outer circular muscle layers. Also, there is an additional ‘oblique’ muscle layer between the circular muscle and submucosa in the stomach, with fibres running at an angle skewed to both the longitudinal and circular directions. This additional layer generates the specialised churning movements of the stomach for breaking down ingested contents. The outermost layer to the GI wall is the serosa, which mainly contains connective tissue and serves as an outer protective coat.

2.1.3 ICC Populations

ICC have been identified in various regions of the GI tract, and several populations of these cells have been classified according to their location within the GI wall [79]. Here, ICC populations relevant to the stomach and small intestine are discussed (Fig. 2.4).

In the stomach, ICC are found within the muscle layers (intramuscular ICC, ICC-IM) and around the MP (ICC-MP) [155]. ICC-IM are spindle shaped cells

Figure 2.4: ICC populations within (a) the stomach and (b) small intestine. (a) In the stomach, a network of ICC-MP lie at the plane of the MP, and ICC-IM are dispersed within the longitudinal (ICC-LM) and circular (ICC-CM) muscle layers. (b) Two networks of ICC exist within the small intestine: ICC-MP and ICC-DMP at the levels of the MP and DMP respectively. Reproduced from [12].
predominantly orientated along the long axis of the surrounding SMC, and can be further classified into cells which lie within the circular (ICC-CM) and longitudinal muscle layers (ICC-LM), although ICC-LM are less numerous [107]. In large mammals including humans, the circular muscle layer is thick and bundles of muscle fibres are often separated by connective tissue septae. ICC are also found within these connective tissue septae (ICC-SEP) as a subpopulation of ICC-CM [89, 131, 138]. ICC-MP are stellate cells which form an interconnected network. These cells exist throughout the stomach except in the fundus, and the network density increases distally along the stomach [13], as well as from the lesser to greater curvature in the antrum [85]. Another type of ICC are present at the interface between the submucosal connective tissue and the innermost circular muscle layer of the pylorus (submucosal ICC; ICC-SM) [162], and these cells, unlike ICC-CM, have branching processes extending perpendicular to the long axis of the circular muscle cells [184].

Two networks of ICC are present in the small intestine. One of these is the ICC-MP network [155], whereas another plane of ICC is located at the DMP (ICC-DMP) [192]. ICC-DMP are also stellate cells, but the majority of their processes are aligned along the long axis of the circular muscle cells [12].

2.2 Identification of ICC

Although ICC were first observed over one hundred years ago, difficulty in identifying them has been one of the greatest impediments to elucidating their significance and contribution to GI motility [174]. This section reviews the tortuous history of ICC discovery in three parts: the beginnings (pre-1889–1950s), the ultrastructural era (1950s–1990s), and the Kit revolution (1990s–).

2.2.1 The Beginnings (pre-1889–1950s)

The great Spanish neuroanatomist Santiago Ramón y Cajal was not the first person to see ICC [174], but was the first to make them widely known since 1889 [16, 17, 18, 19], which justifies the eponym. From the first reports on ICC (pre-1889) until 1958, identification of these peculiar cells relied on light microscopy in conjunction with various imperfect staining techniques, and these imperfections were the root cause of the confusion on the nature of ICC during this period.
Cajal identified ICC using the Golgi silver impregnation and Ehrlich’s vital methylene blue methods (Fig. 2.5), which he thought were staining methods specific to neurons, and hence this led him to believe that ICC were neuronal in nature [174]. Since 1925, studies applying the Bielschowsky-Gros silver impregnation method initiated by Lawrentjew [113, 114] further complicated the situation by thoroughly confusing ICC with Schwann cells. In addition, the Champy (potassium iodide/osmic acid) and Champy-Maillet methods (zinc iodide/osmic acid; ZIO) were also widely used, and since these methods were excellent in staining peripheral nervous tissue, the belief that ICC were neurons was reinforced among investigators [133].

2.2.2 The Ultrastructural Era (1950s–1990s)

In 1958, Richardson [151] presented the first study examining ICC under the electron microscope and defined the ultrastructural features of ICC in the rabbit small intestine (Fig. 2.6). This marked the start of the ultrastructural era, where identification of ICC turned to rigorous ultrastructural assessment. Following Richardson’s pioneering study, ICC were subsequently identified via electron microscopy in the stomach and small intestine of various species (see [28] for a detailed review).
Figure 2.6: ICC under the electron microscope. (a) Reproduced from the first electron microscope study on ICC in 1958 by Richardson [151], identifying ICC (1), two large nerve bundles (N) and a capillary (C) from the rabbit intestine. Longitudinal SMC can also be seen above and below. (b) Reproduced from a ‘modern-day’ study in 2012 by Faussone-Pellegrini et al. [57], reflecting the evolution of electron microscopy over the past few decades. Two ICC (ICC1 and ICC2) are identified from the human stomach, and their processes are in contact with each other (square). ICC1 is also in contact with a SMC via a gap junction (asterisk) and a desmosome-like junction (double asterisk). Scale bar in both (a) and (b) = 1 \( \mu m \).

However, identification of ICC still remained difficult, as there is no single, unique ultrastructural characteristic to distinguish ICC from any other cell type. Moreover, ultrastructural characteristics possessed by ICC show significant regional and interspecies variations [59, 106, 154], ranging from SMC-like to fibroblast-like [155]. Also, ICC ultrastructural characteristics defined in adult animals do not translate to embryonic and postnatal ICC as these properties only develop through maturation [55, 58, 117].
2.2. Identification of ICC

Light microscopy was not abandoned in the ultrastructural era. Numerous attempts were made to correlate light and electron microscopy of ICC, but most of these studies were unfruitful as the light microscopic techniques implemented to stain ICC render the tissue unsuitable for electron microscopy [155]. Continued effort was also invested into searching for a reliable stain for ICC, although none were particularly successful [155].

The ultrastructural investigations on ICC cleared the misconception that ICC were neurons or Schwann cells, but were still unable to settle the question as to the nature of ICC. The initial tendency was to classify ICC as fibroblasts [151], due to their similar light and electron microscopy characteristics as well as the limited experimental techniques at the time, especially the inability to correlate light and electron microscopy of ICC [155]. On the other hand, Taxi proposed ICC to be a separate cell type as early as 1965 [172], and this notion was increasingly supported by subsequent studies later in the ultrastructural era [56, 164].

2.2.3 The Kit Revolution (1990s–)

The discovery of ICC immunoreactivity to the Kit receptor (tyrosine protein kinase) marked the major breakthrough in the field. The first clue for a role of Kit in GI motility was reported by Maeda et al. [126], where they induced severe in-
2. INTERSTITIAL CELLS OF CAJAL

testinal motility disturbance in mice by injecting the monoclonal antibody ACK2, which blocks Kit activation. Furthermore, they observed that the distribution of Kit-immunoreactive cells was reminiscent to that of ICC [126]. Shortly after this seminal work, it was established that animals with loss-of-function mutations in Kit or its ligand stem cell factor (SCF) showed disrupted ICC network formation, indicating the necessity of a functional Kit/SCF signalling pathway for normal ICC development [92, 135, 181, 182].

Knowledge of Kit-immunoreactivity in ICC revolutionised ICC identification. The only Kit-immunoreactive cells in the GI musculature are ICC and mast cells, with the latter being easily distinguishable from ICC as they stain brighter and have a distinct morphology (round cell body with an absence of processes) [73]. Therefore nearly instantly, Kit became the routine marker for ICC due to its specificity, and all other staining techniques faded into obsolescence.

Recently, ICC were found to express Ano1 (previously known as DOG1 and TMEM16A) [49, 186], which is a Ca\(^{2+}\)-activated Cl\(^{-}\) channel [20, 190]. Ano1-immunostaining is also highly specific for ICC [73], but Kit still remains as the widely uptaken unrivaled marker for ICC (Fig. 2.7).

2.3 Roles of ICC

To date, four roles of ICC which contribute to GI motility have been identified: 1) actively generating and propagating electrical pacemaker activity; 2) facilitating neurotransmission; 3) establishing the membrane potential gradient; and 4) acting as a mechanotransducer [51]. Three of these roles were in fact suggested before their proof became available. Cajal himself suggested that ICC were involved in peripheral neurotransmission [18, 19]. Around the same time but independently, Sir Arthur Keith, who discovered the cardiac sino-atrial pacemaker organisation, hypothesised the pacemaker role of ICC [96, 97], apparently not even aware that these were the cells Cajal was advocating. Several decades later, Daniel suggested a mechanotransducer function for ICC [36]. This thesis focuses on the electrical pacemaker role of ICC, but each of these roles are discussed briefly below.

Electrical pacemaker activity is spontaneously generated by ICC-MP in the stomach [40, 143] and small intestine [92, 182]. This pacemaker activity then conducts to adjacent SMC to evoke the ‘slow wave’ depolarisations which initiate the muscular
contractions of the organs [156] (Fig. 2.8). ICC pacemaker activity is thought to be generated via the summation of many small amplitude membrane fluctuations termed ‘unitary potentials’, which result from inward-directed ionic conductances through unique ICC pacemaker channels. When co-ordination and summation of the individual transient unitary potentials reach a certain depolarisation threshold within the ICC, pacemaker activity is initiated with a rapid upstroke depolarisation [156]. Individual ICC generate pacemaker activity at their own intrinsic frequencies [30, 98], but in intact tissue ICC are organised in a syncytium, and all cells are matched to generate pacemaker activity at the highest intrinsic frequency within the syncytium through a process called ‘entrainment’ [34, 104], which likely occurs through voltage-dependent mechanisms similar to those coordinating unitary potential summation [61, 156]. In the stomach, the pacemaker region containing ICC with the highest intrinsic frequency (three cycles per minute (cpm) in humans) is located near the greater curvature of the mid to upper corpus [84, 134], whereas in the small intestine, there is an aborally decreasing intrinsic frequency gradient leading to the formation of step-wise decreases in frequency termed ‘frequency plateaus’.

Figure 2.8: Experimental traces of (a) ICC pacemaker activity recorded from ICC-MP and (b) slow wave activity recorded from SMC from the guinea-pig stomach. Electrical activity within both cell types occurs synchronously at a frequency of three cycles per minute (cpm), but the amplitude of slow wave activity is slightly attenuated in comparison to ICC pacemaker activity. Reproduced from [87].
2. INTERSTITIAL CELLS OF CAJAL

along the organ [38] (12 cpm in the proximal duodenum to 8-9 cpm in the distal ileum in humans [29]). To aid in propagating the pacemaker activity through the thicker circular muscle layer, ICC-CM have been found to actively regenerate slow waves [39, 89]. Furthermore, ICC-IM are thought to have the capability to act as pacemaker cells instead of ICC-MP under certain circumstances such as excitatory vagal nerve stimulation [86].

Several studies have shown that cholinergic and nitrergic neurotransmission is mediated by ICC-IM in the stomach [13, 170] and ICC-DMP in the small intestine [183]. These findings changed the classical concept of neuromuscular transmission in the GI tract, where the neurotransmitter is released and diffuses from nerve varicosities, subsequently binding and activating receptors expressed on neighbouring SMC [15]. Instead, at least for cholinergic and nitrergic neurotransmission, the neurotransmitter emitted from enteric neurons binds to receptors of ICC, and ICC in turn depolarise or hyperpolarise neighbouring SMC through excitatory or inhibitory junction potentials conducted via gap junctions, thereby mediating the process [180].

There exists a membrane potential gradient across the thickness of the circular muscle layer in the GI tract. In the distal stomach and small intestine, the membrane potential in the outer circular muscle layer by the MP is approximately 10 mV hyperpolarised than in the inner circular muscle layer near the submucosal border [4, 80]. This gradient is generated and maintained by ICC through releasing the hyperpolarising factor carbon monoxide [52, 163], and functions as a biological rheostat to allow a graded contractile response based on stimulus strength. That is, a weak stimulus would only recruit the more depolarised layers of circular muscle, evoking only a small contraction, whereas a strong stimulus would also be able to recruit the more hyperpolarised circular muscle layers, generating a large contraction [52, 163].

The non-neural stretch reflex exhibited by gastric muscle which is abolished by an absence of ICC demonstrated the mechanotransduction role of ICC [187]. ICC are known to express various mechanosensitive ion channels, including the $Ca_{v}1.2$ and $BK_{Ca}$ channels (see [110] for a detailed review). More recently, the mechanosensitive $Na_{v}1.5$ sodium channel encoded by $SCN5A$ [169] was identified in ICC from human intestine, but was not found in the intestine of some species including pig and guinea-pig [168]. Delineating the interspecies differences in mechanisms controlling the response of GI electrophysiology to mechanical stimuli therefore remain as an active field of research.
2.4 Mathematical Modelling of ICC

Mathematical modelling has become an attractive strategy in GI research [33, 44]. It offers an alternative to animal and human experiments for verifying hypotheses on normal and abnormal physiology, and predicting the effects of treatment [42]. In particular, multiscale modelling, whereby models spanning across vast spatial and temporal scales are integrated, has become a productive approach to examining the complex interactions between activity of the GI tract at the subcellular, cellular, tissue, organ and body levels [26, 46].

Various models spanning multiple scales of ICC electrophysiological function have been built. This section reviews these models in three categories: cellular scale, tissue scale, and organ and body scale.

2.4.1 Cellular Models

There are two approaches for modelling cellular events in ICC: phenomenological and biophysically-based modelling. Phenomenological models were developed earlier to reproduce the electrical events using relatively simple mathematical equations without a biophysical basis. Since the 1960s, slow wave activity was modelled as a series of coupled Van der Pol relaxation-oscillators [139], and this concept was further expanded to demonstrate entrainment in a network of bi-directionally coupled series of relaxation-oscillators [158, 159]. Subsequently, with the realisation that slow wave activity originates from ICC, an updated oscillator-based model was proposed by Aliev et al. which was the first to distinguish between ICC and SMC function as two interconnected electrical domains [2]. Nevertheless, phenomenological models still presented severe limitations. In particular, Publicover and Sanders argued that the lack of a biophysical basis for phenomenological models restricted their application in investigating the effects of pharmaceutical agents on GI electrophysiology [148].

The alternative biophysically-based modelling strategy builds cellular models by assembling together smaller models of detailed intracellular processes such as individual ion currents, and utilises variables which carry physical quantatity. This modelling strategy was initially proposed by Hodgkin and Huxley in 1952 [88], and its application has significantly advanced neural and cardiac electrical activity simulations over the past few decades [88, 122, 123]. Although comparatively,
biophysically-based modelling of ICC is still in its infancy, four main biophysically-based ICC models (with additional variations, see below) have emerged recently (Table 2.1). The earliest Youm et al. model [191] was partially based on cardiac cells models [123, 130], and simulated intestinal pacemaker activity. The second model constructed was the Corrias and Buist model [32] for simulating gastric pacemaker activity, which contained intracellular \( \text{Ca}^{2+} \) dynamics based on the mitochondria-endoplasmic reticulum relationship proposed by Fall and Keizer [50] (Fig. 2.9). The third Faville et al. model [60] which simulated intestinal pacemaker activity incorporated intracellular \( \text{Ca}^{2+} \) dynamics derived specifically from ICC experimental data, and was the first model to contain a biophysically-based representation of unitary potentials. Recently, Du et al. [41] developed a fourth model for simulating gastric pacemaker activity focusing on computational efficiency to facilitate incorporation into large multiscale simulations.

Three attempts [10, 47, 160] have also been made to modify the Corrias and Buist model [32] to demonstrate entrainment when the cell model is organised in a tissue formation (see Section 2.4.2). Entrainment likely occurs through a voltage-dependent mechanism [156], and hence accordingly, all three studies incorporated a voltage-dependent pathway for modulating intracellular \( \text{Ca}^{2+} \) concentration (\([\text{Ca}^{2+}]_i\)) into the original Corrias and Buist model. The Buist et al. [10] study achieved this by adding an additional voltage-dependent dihydropyridine-resistant \( \text{Ca}^{2+} \) conductance, whereas the Du et al. study [47] incorporated a voltage-dependent inositol 1,4,5-triphosphate (IP\(_3\)) synthesis model proposed by Imtiaz et al. [94]. The simplified ICC cell model later developed by Du et al. [41] also includes this voltage-dependent IP\(_3\)-mediated entrainment mechanism. In the recent study by Sathar et al. [160], the original Corrias and Buist model was combined with a Mealy finite-state machine approach, which split the activity of the cell model into

<table>
<thead>
<tr>
<th></th>
<th>Youm et al. [191]</th>
<th>Corrias and Buist [32]</th>
<th>Faville et al. [60]</th>
<th>Du et al. [41]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ionic conductances</td>
<td>7</td>
<td>10</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>ODEs</td>
<td>14</td>
<td>22</td>
<td>78</td>
<td>6</td>
</tr>
<tr>
<td>Parameters</td>
<td>53</td>
<td>116</td>
<td>640</td>
<td>35</td>
</tr>
<tr>
<td>Simulation time (s)</td>
<td>16.2</td>
<td>21.3</td>
<td>83.9</td>
<td>10.2</td>
</tr>
</tbody>
</table>

Table 2.1: Comparison of biophysically-based ICC cell models. Simulation times correspond to a 60 s period of ICC pacemaker activity simulated on an Intel® Core\(^\text{T M}\) i5-460M CPU using a forward Euler method with a fixed time step of 0.01 ms [41].
2.4. Mathematical Modelling of ICC

Figure 2.9: Simulated trace of ICC pacemaker activity using the Corrias and Buist ICC model [32]. Three subsequent studies modified this model to demonstrate entrainment when the model is organised in a tissue formation (see text for details). Reproduced from [32].

separate active and passive states. Intracellular Ca\(^{2+}\) dynamics remained quiescent in the passive state and were only initiated in the active state, with the switch from the passive to active state occurring when a membrane potential threshold was reached during the non-refractory period, or until the non-refractory period had passed.

2.4.2 Tissue Models

The continuum-based bidomain model has been rigorously employed to simulate electrical activity propagation through cardiac tissue [71, 83, 152], and more recently, through GI tissue as well [47, 149]. This modelling approach conceptualises two interpenetrating cellular domains (i.e., the intracellular cytoplasm and extracellular tissue matrix) and describes the flow of electrical currents within and between these two domains. Therefore, by coupling cell models (see Section 2.4.1) to the bidomain model the interaction of electrical activity presented by individual cell models can be examined at the tissue scale. The pair of equations defining the bidomain model are [44]:

1. \[ \nabla \cdot (\sigma_i \nabla V_m) = -\nabla \cdot ((\sigma_i + \sigma_e) \nabla \phi_e) \quad (2.1) \]
2. \[ A_m (C_m \frac{dV_m}{dt} + I_{ion}) - \nabla \cdot (\sigma_i \nabla \phi_e) = \nabla \cdot (\sigma_i \nabla V_m) \quad (2.2) \]
where $\sigma_i$ and $\sigma_e$ are the conductivity tensors of the intracellular and extracellular domains respectively; $V_m$ and $\phi_e$ are the membrane and extracellular potentials respectively; $A_m$ is the cell surface-to-volume ratio; $C_m$ is the cell membrane capacitance; and $I_{ion}$ is the ionic currents across the cell membrane. Eq. 2.1 calculates the extracellular potential field based on the membrane potential distribution, whereas Eq. 2.2 computes the membrane potential at each time step. Coupling of individual cell models to the bidomain model is achieved through substituting the ionic conductance terms of the cell model into the $I_{ion}$ term in Eq. 2.2.

The bidomain model can be simplified to the monodomain model by assuming either the extracellular domain is highly conducting or that the two domains are of equal anisotropy to remove the extracellular domain [116], and studies have also applied this simplified model to simulate GI electrical activity propagation [3, 115]. Furthermore, an extended bidomain model with multiple intracellular domains sharing a common extracellular domain has been proposed in light of the complex distribution of ICC within SMC [11].

Most studies which employ continuum-based models to simulate GI electrical activity propagation assume homogenous sheets of tissue including only a single cell type. However, the Du et al. study [47] was the first to consider multiple cell types coexisting within the plane of the MP. ICC activity was modelled using the Corrias and Buist ICC model modified to include the voltage-dependent $IP_3$-mediated entrainment mechanism, whereas ‘non-ICC’ tissue was assumed to behave as a current sink. These cell models were embedded into tissue-specific ICC network structures obtained from wild-type (WT) and ICC-depleted ($5-HT_{2B}$ knockout (KO)) mouse jejunum using confocal microscopy, and the bidomain model was solved for electrical activity propagation (Fig. 2.10). This modelling setup thereby allows investigation on the relationships between ICC network structure and GI electrophysiological function, or in short, ICC network structure-function relationships.

Cellular automaton models are an alternative approach to the continuum-based models for simulating electrical activity propagation through excitable tissue. These rule-based models are simplified, low-cost computational tools which have been successful in simulating macroscopic propagation in cardiac muscle [1, 129], and has more recently been applied to simulate GI electrical activity propagation [48, 111].
2.4. Mathematical Modelling of ICC

Figure 2.10: Simulated ICC pacemaker activity propagation sequence over a tissue-specific ICC-MP network structure obtained from the jejunum of four-week-old WT mice. The first image on the left in the top row shows \( V_m \) at time \( t = 0 \) ms and each subsequent image, from left to right then down to the next row, is 40 ms apart. The ICC pacemaker activity was initiated in the top-left corner and in general, propagated towards the bottom-right corner of the network. Reproduced from [47].

2.4.3 Organ and Body Models

Tissue models can be readily upscaled into organ models by incorporating anatomical information of organs obtained from imaging modalities such as magnetic resonance imaging [7, 9]. Modelling of GI electrical activity propagation over entire organ geometries improves our understanding of the electrophysiological basis of the GI tract [25].

However, most studies involving organ models has placed greater focus on the extension to body models due to the potential clinical applications, namely electrogastrography/electroenterography (EGG/EENG), which records GI electrical activity from cutaneous abdominal electrodes [145], and magnetogastrography/magnetoeenterography (MGG/MENG), which records the far-field magnetic field induced by GI electrical activity using the superconducting quantum interference device magnetometer (SQUID) [8]. These studies simulated GI electrical activity over an organ model, then inferred the induced potential and magnetic fields at the surface of the
2. INTERSTITIAL CELLS OF CAJAL

body model in hopes of validating the efficacy of EGG/EENG and MGG/MENG, and better interpreting the recorded signals [43, 105, 149]. Efforts have also been made to solve the inverse problem, where the electrical activity over the organ model is estimated based on the body surface potential or magnetic field [24, 100].
Chapter 3

Quantification of ICC Network Structural Properties

Since the discovery of ICC-specific immunohistochemical stains, confocal microscopy has been an effective tool for imaging and visualising the spatial distribution of ICC throughout the muscularis propria [12, 79]. Loss and injury of ICC has been recognised as a hallmark of several GI functional motility disorders [93], but despite the fact that the complex network structures formed by ICC can be accurately imaged, current descriptors of ICC loss only involve simple cell (nuclei) counts and volume computations (e.g., [74, 173]), and no attempts have been made to quantify spatial changes that may be occurring in the structural properties of ICC networks. Accurately defining the structural characteristics of ICC networks would allow investigators to explicitly contrast and compare aspects of ICC organisation in a consistent and unbiased manner. In addition, the major network structural properties that potentially influence network function and dysfunction could be defined and investigated as, given that ICC generate electrical signals, the organisation of a network may also affect physiology.

To advance this problem, this chapter presents a set of six numerical metrics to automatically quantify structural properties of confocal ICC network images (Section 3.1). These metrics were then applied in proof-of-concept studies to quantitatively determine structural changes in ICC networks that occur during 1) $5$-$HT_{2B}$ receptor and Ano1 KO, as well as postnatal maturation (Section 3.2), and 2) Spry4 KO (Section 3.3).
3. ICC NETWORK STRUCTURE QUANTIFICATION

3.1 Formulation of Numerical Metrics

The 2D formulation of the six metrics: density, thickness, hole size, contact ratio, connectivity, and anisotropy, are described in detail in Sections 3.1.1 to 3.1.6. The metrics were inspired by and partly adapted from existing methods of quantifying structure from alternative fields including bone [140] and textile research [188].

Each of the developed ICC structural metrics was formulated for potential physiological relevance. As this thesis focuses on the pacemaker activity of ICC, the metrics were initially chosen to quantify ICC network structural properties that may influence electrical behavior (Table 3.1). The definitive functional relationships of these structural metrics can be better clarified once structure-function studies utilising these developed metrics are conducted.

An ICC network image from a 5-HT$_{2B}$ receptor KO jejunal ICC-MP data set [173] was used as an example network to illustrate the developed metrics (Fig. 3.1a). See Section 3.2.1 for the detailed description of the example network image.

<table>
<thead>
<tr>
<th>Metric</th>
<th>Potential Physiological Relevance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density</td>
<td>Measures the ICC network volume, reflecting the amount of bioelectrical current generated</td>
</tr>
<tr>
<td>Thickness</td>
<td>Measures the width of cellular structures within the ICC network, which may impact electrical activity propagation through the network</td>
</tr>
<tr>
<td>Hole Size</td>
<td>Reflects the distribution of ICC throughout the tissue (i.e., the ‘tightness’ of the network), and may relate to the uniformity of SMC activation</td>
</tr>
<tr>
<td>Contact Ratio</td>
<td>Indicates the availability of conduction pathways from ICC to non-ICC regions within the MP, which may (through the field coupling conduction mechanism [91]) relate to the efficiency of pacemaker activity conduction into SMC</td>
</tr>
<tr>
<td>Connectivity</td>
<td>Reflects the structural integrity of the ICC network, indicating the cohesion of entrainment pathways [178]</td>
</tr>
<tr>
<td>Anisotropy</td>
<td>Indicates the degree of preferential alignment of ICC structures, and may reflect the dominant pacemaker activity propagation direction, potentially affecting predilection and resilience to dysrhythmia [142]</td>
</tr>
</tbody>
</table>

Table 3.1: Potential physiological relevance of the ICC structural metrics in the context of the pacemaker activity generated by ICC-MP.
3.1.1 Density

The density metric ($\rho$) calculates the relative volume of the tissue occupied by ICC (Fig. 3.1b). This metric is dimensionless and ranges from 0 to 1, with 0 indicating that no ICC were present, and 1 indicating that only ICC were present. The density metric was defined as:

$$\rho = \frac{N_{ICC}}{N_{Total}}$$  \hspace{2cm} (3.1)

where $N_{ICC}$ and $N_{Total}$ are the number of pixels representing ICC and the total number of pixels in the image respectively.

3.1.2 Thickness

The thickness metric ($t$) measures the representative width of the ICC network processes and cell bodies (Fig. 3.1c). This metric, measured in $\mu m$, was calculated as a weighted average of the process and cell body widths over the entire ICC network. In computing this metric, the Euclidian distance transform of the ICC network image was first calculated. This returned a distance map of distance from each ICC pixel to the nearest non-ICC pixel (Fig. 3.2a). The ICC network can be approximated by circles centred on the points of the regional maxima of the distance map, with radii of the regional maximum distances (Fig. 3.2b). Therefore, the regional maximum values of the distance transform signify the ‘radii’ of the processes and cell bodies. The thickness metric was then calculated as a scaled weighted sum of these individual thickness radii:

$$t = 2r \sum_{i=1}^{n} tw_i tr_i$$  \hspace{2cm} (3.2)

where $r$ is the resolution of the image in $\mu m$; $n$ is the number of regional maxima in the distance map; $tr_i$ is the $i^{th}$ regional maximum value or thickness radius in the distance map, and $tw_i$ is the thickness weight applied to $tr_i$. As the thickness radius only reflected half the width, a scaling factor of two was included.
3. ICC NETWORK STRUCTURE QUANTIFICATION

Figure 3.1: Illustration of the six numerical metrics for quantifying ICC network structural properties on a sample ICC network (a) with specific regions magnified in (b-f). (b) Density metric: the proportion of the image that was populated by ICC (red) was calculated. (c) Thickness metric: the representative thickness of the ICC bodies/processes (as indicated by length of red line) was computed. (d) Hole size metric: the representative radius of the non-ICC regions (as indicated by length of red line) was computed. (e) Contact ratio metric: the ratio of ICC that was immediately neighbouring non-ICC (red) to total ICC (white and red) was calculated. (f) Connectivity metric: example ICC network overlaid with red lines that connect the ICC islands together with shortest summed distance. These connections denote the ‘gaps’ or lack of connectivity of the network. Note that as this is a magnified view of the original network in (a), the two seemingly separate islands at the top of the current field of view are actually connected, and hence no connection was drawn to the top island. (g) Anisotropy metric: example ICC network with vectors in the circular (solid) and longitudinal (dashed) directions scaled so the magnitudes were proportional to the respective alignment strengths. (h) Corresponding ICC-DMP network of the example ICC network with vectors in the circular (solid) and longitudinal (dashed) directions as found using PCA. The vectors were scaled so the magnitudes were proportional to the respective eigenvalues (alignment strengths). The background (black) represents non-ICC regions.
3.1. Formulation of Numerical Metrics

Figure 3.2: Illustration of the Euclidian distance transform and its application in the ICC network structural metrics. (a) Euclidian distance transform of the magnified ICC network in Fig. 3.1c, which was used in computing the thickness metric. (b) Approximation of the magnified ICC network in Fig 3.1c, used in computing the thickness metric. This network approximation was constructed by centering circles on the points of the regional maxima of the distance map, with radii of the regional maximum distances. (c) Euclidian distance transform of the non-ICC regions within the magnified ICC network in Fig 3.1d, which was used in computing the hole size metric. The background (black) in (b) represents non-ICC regions.

The thickness weights were defined as:

\[ tw_i = \frac{tr_i^2}{\sum_{i=1}^{n} tr_i^2} \]  

(3.3)

The thickness weights were derived as the proportion of total ICC area represented by the individual thickness radii. Visualising the ICC network image as the circles centered on the regional maxima, the area contributed by the \(i^{th}\) regional maxima is \(\pi tr_i^2\), and the total area of ICC is approximately the sum of the individual circles, \(\sum_{i=1}^{n} \pi tr_i^2\). The ratio of these expressions reduce to the above definition (Eq. 3.3).

Substituting Eq. 3.3 into Eq. 3.2 gave the simplified thickness metric formulation:

\[ t = 2r \frac{\sum_{i=1}^{n} tr_i^3}{\sum_{i=1}^{n} tr_i^2} \]  

(3.4)
3. ICC NETWORK STRUCTURE QUANTIFICATION

3.1.3 Hole Size

The hole size metric \( h \) measures the representative radius of the non-ICC regions in the ICC network (Fig 3.1d). This metric, measured in \( \mu m \), was calculated as a weighted average of the individual radii of all the non-ICC region holes within the ICC network. The calculation process of this metric was identical to that of the thickness metric, but the Euclidian distance transform computed was that of the non-ICC regions. That is, the distance map in the hole size metric returned the distance from each non-ICC pixel to the nearest ICC pixel (Fig 3.2c). Therefore, the hole size metric was defined as:

\[
 h = \frac{r \sum_{i=1}^{n} hr_i^3}{\sum_{i=1}^{n} hr_i^2}
\]

(3.5)

where \( r \) is the resolution of the image in \( \mu m \); \( n \) is the number of regional maxima in the distance map, and \( hr_i \) is the \( i^{th} \) regional maximum value or hole radius in the distance map.

3.1.4 Contact Ratio

The contact ratio metric \( \phi \) calculates the proportion of ICC pixels in contact with (i.e., directly neighbouring) non-ICC pixels (Fig 3.1e). This metric is measured in \( \mu m^{-1} \), and in 2D, is equivalent to the perimeter to area ratio of the ICC network. The contact ratio metric was defined as:

\[
 \phi = \frac{1}{r} \frac{N_{BICC}}{N_{ICC}}
\]

(3.6)

where \( r \) is the resolution of the image in \( \mu m \); \( N_{BICC} \) is the number of border ICC pixels (i.e., ICC pixels directly neighbouring non-ICC pixels), and \( N_{ICC} \) is the total number of ICC pixels. In the actual computation of this metric, the pixels along the edges of the image were discarded as the border ICC pixels could not be differentiated with certainty.
3.1.5 Connectivity

The connectivity metric \( c \) indicates how connected (or disconnected) the ICC network is. This metric, measured in \( \mu m \), was computed as the ratio of tissue area to disconnection or ‘gap’ severity of the ICC network (Fig 3.1f). The ICC network may be formed by more than one group of interconnected ICC pixels, and here these individual groups are termed islands. Assuming there are \( n \) islands, the \( n - 1 \) connections that join the \( n \) islands together with shortest summed distance, or the minimum spanning tree, can be determined using Prim’s algorithm [147]. Starting from the largest island (i.e., the island with the most number of pixels), islands were connected sequentially until all islands were joined. The connectivity metric was then computed as the ratio of image area to the weighted sum of the individual connection distances, and was defined as:

\[
c = r \frac{N_{Total}}{\sum_{i=1}^{n-1} cw_i cd_i}
\]  

(3.7)

where \( r \) is the resolution of the image in \( \mu m \); \( n \) is the number of ICC islands; \( N_{Total} \) is the total number of pixels in the image; \( cd_i \) is the \( i^{th} \) connection distance, and \( cw_i \) is the connection weight of \( cd_i \). The weighted distance sum in the denominator reflects the severity of ‘gaps’ in the network.

The connection weights were defined as:

\[
cw_i = \frac{N_i}{N_{ICC}}
\]  

(3.8)

where \( N_i \) is the number of pixels in the \( i^{th} \) connected island, and \( N_{ICC} \) is the total number of ICC pixels. The weights were the ratios of the added island sizes to the total number of ICC pixels, and hence gauged the size of the added islands. This weighting factor was included since when measuring gap severity, not only are the distances between the islands important, but the relative sizes of the separated islands need to be considered as well. For example, a large island the same distance away as a small island should be considered as a more severe gap. Also for this reason, the starting island was selected to be the largest island, so only the smaller islands were considered as ‘additions’ and the weighted distance sum of the network was not falsely increased.
Combining Eq. 3.7 and Eq. 3.8 gave:

\[ c = r \frac{N_{ICC} N_{Total}}{\sum_{i=1}^{n-1} N_i c d_i} \]  

(3.9)

### 3.1.6 Anisotropy

The anisotropy metric \( \alpha \) reflects the preferential alignment of the ICC network processes in the longitudinal or circular directions of the organ. This metric is dimensionless and ranges from -1 to 1, with a positive value indicating preferential alignment in the longitudinal direction, and a negative value indicating preferential alignment in the circular direction. The magnitude of the metric reflects the strength of the preferential alignment. Several steps were involved in computing this metric:

1. The power spectral density \( P \) of the image was calculated.

\[ P = |F|^2 \]  

(3.10)

where \( F \) is the 2D Fourier transform of the network image.

2. The normalised covariance matrix \( \bar{C} \) of \( P \) was calculated.

\[ \bar{C} = \frac{1}{\bar{\mu}_{00}} \begin{bmatrix} \bar{\mu}_{20} & \bar{\mu}_{11} \\ \bar{\mu}_{11} & \bar{\mu}_{02} \end{bmatrix} \]  

(3.11)

\( \bar{\mu}_{pq} \), the normalised central moments were defined as:

\[ \bar{\mu}_{pq} = \sum_{i=1}^{w} \sum_{j=1}^{h} \frac{(i - \bar{i})^p (j - \bar{j})^q}{((i - \bar{i})^2 + (j - \bar{j})^2)^{p+q/2}} P(i,j) \text{ for } p, q = 0, 1, 2 \]  

(3.12)

where \( h \) and \( w \) are the dimensions (height and width) of the image in pixels; \( \bar{i} \) and \( \bar{j} \) are the \( x \) and \( y \) coordinates of the centroid position of the image respectively, and \( P(i,j) \) is the power spectral density value at position \( (i,j) \). The only difference between Eq. 3.12 and the central moment definition is the division of the normalisation term \( ((i - \bar{i})^2 + (j - \bar{j})^2)^{p+q/2} \). When summing over the individual \( (i,j) \) positions, the standard central moment scales the further away positions by a larger amount. However, in \( P \), these further away
positions correspond to higher spatial frequencies which may in fact be noise. Therefore, all positions were normalised to have equal influence on the final summed value, and only the orientations of the positions were retained.

3. The alignment strengths in the longitudinal ($\lambda_L$) and circular ($\lambda_C$) directions were calculated.

$$\lambda_i = |\bar{C}\vec{V}_i| \text{ for } i = L \text{ or } C$$

(3.13)

where $\vec{V}_L$ and $\vec{V}_C$ are the normalised vectors in the longitudinal and circular directions respectively. These unit vectors were projected onto the mapping matrix $\bar{C}$, and the magnitudes of the resultant vectors reflect the strengths of alignment in the respective directions (Fig 3.1g).

4. The anisotropy metric ($a$) was calculated.

$$a = \begin{cases} \sqrt{1 - \frac{\lambda_C}{\lambda_L}} & \text{if } \lambda_L \geq \lambda_C \\ -\sqrt{1 - \frac{\lambda_L}{\lambda_C}} & \text{if } \lambda_L < \lambda_C \end{cases}$$

(3.14)

3.2 Application 1: $5-HT_{2B}$ Receptor KO, Ano1 KO, and Postnatal Maturation

3.2.1 Imaging Data Sets

The developed metrics were applied in demonstration studies to analyse jejunal ICC network confocal images. The two principal ICC layers in the murine small intestine are in the myenteric plexus (ICC-MP) and the deep muscular plexus (ICC-DMP) [107]. However, as ICC-MP have been identified as the primary pacemakers of the small intestine [182], only the network structure of these cells were analysed.

Two-dimensional bitmap images of the Kit-positive ICC structures were obtained as previously described [47, 173]. Briefly, intestinal sections were dissected quickly, then flushed with ice-cold calcium-free Hanks balanced salt-solution (Invitrogen, Carlsbad, CA), and immediately pinned onto sylgard lined petri dishes to mitigate against tissue deformation. All specimens were handled and prepared in
3. ICC NETWORK STRUCTURE QUANTIFICATION

exactly the same manner. Confocal image slices were sequentially taken throughout the transmural depth of the muscularis propria in fine steps of 0.2-0.3 µm and were volume-rendered in 3D. Bitmaps of the positively labeled structures were then obtained using the Analyze software (AnalyzeDirect, Overland Park, KS) as previously described [136]. Unbiased thresholding algorithms were used to segment the images and determine the volume of the Kit-positive structures, thereby minimising any human influence in the process. The ICC-MP network was relatively thin in the transmural direction (≈10 µm) and the majority of the network laid in-plane. Therefore, the image segments of the ICC-MP were stacked with a maximum intensity projection into 2D images representing the entire ICC-MP network structures.

The 5-HT$_{2B}$ receptor and Ano1 murine ICC network imaging data sets were analysed, and the details are as follows:

1. 5-HT$_{2B}$ receptor data set. This data set contained 23 WT and 23 5-HT$_{2B}$ serotonin receptor KO ICC network images from the slightly proximal to middle jejunum of four-week-old mice (Fig. 3.3a and b). These network images were collected from 11 WT and 12 5-HT$_{2B}$ receptor KO mice. ICC express 5-HT$_{2B}$ receptors, and stimulation with 5-HT (serotonin) increases ICC proliferation and numbers [189]. It has also been demonstrated that a lack of 5-HT$_{2B}$ receptors decrease ICC proliferation, numbers and network volume [173]. These images were 512×512 pixels, and represented physical dimensions ranging from 0.225×0.225 mm to 0.318×0.318 mm.

2. Ano1 data set. This data set consisted of 16 WT and 16 Ano1 KO ICC network images from throughout the jejunum of three-day-old mice (Fig. 3.3c and d). These network images were collected from 4 WT and 4 Ano1 KO mice. Ano1 is a Ca$^{2+}$-activated Cl$^{-}$ channel expressed by ICC [73], and ICC lacking Ano1 channels have been shown to have fewer proliferating ICC [165] but normal numbers of adult ICC. These images were 512×512 pixels, and represented physical dimensions of 0.212×0.212 mm.

The standard biological variability of ICC networks is large, and hence a large number of network structures were included in each group of the imaging data sets ($n = 23$ or 16).
3.2. Application 1

Figure 3.3: Representative ICC network images from the 5-HT$_{2B}$ receptor and Ano1 data sets, as objectively defined by having metric values near the median of the group. Shown are four-week-old (a) WT and (b) KO networks from the 5-HT$_{2B}$ receptor data set, with physical dimensions of 0.318×0.318 mm, and three-day-old (c) WT and (d) KO networks from the Ano1 data set, with physical dimensions of 0.212×0.212 mm. The white regions represent ICC, whereas the background (black) represents non-ICC regions.
3. ICC NETWORK STRUCTURE QUANTIFICATION

3.2.1.1 Orientation of Imaging Data

The computation of the anisotropy metric (see Section 3.1.6) required knowledge on the orientation of the ICC network imaging data relative to the longitudinal and circular directions. However, this orientation information was not directly available from the images, so an alternative strategy to determine the imaging data orientation was developed. ICC-DMP processes are predominantly aligned in the circumferential direction [107], and hence the imaging data of these cells can be used as a reference to determine the relative orientation of the ICC-MP.

Two-dimensional bitmap images of the ICC-DMP network were obtained in the same way as the ICC-MP networks (see Section 3.2.1). The process of extracting the orientation information from the ICC-DMP imaging data proceeded as follows:

1. The normalised covariance matrix ($\bar{C}$) (see Eq. 3.11) of the ICC-DMP image was computed.

2. Principal Component Analysis (PCA) [146] was applied on $\bar{C}$ to find the orthogonal eigenvectors and the corresponding eigenvalues of the matrix.

In 2D, generally an eigenvector pair with two corresponding eigenvalues exists. The projection in the circular direction is expected to be the strongest due to the dominant alignment of the ICC-DMP processes, and since the orientations of features in the frequency domain are orthogonal to the original orientations in the spatial domain [188], the eigenvector with larger (i.e., maximum) eigenvalue points in a direction at right angle to the circular direction. Assuming the longitudinal and circular directions are orthogonal, the eigenvector with larger eigenvalue points in the longitudinal direction, whereas the other eigenvector points in the circular direction (Fig. 3.1h).

3.2.1.2 Pre-processing of Imaging Data

Before computing the metric values of the ICC-MP networks, pre-processing of the images was conducted.

1. *Joining of small gaps.* Gaps with less than one pixel radius were morphologically closed by performing an image dilation followed by erosion using a
circular structuring template with radius of one pixel. This step avoided islands of ICC being separated by small gaps which may be imaging artefacts, and although the joining of these gaps did not visually change the networks much, the metric values may be affected.

2. *Removal of small islands.* Islands with less than four pixels were removed. These islands had area less than $2 \times 2$ pixels, and hence were considered unlikely to be a genuine or significant ICC structure in terms of the network geometry as a whole. Again, the removal of these islands did not introduce obvious visual differences, but the metric values may be influenced.

The ICC-DMP networks were also pre-processed in the same manner.

### 3.2.1.3 Statistical Analysis

The two-sample $t$-test was applied to identify changes in ICC network structure during $5-HT_{2B}$ receptor KO, *Ano1* KO and postnatal maturation. Data points which are below the $1^{st}$ quartile or above the $3^{rd}$ quartile by more than 1.5 times the interquartile range are deemed to be outliers and are excluded from the $t$-tests. In a normal distribution this criteria corresponds to approximately 99.3% data coverage, and hence the identified outliers are indeed rather extreme values. However, the inclusion of these outliers in the statistical analysis is only expected to alter the test results slightly due to the large sample sizes. A $p$-value of less than 0.05 was considered statistically significant.

### 3.2.2 Results

#### 3.2.2.1 $5-HT_{2B}$ Receptor KO

The metric values of the WT and KO networks from the $5-HT_{2B}$ receptor data set are plotted in Fig. 3.4. These data showed that the KO networks had on average a 24% lower density ($p<0.01$), 34% lower thickness ($p<0.01$), 63% higher contact ratio ($p<0.01$), and 96% lower connectivity ($p<0.01$) than that of the WT networks. The KO networks also had on average a higher anisotropy metric value compared to the WT networks (KO mean: 0.44, WT mean: 0.01; $p<0.01$), indicating an increased
3. ICC NETWORK STRUCTURE QUANTIFICATION

preferential alignment of the ICC network processes in the longitudinal direction in the ICC-depleted data set. There was no difference between the hole size of the WT and KO networks ($p=0.15$).

3.2.2.2  *Ano1* KO

The metric values of the WT and KO networks from the *Ano1* data set are plotted in Fig. 3.5. There were no statistically significant differences between the metric values of the WT and KO networks (density: $p=0.31$; thickness: $p=0.27$; hole size: $p=0.59$; contact ratio: $p=0.81$; connectivity: $p=0.14$; and anisotropy: $p=0.65$).

3.2.2.3  Postnatal Maturation

The metric values of the WT networks from the *Ano1* and 5-HT$_{2B}$ receptor data sets were compared to examine ICC network structural changes potentially occurring during postnatal maturation (three-day- and four-week-old respectively), and the results are plotted in Fig. 3.6. These results demonstrated that the four-week-old networks had on average a 38% higher thickness ($p<0.01$), 45% higher hole size ($p<0.01$), 37% lower contact ratio ($p<0.01$), and three times higher connectivity ($p=0.02$) as compared to the three-day-old networks. There was no difference in the density ($p=0.12$) or anisotropy ($p=0.88$) between the three-day- and four-week-old networks.

3.2.2.4  Metric Relationships

A strong non-linear relationship between the thickness and contact ratio metrics was observed (Fig. 3.7). In general, the thinner the network processes the higher the contact ratio of the network. No other obvious metric relationships were identified.
3.2. Application 1

Figure 3.4: Metric values of ICC networks from the 5-HT₂B receptor data set. A * symbol in the figure title indicates a statistically significant difference (p<0.05). A + symbol in the boxplot represents an outlier. Boxplots throughout this thesis are in the following format: the tips of the whiskers represent the maximum and minimum (excluding outliers), and the box marks the 1st quartile, median and 3rd quartile.

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Figure 3.5: Metric values of ICC networks from the Ano1 data set. No statistically significant differences ($p<0.05$) were present. A + symbol in the boxplot represents an outlier.
3.2. Application 1

Figure 3.6: Metric values of three-day and four-week WT ICC networks from the Ano1 and 5-HT$_{2B}$ receptor data sets respectively. A * symbol in the figure title indicates a statistically significant difference ($p<0.05$). A + symbol in the boxplot represents an outlier.
3. ICC NETWORK STRUCTURE QUANTIFICATION

![Figure 3.7: Contact ratio versus thickness metric values of WT and KO networks from both the 5-HT$_{2B}$ receptor and Ano1 data sets. Different symbols indicate different groups of ICC network imaging data.](image)

3.3 Application 2: Spry4 KO

3.3.1 Imaging Data Set

Sprouty homologs (Spry) are negative regulators of Kit receptor tyrosine kinase signalling, and Spry4 is upregulated in a murine model of GI stromal tumours [75]. However, the effect of Spry4 KO has not been characterised in the GI tract. Spry2 KO has been reported to lead to enteric neuronal hyperplasia [171], and hence this metric application aimed to test whether Spry4 KO induces hyperplasia in ICC. Such an animal model would be valuable as, in contrast to the extensively studied effects of a lack of ICC on GI motility, the significance of an excess of ICC is not well understood.

Confocal ICC imaging data were obtained from the proximal jejunum of three-to four-month-old male WT and Spry4 KO mice using immunofluorescence staining procedures as previously described [101]. Briefly, tissue specimens were flushed with phosphate buffered saline (PBS), opened along the mesenteric border, stretched out and pinned flat mucosal side up on the surface of a Sylgard-coated petri dish. Slides were incubated for at least 48 hr at 4°C with the primary antibody against c-Kit (M14) (goat, 1:100; sc-1494, Santa Cruz Biotechnology, Santa Cruz, CA) with constant agitation. Following rinsing in PBS, slides were then incubated for another
Figure 3.8: Representative ICC network images from the proximal jejunum of three-to four-month-old male WT and Spry4 KO mice, as objectively defined by having metric values near the median of the group. The white regions represent ICC, whereas the background (black) represents non-ICC regions. Scale bar (bottom right) = 0.1 mm.

2 hr in the dark at room temperature with the Alexa Fluor 594 donkey anti-goat (1:200; Invitrogen) secondary antibody. Confocal image slices were sequentially taken throughout the transmural depth of the muscularis propria in fine steps of less than 0.9 µm and were volume-rendered in 3D. The ICC-MP network was relatively thin in the transmural direction (≈15 µm) and the majority of the network laid in-plane. Therefore, the image segments of the ICC-MP were stacked with a maximum intensity projection into 2D images representing the entire ICC-MP network structures.

Unbiased thresholding algorithms were then used to segment the images to obtain 2D bitmaps of the Kit-positive ICC-MP network structures (Fig. 3.8), thereby minimising any human influence in the process. Briefly, the general image processing procedure performed was as follows:

1. An average filter was applied on the original (greyscale) image.

2. The averaged image (from Step 1) was subtracted from the original image, and
3. ICC NETWORK STRUCTURE QUANTIFICATION

the range of pixel intensity values of the resultant image was normalised such that the range spanned across the entire spectrum of intensity values (black to white).

3. The intensity values were mapped to new values such that 1% of the pixels were saturated at low (black) and high intensities (white).

4. A threshold value was used to segment the image into a binary image containing only two types of pixels: ICC and non-ICC.

5. If necessary, falsely segmented ICC objects were manually removed.

6. Small ICC objects with a subthreshold number of pixels were removed.

7. The image was morphologically closed by performing an image dilation followed by erosion using a circular structuring template.

8. Small ICC objects with a subthreshold number of pixels were removed (threshold was elevated since Step 6).

9. Small non-ICC objects with a subthreshold number of pixels were removed.

The parameter values of the image processing procedure were selected such that the segmented image appeared visually consistent with the original image. Manual removal of falsely segmented ICC objects in Step 5 was rarely performed (only for less than five network samples in total). Identification of falsely segmented ICC objects was based on object size and morphology.

The orientation information of the ICC-MP network required for the computation of the anisotropy metric was either extracted from the ICC-DMP network as described in Section 3.2.1.1 or from structures identified via non-specific binding. These structures exhibited the general directions in which the LM and CM layers were oriented. The longitudinal direction was therefore extracted from the imaging data at the plane intersecting the LM layer and the MP (using the same method for extracting the circumferential direction from the ICC-DMP networks; see Section 3.2.1.1), and the circumferential direction was assumed to be orthogonal to the longitudinal direction. Before applying the structural metrics, the ICC-MP networks were pre-processed as described in Section 3.2.1.2.

ICC networks images were obtained from seven WT and seven KO mice, and the number of samples obtained from each mouse is shown in Table 3.2. The samples
3.4. Discussion

Table 3.2: Number of ICC network samples taken from each of the seven WT and seven Spry4 KO mice.

<table>
<thead>
<tr>
<th>Mouse</th>
<th>WT</th>
<th>KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>19</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>11</td>
</tr>
<tr>
<td>5</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>10</td>
</tr>
</tbody>
</table>

were 1024×1024 pixels at a resolution of 0.519 ᵁm/pixel, and hence corresponded to physical dimensions of 0.531×0.531 mm. Structural metrics were computed for each sample and were then averaged for each mouse. However, computation of the contact ratio metric was omitted due to its strong relationship with the thickness metric (see Section 3.2.2.4). The entire process of image acquisition, processing and analysis was conducted with the genotype of the mice blinded to the investigators.

The two-sample t-test was applied to identify changes in ICC network structure during Spry4 KO. Outliers were identified as described in Section 3.2.1.3 and were excluded from the statistical analysis. A p-value of less than 0.05 was considered statistically significant.

3.3.2 Results

The average ICC network metric values for the WT and Spry4 KO mice are plotted in Fig. 3.9. There were no statistically significant differences between the average metric values for the WT and KO mice (density: $p=0.46$; thickness: $p=0.95$; hole size: $p=0.28$; connectivity: $p=0.49$; and anisotropy: $p=0.49$).

3.4 Discussion

The current quantitative measures of cell counts and volume computations for ICC networks from full thickness tissue biopsies have identified several clinical conditions involving significant ICC depletion, including gastroparesis [77, 141] and diabetes [81]. However at the same time, there are also patients with significant symptoms but
Figure 3.9: Average ICC network metric values for the WT and Spry4 KO mice. No statistically significant differences ($p<0.05$) were present.
apparently normal ICC numbers (see Fig. 1.3) [77, 141]. This may reflect primary pathological involvement of other cell types including macrophages and neurons, but also may reflect changes in ICC networks that affect function not captured by the relatively simplistic measures currently used. This chapter has presented six numerical metrics for defining the structural properties of ICC networks in confocal images. These metrics enable detailed quantification of the density, thickness, spacing, contact, connectivity and alignment of ICC populations. To show their validity and utility, the metrics were applied in demonstration studies and revealed novel insights into structural changes occurring during ICC depletion and postnatal maturation.

In the demonstration studies in Application 1, ICC networks were found to be depleted and structurally altered in 5-HT$_{2B}$ KO mice but not in Ano1 KO mice. These data support and extend the previous analyses on these animal models using cell counts and volume computations [165, 173], with more detailed structural and statistical evidence. Although Ano1 regulates ICC proliferation [165], alternative mechanisms of proliferation such as Kit [102] and serotonin [173, 189] may compensate in the Ano1 KO model, resulting in structurally normal ICC networks.

During ICC depletion in the 5-HT$_{2B}$ receptor KO mouse model, the network density decreased, the ICC processes thinned and the network became less connected. However, the increased contact ratio in the KO networks may be due to the decreased thickness in contrast to a structural adaptation. The data also revealed, for the first time, that a potential remodelling phenomenon may occur in ICC-depleted networks. Despite the fact that the ICC processes thinned, the hole size metric value of the KO networks did not increase, suggesting a spatial rearrangement of ICC to maintain a consistent spread over adjacent SMC. Spatial remodelling in response to the SMC distribution is feasible, because SMC are primarily responsible for the production of stem cell factor, which is the most important promoter of ICC regeneration and survival [90]. Such a rearrangement could also serve a functional purpose, to optimise the distribution of electrical activity generated by the remaining ICC. If this remodelling did not occur, an increase in the spacing between ICC processes may lead to insufficient or patchy delivery of current to activate neighbouring SMC regions.

Another interesting and novel result from the analysis of the 5-HT$_{2B}$ ICC-depleted networks was the preferential alignment of ICC processes in the longitudinal
3. ICC NETWORK STRUCTURE QUANTIFICATION

direction. The mechanisms for this anisotropic remodelling are uncertain, however, it is possible that it could also convey functional benefits for the ICC-depleted tissue, by retaining the propagation of pacemaker activity down the organ as opposed to around it. If longitudinal coupling of pacemaker activity is disrupted such that the activity is not entrained down the tract, then ICC control over intestinal pacemaker activity frequency plateaus may be impaired or lost [31]. Therefore, during ICC depletion, the ICC network would need to be more intact longitudinally than circularly to deliver consistent entrained activity. Our results of remodelling during ICC depletion await confirmation in other animal models and in disease states before the broader significance of these findings and their meaning can be understood.

The results of comparing the three-day- versus four-week-old WT ICC networks give rise to an additional ‘pruning’ hypothesis that may occur during ICC network maturation. Although the immature networks had the same density as the mature ones, the processes were thinner and more closely spaced. This suggested the immature networks had a larger number of processes. The lower connectivity of the immature networks may be because of the presence of partially pruned processes, and the increased contact ratio may be due to the thinner processes. A detailed study on the postnatal developments of ICC in the murine small intestine was conducted by Mei et al. [132]. Although the quantitative analysis was primarily limited to ICC counts, a thickening of ICC processes during postnatal maturation was also visually observed [132]. An analogous mechanism of synaptic pruning exists in neuroscience, where an initial synaptic overgrowth is followed by judicial pruning during development [22]. This process is thought to serve physiological purposes of reparation and development, and ultimately to maintain efficient brain function [23]. Similarly, the abundance of processes in the immature ICC networks may not all be necessary or efficient, and throughout maturation the redundant or less effective ones would be discarded. As ICC networks only acquire adult morphology in the suckling period [58], pruning may optimise network performance to accommodate for the increasing workload due to organ growth [132] and maturing dietary influences.

The preliminary study in Application 2 did not identify any structural differences between ICC networks from WT and Spry4 KO mice, and hence does not support the hypothesis that Spry4 KO leads to ICC hyperplasia. However, biological variability of ICC networks is large, and thus a larger sample size may be required to confirm the effects, if any, of Spry4 KO on ICC network structure.
3.4. Discussion

It is interesting to note that the anisotropy metric values for the WT mice used in Application 2 were all positive and had rather high magnitudes (over 0.4), unlike the anisotropy metric values for WT mice from Application 1, which varied from approximately -0.5 to 0.5. This indicates that the WT ICC networks from Application 2 consistently displayed strong preferential alignment of processes in the longitudinal direction, whereas the WT networks from Application 1 did not show a consistent direction of preferential alignment. One possible explanation for this discrepancy is the age difference of the mice. The age of the mice from the 5-HT$_{2B}$ receptor and Ano1 data sets from Application 1 were four-week- and three-day-old respectively, whereas the WT mice from Application 2 were three- to four-month old. Mice wean at three weeks of age, and this transition to a solid diet may stimulate further remodelling of the ICC network structure as ICC are stretch-sensitive [187]. This remodelling may serve the functional purpose of strengthening longitudinal pacemaker activity propagation to respond to the increased digestive workload and successfully propel luminal contents along the small intestine. Further investigation on post-weaning changes in ICC network structure and function is necessary to confirm the above hypothesis.

It should be noted that the various changes in ICC network structure identified here do not necessarily correspond to changes in motility. In fact, although 5-HT$_{2B}$ KO mice showed depleted ICC networks, their intestinal transit times were not affected [173]. Lammers et al. also reported that loss of ICC in diabetic rats did not affect the propagation of GI electrical activity [111]. These observations may reflect the purpose of the proposed remodelling phenomenon occurring in ICC-depleted networks, namely to preserve functional performance with reduced ICC numbers and volume. Other possible explanations include the presence of an ICC ‘reserve’ preventing ICC damage from immediately manifesting as motility disorders (see Chapter 1), or, as Lammers et al. suggested, GI dysfunction may be more affected by impairments in systems other than ICC, such as the enteric nervous system or smooth muscle cells [111]. A better understanding of the factors which ultimately determine GI motility is therefore necessary before accurate prediction of motility changes can be achieved.

The 5-HT$_{2B}$, Ano1 and Spry4 KO animal models do not show any physical changes to the organ size, and hence the changes in network properties identified reflect the structural changes specifically occurring within the ICC networks, rather than being an epiphenomenon. In terms of the postnatal maturation analysis, al-
though there is significant growth in organ size from three days to four weeks of age in mice, the identified changes in ICC network structure did not resemble those of stretch, and hence it is expected that the reported observations are genuine developmental changes. However, in studies involving, for example, tissue distension in pseudo-obstruction and similar animal models where the organ is likely to exhibit acute physical changes, additional measures should be taken to account for changes in organ size when applying these metrics on the imaging data. One such strategy, as employed previously [141], is to stain for another unaffected cell type, for example, smooth muscle nuclei as well as for ICC, and then the nuclei counts can be used as a normalising factor to ensure standardised structural comparison between different tissue samples. On the other hand, stretch induced by tissue handling in the experimental procedure may still be present, and hence may have influenced some of the numerical metric values. It is difficult to differentiate between this stretch effect and natural variation in the ICC network structure, both of which may have contributed to the large variance in the reported results. Although measures for minimising errors due to stretch have and should be taken (i.e., meticulous care in tissue handling, multiple samples), methods for further addressing this issue need to be investigated.

A limitation of the thickness and hole size metrics is the lack of sensitivity to tapering effects. That is, the thickness metric does not significantly respond to the rate of decrease of thickness along a cell body or process, and the hole size metric does not effectively reflect the rate of decrease of radius within a non-ICC region. In the computation of these metrics, only the regional maxima of the distance transforms were taken into account. This efficiently captures the general structures of interest, but may ignore the finer details of the tapering regions. However, even if quantified, tapering effects are not expected to have a large influence on the metric values. Also, as demonstrated in Fig. 3.1f, ICC islands may be falsely identified under a limited field of view, which influences the connectivity metric value. That is, a single ICC island may be identified as multiple islands as the ICC pathways connecting them are outside the current field of view. To mitigate this potential error, a larger field of view should be used where possible. This would potentially capture the necessary ICC pathways, and even if not, the larger tissue area would lessen the magnitude of the introduced error. In calculating the anisotropy metric, the longitudinal and circular directions of the organ were assumed to be orthogonal. This assumption is expected to be reasonably accurate, but at the same time it is unlikely these
directions are exactly perpendicular, and hence minor errors would be introduced into this metric value.

The numerical metrics presented in this chapter assess the ICC network as a functional syncytium and individual cell boundaries are not considered. These metrics therefore quantify the structural properties of the network as a whole, rather than that of individual cells. However, for the contact ratio and connectivity metrics in particular, intercellular coupling such as gap junctions [37] and close apposition membranes [79] is likely an important factor related to these aspects of network structure, and yet this information cannot be captured using the current syncytial approach. Therefore, further development of these numerical metrics should also consider identifying individual cells in order to incorporate intercellular coupling information.

The two application studies in this chapter have only applied the structural metrics on ICC-MP networks, but in fact the metrics can be applied on any 2D imaging data, including biological network structures from different organs, regions, or populations. However, it should be kept in mind that the interpretation of the metric results should always be inferred back to the structures being analysed. Also, the definitions of the numerical metrics presented are for 2D analysis, but the concepts are readily extendable to 3D. This extension is mandatory in order to apply the metrics to human imaging data, as the ICC reside in much thicker tissue volumes [77] with processes extending in the transmural direction as well [119], and hence 2D representations of the network structures become inadequate. The current implementation of the numerical metrics was relatively computationally inexpensive for small-scale ICC networks. However, as large-scale imaging data become available [157] or as the metrics are extended to 3D, the computational costs incurred will increase, and hence the implementation may be revised to improve efficiency.

The functional significance of the potential remodelling and pruning phenomena discussed here could be investigated using mathematical simulation techniques, whereby the network structures are embedded with biophysically-based cell models and coupled to an electrical activity modelling framework, as previously demonstrated in GI tissue-specific modeling studies [47, 66]. The simulated pacemaker activity propagation across ICC networks could then be correlated to the structural changes observed in the confocal images.
3. ICC NETWORK STRUCTURE QUANTIFICATION

3.5 Chapter Summary

In summary, this chapter presented a set of six numerical metrics for assessing the structural properties of confocal ICC networks. These metrics allowed for the first detailed automated analyses and unbiased quantitative comparisons of ICC network structures. Their application supported and extended previous studies utilising simplistic analyses and qualitative assessments with more detailed structural and statistical evidence, and provided a quantitative means for testing new hypotheses on factors influencing ICC network structure. The demonstration studies also revealed novel network remodelling and pruning phenomena that may be important for ICC function.

Acknowledgements

I would like to thank Drs. Peng Du and Gregory O’Grady for beneficial discussion and critical review of sections of this chapter; Drs. Gianrico Farrugia and Simon J. Gibbons for providing the ICC network imaging data described in Section 3.2; and Prof. Jean-Marie Vanderwinden for providing the ICC network imaging data described in Section 3.3.
Chapter 4

ICC Network Pacemaker Activity Simulations

This chapter presents two multiscale computational models for simulating pacemaker activity over specific ICC network structures. Such computational models provide a virtual platform for gaining a holistic view encompassing ICC pacemaker activity to GI function, which current experimental techniques cannot capture. Section 4.1 employs the established methodology of coupling biophysically-based cell models to the bidomain model (see Section 2.4.2; hereby termed biophysically-based simulations) in conjunction with the structural metrics presented in Section 3.1 to investigate the structural and functional changes that occur in postnatal development of ICC networks. In Section 4.2, a new cellular automaton model is presented and applied to demonstrate the impact of ICC depletion on pacemaker activity propagation.

4.1 Postnatal ICC Network Developmental Changes

In mammals, nutrition supply via the placenta ceases at birth and the animal becomes reliant upon the GI tract to digest and absorb nutrients from milk [14]. Shortly after, the GI tract has to be prepared for the dietary changes that occur during weaning [82]. The GI tract must therefore undergo adaptations to cope with this changing workload.
In mice, ICC or their precursors first appear in the intestinal wall at embryonic
day 12 (E12) [102, 177]. An intact ICC-MP network with functional pacemaker
activity is formed by postnatal day 2 (P2) [117], whereas ICC-DMP form shortly
after birth, before P5 [176, 177]. Despite the early appearance of ICC, these net-
works continue to develop postnatally. ICC-MP cell density increases after birth
and peaks at approximately two weeks of age before decreasing [102, 132, 176], and
the network morphology adapts throughout postnatal life [132]. Also, the full adult
ultrastructural phenotype of ICC-MP is only obtained by P17 [54] and after P21 for
ICC-DMP [53].

It is well known that the central nervous system (CNS) ensures the development
of a functional neuronal circuitry through the robust mechanism of initial excessive
overgrowth followed by selective pruning of axon branches [120, 121, 124], and a
recent study has also identified postnatal development in the electrophysiological
and morphological properties of neurons in the enteric nervous system [65]. Similar
to these concepts, a potential pruning-like mechanism that occurs during postna-
tal ICC network development was previously identified by comparing three-day-
and four-week-old murine intestinal ICC-MP networks (see Section 3.4) [67]. This
study therefore aimed to identify, quantify, and characterise structural changes in
ICC network imaging data acquired at multiple ages throughout early postnatal life
to test the presence of a pruning-like mechanism, and to elucidate the temporal
course of this process. In addition, biophysically-based computational simulations
of ICC pacemaker activity over the imaged ICC networks [47, 66] were performed to
gain quantitative insights into the functional significance of the identified structural
changes.

4.1.1 Materials and Methods

4.1.1.1 ICC Network Imaging Data

ICC network imaging data was collected using methods as described previously
[108, 132]. All experiments were performed in accordance with the Health Guide
for the Care and Use of Laboratory Animals of the Third Military Medical Univer-
sity (Chongqing, China). In summary, five to ten minutes prior to laparotomy the
calcium antagonist papaverine (0.12 mg/g) was injected intraperitoneally into 29
BALB/c mice at postnatal day 0 (P0), P2, P5, P8, P12, P16, and P24 (P0-P8: n=5
**4.1. Postnatal ICC Network Developmental Changes**

<table>
<thead>
<tr>
<th>Age</th>
<th>Number of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0</td>
<td>8</td>
</tr>
<tr>
<td>P2</td>
<td>9</td>
</tr>
<tr>
<td>P5</td>
<td>5</td>
</tr>
<tr>
<td>P8</td>
<td>11</td>
</tr>
<tr>
<td>P12</td>
<td>10</td>
</tr>
<tr>
<td>P16</td>
<td>10</td>
</tr>
<tr>
<td>P24</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>61</td>
</tr>
</tbody>
</table>

Table 4.1: Number of ICC network samples taken from mice at each postnatal age.

for each age; P12-P24: \(n=3\) for each age) in order to abolish contractile activity of the small intestine. The entire small intestine (from the pylorus to the ileocecal junction) was removed and placed into phosphate buffered saline (PBS) containing papaverine (0.5 \(mg/ml\)) and the diameter and length of the small intestine was measured immediately. To obtain whole-mount preparations, the small intestine was inflated back to these original dimensions and fixed for 30 \(min\) at 4°C with acetone. The longitudinal smooth muscle layer containing the ICC-MP network was prepared under a dissecting microscope, rinsed in PBS and placed in PBS containing 0.3% Triton X-100 at 4°C for 5-10 \(min\). The specimens were first incubated with a blocking solution (4% bovine serum albumin/PBS) for 30 \(min\) at room temperature to avoid non-specific staining, followed by a rat monoclonal anti-\(c\-Kit\) antibody (ACK2, 1:100; eBioscience) for 8 \(hr\) at 4°C. Immunoreactivity was then detected using a Cy3-conjugated secondary antibody (anti-rat IgG, 1:100; Zymed). All specimens were handled in a consistent manner. Confocal image slices of the \(Kit\)-positive ICC-MP structures were sequentially taken throughout the transmural depth of the specimen, but as the ICC-MP network was thin in the transmural direction (\(\approx10\ \mu m\)) and the majority of processes laid in-plane, the image slices were stacked into a 2D representation of network structure containing all positively labelled cells and processes as performed previously [47, 132]. A total of 61 tissue samples were taken, and the number of samples at each age is shown in Table 4.1. The physical dimensions of the samples ranged from 0.363×0.379 \(mm\) to 0.379×0.388 \(mm\), whereas the resolutions ranged from 0.379 \(\mu m/pixel\) to 0.424 \(\mu m/pixel\).

Unbiased thresholding algorithms were used to segment the images and identify the \(Kit\)-positive structures, thereby minimising any human influence in the process. Briefly, the general image processing procedure performed was as follows:
4. ICC NETWORK PACEMAKER ACTIVITY SIMULATIONS

1. An average filter was applied on the original (greyscale) image.

2. The averaged image (from Step 1) was subtracted from the original image, and the range of pixel intensity values of the resultant image was normalised such that the range spanned across the entire spectrum of intensity values (black to white).

3. A threshold value determined using two-means clustering was used to segment the image into a binary image containing only two types of pixels: ICC and non-ICC.

4. The image was morphologically closed by performing an image dilation followed by erosion using a circular structuring template.

5. Small ICC objects with a subthreshold number of pixels were removed.

The parameter values of the image processing procedure were selected such that the segmented image appeared visually consistent with the original image. The segmented images were then pre-processed to remove artefacts for the application of the ICC network structural metrics (see Section 3.2.1.2) [67]. Briefly, small gaps with radius of two pixels ($\approx 0.8 \, \mu m$) or less in the network structure were joined, and small objects of less than four pixels ($\approx 0.65 \, \mu m^2$) were removed.

4.1.1.2 ICC Network Structural Metrics

Four numerical metrics (see Section 3.1) [67] were used to quantify the structural changes that occur in ICC-MP networks throughout postnatal development:

1. **Density**: measures the ICC network volume, representative of the amount of bioelectrical current generated and propagated;

2. **Thickness**: measures the width of cellular structures within the ICC network plane, which may impact electrical activity propagation through the network;

3. **Hole size**: measures the radius of non-ICC regions within the ICC network plane. Reflects the distribution of ICC throughout the MP (i.e., the ‘tightness’ of the network), and may relate to the uniformity of SMC activation;
4.1 Postnatal ICC Network Developmental Changes

4. Connectivity: measures the connectivity of the ICC network. Reflects the structural integrity of the ICC network, indicating the cohesion of entrainment pathways [178].

The anisotropy metric (see Section 3.1) was not computed in this study as the orientation information of the ICC networks was not available.

To quantify the abundance of ICC processes, an additional fifth metric was developed:

5. Branching index: computed as density divided by thickness (i.e., a dense network with thin processes indicates numerous processes).

4.1.1.3 ICC Network Pacemaker Activity Simulations

ICC pacemaker activity simulations over the ICC-MP network structures were conducted using a similar approach to previous studies [47, 66]. Briefly, the ICC networks were discretised into a finite element triangular mesh, with each pixel in the ICC network imaging data corresponding to a node point. The cellular activity of the ICC node points was represented using the biophysically-based Corrias and Buist ICC model [32] modified to incorporate a finite-state machine (FSM) approach [160], whereas the activity of the non-ICC node points was represented using a passive cell model with a zero active ionic current. The FSM modelled a voltage-dependent entrainment mechanism where the cellular activity of the ICC was divided into the two states of Active and Passive, and the transition between the states was determined by the membrane potential ($V_m$), Ca$^{2+}$ dynamics and a non-refractory period which defines the intrinsic frequency of the pacemaker activity. The following rules were defined for the FSM ICC model (Fig. 4.1) [160]:

1. If the cellular activity of a node is Active, it remains Active until the Ca$^{2+}$ dynamics return to a quiescent state. Then it changes to Passive.

2. If the cellular activity of a node is Passive, it remains Passive until $V_m$ exceeds an excitation potential threshold while in the non-refractory period, or until the cellular activity of the node has passed the non-refractory period.
Figure 4.1: State transition diagram for the FSM Corrias and Buist ICC cell model [32, 160]. Q indicates if the Ca\textsuperscript{2+} dynamics are in a quiescent state; T indicates if \( V_m \) exceeds an excitation potential threshold while in the non-refractory period; and \( P \) indicates if the cellular activity of the node has passed the non-refractory period.

The established continuum-based bidomain model widely used to simulate cardiac electrical activity [83] was employed to model the tissue level activity, and the integrated model was solved using the CHASTE computational framework [137]. The solution process of the bidomain model was modified for each ODE time step as described previously [160]:

1. \( V_m \) at each node point was evaluated using the bidomain model.

2. Based on the newly obtained \( V_m \), the cellular activity states of the ICC nodes were updated using the FSM rules.

3. For Passive cellular activity ICC nodes a zero active ionic current was imposed, which is equivalent to the passive cell model used to represent the non-ICC node points.

4. For Active cellular activity ICC nodes the ionic current was evaluated using the Corrias and Buist ICC model [32].

5. When transitioning from Passive to Active, the state variables of the cell model were reinitialised to represent the beginning of a new cycle of Ca\textsuperscript{2+} dynamics.
4.1. Postnatal ICC Network Developmental Changes

ICC pacemaker activity was simulated over each of the 61 networks for 1000 ms with an ODE time step of 0.1 ms and a PDE time step of 1 ms. ICC node points within a square corresponding to 1% of the total network area at the top-right corner of the network were set to activate at time $t = 0$ ms as the initial stimulus to the simulations, whereas the remaining ICC node points were activated via the voltage-dependent entrainment mechanism of the ICC model [160]. The conductivity parameters of the model were selected such that the Ca$^{2+}$ wavefronts propagated through the network at approximately 2 mm/s as observed experimentally [144], and were normalised against the resolution of the network imaging data.

Four measures were used to quantitatively assess the simulated ICC pacemaker activity (Fig. 4.2). One measure was based on the electrical event (average $V_m$ over the network), as this electrophysiological activity contributes to the coordination of small intestinal motility [92], whereas the other three measures were based on the average intracellular calcium concentration over the network ($[Ca^{2+}]_i$):

1. *Activation rate*: computed as the network area divided by the activation time ($t_a$), which was defined as the time taken for the average $V_m$ over the network to reach a threshold of -30 mV. This value was chosen to be slightly lower than the ICC model plateau potential of approximately -25 mV to account for the non-ICC node points;

2. *Peak* $[Ca^{2+}]_i$ ($c_p$);

3. *Time to peak* $[Ca^{2+}]_i$ ($t_p$);

4. *Half peak* $[Ca^{2+}]_i$ time ratio: computed as the ratio of time to half peak ($t_{0.5p}$) to time to peak $[Ca^{2+}]_i$ ($t_p$). This measure gauges the dynamics of the $[Ca^{2+}]_i$ upstroke, with a small value ($\approx 0$) indicating an early initial rise, and a large value ($\approx 1$) indicating a late initial rise.

More emphasis was placed on the Ca$^{2+}$ dynamics as it plays a key role in the initiation of the electrical event [150, 175]. Specifically, Ca$^{2+}$ release through IP$_3$ receptors in the endoplasmic reticulum and subsequent Ca$^{2+}$ entry into mitochondria is a prerequisite of ICC electrical pacemaker activity generation [180].
4. ICC NETWORK PACEMAKER ACTIVITY SIMULATIONS

4.1.1.4 Statistical Analysis

The Kruskal-Wallis one-way ANOVA on ranks followed by multiple comparisons (Dunn’s test) was applied to identify differences in structure and function between sampling age pairs. Outliers were identified as described in Section 3.2.1.3 and were excluded from the statistical analysis. Due to the logarithmic nature of the connectivity metric, the logarithm (base 10) of the connectivity metric values were taken
before conducting the tests. A $p$-value of less than 0.05 was considered statistically significant.

### 4.1.2 Results

Figure 4.3 shows representative ICC-MP networks from the murine intestine at the various postnatal ages, as objectively defined by having structural metric values near the median of the respective groups.

The simulation time over each network was approximately 2 hr using 48 cores from Intel® Xeon® X5660, E5-2680, or E7-2870 CPUs. An example of a simulated Ca$^{2+}$ wave propagation sequence from $t = 200-1000$ ms is shown in Fig. 4.4. The underlying ICC network was 885×909 pixels at a resolution of 0.42 µm/pixel. The Ca$^{2+}$ wave was initiated in the top-right corner and in general, propagated toward the bottom-left corner of the network.

#### 4.1.2.1 Structural Changes

Significant changes through time were identified in the density, thickness, hole size, and branching index metrics (Kruskal-Wallis test: $p<0.01$ for all four metrics; Fig. 4.5), but no change was found in the connectivity metric ($p=0.07$; data not shown). Note that the results reported below and in Section 4.1.2.2 are based on the corresponding statistical analysis and not the general trends of structural metric and functional measure values of ICC networks with postnatal age exhibited in Figs. 4.5 and 4.6 respectively.

The network density from P12 to P24 was higher than that at P5, with the density at P16 being higher than that at P0 as well. The thickness of the ICC processes decreased from P2 to P5, and then increased between P5 and P16. The network hole size decreased by P16 compared to P0, and by P24, the hole size was smaller than at both P0 and P2. The branching index was higher at P12 and P24 compared to P0 and P2, indicating an increased number of processes between these ages.

Integrating these observations, the temporal course of postnatal ICC network structure development is summarised in Table 4.2. In the short period immediately
Figure 4.3: Example ICC-MP networks from the murine intestine at various post-natal ages as revealed by Kit immunoreactivity. The networks in the second and third columns are enlarged views of the bottom-right quarter and sixteenth of the networks in the first column respectively, as indicated by the white squares in the P0 network in the first column. Scale bar (bottom right) = 100 μm, 50 μm and 25 μm for the first, second and third columns respectively.
Figure 4.4: Example simulated Ca\textsuperscript{2+} wave propagation sequence over the P0 network in Fig. 4.3. The black regions represent the non-ICC regions with a [Ca\textsuperscript{2+}]\textsubscript{i} of 0 nM, whereas the blue to red colour field represents the varying levels of [Ca\textsuperscript{2+}]\textsubscript{i} in the ICC over time. The first image on the left in the top row shows the Ca\textsuperscript{2+} distribution at time \( t = 200 \text{ ms} \), and each subsequent image, from left to right, then down to the next row, is 100 ms apart. The Ca\textsuperscript{2+} wave was initiated in the top-right corner and in general, propagated towards the bottom-left corner of the network.

After birth (P0 to P2), the ICC network remained in an immature state. Subsequently, an overgrowth in the number of ICC processes had begun by P5 and lasted until P12, as the branching index was higher at P12 than at P0 and P2, but not P5 onwards. This process overgrowth also resulted in an increased density at P12 compared to P5. The decreased thickness at P5 compared to P2 may facilitate the ICC process overgrowth by allowing more processes to be formed per unit cell volume. The lack of a significant difference in branching index at P16 compared to earlier ages indicated that ICC processes had been discarded. However, the retained pro-
4. ICC NETWORK PACEMAKER ACTIVITY SIMULATIONS

Figure 4.5: Variation in the structural metric values of ICC networks with postnatal age. Cross (×) symbols indicate the respective mean metric values at each age. Asterisk (*) symbols indicate a significant difference in metric values compared to the age noted below the asterisk.

Table 4.2: Summary of the structural and functional changes in ICC networks during postnatal development.

<table>
<thead>
<tr>
<th>Age</th>
<th>P0</th>
<th>P2</th>
<th>P5</th>
<th>P8</th>
<th>P12</th>
<th>P16</th>
<th>P24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structure</td>
<td>-</td>
<td>Increased abundance of ICC processes.</td>
<td>Selected processes are retained and strengthened while others are discarded.</td>
<td>New ICC processes are formed, and the network is adjusted to its adult morphology.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Function</td>
<td>-</td>
<td>Pacemaker activity transmission efficiency and output volume increased.</td>
<td>Pacemaker activity transmission efficiency maintained and output volume further elevated.</td>
<td>Pacemaker activity transmission remained efficient.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
cesses were strengthened due to the increased network density and process thickness at P16 compared to P5, and this strengthening of processes also led to an increased density at P16 compared to P0. Finally, by P24 the higher branching index compared to P0 and P2 again showed formation of new processes, whereas the density remained higher than that at P5. Also, by P16 and P24, the network had smaller holes than at P0 and P2.

### 4.1.2.2 Functional Changes

Significant changes through time were identified in the activation rate, peak $[Ca^{2+}]_i$, and time to peak $[Ca^{2+}]_i$ (Kruskal-Wallis test: $p<0.01$ for all three measures), but no change was found in the half peak $[Ca^{2+}]_i$ time ratio ($p=0.11$; Fig. 4.6).

The activation rate at P12 and P16 was higher than at P5. The peak $[Ca^{2+}]_i$ at P12 was higher than at P5, and was higher than that at both P0 and P5 by P16. The time to peak $[Ca^{2+}]_i$ was shorter between P12 to P24 compared to P5, with P12 and P16 being shorter than P0 as well.

The functional changes are reported in relation to the structural changes, and summarised in Table 4.2. By P12, at the end of the overgrowth of ICC processes, the network had attained higher pacemaker activity transmission efficiency than in the immature state (P0) and before the overgrowth (P5), as seen from the increased activation rate and decreased time to peak $[Ca^{2+}]_i$. The functional output volume quantified by the peak $[Ca^{2+}]_i$ also increased since before the overgrowth (P5). At P16, after the pruning-like phenomenon of ICC processes, the high pacemaker activity transmission efficiency was maintained, and the functional output volume increased since both the immature state (P0) and before the overgrowth (P5). Finally, at P24, after the formation of new ICC processes, the pacemaker activity transmission remained efficient, as the time to peak $[Ca^{2+}]_i$ was still shorter than at the start of ICC process overgrowth (P5), but perhaps not as efficient as the optimal levels observed after the overgrowth (P12) and pruning-like phenomenon (P16).
4. ICC NETWORK PACEMAKER ACTIVITY SIMULATIONS

Figure 4.6: Variation in the functional measure values of ICC networks with postnatal age. Cross (×) symbols indicate the respective mean measure values at each age, and plus (+) symbols indicate outliers. Asterisk (*) symbols indicate a significant difference in functional measure values compared to the age noted below the asterisk.

4.1.3 Discussion

This study was motivated by the hypothesis that the intestinal ICC-MP network undergoes major structural changes during early mammalian postnatal development, serving to adapt and optimise gut function for acquiring nutrients for growth and survival. In particular, the presence of a pruning-like mechanism occurring during postnatal ICC network development (see Section 3.4) [67], similar to that observed in the CNS [120, 121, 124], was tested. Understanding this early postnatal developmental process is important because impaired ICC development has been associated with several early postnatal GI disorders, including transient neonatal pseudo-obstruction [99] and infantile hypertrophic pyloric stenosis [179]. To achieve
4.1. Postnatal ICC Network Developmental Changes

this, numerical metrics for quantifying ICC network structural properties were applied on murine intestinal ICC-MP imaging data at various postnatal ages, and ICC pacemaker activity was simulated over tissue-specific networks using biophysically-based computational modelling to evaluate the structure-function relationships in these networks.

There was excellent correlation between the timing of statistically significant changes in ICC network structure and function throughout postnatal development, as revealed by the separate approaches of structural metric analysis and biophysically-based modelling. Together, these results elucidated the various events and temporal course of ICC network development, as well as the physiological significance of it. It was identified that during postnatal development of ICC networks, there is an initial process overgrowth followed by a pruning-like phenomenon of processes, and subsequently formation of new processes. The concept of axon overgrowth followed by pruning during development is well established in the CNS [120, 121, 124] as a mechanism to ensure the development of a robust neuronal circuitry. Similarly, in ICC network development, an abundance of ICC processes are initially grown to achieve high pacemaker activity transmission efficiency, which may also ensure propagation stability and avoid the development of arrhythmias, as well as to increase the output volume of the pacemaker activity. Subsequently, during the pruning-like phenomenon, the less efficient or redundant processes are discarded without impacting the overall efficiency of the network, and the retained processes are also strengthened to further elevate the pacemaker activity output volume. Mice wean at three weeks of age, and hence the formation of new processes after the overgrowth-pruning-like cycle may offer a chance for the ICC network to adjust to the altered luminal contents and workload. The simulation results showed that pacemaker activity transmission was still efficient after the formation of new processes, but was not as pronounced as after the overgrowth and pruning-like phenomenon. Therefore, these new processes may develop with objectives other than optimising pacemaker activity. One possibility would be development in response to myenteric neurons, which adapt morphologically and electrophysiologically throughout postnatal life until adulthood [65], as ICC are also responsible for integrating neurotransmission with its pacemaker activity [101]. The reduction in hole size observed around the time of weaning (P16 and P24) indicates an improved spatial distribution of the generated pacemaker activity, which may aid in propagating this pacemaker activity through the thickened smooth muscle layers [132].
It is also interesting to note the close correspondence between the structural changes identified in this study and other aspects of ICC and intestinal development presented previously. After birth, the ICC network structure remained in an immature state until P2. It has been shown that ICC-MP only establish mature ultrastructural features and electrophysiological activity during this period [117], and hence development in the network structure may not occur before the individual cells have fully developed (i.e., development during this period focuses on intracellular rather than intercellular aspects). Also, it is known that the Kit receptor is essential for ICC development and intestinal pacemaker activity [92, 126], but W^{bld}/W^{bld} mutant mice with a down-modulation of Kit expression [103] display a normal network of ICC at P5, and the lack of a functional ICC network and intestinal pacemaker activity in adult W^{bld}/W^{bld} mice is due to hampered ICC development after P5 [102]. Therefore, inadequate signalling through the Kit receptor may lead to a failure in the initial overgrowth of ICC processes and consequently disrupts the downstream developmental events.

Structural metric analysis did not show any change in ICC network connectivity from birth through to weaning. This result was as expected since cohesion of the entrainment pathways is crucial for functional pacemaker activity [178], which is present throughout normal postnatal development. Also, the lack of any differences in the half peak $[Ca^{2+}]_i$ time ratio functional measure indicates that the $[Ca^{2+}]_i$ upstroke dynamics do not change throughout postnatal life. That is, the $[Ca^{2+}]_i$ upstroke waveform is approximately linearly scaled at different postnatal ages.

The small intestine grows significantly in size throughout postnatal life [132, 161] which should result in continuous stretching of the ICC network, but the identified non-monotonic variation in network structure through time indicates that these observed changes are not simply an epiphenomenon of growth and the accompanying stretch. On the other hand, stretch to the tissue induced during the experimental procedure may influence some of the structural metric and functional measure values reported. Although precautions to minimise these errors have been taken (i.e., meticulous care in tissue handling, multiple samples), it is difficult to quantify the extent of the induced stretch and hence the magnitudes of the introduced errors. However, as all animals and tissue were treated in exactly the same manner, we can anticipate that the degree of stretch is quite consistent across specimens, therefore reducing the contribution of this variable to the data variance. Nonetheless, methods for further addressing this issue need to be investigated. One such strategy is to co-
stain and count smooth muscle nuclei, which was used in a previous publication to demonstrate that ICC loss in gastroparesis is not a secondary consequence of gastric distension in these patients [141]. Although stretch effects would not be eliminated, smooth muscle nuclei counts can quantify the relative levels of stretch between different tissue samples, and hence can be used as a normalisation factor for standardised analysis.

Both the structural and functional analysis techniques employed in this study view the ICC network as a functional syncytium and individual cell boundaries are not considered. It is expected that this approach is appropriate as ICC-MP networks are highly connected and hence individual cell boundaries (i.e., which processes belong to which cells) cannot be distinguished at the levels of magnification employed in this study [173]. Therefore, the reported changes in the branching of ICC processes throughout postnatal development are relative to per unit cell volume as opposed to per cell, and as the volume of individual ICC may not remain constant throughout development, the results of this study cannot be directly correlated to changes in ICC numbers.

The intervals between the sampling ages were selected to be sparser for the older ages (e.g., only two days from P0 to P2 but eight days from P16 to P24) because it was anticipated that the rate of development decays with time. These sparser sampling ages have been able to exhibit a general trend in the postnatal development of ICC networks, but further studies with a more refined temporal resolution may better capture the details of this developmental process. Another potential limitation of this work is that the ICC networks have been sampled from throughout the small intestine (i.e., from the duodenum, jejunum, and ileum), and comparable developmental patterns across the organ has been assumed. Another avenue of future work will be to examine whether any differences in developmental pattern exist between the different small intestinal regions, and if present, identify the interaction effects between spatial region and temporal development.

4.2 Cellular Automaton Simulations

Previous studies have simulated tissue-specific pacemaker activity propagation over ICC networks by coupling imaging data with a modified biophysically-based Corrías and Buist cell model [32] embedded within a continuum modeling framework
4. ICC NETWORK PACEMAKER ACTIVITY SIMULATIONS

[47, 66]. However, this methodology is computationally expensive [47], mainly due to the large number of equations and parameters present in the cell model, and hence difficulties exist in upscaling these simulations to larger multiscale simulation studies. An alternative strategy of implementing cellular automaton models to simulate pacemaker activity propagation has also been previously conducted [48, 111], but these previous studies focused on a larger spatial scale and did not incorporate structural detail from real ICC networks.

In this section, a new cellular automaton model is presented for simulating the activation phase of ICC pacemaker activity propagation over tissue-specific ICC networks, and numerical metrics for quantitatively measuring the observed propagation patterns are formulated as well. To demonstrate proof-of-concept, these tools were then applied to assess the differences in function between WT and 5-HT$_{2B}$ receptor KO (depleted ICC) networks from the murine jejunum.

4.2.1 Materials and Methods

4.2.1.1 Cellular Automaton Model

The cellular automaton model simulated the activation phase of ICC pacemaker activity propagation over a 2D grid-structure domain by taking discrete time steps. The individual grid nodes were separated into ICC and non-ICC types, and the simulated pacemaker activity propagation was defined by activation values ($a$) between zero and one at the node positions. The activation value is representative of the membrane potential, with zero representing the resting membrane potential (‘off’), and one representing the peak membrane potential (‘on’). As the model focused on the activation phase only, no further activity occurred for a node once it switched to the ‘on’ state. The model incorporated the following two components (Fig. 4.7):

1. Diffusion: This component modelled the passive diffusion effect of membrane potential. Regardless of node type (ICC or non-ICC), all ‘on’ nodes increased the activation values of immediate neighbouring nodes by the diffusion rate parameter $d$ per time step until these neighbouring nodes were also switched to the ‘on’ state (i.e., the activation value reached the maximum of one).
4.2. Cellular Automaton Simulations

Figure 4.7: Schematic diagram of the cellular automaton model, showing the decision processes of the two model components performed every time step to update individual nodal activation values. $a$ is the activation value of the node currently being considered unless otherwise specified, and $d$ and $e$ are the diffusion rate and entrainment threshold parameters described further in the text.

2. Entrainment: Isolated ICC spontaneously generate pacemaker activity at their own intrinsic frequencies [30], but in intact tissue ICC are organised in an electrical syncytium, and the cells become ‘entrained’ to generate activity matching the highest frequency present in the syncytium [178]. This component modelled this ICC characteristic. If at any time-step an ICC node possessed an activation value above the entrainment threshold parameter $e$, the node was immediately switched to the ‘on’ state.
4. ICC NETWORK PACEMAKER ACTIVITY SIMULATIONS

4.2.1.2 Simulation Setup

ICC pacemaker activity propagation was simulated over six ICC-MP networks from the jejunum of four-week-old WT and 5-HT$_{2B}$ receptor KO mice respectively. Normally, serotonin acts on the 5-HT$_{2B}$ receptors to increase ICC proliferation, and a lack of these receptors results in ICC depletion [173]. Two-dimensional bitmaps of the network structures were obtained from confocal imaging data as previously described (see Section 3.2.1) [47, 173], and were then oriented such that the longitudinal axis of the small intestine was vertical (Fig. 4.8). These structures represented physical dimensions of 0.225×0.225 mm with a resolution of 362×362 pixels, and each pixel was mapped to a node in the cellular automaton model to determine the node type (ICC or non-ICC). Therefore, note that numerous ICC nodes would be used to represent a single physical cell.

As an initial stimulus to the simulations, all ICC nodes in the top row of the grid-structure were prescribed to the ‘on’ state at time $t = 0$ ms to imitate a planar pacemaker activity wavefront travelling along the length of the intestine. Pacemaker activity propagation was simulated with a time step of 0.1 ms until all nodes were switched ‘on’. Simulation parameters $d$ and $e$ were both arbitrarily selected to be 0.1, and hence given an ‘on’ neighbouring node, $\text{switch}_{\text{N ICC}}$ and $\text{switch}_{\text{ICC}}$, the times required to switch non-ICC and ICC nodes from the ‘off’ to ‘on’ state were 1 ms and 0.1 ms respectively.

![Figure 4.8: Example WT (left) and 5-HT$_{2B}$ KO (right) ICC networks with the longitudinal axis of the small intestine aligned vertically. These networks represented physical dimensions of 0.225×0.225 mm with a resolution of 362×362 pixels. The white regions represent ICC, whereas the background (black) represents non-ICC.](image-url)
4.2. Cellular Automaton Simulations

4.2.1.3 Propagation Pattern Metrics

In order to quantitatively compare the resulting pacemaker activity propagation patterns simulated over different network structures, two numerical metrics were developed: the ICC and non-ICC activation lag metrics. These metrics measured, in ms, the average time delay in simulated activation times (AT, i.e., the times at which a node switched to the ‘on’ state) from the theoretical minimum AT of the ICC and non-ICC nodes across the entire network, respectively. That is, the differences between simulated and theoretical minimum AT were calculated for each node, and an average value for each of the node types was computed. The theoretical minimum AT for the ICC and non-ICC nodes were defined as follows for the simulation setup of this study:

1. **Minimum ICC AT** (\(\text{minAT}_{\text{ICC}}\)): ICC nodes in the top row were prescribed to ‘on’ as an initial stimulus, and hence these nodes had a theoretical minimum AT of 0 ms. As the diffusion component of the model responsible for the spread of propagation only considered immediate neighbouring nodes, it is only possible to switch ‘on’ a further row of ICC nodes every 0.1 ms (\(\text{switch}_{\text{ICC}}\)). Therefore, the theoretical minimum AT of ICC nodes can be computed as

   \[
   \text{minAT}_{\text{ICC}} = 0.1 \times (n_{\text{row}} - 1) \quad (4.1)
   \]

   where \(n_{\text{row}}\) is the row number starting with one at the top row.

2. **Minimum non-ICC AT** (\(\text{minAT}_{\text{NICC}}\)): Both the top and second-to-top rows were immediate neighbouring nodes to the initial ICC node stimulus, and hence the theoretical minimum AT for non-ICC nodes in these rows were 1 ms (\(\text{switch}_{\text{NICC}}\)). As the pacemaker activity propagated faster through ICC, assuming a pathway of ICC nodes was available to activate the non-ICC nodes in the subsequent rows below, each additional row delayed the theoretical minimum AT by 0.1 ms (\(\text{switch}_{\text{ICC}}\)). Therefore, the theoretical minimum AT of non-ICC nodes can be computed as

   \[
   \text{minAT}_{\text{NICC}} = 0.1 \times \max((n_{\text{row}} - 2), 0) + 1 \quad (4.2)
   \]

   where \(n_{\text{row}}\) is the row number starting with one at the top row.
The two-sample \( t \)-test was applied to compare the propagation pattern metric values of the simulated pacemaker activity propagations over the WT and KO networks. Outliers were identified as described in Section 3.2.1.3 and were excluded from the statistical analysis. A \( p \)-value of less than 0.05 was considered statistically significant.

### 4.2.2 Results

#### 4.2.2.1 Pacemaker Activity Propagation Simulations

The activation phase of ICC pacemaker activity propagation was successfully simulated over tissue-specific ICC network structures using the developed cellular automaton model (Fig. 4.9). It took on average 49.6±1.5 ms and 60.0±4.1 ms (±1 standard error) for the pacemaker activity to propagate across the entire WT and KO networks (i.e., for all nodes to switch to the ‘on’ state) respectively. The computational time required to simulate pacemaker activity propagation over a single network was approximately 3.5 s on a quad-core Intel® Xeon® W3530 CPU.

![Figure 4.9: AT maps of the simulated pacemaker activity propagations over the WT (left) and 5-HT\(_{2B}\) KO (right) ICC networks as shown in Fig. 4.8. The gradient of colours show the AT throughout the network, with red and blue indicating early and late activations respectively.](image-url)
4.2. Cellular Automaton Simulations

Propagation pattern metric values of the simulated pacemaker activity propagations over WT and 5-HT$_{2B}$ KO ICC networks. An asterisk symbol (*) in the title indicates a statistically significant difference ($p<0.01$). A plus symbol (+) in the boxplot represents an outlier.

### 4.2.2.2 Propagation Pattern Metrics

The propagation pattern metric values of the simulated pacemaker activity propagations over the WT and KO networks are plotted in Fig. 4.10. These data showed that pacemaker activity propagation over KO networks had on average a 14 times higher ICC activation lag metric value ($p<0.01$) and a 90% higher non-ICC activation lag metric value ($p<0.01$) compared to that over WT networks.

### 4.2.3 Discussion

A cellular automaton model for simulating the activation phase of ICC pacemaker activity propagation over tissue-specific ICC networks was developed, and as proof-of-concept this model was used to simulate pacemaker activity propagation over normal and depleted murine jejunal ICC networks. Two numerical metrics for quantifying propagation patterns were also developed and used to contrast the simulated propagation patterns over the WT and KO networks. These simulations and metrics showed with quantitative evidence that ICC depletion impaired propagation of ICC pacemaker activity across the network structure. These preliminary results are consistent with, and may help to explain, recent data on slow wave propagation from patients with gastroparesis, a disorder in which the stomach fails to empty normally. ICC depletion in these patients has been found to be associated with reduced slow
wave propagation velocities down the stomach [141]. Note that the formulations of the propagation pattern metrics were not biased by the ratio of ICC to non-ICC nodes. There is no direct relationship between the propagation pattern metrics and the ICC to non-ICC node ratio, so networks with different node ratios can still have the same metric values. Therefore, in the simulation, the quantified impairment in propagation over the KO networks was not purely a side-effect of less ICC nodes, but rather a feature of degradation of the network structure.

The cellular automaton model developed here provides a computationally efficient tool for modelling tissue-specific ICC pacemaker activity propagation in multiscale simulations. This model differs from previous GI electrical activity propagation cellular automaton models [48, 111] in that both ICC and non-ICC node types were explicitly represented, and appropriate cellular automaton rules were applied accordingly. This facilitates investigations of structure on function at much higher spatial resolutions, which is necessary to capture the ICC network structure. The model also produced streamlined results with the previous method of embedding biophysically-based cell models within a continuum modelling framework [47], but with greatly reduced computational expenses. Although not directly comparable, the previous method employed by Du et al. [47] took 2 hr to simulate 400 ms of pacemaker activity propagation over network structures defined by 262,144 nodes, whereas the simulations here only took 3.5 s to simulate approximately 60 ms of pacemaker activity propagation over network structures defined by 131,044 nodes. Assuming simulation time and number of nodes is linearly proportional to computational time, this corresponds to a speed-up of over 150 times. Further studies with a standardised modelling setup will be required to formally discern the similarity and differences between the different modelling strategies.

The developed cellular automaton model worked in time steps, and the time step value was essentially a scaling factor to the simulation output. However, for the simulated propagation to be physiologically realistic temporally, the selection of the time step value was critical. In the current simulation setup, it took a minimum of 361 time-steps to simulate propagation through a distance of 0.225 mm (the longitudinal dimension of the networks). The time step value of 0.1 ms was selected such that the maximum propagation speed across the networks was 6 mm/s, similar to that experimentally recorded in the healthy rat jejunum [111].
4.3. Chapter Summary

The pacemaker activity propagation simulations in this study are still relatively simplistic in comparison to real physiological activity. Basic cellular automaton rules were implemented in the pacemaker activity propagation model to exhibit the general behavior of only the diffusion and entrainment mechanisms; the model parameters ($d$ and $e$) were arbitrarily chosen; and the horizontally uniform initial stimulus to the simulations did not account for potential upstream effects (i.e., influence of network structures above the current field of view). However, as the simulations were all performed under a consistent setup, it is expected that the quantified differences in pacemaker activity propagation over the normal and depleted ICC networks remain valid.

As shown experimentally, transection of the ICC networks in vivo enables the intrinsic frequency of activity in distal parts of the network to be expressed [30]. In the future, an intrinsic component could be included in the model to examine the complex interactions between these different propagation mechanisms. The model and simulation setup can also be calibrated against previous modelling strategies and experimental data to improve the validity of the simulations.

4.3 Chapter Summary

This chapter demonstrated the application of two multiscale computational models to simulate ICC pacemaker activity over tissue-specific ICC network structures. First, quantitative ICC network structural metrics and biophysically-based simulations were employed to assess the structural and functional changes that occur in postnatal development of murine ICC networks. The analysis identified a pruning-like mechanism occurring during postnatal ICC network development which may facilitate mature digestive function, and elucidated the temporal course of this developmental process. The second part of the chapter presented a new cellular automaton model for simulating tissue-specific ICC pacemaker activity propagation, and was used to demonstrate impaired pacemaker activity propagation during ICC depletion. In comparison, biophysically-based simulations represent a more sophisticated and realistic approach as it is established on a sound biophysical basis, but on the other hand, cellular automaton simulations possess extremely high computational efficiency precisely due to its simplicity. The appropriate modelling approach should therefore be selected based on the context of the research, and in any case,
both these multiscale computational models offer a plausible method for demonstrating structure-function relationships of ICC networks across various spatial and temporal scales.

Acknowledgements

Section 4.1: I would like to thank Mr. Shameer Sathar for assisting with the ICC pacemaker activity simulations; Drs. Gregory O’Grady and Feng Mei for beneficial discussion and critical review; Dr. Juan Han for providing the ICC network imaging data; and Dr. Niranchan Paskaranandavadivel for assessing the quality of the ICC network imaging data.

Section 4.2: I would like to thank Drs. Peng Du and Gregory O’Grady for beneficial discussion and critical review; and Drs. Simon J. Gibbons and Gianrico Farrugia for providing the ICC network imaging data.
Chapter 5

Virtual ICC Network Generation

Currently, experimental and technical constraints in imaging ICC networks restrict the data to small fields of view and only having limited variability in network properties. This paucity in imaging data hampers the investigation of ICC network structure-function relationships. To overcome this issue, an alternative computational strategy is proposed for obtaining a comprehensive ICC imaging data set encompassing large-scale ICC networks across a spectrum of network properties. In this chapter, realistic virtual ICC networks are generated in silico using the stochastic Single Normal Equation Simulation (SNESIM) algorithm [66, 69]. The SNESIM algorithm was originally developed in the petroleum industry for building realistic statistical models of the geological formations which host oil reservoirs [166, 167].

5.1 Small-Scale Network Generation

This section aims to validate and demonstrate proof-of-principle of the SNESIM algorithm for generating realistic virtual ICC networks, and hence focuses on networks at a smaller spatial scale. The structural and functional similarity between experimentally-imaged and virtually-generated networks was assessed using ICC network structural metrics (see Section 3.1) [67] and biophysically-based computational modelling (see Section 4.1.1.3) [70]. Following validation, the SNESIM algorithm was also adapted to enable the generation of ICC networks across a spectrum of network properties.
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5.1.1 Materials and Methods

5.1.1.1 The SNESIM algorithm

The SNESIM algorithm generates virtual images of any size with similar structural properties to a user-supplied training image that contains the desired image characteristics. Full details on the algorithm and its user-supplied input parameters can be found in previous reports [118, 166, 167].

In general, the SNESIM algorithm replicates the underlying multiple-point statistics of the training image, which express the conditional probabilities of the values that can be taken by a pixel of interest based on the values of multiple neighbouring pixels. The relative locations of these neighbouring pixels to the pixel of interest are defined in the user-supplied data template, and the set of neighbouring pixels is termed the ‘data search neighbourhood’ (DSN).

To capture large-scale structural properties, the DSN needs to contain far-away neighbouring pixels, whereas for small-scale structural properties close by neighbouring pixels are required. However, as a larger number of neighbouring pixels within the DSN incurs greater computational costs, employing a DSN encompassing both small- and large-scale structural properties is often infeasible. The multi-grid approach overcomes this difficulty by sequentially generating the image over coarse to fine grids while scaling the DSN accordingly. Specifically, the algorithm begins with the coarsest grid where adjacent pixels are further apart, and hence the DSN efficiently covers a larger area. The grid is then recursively refined by including all pixels halfway between any two currently adjacent pixels, thereby shrinking the DSN by a factor of two with each refinement, until the finest grid is reached. With this approach, only close by neighbouring pixels are required in the DSN, as the scaling applied with the coarser grids allow large-scale structural properties to be captured. The coarseness of the initial grid is determined by the user-supplied number of multi-grids. Each time the grid is refined the multi-grid level decreases by one, and a multi-grid level of one corresponds to the finest grid. The DSN and number of multi-grids are therefore important input parameters to accurately replicate structural properties across a range of spatial scales.
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The specific steps taken by the SNESIM algorithm is summarised below (Fig. 5.1):

1. Starting from the highest multi-grid level, each pixel in the training image is scanned using the data template to generate the search tree, which records the multiple-point statistics of the training image for the current multi-grid level.

2. A random path that visits all pixels in the current multi-grid once only is defined.

3. Each undetermined pixel along the random path is scanned by the data template, and the values of the neighbouring pixels within the DSN are used to retrieve the conditional probability density function (PDF) for the current pixel of interest from the search tree generated in Step 1. If the values presented by the neighbouring pixels do not exist in the search tree (i.e., the pattern presented by the pixels in the DSN do not appear in the training image), the pixel in the DSN with furthest distance from the pixel of interest is dropped, and the conditional PDF for the current pixel of interest is re-retrieved. If all neighbouring pixels within the DSN are dropped, the value of the pixel of interest is generated from the user-defined global marginal PDF.

4. The PDF computed in Step 3 is adjusted to account for the local marginal PDF, and a value for the current pixel of interest is simulated from this adjusted PDF. This pixel is added into the image to inform subsequently simulated pixels.

5. Steps 3 and 4 are repeated until all pixels in the random path are simulated.

6. The multi-grid level is decreased by one and Steps 1 to 5 are repeated. All previously simulated pixels in the higher multi-grid levels are retained to inform the subsequently simulated pixels. The algorithm is terminated when multi-grid level one is completed.

In the context of generating virtual ICC networks, the SNESIM algorithm can only generate virtual networks with the same network properties as those of the training image as it simply replicates the multiple-point statistics. Therefore, the SNESIM algorithm was modified to enable the generation of ICC networks across a spectrum of network properties. The specific modifications are as follows:
Figure 5.1: Flowchart of the specific steps taken by the SNESIM algorithm.
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1. Two training images were used instead of one. These two training images represent two points on the spectrum of network properties, and hence by merging the properties of these two training images, ICC networks across a spectrum of network properties can be generated. A search tree was constructed from each of the training images.

2. A merge factor \( \phi \) was computed as:

\[
\phi = \frac{\rho - \rho_1}{\rho_2 - \rho_1}
\]  

(5.1)

where \( \rho \) is the user-supplied global marginal PDF, and \( \rho_1 \) and \( \rho_2 \) are the global marginal PDFs of training images one and two respectively. The user defines the desired network properties to be generated through \( \rho \), with a lower ICC probability corresponding to a state with less ICC. The parameter \( \phi \) determines the state of the desired network properties relative to those of the training images, with \( \phi=0 \) indicating the same network properties as training image one, and \( \phi=1 \) indicating the same network properties as training image two.

3. When simulating a pixel, a PDF was retrieved from each of the two search trees separately. The PDFs retrieved from the search trees corresponding to training image one and two were weighted by \( (1-\phi) \) and \( \phi \) respectively, and the sum of these weighted PDFs was used to simulate the pixel. Any negative probabilities were limited to zero, and probabilities over one were limited to one.

Note that the PDF adjustment to account for the local marginal PDF in the SNESIM algorithm (Step 4) involves the global marginal PDF of the training network, and as the modified SNESIM algorithm utilises two training networks, this PDF adjustment was not implemented.

5.1.1.2 ICC Network Imaging Data

Confocal ICC network imaging data were used as training images to inform the SNESIM and modified SNESIM algorithms [173]. Briefly, two-dimensional bitmap images of the Kit-positive ICC-MP structures were obtained from the jejunum of
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Four-week-old WT (normal) and $5-HT_{2B}$ serotonin receptor KO (depleted ICC) mice. Normally, serotonin acts on $5-HT_{2B}$ receptors to increase ICC proliferation and numbers [189], and a lack of $5-HT_{2B}$ receptors has been demonstrated to decrease ICC proliferation, numbers and network volume [173]. The orientation of the networks relative to the circumferential direction was extracted from the Kit-positive ICC-DMP structures (see Section 3.2.1.1) [67], and the ICC-MP structures were reoriented such that the circumferential direction was horizontal. These networks were also pre-processed to remove artefacts as previously described in Section 3.2.1.2 [67]. Briefly, small gaps with radius of one pixel ($\approx 0.6 \mu m$) or less in the network were joined, and small objects with less than four pixels ($\approx 1.54 \mu m^2$) were removed. One ICC-MP network was obtained from each of six mice (three WT, three KO) as described in Section 3.2.1 [173]. Each network was 362×362 pixels, and corresponded to physical dimensions of 0.225×0.225 mm.

Three virtual ICC networks were generated for each WT and KO training image using the SNESIM algorithm (i.e., nine WT and nine KO virtual networks). The global marginal PDF ICC probabilities of the WT and KO training networks were approximately 0.55 (range: 0.51-0.59) and 0.40 (range: 0.40-0.41) respectively, and the virtual networks were generated with global marginal PDFs matching their training counterparts.

To generate virtual ICC networks across a spectrum of network properties, the WT and KO training images were arbitrarily paired and used to inform the modified SNESIM algorithm. For each of the three training image pairs, three virtual ICC networks were generated with global marginal PDF ICC probabilities of 0.30, 0.35, 0.40, 0.45, 0.50, and 0.55 (i.e., nine virtual networks for each global marginal PDF; a total of 54 virtual networks). The highest ICC probability (0.55) was selected to match the WT training networks, but ICC probabilities lower than that of the KO training networks (0.30-0.35) were selected. This was because although the KO mice showed depleted ICC networks, their intestinal transit times were not affected [173], and hence virtual networks at greater depletion levels were attempted to be generated. All virtual networks generated were the same size and at the same resolution as the training networks.
5.1.1.3 Structural Validation

Five numerical metrics validated in the context of ICC structural analysis (see Section 3.1) [67] were used to quantify the structural properties of the training and virtual ICC networks:

1. **Density**: measures the ICC network volume;
2. **Thickness**: measures the width of cellular structures within the ICC network plane;
3. **Hole size**: measures the radius of non-ICC regions within the ICC network plane;
4. **Anisotropy**: indicates the degree of preferential alignment of ICC structures;
5. **Connectivity**: measures the connectivity of the ICC network.

Prior to the application of structural metrics, the virtual networks were pre-processed to remove artefacts in the same manner as conducted on the training networks (see Section 5.1.1.2). Structural validation of the SNESIM algorithm was performed by comparing the structural metric values of the training and virtual WT and KO networks. For the non-normalised metrics of thickness, hole size and connectivity (i.e., these metric have an infinite range), the relative errors of the metric values were computed, whereas for the normalised metrics of density and anisotropy, the errors were computed relative to the range of the metric (see Section 3.1) [67]. Due to the logarithmic nature of the connectivity metric, the logarithm (base 10) of the metric value was used in the error computation.

5.1.1.4 Functional Validation

ICC pacemaker activity was simulated over the training and virtual ICC networks using the biophysically-based computational model described in Section 4.1.1.3 [70]. The ICC networks were discretised into a finite element triangular mesh, with each node point corresponding to a pixel in the ICC network imaging data. The cellular activity of the ICC node points was represented using the biophysically-based Corrias and Buist ICC cell model modified to incorporate a finite-state machine approach.
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[32, 160], whereas the activity of the non-ICC grid points was represented using a passive cell model with a zero active ionic current. The continuum-based bidomain model was employed to simulate the tissue level activity, and the model was solved using the CHASTE computational framework [137].

ICC pacemaker activity was simulated over each of the training and virtual networks for 1000 ms with an ODE time step of 0.1 ms and a PDE time step of 1 ms. ICC node points within a square corresponding to 1% of the total network area at the top-right corner of the network were set to activate at time $t = 0$ ms as the initial stimulus to the simulations, whereas the remaining ICC node points were activated via the voltage-dependent entrainment mechanism of the ICC model [160]. The conductivity parameters of the model were selected such that the pacemaker activity propagated through the WT training networks at approximately 5 mm/s, as experimentally recorded in the healthy rat jejunum [111].

Four measures were then used to quantitatively assess the simulated ICC pacemaker activity as previously described in Section 4.1.1.3 [70]:

1. Activation rate: measures the rate at which the network achieves an average $V_m$ of -30 mV;
2. Peak $[Ca^{2+}]_i$;
3. Time to peak $[Ca^{2+}]_i$;
4. Half peak $[Ca^{2+}]_i$ time ratio: gauges the dynamics of the $[Ca^{2+}]_i$ upstroke.

Functional validation of the SNESIM algorithm was performed by comparing the functional measure values of the training and virtual WT and KO networks. For the non-normalised measures of activation rate, peak $[Ca^{2+}]_i$ and time to peak $[Ca^{2+}]_i$, the relative errors of the measure values were computed, whereas for the normalised measure half peak $[Ca^{2+}]_i$ time ratio, the errors were computed relative to the range of the measure (see Section 4.1.1.3) [70].

5.1.2 Results

Less than 1 min was needed to generate a virtual network using the SNESIM algorithm on an Intel® Xeon® W3530 CPU, whereas it took less than 2 min to generate a virtual network using the modified SNESIM algorithm on the same machine.
5.1.2.1 The SNESIM Algorithm

The three WT and three KO training networks along with an example virtual network generated from each training network are shown in Fig. 5.2.

The errors in the structural metric values of the virtual networks compared to their training counterparts are shown in Fig. 5.3. The virtual networks accurately replicated the density metric (average absolute errors: WT 0.8%; KO 1.7%), and in the majority of networks the thickness metric as well (WT 5.0%; KO 2.3%). Replication of the anisotropy (WT 4.3%; KO 4.9%) and connectivity metrics (WT 8.4%; KO 5.5%) in the majority of networks was reasonable. The hole size metric was relatively underestimated in the virtual networks (WT 16.0%; KO 16.8%).

The errors in the functional measure values of the virtual networks compared to their training counterparts are shown in Fig. 5.4. The virtual networks accurately replicated the peak [$Ca^{2+}$], (WT 2.0%; KO 2.2%) and half peak [$Ca^{2+}$], time ratio measures (WT 2.1%; KO 3.4%). The activation rate (WT 7.8%; KO 13.9%) and time to peak [$Ca^{2+}$], measures (WT 4.7%; KO 8.0%) were also reasonably replicated for the majority of networks.

5.1.2.2 The Modified SNESIM Algorithm

Example virtual networks generated using the modified SNESIM algorithm are shown in Fig. 5.5. Networks generated with different user-defined global marginal PDFs successfully exhibited a range of network properties, both in between and outside the spectrum presented by the two training networks. The structural metrics and functional measures also varied with respect to the global marginal PDF (Figs. 5.6 and 5.7), demonstrating that by altering the global marginal PDF, virtual networks with a range of structural and functional properties could be generated from two training networks of different properties. In general, the structural metrics and functional measures of the virtual networks displayed a monotonically increasing or decreasing trend with respect to the global marginal PDF ICC probability the virtual networks were generated with. However, the trends differed between the interpolated virtual networks (i.e., generated with global marginal PDF ICC probabilities of 0.40 (KO) to 0.55 (WT)) and the extrapolated ones (i.e., generated with global marginal PDF ICC probabilities of 0.30 or 0.35, below 0.40 (KO)). This is further discussed in Section 5.1.3.
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Figure 5.2: The three WT and three KO training networks (rows one and three) used to inform the SNESIM algorithm, and corresponding virtual networks (rows two and four) generated from each training network. The white regions represent ICC. Each network is $362 \times 362$ pixels, and corresponds to physical dimensions of $0.225 \times 0.225 \text{ mm}$. Scale bar (bottom right) = 0.1 mm.
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Figure 5.3: The percentage errors in the structural metric values of the WT and KO virtual networks compared to their respective training counterparts.

Figure 5.4: The percentage errors in the functional measure values of the WT and KO virtual networks compared to their respective training counterparts.

5.1.3 Discussion

In general, the virtual networks generated by the SNESIM algorithm possessed reasonably similar structural and functional properties to their training counterparts. For the virtual WT networks, four out of five structural metrics and four out of four functional measures had average absolute errors under 10%. For the virtual KO networks, four out of five structural metrics and three out of four functional measures had average absolute errors under 10%. The structural metric associated with the greatest errors was the hole size metric, which focused on examining the non-ICC regions within the ICC network. For a non-ICC region to remain intact, it must
Figure 5.5: Example virtual networks across a spectrum of network properties generated using the modified SNESIM algorithm. The white regions represent ICC. The headings along the left indicate the global marginal PDF ICC probabilities used to generated the virtual networks in that row, whereas the headings along the top denote the training networks used to generate the virtual networks (i.e., 1 denotes that the WT and KO 1 training networks were used to generate the virtual networks in that column). The WT and KO training networks correspond approximately to global marginal PDF ICC probabilities of 0.55 and 0.40 respectively, and hence virtual networks generated at global marginal PDF ICC probabilities within this range were interpolated, whereas those generated outside this range were extrapolated. Scale bar (bottom right) = 0.1 mm.
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Figure 5.6: The structural metric values of the virtual networks generated using the modified SNESIM algorithm against the global marginal PDF ICC probabilities they were generated at. Cross (×) symbols indicate the respective mean metric values at each of the global marginal PDF ICC probabilities.
be void of ICC pixels (otherwise the region essentially divides into multiple smaller regions). The multi-grid approach of SNESIM only simulates sparse pixels initially, leaving the intermediate pixels to be simulated subsequently, but when these intermediate pixels are being simulated the multi-grid level has decreased, and hence the scale of the DSN has also decreased. The algorithm therefore has a more restricted recognition of large-scale structures, and hence probabilistically, ICC pixels may be simulated, consequently reducing the hole size metric. If further accuracy in this metric is required by the operator, an increased DSN may improve the performance of SNESIM in this respect. However, the DSN increase required before obvious performance improvements are gained will likely incur large additional computational expenses.
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The average absolute errors in the time-related functional measures (activation rate, time to peak \([Ca^{2+}]_i\) and half peak \([Ca^{2+}]_i\) time ratio) were slightly higher for the virtual KO networks in comparison to the virtual WT networks. This was because the KO 1 training network was particularly sparse in the top-right corner where the initial stimulus for the ICC network pacemaker activity simulation was applied. Therefore, the onset of the simulated activity was delayed considerably, and the functional measures for the virtual networks generated from the KO 1 training network consistently showed faster activation rates, shorter times to peak \([Ca^{2+}]_i\) and smaller half peak \([Ca^{2+}]_i\) time ratios. The non-time-related measure of peak \([Ca^{2+}]_i\), however, was not affected. Therefore, taking into account these virtual KO networks with particularly high functional measure errors, the functional fidelity of virtual WT and KO networks are comparable.

In practice the accuracy of the algorithm is likely to be even greater than represented in this study, because high fidelity virtual ICC networks may be obtained by applying a further screening step. Numerous virtual networks can be generated and assessed based on their structural metrics, and networks with large structural errors can be discarded. Many of the virtual networks with large functional errors also have large structural errors, and hence virtual networks which are retained through screening will more likely exhibit realistic functional properties.

The pre-processing of the training ICC networks before informing the SNESIM and modified SNESIM algorithms is an important step to generating realistic virtual ICC networks. As these algorithms generate the pixels of the virtual network sequentially, pixels simulated earlier on influence subsequently simulated pixels. Any artefacts present in the training network may be stochastically reproduced in the virtual network, and once reproduced, subsequently simulated pixels may therefore also be affected by this artefact, leading to a cascade of errors.

The modified SNESIM algorithm was capable of generating virtual ICC networks with a range of structural and functional properties by retrieving a PDF from each of the two search trees corresponding to the two training networks, and then simulating pixels from a weighted sum of these PDFs. However, during extrapolation (i.e., when a virtual network is generated with network properties outside the spectrum presented by the two training images), the merge factor takes on values less than zero or greater than one, so a negative PDF weight will be present. The merging of the two PDFs hence becomes the subtraction of one from the other, albeit weighted, as
opposed to simply taking intermediate values in the case of interpolation. Therefore, interpolated virtual networks are likely to be more reliable in terms of fidelity than extrapolated virtual networks.

The PDF adjustment to account for the local marginal PDF in the SNESIM algorithm (Step 4) allows the generated virtual network to match the user-defined global marginal PDF closer by compensating other structural properties [118]. The density structural metric is analogous to the global marginal PDF ICC probability, and hence can be used to assess the degree to which the user-defined global marginal PDF is matched. Although the PDF adjustment was not implemented in the modified SNESIM algorithm, the density metric values of the virtual networks still matched the user-defined global marginal PDF closely. This could be because the merge factor is computed based on the user-defined global marginal PDF and is then used to form the merged PDF for generating the virtual network. Therefore, the user-defined global marginal PDF is inherently present in the virtual network generation process.

The computational cost of using the modified SNESIM algorithm to generate virtual networks was approximately double that of using the SNESIM algorithm as there were two training networks, and for each pixel simulated PDFs need to be retrieved from two search trees. However, with the current input parameters virtual networks can still be generated with reasonably low computational demands using both SNESIM and modified SNESIM. As these algorithms are capable of generating virtual networks of any size, experimentally obtained small-scale ICC network imaging data can be used to inform these algorithms to generate large-scale virtual ICC network imaging data at fields of view extending beyond the limitations presented by experimental imaging.

As future work, the modified SNESIM algorithm can be validated against experimental imaging data of ICC networks in obstruction. During obstruction, ICC networks exhibit a gradient of depletion in the oral direction from the site of obstruction [21], and hence ICC network imaging data can be obtained at various depletion levels throughout this gradient. The networks at the extremes of the spectrum (i.e., normal and severely depleted) can be used as the training networks for the modified SNESIM to test if virtual networks generated at intermediate depletion severities have similar structural and functional properties to those obtained experimentally.
5.2 Large-Scale Network Generation

This section employs the SNESIM algorithm to generate virtual ICC networks at spatial scales beyond the current limits of confocal microscopy. Small-scale spot samples were randomly taken from the large-scale virtual networks, and the structural and functional fidelity of these samples were assessed in the same manner as performed in Section 5.1. In order to simulate ICC network pacemaker activity propagation, the large-scale virtual networks were coupled to the computationally efficient cellular automaton model (see Section 4.2) [68], demonstrating the first multiscale computational framework in the GI field relating cellular structures to tissue level electrophysiology.

5.2.1 Materials and Methods

5.2.1.1 ICC Network Imaging Data

One WT and one KO ICC network as described in Section 5.1.1.2 were used as training networks to inform the SNESIM algorithm (see Figs. 5.9 and 5.10) [173]. Each network was 362×362 pixels, and corresponded to physical dimensions of 0.225×0.225 mm.

Three large-scale virtual ICC networks were generated from each WT and KO training network using the SNESIM algorithm. The global marginal PDF ICC probabilities for the virtual networks were set to match that of their training counterparts (WT: 0.5696; KO: 0.3664). Each virtual network was 40,500×20,000 pixels, and corresponded to physical dimensions of approximately 25.2×12.4 mm, representing the ICC-MP network from a 25 mm segment of the adult murine small intestine [132]. In order to achieve the ‘wrap-around’ effect of the cylindrical small intestine the virtual networks were generated in three steps (Fig. 5.8):

1. A virtual network with 40,500×10,000 pixels was generated first (A+B+C), representing half the small intestinal segment cut along the longitudinal axis.

2. The longitudinal edges of the virtual network generated in Step 1 were used to inform the generation of the second half of the small intestinal segment. The selected input parameters to SNESIM defined a DSN which only included
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Figure 5.8: Schematic diagram of the three steps for generating the large-scale virtual ICC networks around the small intestinal segment with circumferential consistency. Different sections of the virtual network have been labelled and are shaded differently. Refer to text for a description of the three steps. The dimensions represent the number of pixels. The diagram is not to scale.

3. After removing the duplicated appended pixels from the virtual network generated in Step 2 (A+C), the virtual networks from Steps 1 and 2 were concatenated to form the final virtual network around the small intestinal segment (A+B+C+D).
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5.2.1.2 Spot sample validation

Small-scale virtual networks were randomly spot sampled from the generated large-scale networks, and the structural and functional properties of these spot sampled networks were compared to their training counterparts in the same manner as described in Section 5.1.1 to assess fidelity. Five spot samples were taken from each large-scale virtual network (i.e., 15 WT and KO spot samples respectively), and each spot sample was 362×362 pixels, as were the small-scale virtual networks generated in Section 5.1. The locations of the spot samples were determined using a random number generator.

5.2.1.3 ICC Network Pacemaker Activity Simulations

ICC pacemaker activity propagation was simulated over the three WT and three KO large-scale virtual ICC networks using the cellular automaton model described in Section 4.2.1.1 [68]. Note that it would be impractical to perform these simulations using the biophysically-based modelling approach (see Section 4.1.1.3) [70] due to the extremely large computational costs that would be incurred. To imitate the simulation of pacemaker activity over a cylindrical small intestinal segment, the left and right edges of the virtual networks were considered to be immediately adjacent to each other. That is, in the diffusion component of the model (see Section 4.2.1.1) [68], the three pixels on the right edge in the row above, the same row, and the row below a pixel on the left edge were all considered immediate neighbours of the pixel on the left edge, and vice-versa. All ICC nodes within the 100 pixels centred in the middle of the top row of the network were prescribed to the ‘on’ state at time $t = 0 \text{ ms}$ as an initial stimulus to the simulations, which imitated a pacemaker site at that location. Pacemaker activity propagation was simulated with a time step of 0.1 $\text{ms}$ until all nodes were switched ‘on’, and the simulation parameters $d$ and $e$ were both arbitrarily selected to be 0.1.

5.2.2 Results

It took on average 35 $\text{hr}$ and 32 $\text{hr}$ to generate the first (A+B+C) and second halves (D) of a large-scale virtual network respectively on an Intel® Xeon® E5-4650 CPU.
Example spot sampled WT virtual networks are shown in Fig. 5.9, and example spot sampled KO virtual networks are shown in Fig. 5.10. The errors in the structural metric and functional measure values of the spot sampled networks compared to their training counterparts are shown in Figs. 5.11 and 5.12 respectively.

In terms of structure, the virtual networks accurately replicated the density metric (average absolute errors: WT 2.3%; KO 2.2%). For the majority of the networks, the thickness (WT 13.0%; KO 3.1%), anisotropy (WT 8.1%; KO 2.1%) and connectivity metrics (WT 14.0%; KO 10.3%) were reasonably replicated, and the hole size metric was relatively underestimated (WT 9.5%; KO 26.0%).

In terms of function, the virtual networks accurately replicated the peak \([Ca^{2+}]_i\) (WT 4.1%; KO 6.8%) and reasonably replicated the other three functional measures (activation rate: WT 12.5%; KO 11.5%; time to peak \([Ca^{2+}]_i\): WT 9.6%; KO 9.9%; half peak \([Ca^{2+}]_i\) time ratio: WT 3.8%; KO 7.0%) for the majority of networks.

The activation phase of ICC pacemaker activity propagation was successfully simulated over the large-scale virtual networks using the developed cellular automaton model. As there were too many pixels to construct full AT maps, the virtual networks were divided into blocks of 100×100 pixels (corresponding to 62.1×62.1 μm) and the average AT for all nodes within the blocks were used to display reduced-resolution AT maps (Fig. 5.13). It took on average 5.14 s (5.13-5.14 s) and 5.77 s (5.76-5.78 s) for the pacemaker activity to propagate across the entire WT and KO networks respectively, which corresponded to propagation velocities of approximately 4.9 mm/s over the WT networks and 4.3 mm/s over the KO networks. The computational time required to simulate pacemaker activity propagation over a single network was approximately 8 days on a shared memory system with 32 Intel® Xeon® X7560 CPUs.

5.2.3 Discussion

The spot sampled virtual networks possessed similar structural and functional properties to that of their training counterparts. For the virtual WT networks, all five structural metrics and all four functional measures had average absolute errors under 15%, whereas for the virtual KO networks, four out of five structural metrics and all four functional measures had average absolute errors under 15%. Within the structural metrics, the hole size metric displayed the highest errors, which is consistent with, and hence expected to be due to reasons similar to when generating small-scale virtual networks (see Section 5.1.3 for discussion). Although the average
Figure 5.9: Example virtual networks spot sampled from a generated large-scale WT network. The WT training network is also shown for comparison. The white regions represent ICC. The large-scale network is 40,500×20,000 pixels, and corresponds to physical dimensions of approximately 25.2×12.4 mm, representing the ICC-MP network of a 25 mm segment of the adult murine small intestine. The training and spot sampled networks are 362×362 pixels, and correspond to physical dimensions of 0.225×0.225 mm. The arrow (top) indicates consistency in the large-scale network around the circumference of the small intestine.
Figure 5.10: Example virtual networks spot sampled from a generated large-scale KO network. The KO training network is also shown for comparison. The white regions represent ICC. The large-scale network is $40,500 \times 20,000$ pixels, and corresponds to physical dimensions of approximately $25.2 \times 12.4$ mm, representing the ICC-MP network of a 25 mm segment of the adult murine small intestine. The training and spot sampled networks are $362 \times 362$ pixels, and correspond to physical dimensions of $0.225 \times 0.225$ mm. The arrow (top) indicates consistency in the large-scale network around the circumference of the small intestine.
5.2. Large-Scale Network Generation

Figure 5.11: The percentage errors in the structural metric values of the spot sampled virtual networks compared to their respective training counterparts.

Figure 5.12: The percentage errors in the functional measure values of the spot sampled virtual networks compared to their respective training counterparts.

absolute errors of the functional measures were small, a few individual spot sampled networks displayed very large errors in the time-related activation rate and time to peak $[Ca^{2+}]_i$ measures. These large errors arose as the top-right corner of the corresponding spot sampled networks where the initial stimulus to the pacemaker activity simulations was applied coincidentally contained less ICC, thereby delaying the onset of the simulated activity considerably. This also reflects the sensitivity of the pacemaker activity simulations to the initial stimulus, and hence a more robust protocol, such as a planar stimulus along an edge of the network which should present less variation between networks, should be implemented for the stability of simulation results.
Figure 5.13: Reduced-resolution AT maps of the simulated ICC pacemaker activity propagations over the large-scale virtual networks. Headings along the top indicate the different virtual networks over which pacemaker activity propagation was simulated. Refer to text for details on the resolution reduction.

The errors associated with spot sampled virtual networks were in general higher than that of the generated small-scale virtual networks. This is due to the internal heterogeneity of large-scale networks introducing more variation in the network properties when spot sampling. The pixels of the small-scale networks were simulated based on other pixels within the same network, and hence the small-scale networks possess more self-consistency and better control for matching the properties of the training network. On the other hand, the pixels of the spot-sampled networks may be simulated based on pixels outside the field of view of the network (i.e., belonging to the large-scale network but external to the spot sampled network), leading to more variable network properties and hence more pronounced deviations from the training networks.
5.2. Large-Scale Network Generation

The simulated ICC pacemaker activity propagations over the large-scale networks only showed a minor reduction in propagation velocities over KO networks in comparison to WT networks. As $5-HT_{2B}$ KO does not affect intestinal transit times [173], it is logical that the pacemaker activity propagations over the KO networks was not significantly impaired. However, the AT maps of the simulated propagations showed different propagation dynamics over WT and KO and networks. Although in both cases the pacemaker activity propagated radially from the site of the initial stimulus, the circumferential spread of the activity was isotropic over the WT networks but anisotropic over the KO networks. It can be seen from the KO networks that the dominant direction of the ICC processes are, although more preferentially aligned in the longitudinal (vertical) direction, still at an angle to the longitudinal direction. This in conjunction with the little number of processes running in the circular (horizontal) direction, caused the propagation wavefront to slowly spiral around the circumference of the intestine. This phenomenon may in fact be related to the preferential alignment of processes occurring in $5-HT_{2B}$ KO (see Section 3.4 for discussion). The preferential alignment likely served the purpose of preserving longitudinal integrity of the network with reduced network volumes, but the processes were not quite aligned perfectly in the longitudinal direction to potentially serve as a mechanism for the pacemaker activity to traverse across the circumference of the intestine faster, compensating for the anisotropy of the underlying network structures and hence facilitating the generation of motility patterns more similar to luminal contractions.

ICC pacemaker activity propagation patterns were very similar over virtual networks generated from the same training network, especially at the tissue level of the reduced-resolution AT maps. Therefore, at least at the tissue level, the pacemaker activity is rather forgiving of the underlying network structure, or in other words, more drastic alterations in the underlying network structure are required for the pacemaker activity at the tissue level to be influenced.

It should be borne in mind that the cellular automaton model used to simulate ICC pacemaker activity propagation over the large-scale networks remains a simplistic model that has yet to be validated and calibrated against experimental data. Therefore, the specific inferences of the simulation results primarily serve as hypotheses which can be further investigated and tested in detail in future studies.
5. VIRTUAL ICC NETWORK GENERATION

5.3 Discussion

Although ICC depletion is assumed to play a key role in GI dysmotility, a major constraint restricting the investigation of ICC network structure-function relationships is the paucity of imaging data. In particular, there is a lack of ICC network imaging data covering large fields of view and across a range of network properties spanning health and disease states. This chapter addresses these challenges by generating realistic virtual ICC networks \textit{in silico} using the stochastic SNESIM algorithm. The fidelity of the virtual networks generated using SNESIM were validated both structurally and functionally, by applying ICC network structural metrics and biophysically-based computational modelling respectively. Modifications to SNESIM were proposed to facilitate the generation of ICC networks across a spectrum of network properties, and as proof-of-concept, virtual networks were successfully generated with a range of structural and functional properties. Large-scale virtual networks were also generated using SNESIM, and multiscale ICC pacemaker activity propagation was simulated over these networks.

Various studies have attempted to explain and predict GI electrical activity in health and disease states by utilising computer models. Since the 1960s, when the pacemaker role of ICC was still unclear, GI electrical activity was modelled as a series of coupled Van der Pol relaxation-oscillators [139]. This concept was further expanded to demonstrate entrainment in a network of bi-directionally coupled series of relaxation-oscillators [158], and was used to simulate the effects of partial cuts in GI organs on the electrical activity [159]. More recently, a cellular automaton model was developed to investigate the effects of tissue degradation on the overall GI electrical activity propagation, with the degradation being modelled as randomly distributed nodes throughout the simulation grid [111]. Although these studies were insightful in simulating GI electrical activity under a variety of conditions, they lacked the underlying electrophysiological basis of ICC. The generation of virtual ICC networks proposed here can establish a more detailed model of GI tissue, and therefore offers potential for the previous studies to be repeated and extended in a more physiologically realistic manner.

Simulation studies using experimentally-imaged ICC networks have successfully related ICC network structure to function [47]. This framework can be further enhanced by incorporating the virtual network generation algorithms, such that the simulations are not limited to the small spatial scale of the experimental data.
5.4. Chapter Summary

The augmented framework can then be used as a virtual platform to investigate ICC structure-function relationships in, for example, gastroparesis and slow-transit constipation where multiple cellular pathologies and competing theories co-exist [77, 185]. Another potential application of the virtual network generation algorithms is to generate large-scale networks to inform multiscale models [10, 43] as demonstrated in Section 5.2.1.3, which, for instance, can be used to investigate the mechanisms of conduction slowing and dysrhythmias recently observed in a high-resolution electrical mapping study of gastroparetic patients with ICC depletion [141].

5.4 Chapter Summary

In summary, this chapter validated the efficacy of using the SNESIM algorithm to generate both small- and large-scale realistic virtual ICC networks, and presented modifications to the algorithm to enable virtual networks with a range of structural and functional properties to be generated. These algorithms offer an alternative strategy for obtaining the comprehensive imaging data set encompassing large-scale ICC networks across a spectrum of network properties required to elucidate the mechanism relating ICC network structure to GI function. As proof-of-concept, the SNESIM algorithm was employed in conjunction with cellular automaton modelling to demonstrate the first multiscale computational framework in the GI field spanning from cellular structures to tissue level electrophysiology.

Acknowledgements

I would like to thank Dr. Gregory O’Grady for beneficial discussion and critical review of Section 5.1; Drs. Simon J. Gibbons and Gianrico Farrugia for providing the ICC network imaging data described in this chapter; and Dr. Sebastien Strebelle and Prof. Andre Journel for making the SNESIM algorithm source code available.
Chapter 6

Conclusions and Future Directions

Interstitial cells of Cajal (ICC) are instrumental in facilitating normal gastrointestinal (GI) motility [51], with one of its most prominent roles being the electrical pacemaking of the GI tract [92, 182]. Although the pivotal status of ICC in GI health and disease has been recognised, the mechanism relating the complex network structures formed by ICC to GI function remains unknown. Progress in elucidating this mechanism is limited with experimental techniques alone due to factors including: 1) the absence of methods for quantifying ICC network structures; 2) challenges in associating cellular and tissue level activity across multiple spatial and temporal scales; and 3) the lack of a comprehensive ICC imaging data set encompassing large-scale network structures across a range of network properties.

This thesis uses a mathematical modelling approach to devise tools for addressing the aforementioned experimental shortcomings, and furthers current knowledge of ICC network structure-function relationships. A summary of the findings of this thesis and potential future work are presented before concluding.

6.1 Summary of Findings

6.1.1 Quantification of ICC Network Structural Properties

Loss and injury of ICC has been identified as a major hallmark of several GI functional motility disorders [93], but current methods of quantifying ICC network de-
6. CONCLUSIONS AND FUTURE DIRECTIONS

pletion remain simplistic and are unable to capture intricate variations in network structure. Therefore, a set of six numerical metrics were developed to assess complex ICC network structures which may affect pacemaker activity generation in a consistent and unbiased manner:

1. **Density**: measures the ICC network volume;

2. **Thickness**: measures the width of cellular structures within the ICC network plane;

3. **Hole size**: measures the radius of non-ICC regions within the ICC network plane;

4. **Contact ratio**: indicates the availability of conduction pathways from ICC to non-ICC regions within the ICC network plane;

5. **Anisotropy**: indicates the degree of preferential alignment of ICC structures;

6. **Connectivity**: measures the connectivity of the ICC network.

As proof-of-concept, these metrics were applied to determine the effects of 5-HT$_{2B}$ knockout (KO), *Ano1* KO, postnatal maturation, and *Spry4* KO on ICC-MP networks from the murine jejunum, facilitating the first sophisticated analyses of ICC network structure. In the case of examining 5-HT$_{2B}$ KO, *Ano1* KO and postnatal maturation, the metrics supported and extended the previous simplistic analyses and qualitative assessments with more detailed structural and statistical evidence, whereas in the case of examining *Spry4* KO, provided a quantitative means for testing new hypotheses on factors influencing ICC network structure.

Analysis results showed that 5-HT$_{2B}$ KO networks had, on average, a lower density, lower thickness, higher contact ratio, and lower connectivity than that of the WT networks. The KO networks also had, on average, a higher anisotropy metric value compared to the WT networks. These results revealed a novel remodelling phenomenon occurring during ICC depletion, namely a spatial rearrangement of ICC and preferential longitudinal alignment, and these network alterations may ultimately serve functional purposes of optimising electrical pacemaker activity in ICC-depleted states.

Consistent with previous studies [165], metric analysis did not find any differences between ICC networks from *Ano1* KO and WT mice. Although *Ano1* regulates ICC...
proliferation [165], alternative mechanisms of proliferation such as Kit [102] and serotonin [173, 189] may compensate in Ano1 KO animals, resulting in structurally normal ICC networks.

To investigate postnatal developmental changes in ICC network structure, metric values of ICC networks from three-day- and four-week-old WT mice were compared. It was discovered that four-week-old networks had, on average, a higher thickness, higher hole size, lower contact ratio, and higher connectivity compared to the three-day-old networks. There was no observed difference in the density or anisotropy between the three-day- and four-week-old networks. These results give rise to a ‘pruning’ hypothesis that may occur during ICC network maturation, whereby there is a larger number of processes in the immature ICC networks which may not all be necessary or efficient, and throughout maturation the redundant or less effective ones are discarded. This hypothesis and its functional significance is further tested and expanded in conjunction with ICC network pacemaker activity simulations in Section 4.1, and the findings of which are summarised in Section 6.1.2.

There were no observed differences between the average metric values for the WT and Spry4 KO mice, and hence the preliminary metric application performed in this thesis does not support the hypothesis that Spry4 KO leads to ICC hyperplasia. However, as biological variability of ICC networks is large, a larger sample size may be required to confirm the effects, if any, of Spry4 KO on ICC network structure.

In summary, the development of the ICC network structural metrics presents a quantitative approach for describing ICC network structural properties, and facilitates the discerning of subtle differences between networks in various states of health and disease.

### 6.1.2 ICC Network Pacemaker Activity Simulations

A major challenge in relating ICC network structure to GI function arises from the vast spatial and temporal scales over which these activities occur. Experimental techniques for capturing the corresponding activity at a single scale are being developed (e.g., Ca^{2+} imaging at the cellular level [144]; high-resolution electrical mapping at the tissue level [141]), but no techniques are on the horizon for simultaneously capturing activities at multiple scales. Therefore, the feasibility of employing multi-scale models to relate ICC network structure to its electrical pacemaker activity was
evaluated. Provided sufficient computational resources, the models can be upscaled to span multiple spatial and temporal scales.

The performance of the established biophysically-based simulations, where biophysically-based cell models are coupled to the bidomain model, was inspected in the context of postnatal ICC network development. The numerical metrics for quantifying ICC network structural properties (described in Sections 3.1 and 6.1.1) were applied on confocal ICC network imaging data obtained from the murine small intestine at postnatal ages spanning birth to weaning. These imaging data were also used to inform the biophysically-based simulations to simulate pacemaker activity in the networks and quantify how changes in structure may alter function. Four measures based on the average membrane potential ($V_m$) and intracellular calcium concentration over the network ($[Ca^{2+}]_i$) were used to quantitatively assess the simulated ICC pacemaker activity:

1. **Activation rate**: measures the rate at which the network achieves an average $V_m$ of -30 mV;
2. **Peak $[Ca^{2+}]_i$**;
3. **Time to peak $[Ca^{2+}]_i$**;
4. **Half peak $[Ca^{2+}]_i$ time ratio**: gauges the dynamics of the $[Ca^{2+}]_i$ upstroke.

The results showed a pruning-like mechanism which occurs during postnatal development, and the temporal course of this phenomenon was defined. There was an initial ICC process overgrowth to optimise network efficiency and increase functional output volume. This was followed by a selective retaining and strengthening of processes, while others were discarded to further elevate functional output volume. Subsequently, new ICC processes were formed and the network was adjusted to its adult morphology. These postnatal ICC network developmental events may be critical in facilitating mature digestive function.

In contrast to the computational resource-demanding biophysically-based simulations, a new computationally efficient cellular automaton model for simulating tissue-specific ICC network pacemaker activity propagation was developed. Although cellular automaton models for simulating GI electrical activity have been
proposed previously [48, 111], the developed model was the first to explicitly repre-
sent ICC and non-ICC node types and apply appropriate cellular automaton rules
accordingly. This facilitates investigations of structure on function at much higher
spatial resolutions, which is necessary to capture complex ICC network structures.
The developed model was applied on ICC network structures from WT and 5-HT_{2B}
KO (ICC depleted) mice, and successfully simulated pacemaker activity propaga-
tion over these networks with greatly reduced computational time compared to the
biophysically-based simulations. The pacemaker activity simulations demonstrated
an impaired propagation during ICC depletion. This cellular automaton model
therefore offers a computationally efficient framework for relating ICC structure to
function, and holds promise for further application to define ICC structure-function
relationships across various spatial and temporal scales.

To summarise, multiscale modelling was demonstrated to be a plausible approach
for relating ICC network structure to its electrical pacemaker activity. In compari-
son, biophysically-based simulations possess a more realistic basis, whereas cellular
automaton simulations trade the biophysical details for computational efficiency.
The more suitable modelling approach should therefore be selected based on the
context and purpose of the research.

6.1.3 Virtual ICC Network Generation

Currently, the lack of a comprehensive ICC network imaging data set encompassing
large-scale ICC networks across a spectrum of network properties hinders progress
in defining ICC network structure-function relationships. Experimental imaging of
these large-scale networks remains challenging because of technical constraints, and
hence an alternative strategy of acquiring this comprehensive imaging data set by
generating realistic virtual ICC networks in silico using the stochastic Single Normal
Equation Simulation (SNESIM) algorithm is presented.

Initially, to validate and demonstrate application of the SNESIM algorithm for
generating realistic virtual ICC networks, focus was placed on networks at a smaller
spatial scale. ICC network imaging data obtained from WT and 5-HT_{2B} KO (ICC
depleted) mice were used to inform the algorithm, and the virtual networks generated
were assessed using ICC network structural metrics and biophysically-based ICC
network pacemaker activity simulations. When the virtual networks were compared
to the training networks, there was less than 10% error for four out of five structural metrics and all four functional measures. The SNESIM algorithm was then modified to enable the generation of ICC networks across a spectrum of network properties from limited experimental imaging data, and as proof-of-concept, virtual networks with a range of structural and functional properties were successfully generated.

The SNESIM algorithm was further applied to generate large-scale virtual networks with fields of view extending beyond the limitations presented by experimental imaging. WT and 5-HT$_{2B}$ KO virtual networks with a resolution of 40,500×20,000 pixels were generated, representing the ICC-MP network within a 25 mm segment of the adult murine small intestine. The fidelity of the large-scale virtual networks was reconfirmed by applying the structural metrics and biophysically-based simulations on selected small-scale spot samples of the networks. There was less than 15% error for four out of five structural metrics and all four functional measures when comparing the spot sampled virtual networks to the training networks. The large-scale virtual networks were then coupled to the cellular automaton model for simulating ICC network pacemaker activity propagation, and successfully demonstrated the first multiscale computational framework in the GI field relating cellular structures to tissue level electrophysiology. Simulation results showed only a minor reduction in propagation velocities over KO networks (4.3 mm/s) in comparison to the WT networks (4.9 mm/s), which is consistent with the experimental finding that 5-HT$_{2B}$ KO does not affect intestinal transit times [173].

The SNESIM and modified SNESIM algorithms therefore provide a viable method for obtaining large-scale ICC network imaging data across a spectrum of network properties.

### 6.2 Future Directions

The definitions of the ICC network structural metrics presented are for 2D analysis, as the ICC network imaging data utilised were from the murine small intestine, which is thin in the transmural direction (≈15 µm). These metrics need to be extended to 3D to enable their application on, for example, gastric ICC networks or larger animals including humans, where the ICC reside in much thicker tissue volumes [77] with processes extending in the transmural direction as well [119]. Complimentary techniques for supporting the metric analyses will also need to be
developed. These include methods for delineating the interwoven ICC populations within thick tissue volumes [119], and a more standardised approach for segmenting ICC network structures from confocal imaging data. Another idea that can be pursued is to broaden the use of the structural metrics to neuronal networks of the enteric nervous system, which are often simultaneously affected with ICC in GI functional motility disorders [77, 185]. That is, the structural properties of enteric neuronal networks can also be assessed using the structural metrics to offer the first quantitative analyses of the neuronal network structures contributing to normal GI motility. Further advancement of the structural metrics may transform their utility from being tools for pure scientific research to diagnostic indicators in the clinical setting. For instance, the complex 3D ICC network structures sampled from biopsies of patients can be gauged with the metrics in routine clinical assessments of GI functional motility disorders and to assist in deciding treatment protocols.

The multiscale models for simulating ICC network pacemaker activity propagation await further validation and calibration against experimental data in order to transform them into powerful predictive tools. The modelling approach presented in this thesis views the ICC network as a functional syncytium and neglects details on intercellular coupling such as gap junctions and close apposition membranes. The importance of intercellular coupling in accurately predicting ICC network function from structure should therefore be confirmed by validating and calibrating the simulation models against Ca\textsuperscript{2+} imaging data over small-scale networks.

In order to achieve validation and calibration over large-scale networks, the multiscale models coupled with the SNESIM algorithm can be compared to high-resolution (HR) electrical mapping [45, 112, 141]. Specifically, small-scale ICC networks experimentally imaged from GI tissue can be used to generate large-scale virtual networks using SNESIM. The multiscale models can then be validated and calibrated by matching the simulated ICC pacemaker activity propagations over the large-scale virtual networks with HR electrical mapping data recorded from the tissue. Together with the continuous evolution of computational resources, the multiscale models in conjunction with the SNESIM and modified SNESIM algorithms can be applied to output increasingly detailed and realistic simulations for unravelling the mechanism through which ICC network structure relates to GI function.
6. CONCLUSIONS AND FUTURE DIRECTIONS

6.3 Concluding Remarks

Interstitial cells of Cajal were first observed over one hundred years ago, but only in the past two decades or so has their paramount significance in facilitating normal GI motility been realised. Within these recent years, there has been an exponential growth in the understanding of these specialised cells, which can mainly be attributed to experimental breakthroughs such as the discoveries of ICC immunoreactivity to Kit and the electrical pacemaker role of ICC. However, currently a key unanswered question in the field of ICC research which experimental techniques alone struggle to address is defining the mechanism relating ICC network structure to GI function.

This thesis presents novel mathematical and computational tools, which in conjunction with experimental data, form a comprehensive framework providing unprecedented analyses and a virtual platform for investigating ICC network structure-function relationships. The application of this framework has already shed light on structure-function relationships in various health and disease states. Further refinement of this framework combined with concurrent improvements in computational resources will enable the elucidation of these key structure-function relationships, leading to a more extensive understanding of GI physiology and pathology, as well as holding promise for the clinical utility of patient ICC network information to transform the diagnosis, prognosis and treatment of GI functional motility disorders.
Appendix A

Publications, Presentations and Awards

The following publications, presentations and awards were produced during the course of this thesis.

A.1 Peer-Reviewed Publications


* Selected as issue cover


### A.2 Book Chapters

A.3 Oral Presentations


* Awarded Young Investigator’s Award

A.4 Conference Abstracts


*Presented as poster at Digestive Disease Week 2012, San Diego, CA, USA.*


*Presented as poster at Digestive Disease Week 2011, Chicago, IL, USA.*

* Awarded Poster of Distinction

A.5 Awards

2013 Riddet Student Travel Award
*Riddet Institute, Palmerston North, New Zealand*

2013 R. H. T. Bates Postgraduate Scholarship
*Royal Society of New Zealand, Wellington, New Zealand*
2012 Short-Term Stay in Japan Scholarship
*Japan Student Services Organization, Yokohama, Kanagawa, Japan*

2012 Freemasons Postgraduate Scholarship
*Freemasons Charity, Freemasons New Zealand, Wellington, New Zealand*

2012 Young Investigators Award
*International Electrogastrography Society*

2011 Poster of Distinction
*Digestive Disease Week 2011, Chicago, IL, USA*

2011-2014 University of Auckland Health Research Doctoral Scholarship
*University of Auckland, Auckland, New Zealand*
Appendix B

Biophysically-Based Simulations

Additional details of the biophysically-based simulations of ICC network pacemaker activity conducted in Sections 4.1 and 5.1 are summarised below.

The values used to reinitialise the state variables of the finite-state machine Corrias and Buist ICC model when it transitions from Passive to Active are listed in Appendix A of [160], and the equations and corresponding parameters governing the Active cellular activity can be found in [32]. The excitation potential threshold and non-refractory period were set to -55 mV and 8 s respectively.

The derivation of the bidomain model is covered in [44]. $A_m$ and $C_m$ were set to 2000 cm$^{-1}$ and 2.5 $\mu$Fcm$^{-2}$ respectively. The conductivity parameters used in Section 5.1 are listed in Table B.1. In Section 4.1, these parameters were scaled by $r/0.379$, where $r$ is the resolution of the ICC network (i.e., these parameters were used for simulations over networks with a resolution of 0.379 $\mu$m/pixel, and parameters used for simulations over the other networks were scaled based on resolution accordingly).

<table>
<thead>
<tr>
<th>Node</th>
<th>$\sigma_i$</th>
<th>$\sigma_e$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICC</td>
<td>0.12</td>
<td>0.02</td>
</tr>
<tr>
<td>Non-ICC</td>
<td>0.0012</td>
<td>0.0002</td>
</tr>
</tbody>
</table>

Table B.1: Conductivity parameters used in the bidomain model for simulating ICC network pacemaker activity in Section 5.1. In Section 4.1, these parameters were scaled by $r/0.379$, where $r$ is the resolution of the ICC network. Listed values are in mScm$^{-1}$. 
Biophysically-based Simulations

ICC network imaging data were discretised into a linearly interpolated regular triangular finite element mesh using the CHASTE computational framework [137]. Each pixel of the imaging data corresponded to a node in the mesh. The initial value of $V_m$ for both the ICC and non-ICC nodes was $-67 \text{ mV}$, and the no flux condition was set at the boundary of the mesh.
References


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