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Super Resolution Imaging of Cardiac Ventricular Myocyte Calcium Handling Systems

Yufeng Hou

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Biomedical Sciences, the University of Auckland, 2016.
Acknowledgements

Firstly I’d like to thank my supervisor Professor Christian Soeller for his guidance over the past 4 years. Whose readiness to share his expertise and wisdom as well as his perseverance - often late on his evening in England - meant I could make the most of the opportunity to work at this lab. Also I’d like to thank him for the numerous opportunities to present findings and meet other experts of the cardiac field at conferences which I would not have been able to attend without his help.

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Abstract

The ventricular cardiac myocyte is the most prominent cell of the heart by volume and weight and is responsible for the contractive force necessary to pump blood around the body. Operation of the ventricular myocyte centre occurs through the coupling of electrical activity and the subsequent mechanical contraction, a process known as excitation contraction coupling (EC Coupling). EC coupling centre around the precise handling of both extracellular and internal calcium ion stores in a process called calcium induced calcium release (CICR). This process is dependent on the precise nanometer scale arrangement of the Ryanodine receptor (RyR) protein within the SR membrane which act as the primary calcium release channel for the SR calcium stores. This thesis aims to investigate the ryanodine receptor and associated structures using super resolution imaging modality to investigate nanoscale changes in structural distribution. Section one focuses on the imaging of the Z disc – the region of localisation for RyR release sites. Results show thicknesses of z disc were measured at 100 nm with transverse arrangement showing all fibril cores being within a unified 500 nm distance band. The second section demonstrates the distribution of RyR proteins in transverse rat cardiac myocyte tissue sections. Results demonstrated a large variation in cluster sizes with approximate exponential distribution. Mean cluster size was 63 RyR with mean edge to edge separation of 130 nm in general agreement with previous confocal data. Colocalisation of RyR with the T-tubule – SR crosslinking protein JPH showed a high level of colocalisation compared with an idealised colocalisation simulation of RyR vs RyR (61% vs 78%). Data is consolidated with diffraction limited data highlighting the detection falloff at smaller cluster sizes. The final section investigated differences in human RyR distribution for normal, idiopathic dilated cardiomyopathy, and rat cells. Results showed little change in cluster size distribution between all three cases, however a change in nearest adjacent neighbour distances were observed. A reduction in colocalisation between JPH and RYR is further seen in humans as compared with rats. These results highlight the subtle structural features seen in the arrangement of RyR clusters, and how super resolution imaging provides greater clarity and additional details on top of the previous attempts at analysis with conventional microscopes.
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<tbody>
<tr>
<td>AB</td>
<td>Antibody</td>
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<tr>
<td>CICR</td>
<td>Calcium induced calcium release</td>
</tr>
<tr>
<td>CRU</td>
<td>Calcium release unit</td>
</tr>
<tr>
<td>Cav-3</td>
<td>Caveolin 3</td>
</tr>
<tr>
<td>DHPR</td>
<td>Dihydropyridine receptor</td>
</tr>
<tr>
<td>DCM</td>
<td>Dilated cardiomyopathy</td>
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<tr>
<td>dSTORM</td>
<td>Direct stochastic optical reconstruction microscopy</td>
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<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>EMCCD</td>
<td>Electron multiplying charge coupled device</td>
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<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>EC Coupling</td>
<td>Excitation contraction coupling</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>HF</td>
<td>Heart failure</td>
</tr>
<tr>
<td>IDC</td>
<td>Idiopathic dilated cardiomyopathy</td>
</tr>
<tr>
<td>Event</td>
<td>Individual fluorescent molecule blink event</td>
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<tr>
<td>JPH</td>
<td>Junctophilin</td>
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<td>LV</td>
<td>Left ventricle</td>
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<tr>
<td>LTCC</td>
<td>L-type calcium channel</td>
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<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MEA</td>
<td>Mercaptoethanol</td>
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<tr>
<td>NA</td>
<td>Numeric aperture</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PALM</td>
<td>Photoactivated localisation microscopy</td>
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<tr>
<td>ROI</td>
<td>Region of interest</td>
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<tr>
<td>RyR</td>
<td>Ryanodine receptor</td>
</tr>
<tr>
<td>Clusters</td>
<td>Ryanodine receptor clusters</td>
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<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
</tr>
<tr>
<td>SERCA</td>
<td>Sarcoplasmic/endoplasmic reticulum calcium ATPase</td>
</tr>
<tr>
<td>NCX</td>
<td>Sodium calcium exchange</td>
</tr>
<tr>
<td>STED</td>
<td>Stimulated emission depletion microscopy</td>
</tr>
<tr>
<td>STORM</td>
<td>Stochastic optical reconstruction microscopy</td>
</tr>
<tr>
<td>T-tubule</td>
<td>Transverse tubule</td>
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<tr>
<td>WGA</td>
<td>Wheat germ agglutinin</td>
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Thesis chapter: Super-resolution fluorescence imaging to study cardiac biophysics: α-actinin distribution and Z-disk topologies in optically thick cardiac tissue slices


CO-AUTHORS

<table>
<thead>
<tr>
<th>Name</th>
<th>Nature of Contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Christian Soeller</td>
<td>Project Supervisor, provided details on research direction, correction of drafts and text, and main funder of research.</td>
</tr>
<tr>
<td>Isuru Jayasinghe</td>
<td>Provided analysis of myofibrillar diffusion distances, proofreading of text and changes to text material.</td>
</tr>
<tr>
<td>David J Crossman</td>
<td>Assistance with acquisition of confocal images.</td>
</tr>
<tr>
<td>Vijay Rajagopal</td>
<td>Provided electron microscopy images used in modality comparison analysis</td>
</tr>
<tr>
<td>David Baddeley</td>
<td>Construction and maintenance of dSTORM system, provided technical support for its function and image acquisition. Provided software suite for data analysis.</td>
</tr>
</tbody>
</table>

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The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate’s contribution to this work, and the nature of the contribution of each of the co-authors; and

- that the candidate wrote all or the majority of the text.

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<td>13/1/16</td>
</tr>
<tr>
<td>Isuru Jayasinghe</td>
<td></td>
<td>25/1/16</td>
</tr>
<tr>
<td>David J Crossman</td>
<td></td>
<td>26/1/16</td>
</tr>
<tr>
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<td></td>
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Chapter 2: Nanoscale analysis of ryanodine receptor clusters in dyadic couplings of rat cardiac myocytes


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<tr>
<th>Nature of contribution by PhD candidate</th>
<th>Majority of experimental work and data, Drafting and writing of methods, results and figure legends. Partial contribution in introduction and discussion, proof reading, and construction of supplementary figures. Drafting of figures.</th>
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**CO-AUTHORS**

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<th>Nature of Contribution</th>
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<tbody>
<tr>
<td>Isuru Jayasinghe</td>
<td>Writing of introduction and discussion, proofreading and correcting of other sections. Reworking of figures, advanced analysis of datasets for colocalisation and cluster distributions, analysis of myofibrillar perimeter work. Supplied some of the dSTORM image data.</td>
</tr>
<tr>
<td>Christian Soeller</td>
<td>Assistance in writing of introduction and discussion, checking drafts and rewriting for all sections. Guidance on direction of data analysis and paper structure, funding for project.</td>
</tr>
<tr>
<td>David Crossman</td>
<td>Assistance with obtaining diffraction limited data.</td>
</tr>
<tr>
<td>David Baddeley</td>
<td>Construction and maintenance of dSTORM system, provided technical support for its function and image acquisition. Provided software suite for data analysis.</td>
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</table>

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<td>David Baddeley</td>
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Chapter three: Analysis of Calcium release unit in humans: Comparison of rat, normal human and diseased human samples.

From work: Unpublished

Nature of contribution by PhD candidate: Majority of experimental work, acquisition of dSTORM images, some confocal images. Writing of main text blocks, construction of figures, statistical analysis of data.

Extent of contribution by PhD candidate (%): >70%

CO-AUTHORS

<table>
<thead>
<tr>
<th>Name</th>
<th>Nature of Contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>David Crossman</td>
<td>Providing human samples, assistance and acquisition of confocal images, direction of research. Proofreading and revision of text. Providing Western blot data for comparison of quantification.</td>
</tr>
<tr>
<td>Xin Shen</td>
<td>Assistance of confocal image acquisition, carried out western blot experiments presented in results section. Assisted in tissue extraction for human samples.</td>
</tr>
<tr>
<td>David Baddeley</td>
<td>Construction and maintenance of dSTORM system, provided technical support for its function and image acquisition. Provided software suite for data analysis.</td>
</tr>
<tr>
<td>Christian Soeller</td>
<td>Provided research direction, proofreading and editing on text, and funding for project.</td>
</tr>
</tbody>
</table>

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<td>Christian Soeller</td>
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# Introduction

In order to pump blood around the body, the heart operates continually throughout a person’s lifetime beating upwards of two billion times. The central role of the heart and the stresses placed upon it by such continuous activity results in a plethora of different diseases when problems arise. As such, within the developed world, cardiovascular disease is one of the top three contributors to morbidity and the singular top contributor to mortality (Roger et al., 2012); a majority of conditions fall under the ischemic heart disease (IHD) classification.

Heart attacks (myocardial infarctions or MI) are possibly the most well-known type of ischemic heart disease. Due to improvements in detection and intervention strategies, there is a reduction of direct mortality from MI (Smolina, Wright, Rayner, & Goldacre, 2012), the majority of patients who survive such infarcts will however still have a significantly elevated risk of a subsequent infarct and development into heart failure (Steg et al., 2012). In order to develop new techniques for treating such diseases, research into the underlying causes and mechanisms for these diseases is first necessary before the development and implementation of new treatment options.

Current research into cardiac function is often focused around the subcellular interactions between different components and organelles. Specifically, calcium ions hold a central position as the activator and regulator of cell function. Within cardiac myocytes, calcium is responsible for the direct coupling between sarcolemmal depolarisation and subsequent contraction, a process known as excitation contraction coupling (EC coupling). Three organelles can be considered central components in EC coupling and cell calcium cycling. The T-tubular system (T-tubules) act as surface membrane extensions deep into the cell cytoplasm for conduction of action potentials; the sarcoplasmic reticulum (SR), an extension of the endoplasmic reticulum (ER), that acts as the primary intracellular calcium storage; and the myofibrils, the organelle responsible for calcium dependent force generation.

Evidence continues to mount regarding the role of calcium in manifestation of disease. It is now apparent that alterations in key calcium handling mechanisms are a hallmark of disease progression. Unfortunately the underlying mechanisms behind the changes in calcium
regulation between normal and diseased cells are still not clear. Multiple groups have additionally made clear the requirement for nanometer scale arrangement necessary for maintaining normal function (M. B. Cannell & Kong, 2012; D. J. Crossman, P. N. Ruygrok, C. Soeller, & M. B. Cannell, 2011; M. D. Stern, 1992). Unfortunately, structure on this scale is difficult to directly image, due to the resolution of conventional light microscopes being limited by diffraction to roughly 200 nm. Recent developments of super resolution microscopy however, provide a much needed increase in resolution while maintaining many of the practical aspects from conventional fluorescence microscopy.

**Figure 1:** Stylised drawing of a typical myocyte labelled with key subcellular components. Myofibrillar bundles are marked in red; Sarcoplasmic Reticulum processes are marked in green; and T-tubular and surface membrane is marked in yellow. Image adapted from (Fawcett & McNutt, 1969).

Therefore, to better understand alterations to calcium handling in disease, it is important to first understand how normal cellular handling of calcium occurs. Progression can then be made into analysing alterations in diseased states with the overall goal of identifying points
of change and possible targets and markers that may potentially be used in identification of treatment for heart disease in the future.

The aim of this thesis is for the use of super resolution microscopy in imaging cardiac myocytes and the organelles associated with calcium handling during contraction. Particular emphasis will be on validation of super resolution microscopy on tissue samples in rat and humans focusing on the imaging of the ryanodine receptors (RyR) - the main sarcoplasmic reticulum calcium channel and its associated proteins.

Specifically, the aims centre; firstly in the visualisation of the myofibrillar Z disc using dSTORM super-resolution microscopy in comparison with Electron microscopy and confocal microscopy in order to demonstrate the additional details possible in super-resolution microscopy. Secondly, the usage of dSTORM imaging in the visualisation of RyR calcium release clusters and its associated protein junctophilin 2 (JPH) for the identification of nanoscale features in structural calcium control. Finally, the imaging of human normal and diseased samples (idiopathic dilated cardiomyopathy) in order to compare differences between species as well as changes in protein distribution in pathology development.

**Calcium induced calcium release and myofibrillar activation**

The main constituent cell of the heart by volume and mass is known as the cardiac myocyte. It is a specialised striated muscle fibre similar to skeletal muscle although with important distinguishing features involving the branching nature of the cell and the separation of cell bodies by the intercalated disks (D. M. Bers, 2000). The mechanism for EC coupling also varies, with cardiac cells relying on calcium induced calcium release while skeletal muscle having direct coupled gating (Fabiato, 1983).

Central to the operation of the cardiac myocyte is the mechanism of activation for the myofibrils in a highly co-ordinated manner referred to as excitation contraction coupling (EC coupling). Additionally, within cardiac muscle, EC coupling is unique in that the mechanism involves the activation of sarcoplasmic reticulum calcium channels by presence of intracellular calcium itself, a process referred to as calcium induced calcium release (CICR). As with all striated and some smooth muscle types, cardiac muscle contraction is first
signalled by an electrical action potential that propagates along the sarcolemmal membrane.

**Figure 2:** Schematic diagram of calcium induced calcium release. 1; Arrival of the action potential along the sarcolemmal membrane. 2; Depolarisation of T-tubular membrane leads to opening of L-type calcium channel resulting in initial influx of calcium ions from the extracellular space. 3; calcium released from the LTCC diffuse across the dyadic space binding to the RyR on the SR membrane leading to their opening and subsequent release of SR calcium stores. 4; Released calcium diffuses through the cytoplasm binding to troponin c on myofilaments initiating contraction of the myofibrils. 5; contraction ends through the reuptake of calcium ions across the SR through the SERCA protein and through the sarcolemma and T-tubular membrane through the sodium calcium exchange (NCX).

Within the membrane the T-tubules act to assist the propagation of the action potential deep into its core synchronising the contraction of inner myofibrils with the outer ones (Ibrahim, Gorelik, Yacoub, & Terracciano, 2011). The t-tubular system was first hypothesised
to exist by Huxley (1958) who identified within skeletal muscle that depolarisations resulted in rapid propagation through the cell. At the time he hypothesised that a network of tubular networks should be present in order to explain this phenomenon. The extent of the transverse tubule system and its formation varies between species (Isuru D. Jayasinghe et al., 2014) with mammalian skeletal muscle T-tubules running along the AI border while mammalian cardiac T-tubules running approximately along the Z disc region. Contrary to what its name implies, the T-tubules do not exclusively run transverse to the cell long axis, but rather also contain considerable amounts of longitudinal connections especially within skeletal muscle (C. Soeller & Cannell, 1999). Along the walls of the T-tubules L-type calcium channels (LTCC) reside, where the depolarisation of the membrane triggers the opening of voltage gates within the channel enabling a transient calcium flux into the cell (figure 2 part 2).

The initial calcium flux from the T-tubules is insufficient to fully activate contraction, and so a secondary calcium current from the sarcoplasmic reticulum is required. Opposing the T-tubular surface is the corresponding membrane of the junctional sarcoplasmic reticulum containing dense semi-crystalline arrays of calcium channels - Ryanodine receptors (RyR). The calcium activated nature of the cardiac RyR isotype means the transient flux of calcium will trigger their opening resulting in the release of stored calcium ions within the SR lumen. As the SR holds calcium in very high concentrations (in addition to buffering by chelators such as calsequestrin), the release of its calcium store leads to significant cell wide increases in calcium concentration up to the µM range. The relative contribution from the different release pathways vary between species, with humans contributing roughly 50% : 50% ratio of EC calcium and SR calcium (Pieske, Maier, Bers, & Hasenfuss, 1999), rabbits, roughly 30% : 70% EC and SR ratio, and rats a 8% : 92% ratio of EC and SR calcium (Bassani, Yuan, & Bers, 1995).

RyR exists as a large tetrameric channel protein of four 550 kDa monomers and acts as the primary calcium efflux protein of the SR (Lanner, Georgiou, Joshi, & Hamilton, 2010). Currently 3 main isotypes of RyR are known localising to different regions of the body; RyR1 is found in the skeletal muscle and operate in a directly coupled mechanism where depolarisation of the LTCC mechanically triggers the opening of the RyR via foot processes. RyR2 is found exclusively in the heart and activate via calcium induced calcium release as
detailed here. RyR3 is the least well known of the isotypes and has been implicated in roles ranging from smooth muscle regulation to neuronal function and neonatal skeletal muscle function. The completed tetrameric formation spans 27 nm X 27 nm wide and 19 nm high with a 10 nm protrusion into the cytosol accounting for the majority of the span of the dyadic cleft. Electron micrographic imaging and tomography have managed to create 3 nm resolution images of RyR2 and 2 nm resolution image of RyR1 both detailing possible sites for calcium binding (Liu, Zhang, Li, Chen, & Wagenknecht, 2002; Liu, Zhang, Wang, Wayne Chen, & Wagenknecht, 2004; Radermacher et al., 1994). More recently, near atomic level resolution images of RyR1 have become available shedding light on various key residues relating to overall regulation of the channel (Yan et al., 2015). Interestingly, while RyRs in general are selectively permeable to calcium ions, they also possess high conductivities for monovalent ions such as potassium and lithium (Inui, Saito, & Fleischer, 1987). This conductivity may assist with charge countering during calcium release where a large net flux of positive calcium ions is observed crossing the SR membrane.

Contraction of the myocyte is in turn mediated by an assortment of proteins affiliated with the thin contractile filaments. In order to prevent permanent activation of the myofibrils, the troponin and tropomyosin complex binds to the actin thin filaments of the myofibrils preventing access of the myosin binding sites by the thick filament myosin heads (Farah & Reinach, 1995). This prevents the power stroke of the contraction cycle stopping contraction from occurring. The elevated levels of calcium released during excitation leads to binding of free calcium to troponin C, a subunit of the troponin protein, which then triggers a conformational change troponin I opening the myosin binding sites on the thin filaments causing contraction to occur (Potter & Gergely, 1974).

Termination of the transient activation occurs through calcium reuptake into the SR and extrusion through the plasma and T-tubular membrane. Within the SR, The sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) is the primary protein responsible (Fabiato & Fabiato, 1975). As an ATPase, SERCA utilises the hydrolysis of an ATP molecule to drive 2 calcium ions across the sarcoplasmic membrane. Within the plasma membrane, the sodium calcium exchange (NCX) is the primary method of extrusion (Bassani, Bassani, & Bers, 1994) along with other contributors such as the plasma membrane calcium ATPase (Monteith & Roufogalis, 1995).
Therefore, essential for the correct operation for heart cells is the internal organelle system which is highly specialised for its function. This precise spatial arrangement of proteins is what enables the rapid conversion of electrical depolarisation to contraction (D. M. Bers, 2008). Of these organelles the most important are as follows.

**Myocyte internal organelle systems**

**Contractile apparatus**

Classified as striated muscle, the cardiac myocytes possess highly ordered arrays of contractile machinery comprised of actin and myosin polymers forming thin and thick filaments respectively (Simpson et al., 1996). During contraction, the myosin heads of the thick filament bind to the actin thin filaments in a cross bridge cycle to allow the sliding of the filaments past each other shortening the muscle and producing force - a process known as the sliding filament theory (Fabiato & Fabiato, 1975). The thin filaments are in turn anchored at the Z discs where numerous different structural and regulatory proteins reside to transfer force both along the cell and into the sarcolemmal membranes (Frank, Kuhn, Katus, & Frey, 2006). A central component of various disease states for the heart is the progressive loss of force generated by myocytes. It is therefore important to investigate the structure and function of the contractile apparatus to understand possible changes that may occur to lead to pathology.

The myofibrillar Z disc is seen in electron micrographs as a dark electron dense line within the myofibrils when imaged in longitudinal arrangement (Hoshijima, 2006; Y. Hou et al., 2014b; Knappeis & Carlsen, 1962). Because of the readily apparent visualisation of it, the Z disc is often classified as the boundary between individual sarcomeres. In addition to its anatomical significance, the electron dense nature belies the complex protein-protein interaction systems present within this region.

Three central proteins are associated with the Z disc comprising of actin, α actinin, and titin. Actin is the primary protein of the sarcomeric thin filaments which at the point of the Z disc, is nearing its terminus (Lu et al., 1992). Actin thin filaments act as passive rails for which the active ATP consuming myosin heads of the thick filaments bind and move along during contraction, whilst being associated with the troponin and tropomyosin complex which acts
to regulate contraction through opening and closing of the actin – myosin binding sites through calcium binding troponin C (Ahuja, Sdek, & MacLellan, 2007; D. M. Bers, 2008).

α actinin, the antiparallel homodimeric crosslinking protein which binds the opposing actin of the thin filaments enabling the linking of opposing polarity filaments (Franzot, Sjoblom, Gautel, & Djinovic Carugo, 2005; Ribeiro Ede et al., 2014; Sjoblom, Salmazo, & Djinovic-Carugo, 2008). Found not only within the Z disc, alternative isoforms of α actinin act as anchoring sites for the cytoskeletal thin filament framework of other cells(Knudsen, Soler, Johnson, & Wheelock, 1995; E. Lazarides, 1976). Within cardiac myocytes, α actinin holds a key role both as the thin filament cross-linker and as a site of multiple protein interactions regulating contractility (Otey & Carpen, 2004; Ribeiro Ede et al., 2014; Schultheiss et al., 1992).

Finally, titin, acts as a major point of contact between the Z disc and other essential sarcomeric structures(H. L. Granzier & Labeit, 2004). As the longest known protein, during development, titin acts as a ruler for the growth of the thick filaments while during contraction, titin’s elastic elements provide the majority of passive force for the sarcomere(H. Granzier, Kellermayer, Helmes, & Trombitas, 1997). These elastic element tensions further can be regulated through numerous binding domains along the titin molecules, including sites of phosphorylation, and binding to adjacent actin filaments. Spanning from Z disc to Z disc, titin interacts with itself at the Z disc to link adjacent sarcomeres and is capped by the T-cap protein (Faulkner, Lanfranchi, & Valle, 2001; Yamashita, Maeda, & Maeda, 2003).

**Z disc Signalling**

The Z disc’s importance further also extends as a signalling hub of the myocyte, possessing many different interacting protein complexes regulating a variety of functions ranging from cell growth to calcium homeostasis (Frank et al., 2006; Luther, 2009).

The costameric complex is found along the edges of the Z disc and acts as both a structural link between the sarcolemma and the force generating sarcomeres (Hoshijima, 2006; Samarel, 2005). Within the costameric complex the protein desmin acts as the functional link to the sarcomere. With a variety of structural and signalling roles, desmin, an
intermediate filament component, plays a key role as the primary linker of the myofibril Z disc to the costameric complex (E Lazarides et al., 1982).

An important component of Z disc signalling function occurs through mechanotransduction, where the cell senses the mechanical stresses experienced during contraction and appropriately modifies both cell hypertrophy (as seen in heart failure) and sarcomeric growth (Frank & Frey, 2011; Knoll, Hoshijima, & Chien, 2003; Lammerding, KAMM, & LEE, 2004; Samarel, 2005). The location of these sensors within the Z disc varies considerably with costameric localised proteins such as melusin and Z disc localised proteins such as calsarcin-1 being implicated in regulating growth.

**T-tubules**

The T-tubules show distinct protein distribution in comparison to the sarcolemma membrane that it extends from. In addition to potassium and sodium channels necessary for action potential propagation, there is an increased amount of LTCC for trigger calcium currents (Kawai, Hussain, & Orchard, 1999), high levels of NCX (Scriven, Dan, & Moore, 2000) for calcium extrusion, and elevated β2 adrenergic receptor distribution (Nikolaev et al., 2010). These proteins also show non homogeneous distribution within the walls of the tubule. The primary inhomogeneity occurs at the sites of interaction with the sarcoplasmic reticulum where L-type channels are found in high concentrations in order to allow sufficient influx of calcium ions for the subsequent activation of the ryanodine receptor on the sarcoplasmic side of the interaction.

The T-tubules shows multiple external interactions with both regulatory and structural proteins associated with calcium control. Junctophilin (JPH), currently understood to be a primarily structural protein contains an interaction zone in the c-terminus which binds to the T-tubular membrane (Takeshima, Komazaki, Nishi, Iino, & Kangawa, 2000). With the n-terminus anchored in the SR membrane, this effectively allows JPH to act as a linker between the SR and T-tubules forming a close nanometer interaction point between the two organelles. This linkage is of particular importance in excitation contraction coupling with experiments having shown possible interaction structurally and functionally with the LTCC of skeletal muscle (Golini et al., 2011). However, as JPH has only been recently identified, much of its additional functions are still uncertain.
Interestingly, the t-tubular network shows spatial arrangement variation between similar cells. Within mammals, the t-tubules reside primarily in line with the z disc in cardiac myocytes whereas skeletal myocytes show t-tubules in line with the A1 band of the sarcomere. Possible reasons for this discrepancy between similar cells types is not readily clear, however, the mechanism of excitation contraction coupling likely plays a role, where skeletal muscle relies on mechanical gating while cardiac myocytes rely on calcium induced calcium release.

**Sarcoplasmic reticulum**

The Sarcoplasmic reticulum is found within the cytosol as an extension of the endoplasmic reticulum. Filled with concentrations of calcium at roughly 0.5 to 1.5mM free calcium (~15mM total), the release of this calcium store act as the activating trigger for contraction of the myofibrillar complex. The overall distribution of the SR is less variable between species when compared to the T-tubules. Extensively distributed around the myofibrils, the main concentration for mammals such as rats and humans occurs around the Z disc, where the T-tubular system also resides.

The SR can be subdivided into two main sections, the junctional and non-junctional SR. The junctional SR (jSR) is found primarily at the Z disc and is the area where the SR comes to close proximity to the T-tubular system. The non-junctional SR (njSR) is all other areas excluding the jSR. Though membrane continuity wise there is no clearly defined separation between jSR and njSR, they nonetheless possess differences in protein distribution (I. D. Jayasinghe, Cannell, & Soeller, 2009) which leads to distinct functional regions.

One of the main protein features of the jSR is the presence of semicrystalline arrays of ryanodine receptors (RyR). The Ryanodine receptors are calcium activated calcium channels essential for the activation of cellular contraction via the release of calcium from the SR stores. These jSR arrays are held at very close proximity to the T-tubular system (T. Hayashi et al., 2009) to enable tight coupling between the activating current from the LTCC and opening of the RyR arrays. The combined region of the RyR array and the LTCC occupied T-tubule area is known as a dyad. Modelling as well as observational evidence have shown that the interface between the jSR and the T-tubules must be regulated with nanometer
level precision for effective coupling of T-tubular calcium flux and the subsequent activation of SR calcium release (M. B. Cannell, Crossman, & Soeller, 2006).

Regulation of RyR open probability is directly influenced by the proximity and release flux from the LTCC. Cardiac RyR and LTCC differ markedly from what is found in skeletal muscle where a direct coupling exists between the proteins. This lack of direct coupling means that dyadic dimensions can vary in terms of membrane separation (M. B. Cannell et al., 2006). In reality however, the dyadic cleft remains an approximately consistent 10 nm gap through the role of the linker protein junctophilin (Takeshima et al., 2000; van Oort et al., 2011). In addition, the opposing membrane proteins both have protrusion into the cell cytoplasm bringing the interface between channels even closer (Lanner et al., 2010).

In addition to the RyR arrays, further found within the jSR is a series of calcium sequestration and sensing complexes. Central to this is calsequestrin (Beard, Laver, & Dulhunty, 2004) a protein found within the lumen of the SR which acts as a buffer for calcium ion concentrations. Calsequestrin is capable of binding 50-60 mol calcium per mol of protein (Mitchell, Simmerman, & Jones, 1988; Slupsky, Ohnishi, Carpenter, & Reithmeier, 1987) and therefore is the primary store for calcium within the jSR. The large buffer of calcium ions means a lower free ion concentration enabling more calcium to be stored within the region (Shannon & Bers, 1997) because of this, during release events; the jSR does not typically undergo full calcium depletion (Bassani et al., 1995).

To assist in coupling between the RyR and Calsequestrin, a series of auxillary protein are present within the lumen of the jSR. Junctin and triadin bind with calsequestrin and the luminal side of the ryanodine receptor to form a complete release unit (I. Gyorke, Hester, Jones, & Gyorke, 2004; S. Gyorke, Gyorke, Terentyev, Viatchenko-Karpinski, & Williams, 2004; Lanner et al., 2010; Terentyev et al., 2007). Suggestions have been made for this protein complex to act as a luminal calcium sensor which can assist in the regulation of ryanodine receptor activation (I. Gyorke et al., 2004) supplementing stochastic attrition in the termination of release.
Graded response and termination of release

Important of note is the graded response of the cardiac myocyte, in that the force of contraction and the overall calcium transient from the RyR is dependent on the activating transient from the LTCC (New & Trautwein, 1972; M. D. Stern, 1992). This means, that the calcium released by the RyR is directly related to the level of activation of the LTCC making it a function of the overall cell depolarisation level. By voltage clamping the cell to different membrane potentials, variable calcium release from the RyR (as a whole not individually) are detected. Therefore, by regulating the process of CICR, control can be established over the force and time dynamics of a single heartbeat.

Stoichiometrically however, the proteins are not distributed at a 1:1 ratio and can vary considerably between clusters. Rat’s typically have smaller influx currents from the LTCC to match the extrusion quantity by the NCX while humans and rabbits show comparatively higher currents (Shattock & Bers, 1989). Although there is no fixed ratio, there is still nonetheless graded control of calcium release from the RyR clusters in response to the LTCC trigger current; the greater the trigger current – the greater the release from the CRU (New & Trautwein, 1972). This scalability of calcium release was until the discovery of calcium sparks and local control theories not well understood in regards to CICR as the nature of RyR’s calcium activated calcium release should be self-regenerating (M. B. Cannell, Cheng, & Lederer, 1994; M. D. Stern, 1992).

It was suggested in 1992 that due to the close proximity of the T-tubular surface and the SR surface, the cleft in which the RyR and LTCC interact can be considered spatially excluded from the surrounding cytoplasm creating a local calcium signalling domain (M. D. Stern, 1992). This is in contrast to the typical common pool models used at the time which incorporates the entire cell as a single calcium domain. This local domain occupies a very small volume allowing even small calcium transients to elicit large changes in concentration (M. B. Cannell & Kong, 2012). Because of this feature, gating sensitivity can be more easily adjusted to be less sensitive thereby removing the problem of spontaneous contractions. This also offered an explanation for the perceived graded response as while locally there may be regenerating release, the small size of the local region means that concentration
within the cleft will dissipate quickly and grading can be achieved based on how many local domains are activated.

Confirmation of local control models and the graded response explanations came with the discovery of calcium sparks (Cheng, Lederer, & Cannell, 1993). Calcium sparks are what appear to be quantal calcium release events of otherwise quiescent myocytes. It was thought the summation of these events leads to the complete calcium transient seen in a contraction and that abnormal quantity or size of the quantal releases can lead to pathological calcium wave formation (Coombes, Hinch, & Timofeeva, 2004). This occurrence is most clearly demonstrated when SR calcium loads were changed relative to standard homeostatic levels (Coombes et al., 2004; J. Keizer & Smith, 1998; Joel Keizer, Smith, Ponce-Dawson, & Pearson, 1998; Sobie et al., 2006).

The exact origin of calcium sparks were under contention for a long period. However, improvements in temporal-spatial imaging of calcium transients as well as single channel measurements from RyR molecules have suggested that the spark fluxes originate from individual clusters ranging from 6 to 20 individual channels (M. B. Cannell & Kong, 2012) and not individual receptors. This supports the idea that while local regenerative behaviour occurs within clusters, a large spatial separation between these clusters acts to dilute the calcium transients preventing activation of neighbouring clusters.

The size of the locally regenerating clusters therefore becomes a key variable in the termination of calcium release alongside receptor sensitivity (Michael D Stern & Cheng, 2004). Clusters too large will lead to a regenerating transient that is sufficient to activate neighbouring clusters while smaller clusters will be easier to control but may have problems activating adjacent clusters without sufficient current.

Another factor which cluster size contributes to is the time interval for termination. The mechanism proposed by (M. D. Stern, 1992) is one of stochastic attrition where closing of RyR is primarily determined by initial probabilistic closing of a few receptors within the dyad. Upon closing, the channel will be in a closed state for a short period of time in which the calcium concentration within the cleft will fall thereby increasing likelihood of nearby RyR channels also closing (Sham et al., 1998). From this, smaller clusters will tend to auto terminate quicker than large clusters as sufficient proportion of the activated cluster must
close before the concentration within the cleft noticeably reduced open probabilities. Larger clusters therefore will require more channels to stochastically close at the same time while smaller clusters will require less and thereby require on average less time to terminate (given all other parameters remain the same.)

SR depletion has also been investigated in depth in regards to both termination of release and potential pathological manifestations of SR calcium load. The progress in understanding of cell calcium dynamics came in parallel with the development of better calcium sensing dyes such as the assorted Fura/Fluo AM ester dyes used for cell loading (Hilgemann & Langer, 1984). These dyes have allowed through visualisation the ability to gauge the relative calcium levels within the cell and SR. When applied to cells during contraction, the general calcium fluorescence of the SR does not show significant change, suggesting that at least SR wide, there is no sign of major calcium depletion (Bassani et al., 1995). However this does not preclude the possibility of local junctional SR depletion, due to the large channel quantity, although, it should be noted that the presence of calsequestrin within this region will act to buffer any significant calcium concentration change (S. Gyorke et al., 2004).

More recently, stochastic attrition and SR depletion have been consolidated together in a process now termed induction decay (Laver, Kong, Imtiaz, & Cannell, 2013). This process considers the increase in closing probability due to local SR depletion alongside the probabilistic nature of multiple channels closing concurrently to prevent the regeneration of calcium release. This model which incorporated the major buffers as well as ions in a structure based on electron microscopy images of membrane networks produced sparks with calcium profiles which were less dependent on overall cluster size. The key requirement for this model however is the extensive depletion of local calcium concentrations within the dyadic space coupled with the time for refilling via diffusion from adjacent SR. Observations to this effect have been previously noted and referred to as blinks (Brochet et al., 2005) and show a reduction in local SR calcium of approximately 65% as well as local refractory period where subsequent blinks are of reduced magnitude. Calcium blinks are seen as distinct from the slower calcium scraps which is of reduced spatial resolution and show a much longer time course and smaller depletion (Shannon, Guo, & Bers, 2003). To further complement depletion and stochastic attrition, the possibility of direct receptor to receptor coupling has also been investigated. There is increasing evidence to suggest that
there is increased affinity for receptor to receptor interaction. This affinity suggests a possible underlying interaction between channels where the channels within arrays are able to co-activate or terminate adjacent channel releases (M. B. Cannell & Kong, 2012). Increasing evidence has been found in regards to coupled gating with the identification of possible ions supporting such interaction (Gaburjakova & Gaburjakova, 2014). A likely method of coupled gating is that the inactivation of one channel leads to the corresponding decreased open probability of the adjacent channel. In this way, stochastic attrition can be amplified and a more timely termination can be facilitated.

A possible source of coupled gating also identified is the RyR associated protein FKBP 12.6. Experiments show that a knockdown of this protein results in the corresponding increase in spark frequency while an overexpression tends to restrict spark formation. FKBP is also implicated in numerous calcium interactions, a topic which is still hotly debated (Shou et al., 1998; Timerman et al., 1996; Wehrens et al., 2003).

Analysis of protein sequences has further identified numerous potential sites of regulatory interaction within the RyR molecule. These include both possible SR Luminal and Cytoplasmic calcium sensors for the termination of function (I. Gyorke et al., 2004). Furthermore, it has been found that the sudden spike in positive calcium ion concentration would cause the eviction of other positive ion types. This has been found to be likely the case for magnesium ions where not only is there magnesium ion binding sites on the RyR, these sites seem to alter the RyR gating kinetics so that activation is very difficult (Laver, Baynes, & Dulhunty, 1997). However, the range of concentrations required for this activity is higher than what is anticipated for the dyadic cleft.

**RyR distribution within the myocyte**

The size and spatial distribution of the individual release clusters is of importance to the overall function of the cell. The distribution of sizes can however be altered in certain circumstances and are dependent on various factors. An important regulator of cluster arrangement recently implicated is the junctophilin protein which has been shown to alter cluster arrangements depending on level of expression (Munro et al., 2016) as well as various ionic compositions within the extracellular space altering possible fluidity of the
crystalline array (Asghari et al., 2014). However, currently we have little detailed information on the time scales over which RyR clusters rearrange – it has been suggested that phosphorylation may alter cluster arrangements fairly rapidly (Asghari et al., 2014).

Within cardiac myocytes, these RyR release clusters can be seen as fitting three broad groups with slight differences in operation. These different subsets are termed peripheral cluster, T-tubular associated cluster, and non T-tubular associated clusters.

**Peripheral clusters**

The first type of release cluster is the peripheral release sites where the SR and the sarcolemma interface at the cell surface. Characterisation of the release sites can be easily carried out through the use of isolated myocytes. These studies (D. Baddeley, Jayasinghe, Lam, et al., 2009) involving super resolution microscopy has shown that these rat couplons are typically small (14 RyR down from ~100 from EM estimates of all clusters) and stretch more or less as a ring around the z disk. In comparison with interior cell couplons, under confocal microscopy they are more scattered in line with the Z disc at times forming double rows flanking the line whereas interior images show the RyR typically sitting along the Z disc (Chen-Izu et al., 2006).

Distance and dimension wise, they are not circular as expected from confocal images, but rather elongated with average ratios of 1 : 9 based on major and minor axis. This elongation extends to measurements for nearest adjacent neighbour where distances of <50nm is commonly present suggesting high level of intercluster interaction (I. D. Jayasinghe et al., 2012). These nearest neighbour interactions occur typically along the length of the t-tubule with greater separation between Z discs. Inter Z disc clusters are also present though at a lower frequency with typically larger separation between clusters (Chen-Izu et al., 2006).

Functionally however, there is little to differentiate peripheral and central receptors, activation and termination of CICR occurs via the same mechanism and separation of peripheral and central membranes remain approximately the same (C. Soeller, Crossman, Gilbert, & Cannell, 2007). Possible differences between the clusters however exist in the sensitivity to second messenger systems with higher population of beta adrenergic receptors found in the T-tubular membrane potentially decreasing the sensitivity of peripheral receptors to changes in cellular signalling (Nikolaev et al., 2010).
Finally, peripheral receptors are the first CICR components to form in developing ventricular myocytes (Dan, Lin, Huang, Biln, & Tibbits, 2007) making them responsible initially for the majority of calcium release. Time for formation of the T-tubular system vary between species, however, they all tend to occur late in cardiac development as a possible last point of cell differentiation (Brette & Orchard, 2003). This is also seen in atrial tissue where T-tubular systems are less well developed (Smyrnias et al., 2010).

**T-tubule associated release clusters**

T-tubular associated clusters also known as central release clusters tend to lie along the Z disc with confocal images of Z disc regions in longitudinal view showing very high levels of colocalisation between a Z disc marker such as α actinin and RyR. This close association then breaks into a dual line arrangement near the periphery (Chen-Izu et al., 2006). The reason for this close arrangement to the Z disc within mammalian T-tubules is uncertain however; it is possible that due to the complex organisation of the Z disc as an intracellular signalling site positioning of calcium release units in close proximity allows for a more rapid response to changing cellular environments.

The central calcium release unit functions via a near identical method to the peripheral clusters through CICR. Morphologically however, they tend to be larger than the peripheral clusters (Clara Franzini-Armstrong, Feliciano Protasi, & Venkat Ramesh, 1999; Isuru D. Jayasinghe et al., 2014). Confocal microscopy shows typically circular diffraction limited blobs with very little sub structural detail (T. Hayashi et al., 2009). Additionally, most analysis takes place using longitudinal isolated cells with little transversely sectioned cells for comparison (though more recently transverse imaging is more common.) From confocal data, an estimate of an average of 70 – 80 RyR/CRU is predicted with a predisposition for smaller sizes (Chen-Izu et al., 2006). Larger sizes however are also present with some clusters being >200 RyR. As the RyR orientation along a T-tubule cannot be determined by the two dimensional confocal images, a correction factor was also needed over this data resulting in a final estimate with mean of 180 RyR per cluster (C. Soeller et al., 2007).

Measurement of size has also been extensively done in electron microscopy to supplement the confocal data. One of the earlier comparisons was made by Franzini-Armstrong across a number of different species skeletal and cardiac myocytes (Clara Franzini-Armstrong et al.,
1999). This study did not employ 3D methods such as tomography and serial sectioning and so relied on expecting a circular shape descriptor for the identified clusters and using the counting of cytoplasmic feet of RyR to identify the quantity of receptor present. A small sample number of only 30 analysed clusters increase the uncertainty in the measurement. Even so, this method gives a good starting basis for the quantity of receptor in clusters which the Franzini-Armstrong paper reports to be approximately 120 receptors on average within rats.

More recently, the full suite of electron microscopy techniques were implemented in the imaging of mouse central dyadic clusters to obtain estimates of size of the release cleft (T. Hayashi et al., 2009). This study employs serial sectioning and tomography to obtain a 3D structure of the dyad. Though only mice were examined in this study, it was found that their results were quite comparable; though lower than the previous Franzini-Armstrong findings. These measurements possess reduced sources of error as all three spatial axis is able to be measured. However, as this is based on the Identification of closely associating membranes, the ability to differentiate between membranes with high RyR content and little RyR presence is not readily possible.

**Non t-tubule associated release clusters**

Non T-tubular associated RyR release sites have been proposed to play an important part in the formation of cardiac disease (Song et al., 2006). Their distance away from the T-tubular interface means standard activation via LTCC is not possible. These receptors therefore rely on nearby coupled release units for activation and calcium release in a system known as fire-diffuse-fire (Dawson, Keizer, & Pearson, 1999). This manner of release typically results in a delay between the main release clusters and the non T-tubular associated clusters. Subsequently this delay will prolong the overall calcium transient (M. B. Cannell et al., 2006) and can possibly be attributable to disease models showing similar increases in duration of calcium release (Heinzel et al., 2008).

The extent of non-tubule associated clusters found within a given cell therefore has direct implications on the function of the cell. Larger portions of non T-tubular cluster will result in a generally longer calcium transient limiting the minimum time required for contraction and a reduction in the overall efficiency of contraction (M. B. Cannell et al., 2006). Between
species this distribution will vary considerably depending on the requirements placed on cardiac tissue, however, the greater implication is the changes that occur within tissues that has been damaged by disease (Lyon et al., 2009). It’s been shown that in pathological tissue, there may be an increase in the overall amount of non T-tubule associated RyR possibly as consequence of loss of coupling at dyadic sites (Song et al., 2006). Modelling of such a situation has shown that the increasing the amount of non-tubule associated cluster along with wider spacing between release sites tended to result in a comparative heightened risk of calcium wave formation (Coombes et al., 2004; Joel Keizer et al., 1998). This increase due to non-dyadic RyR having higher leak tendencies resulting in rises in local calcium content. As calcium waves are the primary cause for delayed after depolarisations in cells (De Ferrari, Viola, D’Amato, Antolini, & Forti, 1995), this will result in an increase in chance of cardiac arrhythmia.

Regulation of the Ryanodine receptor protein

The control of the calcium release units lie in the control and regulation of its main constituent protein, the RyR. As previously mentioned, due to its size and importance of its operation, there is a plethora of associated points of interaction, many of which are still poorly understood. Of these, luminal calcium sensing, phosphorylation and, self interaction via a process of coupled gating has been investigated as possibly the central contributors to overall RyR function. These points of interaction both contribute to normal regulatory function in cardiac release as well as sources of many problems in cardiac disease.

On the opposing side to the T-tubule membraneinterface of the calcium release unit is the corresponding cytoplasm - jSR interface. This interaction is of importance in understanding possible luminal calcium sensors and control of SR calcium content.

The combination of junctin, triadin and calsequestrin acts to buffer the terminal SR luminal calcium levels enabling a higher density of calcium ions in this region for release (Shannon & Bers, 1997). In addition to calcium sequestration, this complex has also been hypothesised to act as a luminal sensor of calcium content (I. Gyorke et al., 2004). During release, a fall in concentration of calcium within the luminal region leads to the reduction in binding of calcium to the calcium sensing protein complex. This reduction in binding subsequently
reduces the open probability of the RyR channel increasing chance of a given channel to close. This can act in combination with stochastic attrition and coupled gating to facilitate a rapid termination of release (Michael D Stern & Cheng, 2004). This has been investigated via the induction decal model of release termination (Laver et al., 2013).

The direct RyR interaction with junctin or triadin is likely an important factor in SR calcium sensing. These two proteins act as a link from the RyR to Calsequestrin which is the primary calcium chelator with all three combined acting as the luminal sensor of calcium (I. Gyorke et al., 2004; L. Zhang, Kelley, Schmeisser, Kobayashi, & Jones, 1997). The hypothesised site for this linkage occurs along a KEKE alternating positive negative motif on the luminal side of the RyR. Further evidence for these mechanisms exist in numerous complex models that simulate this situation (M. B. Cannell, Kong, Imtiaz, & Laver, 2013) as well as measured depletion rates from the terminal SR which show a reduction in calcium concentration of 59% (Bassani et al., 1995). This suggests that there are likely significant changes to the SR calcium load. However, within the same study evidence also show that when pushed, the cell is capable of much longer phases of depletion indicating that alone passive SR depletion may not be enough to facilitate spark termination. Mutation studies of Calsequestrin (CSQ) have shown that changes to this molecule results in direct impacts to the formation of sparks (I. Gyorke et al., 2004; S. Gyorke et al., 2004; Terentyev et al., 2007) as well as identified mutation sites for causing CPVT phenotypes with is directly related to overall cell calcium handling through higher calcium release due to higher open probability of the channel.

In addition to CSQ as a luminal sensor, there is also increasing evidence of a luminal sensor on RyR itself with numerous identified calcium binding sites and bilayer studies of isolated receptors producing a luminal calcium dependent response (I. Gyorke & Gyorke, 1998). Current debate continues as to the precise arrangement of the luminal calcium sensor. Evidence continues to be gathered demonstrating that the RyR2 channel when isolated from the calsequestrin complex still can produce luminal dependent calcium release (Chen et al., 2014; Sitsapesan & Williams, 1994). Chen, et al (2014) further showed that within the RyR2 channel mutation of residue E4872 to a positive or neutral amino acid resulted in loss of luminal calcium response indicating a likely candidate region for direct luminal calcium regulation.
In pathological circumstances, this calcium sensor (whether it is via calsequestrin or innately within RyR) can act to facilitate greater basal leak of calcium ions during the quiescent state. Genetic mutations within the calcium sensing system leads to an increased effect on RyR leading to higher leak rates for a given luminal concentration resulting in higher chances of spontaneous release events (Terentyev et al., 2003; Viatchenko-Karpinski et al., 2004). This is most evidenced with CPVT sufferers where point mutation within the RyR localised to the region identified as a candidate for the luminal calcium sensor produces proteins which are more likely to open resulting in a reduction in store overload calcium release events (SOICR) (Jiang et al., 2004). Experimental proof for the existence of such a sensor system and the effects on spontaneous wave formation is shown in cases where the sensor system is selectively knocked down or up resulting in distinct changes to the overall cell calcium handling (S. Gyorke & Terentyev, 2008).

The phosphorylation of the RyR protein is seen as a central regulator of function. The results of phosphorylation and the lack thereof have been most intensely studied in cases of disease formation. Central to the phosphorylation regulation of RyR and many other calcium regulatory proteins is the calcium/camodulin dependent protein kinase 2 (CaMK2) which acts to trigger the phosphorylation of RyR and associated proteins. Interestingly, while its actions throughout the cell is quite prolific being triggered from many sources and signalling cascades (Ai, Curran, Shannon, Bers, & Pogwizd, 2005), the phosphorylation effect of CaMK2 on the RyR typically can be seen as detrimental to overall function. Implications include the increase of resting calcium leak (Curran, Hinton, Ríos, Bers, & Shannon, 2007), and spark generation both leading to higher arrythmiogenic tendencies for the cell. Even more interesting is that the selective knockdown of CaMK2 shows no immediate detrimental effects on cellular function. In many cases, the reduction in CaMK2 results in better calcium regulation within the cell (T. Zhang & Brown, 2004). Therefore a pressing question remains on what the functionally significant role CaMK2 plays within the cell. It is possible that the up regulation of CaMK2 is in order to trigger some other non RyR associated pathway with the pathological alterations on RyR being a result of excessive up regulation.
**Visualisation of RyR clusters**

Light microscopy has been the mainstay for biologists since the invention of the microscope in the 16th century. As time progressed, refined techniques in constructing objectives and condensers allowed the steady increase in resolution attainable. In the late 19th century, Ernst Abbe discovered that there was a fundamental limit to resolution using light microscopy (Abbe, 1873).

Termed the Abbe limit (more typically referred to the diffraction limit), the fundamental resolution limit for a given light microscope setup is dependent on the wavelength of light being detected and the numeric aperture of the objective and condenser. With current oil immersion lenses, the maximal numeric aperture for an objective lens sits at around 1.5. With these types of objective lenses, the approximate resolution would be half of the wavelength used (~250nm for the standard 488nm excitation light).

Direct visualisation of RyR clusters with microscopy systems and immunohistochemistry remain as the simplest method of identifying cluster distributions and quantity. Confocal microscopy, with its 200nm resolution, is able to just resolve enough detail for the estimation of cluster sizes. Analysis of these images show that the average cluster size should be on the order of 150 RyRs per cluster (Chen-Izu et al., 2006). Alternatively, electron microscopy can also be employed to visualise the presence of RyR within dyadic regions producing results similar to light microscopy (Clara Franzini-Armstrong et al., 1999).

**Confocal Microscopy**

Optical microscopy has been used extensively since its discovery on visualisation of the cellular domain. Subsequently an extensive array of tools and techniques has been developed for better visualisation and quantitative analysis of structures (McNally, Karpova, Cooper, & Conchello, 1999; Stephens & Allan, 2003). Of these, currently one of the most used is the immunofluorescence confocal microscope.

Immunofluorescence confocal microscopy differs from standard transmitted light microscopy firstly through the method of illumination. Instead of transmission through the sample, with contrast caused by attenuation and scattering by the sample, the illumination occurs via an excitation laser (of either arc-lamp for standard fluorescence or laser for
confocal.) The samples for confocal microscopy are prepared through immunohistochemical labelling of samples with antibodies that have fluorescent fluorophores attached. The excitation beam energises the fluorophore, which then releases the absorbed energy as light of a longer wavelength than the excitation beam. A dichroic mirror and emission filter then redirects and filters the emitted light so that theoretically only the fluorescence from the labelled targets will pass through. In practice however, auto-fluorescence from the tissue typically occurs in a wide range of wavelengths can factor in as a problem of background in images (Rosen et al., 2007).

Confocal microscopy further add background reduction through the use of a pinhole system when compared with standard fluorescence setups (Shotton, 1989). The pinhole before the sensor acts to restrict entry of out of focus light through effectively blocking (if properly calibrated) non 0th order maxima of a microscopes point spread function (PSF – also referred to commonly as the airy disk). The point spread function of a microscope is defined as the distortion of a point source of light by the microscope lens system. This has an additive effect of assisting in axial resolution by eliminating the out of focus light from other Z levels thereby increasing the sharpness of the in focus plane. It is important to note however, that this method of visualisation does not surpass the diffraction limit.

Confocal microscopy while having good subject to back ground contrast (the labelled targets are illuminated against a black background) is still none the less limited by the diffraction limit of light at ~200 nm (Abbe, 1873). While RyR is considered a large protein with a single protein footprint of 27 nm X 27 nm (Inui et al., 1987; Lanner et al., 2010), within a resolvable region of ~200 nm X 200 nm up to 36 RyR proteins can be found making the smallest cluster size estimates at about that range. Work based on measurement of releases have shown that - at least for calcium sparks – the ion flux only equates to between 6 and 20 individual molecules. At this range, resolving of structure and size would be very difficult if not impossible with confocal microscopes. Demonstration of confocal microscopy limitations can be seen in (T. Hayashi et al., 2009) where a sharp detection fall off for RyR clusters is seen below the resolution limit of the confocal microscope.

However, due to the easily accessible nature and ability to distinguish between target and background, confocal was still widely used to show potential cluster sizes within myocytes.
(C. Soeller et al., 2007) showed estimates of approximately 170 RyR per couplon on average. This estimate is considerably more than what was expected based on channel recordings which suggest somewhere on the order of 6 - 20 individual RyR activating during spark generation (M. B. Cannell & Kong, 2012). At this estimate, a large portion of the RyR stays inactive during a spark.

**Electron Microscopy**

Due to the much shorter wavelengths of electrons, the resolution of electron microscopes is unparalleled with other imaging systems. The electron microscope however possess significant complications in terms of sample preparation, typically involving resin embedding or freeze fracturing, which can – if not handled correctly – cause damage to the fine sub structures of cells (Holt & Hicks, 1961; Shelton & Mowczko, 1978). Additionally, due to the intense ionising radiation and a high vacuum imaging chamber, there is no possibility of imaging of live cells. As most imaging is also done using differences in structural electron attenuation (different structures reduce electron transmission by different degrees), the resultant image is similar to transmitted light images.

This low contrast result means considerable effort must be made to segment out the regions of interest. In identifying RyRs additional problems arise with attempting to section exactly along the plane of the clusters. The method for identifying RyR within micrographs involve counting “feet” or the cytoplasmic protrusions visible at the SR/T-tubule interface which at times can be difficult (Clara Franzini-Armstrong et al., 1999). Because of the difficulties in cutting entire couplons (sections of EM are typically ~50nm thick, thinner than optical confocal sections) within a single slice, most estimates on cluster sizes rely on assuming a circular cluster arrangement, which when applied to a tubular structure, may not be fully correct.

When used with these estimates, it was found that there would be approximately 120 RyR per couplon. At these numbers stochastic attrition would require considerable time for deactivation of the entire cluster once it has been triggered (M. B. Cannell & Kong, 2012; Sobie, Dilly, dos Santos Cruz, Lederer, & Jafri, 2002; Michael D Stern & Cheng, 2004). Further, it also suggests that the vast majority of RyR within a typical couplon would remain inactive in a calcium spark event.
Super-Resolution Microscopy

Recent development in microscopy techniques has led to the development of new novel techniques for the visualisation of samples. One of the most promising has been the ability to break the diffraction limit for light microscopes known as super resolution microscopy. Super resolution microscopy can be broken down into two main techniques, stochastic reconstruction such as stochastic optical reconstruction microscopy (STORM) (Rust, Bates, & Zhuang, 2006), and photoactivated localisation microscopy (PALM) (Hess, Girirajan, & Mason, 2006); and PSF constraining using activation and inactivation light beams seen in stimulated emission depletion microscopy (STED) (Hein, Willig, & Hell, 2008).

Both of these methods attempt to circumvent the diffraction limit through different methods of constraining the point spread function so that fluorophore positions can be determined at accuracy higher than what conventional microscopy is able to (Thompson, Larson, & Webb, 2002). This order of magnitude improvement in resolution is of particular interest in cardiac science as the essential dyadic structures fit within this new resolution range (D. Baddeley, Jayasinghe, Lam, et al., 2009), enabling the application of many light microscopy techniques while still being able to visualise the likely presence of individual bound fluorophores.

With STED, constraint of the PSF is achieved through restricting the point of illumination through the application of a central activator laser surrounded by a donut of inactivation laser. It was found that the interaction interphase between the lasers showed a nonlinear response by the emitted fluorophores (they can be deactivated very easily) (Hell & Wichmann, 1994). As of such, at the edges of the ring, the location of fluorophores can be detected to levels beyond the diffraction limit. In practice, the resolution of STED falls somewhere around 20-30 nm laterally (Hein et al., 2008).

The alternative to STED microscopy is the localisation super resolution setups (Hess et al., 2006; Rust et al., 2006). These setups rely on the ability of digitally identifying centroids at much greater accuracy than the diffraction limit. In standard fluorescence, the illuminating beam typically activates the majority of labelled fluorophores; because the PSF of all fluorophores overlap they create a continuous image seen in typical images. However, by restricting the amount of fluorophores are on at any one time individual fluorophore
outputs can be isolated and the centroids (the central point of the fluorescent event) of each of the fluorescent events (hence forth referred to as just events) identified at very high spatial resolution; the process in which fluorophores can be switched on and off in sequence is known as photoswitching. By alternating the fluorophores on at any one time, a series of images can be acquired with the complete event centroids of the majority of fluorophores the resultant image of localised events therefore has a resolution greater than the diffraction limit.

Whilst both PALM and STORM fundamentally rely on localisation methodology, they are held distinct by the way in which they initiate photoswitching. Whereas STORM uses standard immunohistochemical techniques of conventional fluorophores associated with target structure specific antibodies (D. Baddeley, Jayasinghe, Lam, et al., 2009; Huang, Wang, Bates, & Zhuang, 2008; Rust et al., 2006), PALM relies on the intrinsic genetic exhibition of modified protein (green, cyan fluorescent proteins etc) (Hess et al., 2006). Both techniques in their initial development however, relied on the concept of dye pairs for photo switching where a dye combination consisting of an activation and inactivation dye of a different excitation wavelength are used. The initial laser excitation wavelength results in the shunting of fluorophores into a stable dark state which is then pulled out stochastically through the use of the alternative wavelength illumination. Repeating this step many times then allows the collection of a sequence for single molecule localisation and reconstruction. More recently however, the two techniques have become more interconnected and are now relying on conventional fluorophores and chemically induced photo switching (van de Linde et al., 2011). The use of chemical switching allowed for much wider adoption of the technique as complex dual fluorophore conjugate antibodies are no longer necessary for super resolution imaging.

To date, groups have employed super resolution for the imaging of a variety of cellular structures within different cell types (Huang, Babcock, & Zhuang, 2010; J. Wu et al., 2013). Within cardiac cells work has been primarily focused on fixed isolated myocytes which provided low background and good antibody access. Imaging of peripheral clusters showed that using dSTORM, an accurate count and view of the morphology of ryanodine receptors was possible (D. Baddeley, Jayasinghe, Lam, et al., 2009). Further progress however, needed to be made in imaging cells within the tissue structure. Tissue sections provide additional
complexity in the form of background auto fluorescence which can considerably reduce both the ability of detecting fluorescent events and the precision of the localisation.

### dSTORM Microscopy

#### Rationale of dSTORM

Within the stochastic methods for single molecule localisation, the initial step involves the identification of a centroid from an isolated fluorophore (Thompson et al., 2002). This process of centroid identification was first pioneered as single molecule tracking where it enabled the precise tracking of protein movements (e.g. myosin cycling (Yildiz et al., 2003)).

The accuracy in which single molecules can be tracked is dependent on numerous acquisition variables. Importantly, the accuracy of single molecule centroid detection is significantly improved over the diffraction limit and is a measure not restricted purely by the numeric aperture and emission wavelength. Instead, the ratio of background to foreground intensity, the photon count of emitting fluorophore and the overall pixel resolution of the acquisition system plays a bigger role in determining the accuracy in which the centroid is localised to (Thompson et al., 2002). In practice, single molecule can be localised typically down to the low nano meter ranges of 1-5 nm (Ober, Ram, & Ward, 2004; Tahmasbi, Ward, & Ober, 2015). At this point of resolution, larger protein complexes can have individual subunit movements analysed (Yildiz et al., 2003) and smaller molecule movements can further be accurately tracked.

#### dSTORM imaging

The methodology of dSTORM differs from the original STORM concept primarily through the mechanism in which the dark and light states of the fluorophore are induced. The original STORM setup relied upon specific interacting dye pairs. Initial work by (Rust et al., 2006) showed that by using a combination of cy5 and cy3 dyes, red illumination was able to place the cy5 fluorescence into the dark state and through a non-FRET dependent proximity energy transfer, the use of a green cy3 excitation beam allowed the conversion of the cy5 from its dark state back to the light state. By controlling intensity of the dark state inducing red beam and the reactivation green beam, as well as the proximity of the fluorophores, illumination can be controlled so that at any given imaging frame, individual activated
fluorophores can be isolated and through single molecule localisation, a sub diffraction image can be created.

**Figure 3:** Detailing the principle behind dSTORM imaging. Single molecule localisation is used across multiple frames of reswitching fluorophores (red diffraction limited spots) to produce co-ordinates for the centroids of each event (green points). All frames of centroid locations are then combined to create a map of all identified points resulting in an image with resolution below the diffraction limit.
The dSTORM method however, foregoes the requirements of two fluorophores and instead uses only a single cyanine based fluorophore (D. Baddeley, Jayasinghe, Cremer, Cannell, & Soeller, 2009; Heilemann et al., 2008; Lemmer et al., 2009; van de Linde et al., 2011). dSTORM further simplifies the mechanism of illumination by relying on only a single activation laser which also acts as the inactivation laser. To achieve this, optical photoswitching was replaced by chemical photoswitching through the use of specific reducing mounting media for samples (Dempsey et al., 2009; van de Linde, Wolter, Heilemann, & Sauer, 2010). When under intense illumination, fluorophores typically undergo a process of irreversible photobleaching (Widengren & Rigler, 1996). This occurs through firstly the formation of an excited triplet state; the high energy electron within this state is capable of reaction with most reducing molecules, with particular affinity for oxygen within the mounting media. A sulfhydryl group on the reducing media allows a competitive reaction with the photo bleaching pathway by producing an alternative and reversible sulfhydryl intermediate (Dempsey et al., 2009). By controlling the intensity of illumination and composition of the reducing media, the rate in which photoswitching occurs can be controlled. Therefore, to obtain the maximal amount of events, the level of oxygen within the sample must be tightly controlled. Too much oxygen leads to an increased decay through the photobleaching pathway while too little means the step required for the reactivation is slowed leading to few activated fluorophores at any one time.

3D dSTORM

Standard localisation setups focus on improvements in the lateral plane of focus. However, this neglects the axial plane when imaging, producing sub diffraction limit resolution only in one plane. Improvement in localisation to include the axial resolution relies on the 3D visualisation of the imaging systems PSF to correlate the fluorescent events with a different z level. Unfortunately, the PSF is symmetrical about the xy plane meaning, the precise z identification of above or below the imaging plane is not readily possible (Huang et al., 2008; Shechtman, Sahl, Backer, & Moerner, 2014). To overcome this limitation, a number of methods have been developed.

One of the first method developed for 3D imaging was the insertion of a cylindrical astigmatic lens into the primary image output pathway. This acts to distort the psf so that it becomes lengthened in either the x or y axis depending on whether the plane is above or
below the fluorophore location with this information it now becomes possible to determine the precise fluorophore 3d location (D. Baddeley et al., 2011; Biteen, Goley, Shapiro, & Moerner, 2012; Huang et al., 2008). The downsides of an astigmatic lens however involves the sacrifice of photon count (due to the transmission of light through the additional optical element) and general resolution (due to additional blurring by the astigmatic lens) (Badieirostami, Lew, Thompson, & Moerner, 2010). In addition, as distortion is only single axis the PSF is highly distorted at extreme z positions and can lead to a large mismatch between x and y axis accuracy. Furthermore, the elliptical distortion imparted by the astigmatic lens also means less events can be detected per frame before significant overlap causes non single fluorophore events (D. Baddeley et al., 2011). However, these trade-offs under the correct circumstance will still be worth it for the gain in axial resolution allowing precise identification of structures.

Alternative to the use of astigmatism is the use of biplane focusing to allow 3d localisation. By focusing on a plane above and below the position of the PSF, the comparison of shape differences in the PSF then allows the localisation of the z position. The difficulty with the biplane method lies in the complexity in its setup. Co-ordination of calibrated two image planes at the precise distances is difficult. Further, the need to image two planes means that the output light path must be split, leading to halving of the emission intensity. However, an advantage over the astigmatic method is that there is no other distortion to the PSF meaning accuracy in localisation will not be one dimensionally affected.

**dSTORM structural resolution**

Whilst much work has been done into improving and analysing the precision of single molecule tracking, the overall resolution of structures imaged through STORM is not as well understood. Typical resolution of dSTORM images range from 5 nm to 30 nm (D. Baddeley, Jayasinghe, Cremer, et al., 2009; Huang et al., 2008) and are determined by the smallest structure resolvable within acquired images.

More quantitatively however, the final resolution of a dSTORM image is dependent on a number of factors. The resolution obtained with the isolation of a single molecule event is not the resolution of the entire system as it is the culmination of many individual points which produces the final image. This however, does not mean individual point resolution
has little impact on the final image resolution. The individual point resolution firstly provides a maximal limit on the resolution of the setup and can be seen as the maximal resolution of the setup provided event sampling on average is denser than 2 times the point resolution for the Nyquist criterion (Ober et al., 2004). In practice, this is a highly unlikely situation except for small local regions of images. In more general terms, the uncertainty of the point resolution will be additive on other factors determining resolution.

The overall density of events within a given region is possibly the main determinant of overall image resolution (Y. Hou et al., 2014b). For a given structure, greater density of labelling represents a denser sampling of the structure, by applying the Nyquist sampling criterion, where the maximal resolvable structure is the smallest distance where >2 sample points are present (Y. Hou et al., 2014b; Y. Hou, Jayasinghe, Crossman, Baddeley, & Soeller, 2015). The difficulty with quantifying this using images is that due to the stochastic nature and the at times non homogenous labelling of the samples leading to a non-even distribution of events. This effectively means, some areas of the image may have greater resolution than others, however, in general better single point localisation accuracy and high event densities are the key requirements to a high resolution image.

In addition to raw event densities, the actual labelling density further plays a part in overall resolution of the setup (D. Baddeley et al., 2011; Y. Hou et al., 2014b; Y. Hou et al., 2015). Imaging in dSTORM relies on the detection of attached fluorophores on the target proteins. With poor targeting, the wrong structure may be labelled or there is not enough binding affinity between the antibody and target resulting in a final labelling pattern not representative of the sample imaged. Finally, the structure of the primary secondary complex itself can create an alteration in resolution. Typically around 8-10nm in length (Valentine & Green, 1967), this creates a spherical region around the bound target where this antibody tail may be present. As the fluorophore is attached only to the back of the secondary antibody, this sphere of uncertainty will further limit the effective resolution.

**dSTORM in optically thick sections**

Whilst much work has been done into the super resolution imaging of cardiac myocyte biophysics in an isolated or cultured myocyte setting (D. Baddeley, Jayasinghe, Cremer, et al., 2009; D. Baddeley, Jayasinghe, Lam, et al., 2009; Huang et al., 2008; Lemmer et al., 2009;
Rust et al., 2006; Tahmasbi et al., 2015), less has been achieved using optically thick tissue sections. Tissue sections offer additional information in the form of cell to cell interactions and matrix structure, both of which are removed during isolation procedures. Further, potentially cell damaging steps such as enzymatic digestion can be avoided with tissue sections, preserving tissue in a more as is state when labelling and imaging.

One of the main reasons for the difficulty in imaging tissue sections is the presence of background auto fluorescence of the tissue. This is particularly prevalent in the extracellular space where collagen bundles can often be seen unlabelled (D. Baddeley et al., 2011). During imaging, general auto fluorescence increases the background of captured frames leading to greater difficulty in identifying the weaker fluorescent events (Chao, Ward, & Ober, 2012; Ober et al., 2004). It may also lead to inaccurate detection of background; increasing the amount of false positive events. Increased background is also directly correlated with decreased localisation precision further reducing the effective resolution of the image (Thompson et al., 2002).

**Some open questions in cardiac excitation contraction coupling**

Currently, many unanswered questions still remain in regards to cardiac function. As previously highlighted, the mechanism for termination of release at the dyad level has still not been experimentally visualised at high resolution. While many mechanisms have been shown to contribute to the channel closing, such as stochastic attrition and coupled gating (M. B. Cannell et al., 2013; Laver et al., 2013; Marx et al., 2001), the lack of certainty regarding the layout of the release unit makes pinpointing the relative contributions of the methods difficult. Further high resolution visualisation of the RyR clusters would provide evidence into the nature of the arrangement of dyads and determining the underlying mechanisms for control of RyR calcium release.

Furthermore, there is still little understanding regarding mechanisms of pathology and function at the cellular level. It is understood that proteins such as CaMKII, Junctophilin, and FPKB, are all proteins contributing to the manifestation of disease progression of ventricular arrhythmia and ventricular heart failure (Ai et al., 2005; Ibrahim et al., 2011; Nikolaev et al.,...
2010; Shan et al., 2010; C. Y. Wu et al., 2014) However, the precise mechanisms leading up
to various disease pathology is not yet well understood. The loss of calcium regulation is
believed to be a big contributor to cellular dysfunction as evidenced by numerous
measurements of altered calcium transients as well as the plethora of pathological
mutations seen in the RyR protein. Additionally, very complex underlying causes including
subtle nanoscale changes in structural distribution of the T-tubules and release sites (A.
Guo, Zhang, Wei, Chen, & Song, 2013; Ibrahim et al., 2011; van Oort et al., 2011; H.-B. Zhang
et al., 2013), are known to trigger changes in cell calcium regulation. While some of these
changes has been characterised in animal models, difficulties in culturing and maintaining
human myocytes mean many functional details are still unknown. Furthermore, because of
the small size of some structural feature changes, targeted fluorescent imaging of these
proteins using confocal microscopy does not provide sufficient resolution to elucidate and
quantify the changes (T. Hayashi et al., 2009). This is however not to say that calcium is the
only trigger in ventricular pathology. In heart failure it has been recorded that there are
significant alterations in other ion channels and proteins such as the potassium channels
which also contribute heavily to arrhythmogenic behaviour of the diseased cell (Lubbe,
Holland, Gilchrist, & Pybus, 1986; Willis, Ponce-Balbuena, & Jalife, 2015).

**Scope of the thesis**

In relation to the aims as outlined earlier, the initial focus will be on validation of using
dSTORM over conventional electron and confocal methodologies within tissue sections. This
is achieved through direct imaging comparisons between the different methodologies on
α actinin, a protein found in high density at the Z disc regions of myocyte myofibrils - a
region closely associated with cellular signalling and calcium activity. The comparisons will
further involve simulations based on observed individual event characteristics to see how
event density will affect overall image resolution. Further, a comparison between standard
localisation and 3d localisation will be undertaken to determine the axial resolution gain.
Finally, post processing techniques will be analysed to determine which methods allow best
representation of data observed.

Part two of this work will then use data obtained in part one to further examine the
distribution of internal calcium release clusters (labelled as the RyR protein) within rat
healthy cardiac tissue sections; focusing on the overall layout of RyR arrays and their proximity relationship with the SR – t-tubule linking JPH protein. Mean sizes and distances between clustered will be calculated and compared with previous data from other modalities. The understanding of distribution at close to the single channel level is then related to current understanding of calcium handling.

The final section will examine clusters in healthy and diseased human cardiac tissue, with emphasis on the use of dSTORM for the visualisation of possible nanoscale changes to RyR and JPH2 spatial distribution to the cell. This section will also contrast the differing subcellular structures between human and rat samples to relate identified species variation which could impact the overall understanding of CICR and how animal models can be related to human disease.
Chapter 1

Super-resolution fluorescence imaging to study cardiac biophysics: α-actinin distribution and Z disc topologies in optically thick cardiac tissue slices

Yufeng Hou\textsuperscript{a}, David J Crossman\textsuperscript{a}, Vijay Rajagopal\textsuperscript{b}, David Baddeley\textsuperscript{a,c}, Isuru Jayasinghe\textsuperscript{d} and Christian Soeller\textsuperscript{a,d}

\textsuperscript{a} Department of Physiology, University of Auckland, Auckland, New Zealand; \textsuperscript{b} Dept. of Electrical and Electronic Engineering, University of Melbourne, Australia; \textsuperscript{c} Department of Cell Biology, Yale University, New Haven, USA; \textsuperscript{d} Biomedical Physics, University of Exeter, UK
Abstract

A major motivation for the use of super-resolution imaging methods in the investigation of cardiac biophysics has been the insight from biophysical considerations and detailed mathematical modelling that the spatial structure and protein organisation at the scale of nanometers can have enormous implications for calcium signaling in cardiac muscle. We illustrate the use of dSTORM based super-resolution in optically thick (~10 μm) tissue slices of rat ventricular tissue to visualize proteins at the cardiac Z disc and compare those images with confocal (diffraction-limited) as well as electron microscopy (EM) data which still provides a benchmark in terms of resolution. α-actinin is an abundant protein target that effectively defines the Z disc in striated muscle and provides a reference structure for other proteins at the Z-line and the transverse tubules. Using super-resolution imaging α-actinin labelling provides very detailed outlines of the contractile machinery which we have used to study the properties of Z discs and the distribution of α-actinin itself. We determined the local diameters of the myofibrillar and non-myofibrillar space using α-actinin labelling. Comparison between confocal and super-resolution based myofibrillar masks suggested that super-resolution data was able to segment myofibrils accurately while confocal approaches were not always able to distinguish neighbouring myofibrillar bundles which resulted in overestimated diameters. The increased resolution of super-resolution methods provides qualitatively new information to improve our understanding of cardiac biophysics. Nevertheless, conventional diffraction-limited imaging still has an important role to play which we illustrate with correlative confocal and super-resolution data.

Keywords: EC coupling, microscopy, super-resolution imaging, heart, myofibril
Introduction

Progress in cardiac biophysics has often been closely linked with methodological advances, in particular with improvements in fluorescence imaging techniques. The introduction of fluorescent Ca$^{2+}$ indicators with suitable affinity and rapid kinetics (Grynkiewicz, Poenie, & Tsien, 1985) has contributed greatly to the study of calcium fluxes. To date such studies are arguably most advanced in muscle, and perhaps specifically in cardiac muscle. Similarly, the insight that the view of the cardiac myocyte cytosol as a well-stirred compartment in which Ca$^{2+}$ is essentially uniform is problematic had first been suggested on mostly theoretical grounds (M. D. Stern, 1992) but the crucial breakthrough was the discovery of microscopic Ca$^{2+}$ release events (Cheng et al., 1993). The technical advancement that enabled this discovery was the introduction of confocal microscopy to biomedical research as well as the availability of indicators from the Fluo-3 family (Minta, Kao, & Tsien, 1989) which are distinguished by a very large modulation of fluorescence with increasing Ca$^{2+}$ (albeit at the cost of not being ratiometric). The resulting understanding has now led to a fairly well-developed theory of local control (for a review, see e.g. (M. B. Cannell & Kong, 2012)) which emphasizes the role of gradients in Ca$^{2+}$ throughout the cell and the existence of local signalling domains. Such local control enables the cardiac muscle cell to work as a graded system based on calcium-induced calcium release (Fabiato, 1983) that is both robust and exhibits high amplification of a trans-sarcomeric trigger Ca$^{2+}$ influx to allow intricate control of the cardiac Ca$^{2+}$ transient, the primary modulator of cardiac contractility at the cellular level.

The local control view of cardiac EC coupling recognises the primary importance of spatial organisation and reveals the important role of the dyadic junctions between the surface membrane (and its extensions, the transverse (t-) tubules) and the membranes of the terminal sarcoplasmic reticulum (SR). At junctions the membranes oppose each other and the gap is only ~15 nm in width (Fawcett & McNutt, 1969) which is critical to confining the signalling between surface L-type Ca$^{2+}$ channels and ryanodine receptors (RyRs) which are the SR Ca$^{2+}$ release channels. In the junctional signalling space Ca$^{2+}$ concentration reaches peak levels that are several orders of magnitude higher than in the bulk cytosol. Junctions may also be small in lateral extent, electron tomography suggests complex 3D arrangements with few tens of nanometre extent along the shorter axes (T. Hayashi et al., 2009). In
addition, the T-tubules themselves are sub-resolution in diameter (~50-250 nm in the rat (C. Soeller & Cannell, 1999)) and the clusters of RyRs that are thought to underlie the microscopic Ca\textsuperscript{2+} sparks may be as small as ~30 nm along their shortest axis (D. Baddeley, Jayasinghe, Cremer, et al., 2009; T. Hayashi et al., 2009) (but large clusters can reach micrometer sizes). These are just a few examples illustrating that there are many features of the cardiac Ca\textsuperscript{2+} signalling apparatus that are “nano-objects” and generally not resolved by diffraction-limited imaging approaches, such as confocal microscopy (with a “best” lateral resolution of ~200-250nm). Accordingly there has been considerable interest in utilizing higher resolution methods, ideally compatible with fluorescence stains and indicators due to their high contrast and specificity.

This gap in our imaging capabilities has recently been filled with the advent of so called optical super-resolution microscopy, of which there are a number of different ‘flavours’. STED (or stimulated emission depletion) microscopy, uses a saturable fluorescence depletion effect to selectively darken all molecules but those within the centre of a laser spot that is scanned through the sample similar to confocal microscopy (Hell & Wichmann, 1994). Single molecule localisation microscopy, by contrast, is a widefield technique which relies on darkening all but a few fluorophores at any given time (Betzig et al., 2006; Hess et al., 2006; Rust et al., 2006). This allows the few fluorophores that are “bright” to be observed as isolated, diffraction limited spots whose centre can be localised with an accuracy that is only limited by the number of photons that we can collect (and the brightness of the background). By detecting and localising subsets of fluorochromes through typically thousands of frames a composite image can be constructed and rendered into a format familiar to conventional fluorescence micrographs using algorithms to estimate local marker density (D. Baddeley, Cannell, & Soeller, 2010). These methods are known under a variety of acronyms such as PALM, fPALM, STORM etc and achieve lateral resolutions of ~20-30 nm. In one variation, known as dSTORM (direct STORM (Heilemann et al., 2008)), conventional fluorochromes become photoswitchable under suitable conditions and this approach is particularly practical as normal imaging and labeling protocols can be followed to a large extent. We have previously extended dSTORM for multi-colour 3D super-resolution imaging, a method that we termed d\textsuperscript{4}STORM (D. Baddeley et al., 2011).
Several studies employing super-resolution imaging for the study of cardiac myocytes have clearly demonstrated its utility for studies of cardiac biophysics. The near-molecular resolution of STORM based super-resolution methods and the comparatively large size of RyRs (ca 30 nm (Meng et al., 2007)) allowed a first quantitative estimate of the number of RyRs per cluster (see Fig. 1A) which revealed an unexpectedly broad size distribution that is compatible with a stochastic self-assembly process (D. Baddeley, Jayasinghe, Cremer, et al., 2009). Similarly, the resolution provided by STED microscopy has revealed a direct view of the complex membrane topologies in mouse T-tubules and highlighted changes in tubules associated with the development of heart failure in a mouse model (Wagner et al., 2012). The ability to reveal T-tubules in fluorescence microscopy is shown here with prominent tubules in a horse ventricular myocyte stained with the membrane marker wheat germ agglutinin (WGA), a useful marker of cardiac T-tubules (Mark B. Cannell, McMorland, & Soeller, 2006). Further uses of super-resolution microscopy for the study of cardiac biophysics have recently been reviewed (C. Soeller & Baddeley, 2013).

![Figure 1](image1.png)

**Figure 1.** Super-resolution imaging of RyR clusters and cardiac T-tubules. (A) A collage of randomly selected dSTORM images of RyR clusters from peripheral couplons of rat ventricular myocytes illustrates the diverse morphologies and cluster sizes. From (Baddeley et al., 2009), reproduced with permission. (B) A transverse dSTORM image of a horse ventricular myocyte stained with wheat germ agglutinin-Alexa 680 reporting tubule connectivity and variations of the local tubule diameter. Scale bars: A: 200 nm, B: 1 μm.

An important structural component of cardiac myocytes (and other striated muscle cells) are the boundaries of sarcomeres called Z-lines (as they appear as lines in 2D micrographs) or, taking the full 3D structure of myofibrills into account, Z discs, as in 3D the sarcomere boundaries are thin disks coinciding with the cross-section of myofibrillar bundles. A
number of protein involved in excitation-contraction (EC) coupling are located around the edges of Z discs such as RyRs and other junctional proteins (I. D. Jayasinghe et al., 2009; C. Soeller et al., 2007) as well as the T-tubules that are largely found in close proximity to the edges of myofibrillar Z discs. One protein that is greatly concentrated within Z discs and serves as a distinctive Z disc marker is α-actinin. α-actinin anchors thin filaments at the Z-line and interactions with the sarcomeric protein titin and actin in thin filaments are critical for the development and maintenance of normal sarcomeric structure (Sjoblom et al., 2008).

We have previously demonstrated the use of super-resolution microscopy to achieve improved contrast and resolution in optically thick tissue sections (David Baddeley, Cannell, & Soeller, 2011). In the present study we provide a more extended study of tissue-section based dSTORM imaging to resolve the details of myofibrillar Z discs and the distribution of the protein α-actinin. Detailed comparison with both diffraction-limited widefield and confocal data as well as electron micrographs reveals the resolution and associated contrast improvement but also emphasizes remaining limitations. Our results support the use of super-resolution microscopy for nanoscale investigations of structures and protein distributions that underlie the biophysics of cardiac muscle, in particular Ca\textsuperscript{2+} handling and production of force.
Materials and Methods

Tissue Preparation.

All use of rats and rat tissue was approved by the Auckland University ethics committee prior to experimentation. Healthy adult Wistar rats in the weight range of 250-300g were sacrificed by lethal injection of sodium pentobarbitorne followed by cervical dislocation. The heart was removed and mounted on a Langendorff perfusion apparatus and perfused with 2% paraformaldehyde in phosphate buffered saline (PBS). Upon removal from the perfusion setup, the atria were first removed and the ventricular portion was cut into three chunks (which included the septal region, and the 2 upper ventricular regions, respectively.) The tissue chunks were cryo protected (in solutions containing increasing sucrose concentration, up to 30% sucrose) and frozen in isopentane cooled by liquid nitrogen. Cryosections were cut from the hearts at a measured thickness of 5 to 10 µm and allowed to thaw onto poly-l-lysine precoated number 1.5 glass coverslips. Horse ventricular tissue was freshly obtained from an abattoir near Taunton, UK, and 3-5 mm thick tissue blocks were fixed by immersion in 1% paraformaldehyde for an hour and further processed as described for rat tissue above.

Immuno Labelling

All immunolabelling procedures were done at room temperature (20 ⁰C) unless otherwise stated. Sections were first hydrated with PBS for 5 minutes before permeabilisation with 100µL of PBS containing 1% triton X-100 for 15 minutes, followed by washing with 150 µL PBS for 15 minutes. Antigen blocking was carried out using Image-iT FX Signal Enhancer (Life Technologies) which was applied for 1 hour.

To label sections for α-actinin mouse monoclonal anti-α-actinin (IgG1) primary antibody (Sigma-Aldrich) was applied at 1:200 dilution from stock. For dual colour imaging an additional mouse monoclonal anti-SERCA2a (IgG2a; Abcam) was simultaneously applied at a 1:200 dilution. Primary antibodies were incubated overnight at 4 ⁰C. After incubation the sections were washed 3 times with 150 µL PBS.

Secondary antibodies by Life Technologies Ltd. were applied at 1:200 dilution from stock and incubated for 2 h at room temperature. For dual α-actinin and SERCA2a labelling, secondary antibodies were applied in sequence with the IgG2a applied first and anti-IgG
(H+L) second, after washing, to reduce cross talk between same host species but different isotype primary antibodies.

For correlative imaging using widefield illumination, Alexa 488 phalloidin was used to label myofibrillar regions. To record correlative confocal and super-resolution images of α-actinin in sections both Alexa 488 and Alexa 680 conjugated secondary antibodies were used. Following application of anti-α-actinin primary antibody at 1:200 dilution (as in previous experiments) sections were incubated with a mixture of anti-mouse secondaries conjugated to Alexa 680 (1:100) and Alexa 488 (1:400).

**Super-Resolution Imaging (dSTORM)**

Samples were mounted using mounting media prepared for dSTORM imaging consisting of 90% glycerol, saturated glucose, 5 mM Cysteamine (Sigma-Aldrich), and an oxygen scavenging system of catalase (at 0.005mg/mL; Sigma-Aldrich) and glucose oxidase (0.05mg/mL; Sigma-Aldrich).

Imaging was carried out on a modified Nikon TI Eclipse inverted fluorescent TIRF microscope capable of dual colour super resolution imaging with 670 nm laser excitation, and full electronic stage and focus control. The system is controlled by the PYME acquisition program developed by Dr David Baddeley (code.google.com/p/python-microscopy/).

Images were acquired after an initial ~6 s pre-exposure (to allow the transition of the majority of fluorophores into a meta-stable dark state) followed by a 15-30 minute acquisition time at 50ms/frame, i.e. 15000 – 40000 frames. 3D super resolution images were acquired with a cylindrical lens inserted in the detection light path to enable localisation along the z axis. Super-resolution images were rendered from the localised event data using the greyscale rendering algorithm described previously (D. Baddeley et al., 2010).

**Widefield Epi-Fluorescence Imaging**

Wide field image stacks were obtained on the same set up as in dSTORM imaging. Upon selection of the region of interest, the widefield images were captured prior to imaging via dSTORM. The samples were illuminated with high angle 491 nm laser excitation. This results in a higher contrast image allowing for better subsequent deconvolution. Z stacks of the entire tissue section were acquired with exposure time of 500ms and step size of 200 nm.
**Confocal Imaging**

Confocal images were obtained on a Zeiss LSM 710 inverted confocal microscope using a 63x 1.4 NA oil immersion objective. Imaging and scan parameters were adjusted to ~89 nm/pixel. Pinhole size was set at one Airy unit. Point-spread functions (PSFs) were recorded using 200 nm Tetraspeck beads (Life technologies). PSF stacks were acquired as z series of 63 optical slices with a step size of 50 nm. Responses from 4 individual beads were averaged to produce the final PSFs for deconvolution.

Confocal stacks were recorded onto series of 800x800 pixel TIF images using z-steps of 300 nm through the entire thickness of the tissue section.

**Correlative imaging of diffraction-limited and super-resolution data**

In order to locate cells on the confocal microscope following the initial super-resolution imaging of Alexa 680 labelling, outlines of the section where optically traced and the locations of cells that were imaged noted with the aid of position coordinates of the motorised stage. Epi-fluorescence data for correlative widefield imaging of Alexa 488 signals and super-resolution imaging of Alexa 680 labels were obtained on the same setup so that no further image alignment was required.

Correlative volume images were first aligned laterally. All imaging manipulations were carried out in the image manipulation environment ImageJ. As higher resolution detail of the super resolution setup needed to be preserved, confocal images were scaled up to match the pixel scaling of the super-resolution images. Initial scaling was done via direct image pixel size comparison. From this a scaling factor was determined and applied to the confocal and wide field images using bicubic interpolation. Due to chromatic shift and minor variation between microscope calibrations, a secondary fine alignment was necessary. This process included minor rotation, re-scaling and x-y translations.

For 3D comparisons, after lateral (2D) alignment of images, the stacks were re-sliced along the axial direction and the confocal stacks were expanded to match axial pixel sizes of the super-resolution data. A final translation was applied to the confocal volumes in the axial dimension to achieve correct 3D alignment.
Deconvolution

All deconvolution was carried out using our custom image processing software that is part of the python-microscopy environment (code.google.com/p/python-microscopy/). Wide field images were deconvolved using a modified ICTM method (Van Kempen, Van Vliet, Verveer, & Van Der Voort, 1997) using 20 iterations and a regularization parameter of 0.01. For confocal images, a Richardson Lucy algorithm (Richardson, 1972) was applied for 30 iterations using the measured PSF.

Simulation of dSTORM stochastic effects

Simulation data was constructed and rendered based on a 2D binary mask of the myofibrils that was generated by segmenting thin section EM data. Fluorophores staining the Z disc of the entire myofibrillar area uniformly were simulated at a density of 0.05 fluorophores per mask pixel (2x2 nm area). Based on prior experimental measurements, the mean photon counts were set to 300 per background pixel and 1300 per localised event, each repeating an average of 1.5 times. These parameters appeared to reliably recreate the time course and a localisation accuracy of single molecule events observed in experimental data series acquired from tissue sections stained for α-actinin. The coordinates of the events detected from simulated data were rendered in the same way as the actual super-resolution data using the algorithm described previously (D. Baddeley et al., 2010).

3D rendering

Greyscale super-resolutions were visualised in 3D using surface rendering implemented using the python Mayavi 3D scientific data visualization environment that has been integrated into the python-microscopy environment. The correlative data was isosurface rendered where confocal stacks were thresholded using isodata and the super-resolution isosurface was rendered at an intensity threshold corresponding to the 60\textsuperscript{th} percentile determined such that 60% of the pixel intensities. total signal was included within the mask.

EM imaging

EM tomograms of ventricular cardiomyocytes from mice were prepared from 3-6 months old 129/Sv mice as described previously (T. Hayashi et al., 2009), and EM tomograms of rat ventricular cardiomyocytes were prepared using an equivalent approach. Briefly, sections cut at 500 nm thickness were imaged at 15,000× magnification and angular increments of 2°
from −60° to +60° using a JEOL 4000EX intermediate voltage electron microscope operated at 400kV. Tomograms were reconstructed using a transform-based back projection algorithm, TxBR (Lawrence, Bouwer, Perkins, & Ellisman, 2006).

**Image analysis for local myofibrillar / inter-myofibrillar space diameter**

A binary mask that encompassed 85% of the local event densities within the myofibrillar areas was constructed from the 2D greyscale dSTORM images that were first projected across 300 nm in the axial direction and a median filter was applied (45 x 45 nm kernel). The mask was subject to an open function to target segregation of weakly separated regions of α-actinin staining. The inverse of this myofibrillar mask was used to analyse the inter-myofibrillar spaces. An automated algorithm that determined the local thickness of a given myofibrillar or intermyofibrillar region was applied using ImageJ 1.48k and the ‘Local Thickness’ plugin. The output of this analysis included a mask of the myofibrillar/inter-myofibrillar regions colour-coded for the local width and a histogram of the proportions of the regions as a function of the local width.
Results

α-actinin is an actin-binding protein that is present in high density at the boundaries between sarcomeres, the Z discs. It therefore is a prominent marker of myofibrillar bundle structure when viewed in cross-section. This can be seen in Fig. 2 which shows transverse (cross-sectional) views of rat cardiac ventricular myocytes in three different imaging modalities. In a thin section electron micrograph myofibrils (and mitochondria) can be readily identified in cross-section (Fig. 2A). The effective resolution is on the order of a few nanometres and boundaries between adjacent myofibrils are clearly distinguishable. Using fluorescence microscopy myofibrils can be effectively visualised using α-actinin which clearly outlines myofibrilar Z discs, i.e. the ends of sarcomeres that accurately define the cross-section of myofibrillar bundles at Z-lines, i.e. every ~1.8 µm in a quiescent myocyte. The resulting Z-line pattern as obtained with a confocal microscope in a transverse section through a rat myocyte (Fig. 2C) shows a similar pattern but the resolution by comparison to the EM dataset is clearly reduced. We conducted dSTORM based imaging in similar rat heart sections (using the fluorochrome Alexa 680 rather than Alexa 488 for its good photoswitching properties) and we found that there is sufficient optical contrast when using

![Image](image-url)
~10 µm thick sections to effectively detect fluorescence “blinks” from single marker molecules and generate high quality super-resolution images (Fig. 2B).

To investigate the achievable detail in conventional diffraction-limited imaging with that in super-resolution images we conducted direct comparisons between images of the same cell and correlated the imaging modalities. In a direct comparison of a deconvolved wide-field

Figure 3: Correlative dSTORM and diffraction-limited microscopy of cardiac myofibrils. (A) A projection of a 800 nm deep dSTORM volume within a rat ventricular tissue section stained for α-actinin/Alexa 680 (B) Deconvolved widefield fluorescence of Alexa 680 of the same volume (C) Overlay of these images emphasise the sharper myofibrillar edges, finer texture of labelling and the finer axial resolution (yellow colouration of in focus regions) achieved
with dSTORM. (D) 800 nm deep projection of dSTORM volume of a similar sample. (E) Projection of the corresponding deconvolved confocal volume. (F) The overlay of the correlative images illustrate the similarities in the axial resolution (uniform density of yellow colouration between myofibrils) and the accentuated edge intensity in the deconvolved confocal image. (G&H) The Gaussian spikes of the edge intensity introduced by deconvolution are further illustrated by plotted intensity profiles of the fiduciary lines indicated in panels D&E. Note the Gaussian shape of the intensity profile of smaller myofibrils in the deconvolved images that in the dSTORM data show steeper edge transitions indicative of the higher resolution.

microscope stack and the corresponding super-resolution image (Fig. 3A,B) the myofibrillar outlines are not well-defined in the widefield image even after deconvolution. By contrast, the myofibrillar Z discs are much clearer in the super-resolution image which is especially apparent in an overlay of images from the same cell (Fig. 3C). The appearance of the Z discs is much improved in an image from a deconvolved confocal data stack (Fig 3D-F). We investigated the differences between the dSTORM data and the confocal slice in more detail to reveal systematic differences resulting from the different modalities. Intensity profiles were extracted from corresponding image regions. These comparisons highlight differences particularly at the edges of myofibrillar Z discs. The deconvolved data exhibit systematic overshoots at these edges that are not present in the super-resolution data. Similar edge effects were observed when using a different deconvolution algorithm (ICTM) and the confocal point spread function was re-measured to exclude a simple measurement error as the origin of the edge effect. In addition, a hole or gap in the myofibrillar arrangement is enlarged in the deconvolved confocal data set. The super-resolution data provides an ideal test to evaluate the limits of information that can be deduced from diffraction-limited data and the comparison suggests that the edge effects in the deconvolved data are at least partially an artefact of the deconvolution procedure associated with the relatively steep transition from the edge of the α-actinin distribution to the surrounding non-myofibrillar space.

These results confirm, as expected, that super-resolution imaging provides a significant improvement over diffraction-limited approaches. The limitations in resolution become clearer when detailed views of super-resolution data are compared with similar thin-section electron micrographs. Boundaries between myofibrils are distinctive in an EM image (Fig.
4A) which is both due to the higher resolution that is still accessible in EM versus super-resolution data but also due to dark electron-dense lines between adjacent myofibrillar

![Figure 4: Detection of nanometer-scale inter-myofibrillar spaces. (A) Boundaries of closely-arranged myofibrils are readily detected in thin-section electron micrographs (arrows). (B) A 800 nm-deep projection of a dSTORM volume of a rat ventricular section stained for α-actinin also reports similar inter-myofibrillar spaces (arrows) unresolvable with standard optical microscopies. (C) Dual colour dSTORM data of α-actinin (red) and SERCA2A (green) reports SR tubules occupying these narrow spaces between myofibrils (arrow). Scale bars 1µm.]

bundles which represent components of the SR surrounding the myofibrils. In super-resolution data small gaps can be identified where the α-actinin density is slightly lower than in the surrounding areas although the contrast is not as high as in the EM data. This may be partially due to the 3D extent of the data in the tissue sections as compared to the thin EM data that avoids blurring resulting from axial heterogeneities (i.e. in the direction along the axis of the cell) of α-actinin distribution at the edge of myofibrillar bundles. An advantage of fluorescent methods is the molecular specificity of fluorescent markers. By combining α-actinin imaging with imaging of the SR protein SERCA an effect similar to that in the EM micrograph can be achieved where the boundaries of Z discs (i.e. patches of α-actinin staining) are clearly identified using the bands of SERCA staining surrounding myofibrils (Fig. 4C). The dual-label image also emphasizes the advantage of single-molecule detection based super-resolution to reduce the spectral-crosstalk between markers to a very small number (typically < 1%, (D. Baddeley et al., 2011)) although some residual crosstalk may result from the staining procedure as both primary antibodies are from the same host species and specificity was achieved with isotype-specific secondary antibodies. Nevertheless, the improved contrast in the double-label super-resolution data is clear and
can make up some of the resolution-deficit as compared to EM micrographs, particularly in cases where the relationship between electron-densities and their molecular make-up may not be very clear.

**Figure 5:** dSTORM image texture of the α-actinin labelled regions. (A) A dSTORM volume of a rat ventricular tissue section stained for α-actinin illustrates a ‘grainy’ texture reflecting nanometre-scale non-uniformities of the event densities within myofibrillar regions. (B) A similar texture was observed in a synthetic dSTORM image obtained by simulating a uniform antibody labelling density, stochasticity of fluorophore photoblinking and the localisation accuracy achievable in a 5 µm thick tissue section. (C) Overlaid line plots compare the intensity profiles of these local in homogeneities in the regions indicated in panels A (blue, experimental image) and B (green, simulated). Scale bars 1µm.

While the previous data shows that dSTORM super-resolution allows greatly enhanced identification of Z-line extent and topology as compared to diffraction-limited imaging, it also has some features that may complicate data interpretation. One feature that is quite apparent in super-resolution imaging of extended structures, such as the Z disc associated distribution of α-actinin, are the granular appearance of the Z discs. We believe this reflects both the stochastic binding of markers to the target as well as some of the stochastic properties of the photo-blinking that underlies STORM type (and also PALM type) super-resolution principles. To test the idea that the majority of the variation in the α-actinin signal can be explained in that way we compared actual super-resolution recordings of α-actinin (Fig. 5A) with a simulated data set (Fig. 5B). The simulated data set was generated by assuming that there was a fixed average density of α-actinin markers across a mask of myofibrillar cross-sections which was manually segmented from an electron micrograph (as shown in Fig. 4A). From these markers simulated localisation events were generated assuming an average brightness of 1300 photons and each marker showing up in events on average 1.5 times against a background of 300 photons. These values are broadly
representative of the photon statistics we observed in actual data series. On the basis of these photon counts localisation errors were simulated and the resulting set of detected and localised marker events was processed analogous to actual experimental data sets, i.e. local event densities were estimated as described in Methods. The resulting simulated super-resolution image is shown in Fig. 5B and qualitatively shares many of the features of the actual super-resolution images of α-actinin across Z discs. The good quantitative agreement between simulation and actual imaging data is shown in an overlay of intensity profiles from the two data sets (Fig. 5C). This analysis suggests that the variation in signal intensity across individual Z discs is largely stochastic in nature and the intensity pattern that we observe is compatible with a largely uniform lateral distribution of α-actinin across Z discs.

Figure 6: Resolving the longitudinal thickness of α-actinin distribution at the Z discs. (A) α-actinin of vertically oriented myocytes within rat ventricular sections appear as ~700-1000 nm thick bands in axially re-sectioned standard 2D-localisation dSTORM volumes that offer confocal-like axial resolution. (B) The staining appears in notably better-resolved bands (width of ~100 nm) in identical regions imaged through the 3D super-resolution dSTORM (C) A similar longitudinal width of the α-actinin bands is observed at Z discs oriented strictly orthogonally to the image plane. (D) Z discs in longitudinally sectioned tomographic high voltage EM images appeared as electron-dense bands similar in morphology and thickness.
(E) Overlaid line profiles compare the staining density across the Z discs along the long axis of the myocytes based on axially re-sectioned standard dSTORM volumes (green), re-sectioned 3D super-resolution dSTORM volumes (blue), in-plane standard 2D dSTORM images (red) and tomographic EM (yellow). (F) 3D rendering of confocal (green) and dSTORM (red) volume data. Scale bars A-C: 1µm, D: 500 nm.

Beside the lateral distribution of α-actinin across Z discs the distribution in the axial direction is also of considerable interest. The ability to resolve the distribution profile of α-actinin in the axial direction requires super-resolution along the axis of the cell. When using lateral super-resolution to image tissue sections that are taken across the cross-section of myocytes the resolution in the axial cell direction is essentially diffraction-limited (i.e. similar to that of a confocal microscope). The optical sectioning ability of 2D localisation microscopy arises from the detectability of single molecule fluorescence which quickly drops with distance from the focal plane. This is illustrated by reslicing a dataset that was recorded in this way which shows the poor resolution orthogonal to the Z disc plane (Fig. 6A). This can be overcome by using 3D super-resolution, for example, using astigmatism-based localisation. Using this modality the axial thickness of the α-actinin distribution at the Z-line appears greatly reduced (Fig. 6B), similar to the apparent diameter when imaging a section of cardiac tissue that was cut parallel to the axis of myocytes (Fig. 6C) so that the high lateral super-resolution is used to observe axial α-actinin distribution. We compared this to the apparent thickness of the Z-line in an electron micrograph of a mouse myocyte in which the electron dense bands at the sarcomere boundaries, i.e. the Z-lines were ~100 nm in diameter Fig. 6D. The resulting axial profiles from the various ways of imaging α-actinin (Fig. 6E) compare the apparent axial distribution showing that the FWHM of axial profiles when using 3D super-resolution is ~100 nm, similar to the thickness observed in the EM micrograph suggesting that 3D super-resolution is capable of resolving the axial distribution of α-actinin.

The shape and local diameter of myofibrils determines the diffusion distances for calcium following release around the edges of Z discs (where RyRs and junctions are located). This in turns relates to the lag with which calcium arrives at the centre of myofibrils versus their peripheral regions thus affecting the heterogeneity of Ca²⁺ and contractile activation. We therefore measured the local diameter of both the myofibrillar and inter-myofibrillar space from the lateral super-resolution data of α-actinin distribution (Fig. 7A, B). The diameter was
measured by a ‘local diameter’ algorithm that determines the diameter of the largest circle that locally fits fully within the structure of interest as shown for both myofibrillar and inter-myofibrillar space in Fig. 7C,D. We used this approach to quantify diameters based on 6 super-resolution data sets from 6 cells and constructed histograms as shown in Fig. 7E and 7F, taking care to avoid any potentially nuclear or peri-nuclear regions (which might have biased inter-myofibrillar diameters). The histogram of myofibrillar diameters had a mode at 0.5 μm, was approximately symmetric and bell-shaped. Note that no regions with diameters greater than 1 μm were present. The mean diameter was 0.55 ± 0.23 μm (SD). By contrast, the mean diameter of the inter-myofibrillar space was larger, 0.66 ± 0.44 μm (SD). A considerable fraction of the inter-myofibrillar space had diameters > 1 μm and the distribution was broader, compatible with the idea that the requirement for short diffusion distances is not as severe as within myofibrils themselves (more so as most of the space is taken up by mitochondria).
Figure 7: Measurement of myofibrillar and intermyofibrillar spaces using dSTORM images. (A) Transverse view of a rat ventricular myocyte labelled for α-actinin illustrates the varying cross-sectional shapes and sizes of the myofibrils. (B) The magnified view of the region indicated (box) in panel A illustrates the anisotropic intermyofibrillar spaces, some well below the diffraction-limit. Colour-coded binary masks of the same region illustrate the local width of the (C) myofibrillar spaces and (D) intermyofibrillar spaces in nanometres. These analyses are graphically represented in histograms of the percentage of the (E) myofibrillar and (F) intermyofibrillar cross-sectional area shown as a function of the local width. Scale bars A: 2 µm and B: 1 µm.
Discussion

Use of super-resolution for cardiac biophysics studies

Super-resolution imaging of cardiac proteins and membrane structures has allowed us and others to probe nanoscale features of cardiac myocytes that are thought to be critical for cardiac EC coupling and biophysics. Important examples include RyR clusters (D. Baddeley, Jayasinghe, Lam, et al., 2009) and cardiac T-tubules (Wagner et al., 2012). In this study we have focused on the use of dSTORM based tissue imaging of α-actinin. dSTORM, as one of the single-molecule localisation based super-resolution approaches relies on the detection of fluorescence bursts from single (or small groups of) molecules. The achievement of sufficient contrast to reliably detect these flashes or “blinks” in optically thick tissue sections is a challenge but can be overcome in a number of ways. Here we used a highly inclined illumination geometry (HILO,(Tokunaga, Imamoto, & Sakata-Sogawa, 2008)) that results in sufficient contrast as we previously demonstrated (D. Baddeley et al., 2011). In addition, the whole depth of the section may be pre-bleached to increase focal plane contrast(Dani, Huang, Bergan, Dulac, & Zhuang, 2010). Simultaneous excitation of several fluorochrome species and ratiometric detection enabled multi-colour detection with very little cross-talk (D. Baddeley et al., 2011) as demonstrated here by imaging α-actinin and SERCA at Z discs (see Fig. 4C).

A feature of single-molecule based super-resolution imaging that is apparent in our data (see Fig. 5) is the stochastic granularity in the recorded event-density that may be unfamiliar to users of diffraction-limited imaging approaches (although the photon-noise associated with faint signals is a stochastic signal modulation that may be encountered). This feature is more noticeable since α-actinin is extended in the lateral direction across Z discs and our simulations confirm that the expected stochastic variations are relatively large when assuming a uniform average concentration of α-actinin across the Z disc. This is in part due to the fact that at the resolutions achieved in dSTORM (and similar techniques) the number of marker molecules per “resol” (resolution volume) is comparatively low. This by itself leads to stochastic variation (similar to the stochastic variation in photon counts) and is a hallmark of working in a regime where single or groups of molecules are accessible. A similar effect is observed in immuno-gold studies in EM where, were one to calculate the
density of gold particles per unit area/volume, large fluctuations would be observed. In this sense some fluctuations are intrinsic to highly resolving methods that approach the molecular scale and equally apply to other super-resolution methods, e.g. STED. In addition, the statistics of photo-switching in dSTORM and PALM add further stochastic variation. The reason that the experimenter needs to be aware of these variations reside in the interpretation of the recorded distribution patterns: it is not a priori clear how a uniform distribution of target molecules can be distinguished from a variable distribution of target molecules and we suggest that new statistical methods are required to establish robust evaluation procedures. Note that this variation is quite apparent in the case of an extended target distribution, as seen here with α-actinin, but similar considerations apply when observing apparent target densities of structures that are sub-resolution size in all dimensions. In general, the density of markers is an important determinant of both the resolution that can be achieved (D. Baddeley et al., 2010; Shroff, Galbraith, Galbraith, & Betzig, 2008) and in conjunction with other stochastic effects may limit if a specific protein distribution pattern is actually detectable.

**Correlative imaging of α-actinin with super-resolution and diffraction-limited confocal microscopies**

We made use of correlative confocal and super-resolution imaging to directly compare the resolution of features between these modalities. In addition, by comparing deconvolved confocal data to its super-resolution counterpart in the same cell we showed that some of the features introduced by the deconvolution procedures must be interpreted with care. The use of deconvolution in diffraction-limited microscopy is well-established (Mark B. Cannell et al., 2006; Van Kempen et al., 1997) but may be prone to subtle artefacts. We suspect that the transition edges of Z discs where α-actinin density declines sharply is the main reason that the non-linear deconvolution algorithms are likely to generate excessive signals at these boundaries. Super-resolution imaging provides an ideal tool to study these manipulations of diffraction-limited data critically.

The ability to combine diffraction-limited and super-resolution imaging modalities offers a number of advantages beyond such tests. The high-resolution data in a 3D region can be complemented by the much larger extent of the volume typically accessible with a confocal approach. This can be very useful both due to the multi-scale nature of cardiac biophysics
problems and to reduce the high sampling requirements that are necessary in a scanning super-resolution technique such as STED. Beside the correlative imaging between fluorescence techniques we also used qualitative comparisons with EM data to illustrate the fidelity achievable with super-resolution imaging. The further development of correlative EM and fluorescence super-resolution imaging offers the promise to combine the very high resolution of EM with the high contrast and specificity of fluorescent imaging and therefore should be high on a wish list of cardiac biophysicists.

Resolving sarcomeric structure and non-myofibrillar components

α-actinin plays a vital role in helping arrange and maintain sarcomeric structure by anchoring actin that is in thin filaments at the Z disc. It also interacts with titin along its z-repeats and it is thought that the interactions between titin, α-actinin and globular actin (in thin filaments) are the key molecular units that ensure the integrity of thin filament bundles and their anchoring at the Z disc (Sjoblom et al., 2008). There are a great number of additional proteins that have been implicated in the assembly process itself as well as sarcomeric maintenance, for example, N-RAP (Manisastry, Zaal, & Horowits, 2009) and other cross-linking or capping proteins, e.g. MLP (Knöll et al., 2002). In the super-resolution imaging experiments presented here one feature of α-actinin is its suitability as a marker of Z discs and myofibrillar cross-sectional area. The comparison of diffraction-limited and super-resolution modalities clearly shows that the higher spatial resolution of dSTORM enables greatly improved ability to find edges and distinguish nearby bundles. EM methods still have a resolution edge (i.e. <1 nm resolution is very achievable) over super-resolution imaging approaches (20-40 nm resolution) for this purpose but the increased sample preparation requirements should be weighed against that. In addition, the fluorescence methods underlying the super-resolution approach achieve molecular specificity whereas in EM micrographs the molecular origin of electron-densities is typically much less clear. The ability to properly segment myofibrils is critical in determining the local diameter of the myofibrillar space as we tested by comparing diameters based on confocal data with those determined from the same cell region but images using super-resolution of α-actinin. The lower resolution in confocal data (even following deconvolution) led to erroneous fusion of adjacent myofibrillar bundles which distorted the diameter histogram towards larger diameters (see. Supplementary Fig. S1) and caused a ~30% increase in the mean diameter.
for the cell that was analysed. In connection with this point, we had previously observed that SERCA labelling appeared to bisect myofibrillar regions where there was no detectable drop in the myofibrillar stain phalloidin (I. Jayasinghe, Crossman, Soeller, & Cannell, 2012). In our super-resolution data bands of SERCA coincide with decreases in α-actinin labelling density (Fig. 4C) and we can now resolve our earlier observation as an artefact of the diffraction-limited imaging modality. Another interesting feature of the sarcomeric structure of cardiac myocytes are the bifurcations of myofibrils that give rise to misregistrations between Z discs and a helicoid topology of Z discs across the whole myocyte (or large regions) (I. D. Jayasinghe, Crossman, Soeller, & Cannell, 2010; Peachey & Eisenberg, 1978). Based on confocal data we hypothesized that myofibrils may split to, for example, to accommodate large structures (such as a nucleus) and run at different angles or twist. It was difficult to detect direct evidence for such splitting in the confocal data sets, presumably due to diffraction-limited resolution. It should be possible to investigate this with super-resolution imaging of α-actinin. In our data sets to date, we could not readily detect any evidence or splitting, although this can be explained by the limited longitudinal extent of the super-resolution data sets which reduced the likelihood of locating such regions. A strategy to increase the probability of finding candidate areas would use the correlative method introduced above where extensive confocal 3D data sets would be used to identify suitable regions that would then be imaged using dSTORM based super-resolution.

The effective local diameter of myofibrils is important as it is one factor that affects the diffusion distance of Ca^{2+} from local release sites to the core of myofibrillar bundles. A full analysis takes into account the placement of release sites (i.e. RyR clusters) at the periphery of myofibrils (e.g. (C. Soeller et al., 2007)) but the diameters place upper bounds on these diffusion distances. Notably, myofibrillar diameters were $\leq 1\mu m$ implying that diffusion distances are not larger than 500 nm given sufficiently high density of release sites. The non-myofibrillar space is largely occupied by mitochondria (Fig. 2A) which can be effectively detected purely morphologically in EM micrographs. It is more difficult to identify other organelles that occupy a much smaller volume fraction and for this purpose the high resolution and specificity of super-resolution approaches may be very useful.
Alpha-actinin, distribution and relationship to sarcomere assembly

There is agreement that the detailed arrangement of cross-linking regions at the Z-line/Z discs is complex and a number of models have been suggested (Hampton, Taylor, & Taylor, 2007; Taylor, Taylor, & Schachat, 2000), often based on in vitro molecular data of physical and/or biochemical interactions between Z-line proteins. Actual 3D imaging data that observes this in more detail is to our knowledge still mostly lacking. This gap could be filled by super-resolution imaging of the key candidate proteins. Here we investigated the axial distribution of α-actinin around the axial centroid of the Z disc. 3D super-resolution based axial profiles suggest that the α-actinin distribution is ~100 nm wide, a value similar to that obtained with electron microscopy on the basis of Z-line electron density. The electron dense band (i.e. the Z-line) should be a good proxy for the α-actinin distribution since immune-electron microscopy of ferritin-labelled anti-α-actinin shows good agreement between the electron-dense area and the distribution of α-actinin antibodies (Knöll et al., 2002; Lemanski et al., 1985; Tokuyasu, Dutton, Geiger, & Singer, 1981). It is thought that the region where α-actinin cross-links actinin coincides with the Z-repeats of titin (P. Young, Ferguson, Banuelos, & Gautel, 1998). In other words, the longitudinal extent of this titin region effectively sets the longitudinal spacing of the cross-linked actin filaments. In a variety of pathological conditions the integrity of the Z-line is compromised, either as a result of putative mechanical forces or as a result of primary defects in molecular assembly. For example, functional mutations in Muscle LIM Protein (MLP) (Arber et al., 1997), actinin-associated LIM Protein (ALP) family (Pashmforoush et al., 2001) and the enigma family of proteins (enigma, ENH and cypher) (Cheng et al., 2010) all lead to muscle pathologies including cardiomyopathies at varying severities. Disruption of their fundamental role in these proteins in the development or maintenance of the sarcomeric organization manifests in morphological changes such as loss of regularity and the laminar structure of the myofibrils, thickening and/or lateral misregistration of Z-lines between adjacent myofibrils (although later observed in the healthy myocardium (I. D. Jayasinghe et al., 2010; Christian Soeller, Jayasinghe, Li, Holden, & Cannell, 2009)). The super-resolution analyses presented in this paper are sensitive in detecting such nano-scale changes in the form of thickening of the α-actinin staining bands (Figure 6E), non-uniformities in their spacing and a broadening/skewing of the distribution of myofibrillar diameters (Figure 7E) in non-dehydrated tissue samples. An advantage of immuno-super-resolution microscopy is the
ability to directly correlate such changes with the location of putative proteins implicated in the pathology. For example, the loss of interaction between Tcap (located at the Z-line) and titin in certain dilated cardiomyopathies (Takeharu Hayashi et al., 2004) could be quantified at nanometre precision by using antibodies targeting specific sites of the two proteins.

Transverse super-resolution images of myocytes stained for α-actinin also allowed us to resolve closely packed myofibrils that were previously un-resolved using diffraction-limited microscopy methods. The narrow inter-myofibrillar boundaries that could not be seen in diffraction-limited data (I. Jayasinghe et al., 2012; C. Soeller et al., 2007) are likely to provide fast para-myofibrillar pathways for the transverse diffusion of calcium from the calcium release sites. Quantitative analysis of the myofibril diameters (similar to Fig. 7) using super-resolution images would be particularly useful in understanding the myofibril proliferation during physiological growth and load-induced hypertrophy (Anversa, Hiler, Ricci, Guideri, & Olivetti, 1986) while maintaining the myofibril diameter (Toffolo & Ianuzzo, 1994).

The nanometre-scale analysis of the α-actinin staining and the SR architecture (Figure 4) at the Z-line is also a demonstration of the robustness of this technique in resolving nano-scale pathological remodelling of other ultrastructural features found near the Z-lines (e.g. T-tubules, dyads). Specifically, departure of T-tubules from the Z-lines in a range of heart pathologies (D. J. Crossman et al., 2011; Wei et al., 2010) is thought to result from the loss of structural (and functional) links to structural proteins (e.g. BIN-1, Junctophilins and potentially Tcap) (Nicot et al., 2007; Wei et al., 2010) found at Z discs. We therefore expect super-resolution microscopies to provide vital information on such remodelling and the mechanisms underlying impaired EC coupling and generation of force.

**Conclusion**

The recent introduction of practical super-resolution imaging techniques has enabled the observation of features of cardiac myocytes that were previously difficult to assess with conventional techniques. This includes features on the scale between ~30 nm and 200 nm where cardiac myocytes have a rich subcellular structure including RyR clusters, T-tubules and details of the sarcomeric makeup. Here we demonstrated this with data on α-actinin and the structure of Z discs. There is a wealth of information on proteins concentrated at
the Z-lines, mostly from molecular studies, but it is not always clear how these complexes are spatially organized at and around the Z disc. We expect that super-resolution microscopy in its many variations (e.g. STED, PALM, STORM, structured illumination etc) will help fill these gaps in our knowledge and improve our understanding of cardiac biophysics.

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Figure S1: Analysis of local myofibrillar widths based in correlative confocal and dSTORM images. Transverse confocal and dSTORM projections of α-actinin staining across a 250-nm deep volume in a rat ventricular myocyte are shown in (A) and (B) respectively. The masks of the (C) confocal images and (D) dSTORM images of the myofibrillar spaces are colour-coded to indicate the local width measured (in microns). The histograms of the pixels of the masks as a function of local width measured from (E) confocal and (F) dSTORM data. Note that the former distribution is distinctly wider and reports local widths in excess of 1 µm. These regions correspond to myofibrillar spaces consisting of unresolved myofibrillar boundaries in
the confocal images. Mean diameters increased from 0.62 ± 0.25 (dSTORM analysis) to 0.82 ± 0.26 um (confocal analysis) for the cell that was analysed.
Chapter 2

Nanoscale analysis of ryanodine receptor clusters in dyadic couplings of rat cardiac myocytes

Yufeng Hou\textsuperscript{a,\#}, Isuru Jayasinghe\textsuperscript{b,\#}, David J Crossman\textsuperscript{a}, David Baddeley\textsuperscript{a,c}, and Christian Soeller\textsuperscript{a,b,*}

\textsuperscript{a} Department of Physiology, University of Auckland, Auckland, New Zealand; \textsuperscript{b} Biomedical Physics, University of Exeter, UK; \textsuperscript{c} Department of Cell Biology, Yale University, New Haven, USA

\#these authors made equal contributions to this work

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Abstract
The contractile properties of cardiac myocytes depend on the calcium (Ca^{2+}) released by clusters of ryanodine receptors (RyRs) throughout the myoplasm. Accurate quantification of the spatial distribution of RyRs has previously been challenging due to the comparatively low resolution in optical microscopy. We have combined single-molecule localisation microscopy (SMLM) in a super-resolution modality known as dSTORM with immunofluorescence staining of tissue sections of rat ventricles to resolve a wide, near-exponential size distribution of RyR clusters that lined on average ~57% of the perimeter of each myofibril. The average size of internal couplons is ~63 RyRs (nearly 4 times larger than that of peripheral couplons) and the largest clusters contain many hundreds of RyRs. Similar to previous observations in peripheral couplons, we observe many clusters with one or few receptors; however ≥80% of the total RyRs were detected in clusters containing ≥100 receptors. ~56% of all clusters were within an edge-to-edge distance sufficiently close to co-activate via Ca^{2+}-induced Ca^{2+} release (100 nm) and were grouped into ‘superclusters’. The co-location of superclusters with the same or adjacent T-tubular connections in dual-colour super-resolution images suggested that member sub-clusters may be exposed to similar local luminal Ca^{2+} levels. Dual-colour dSTORM revealed high co-localisation between the cardiac junctional protein junctophilin-2 (JPH2) and RyR clusters that confirmed that the majority of the RyR clusters observed are dyadic. The increased sensitivity of super-resolution images revealed approximately twice as many RyR clusters (2.2 clusters/µm³) compared to previous confocal measurements. We show that, in general, the differences of previous confocal estimates are largely attributable to the limited spatial resolution of diffraction-limited imaging. The new data can be used to inform the construction of detailed mechanistic models of cardiac Ca^{2+} signalling.
Introduction

The ryanodine receptor (RyR) is the primary calcium (Ca\(^{2+}\)) release channel responsible for the release from internal stores in many different cell types including cardiac muscle (Franzini-Armstrong & Protasi, 1997). The arrangement of these giant tetrameric channels has been classically described as a quasi-crystalline clustered organisation at the junctional (dyadic) clefts between the sarcoplasmic reticulum (SR) and T-tubules (Loesser, Castellani, & Franzini-Armstrong, 1992). Such clusters are thought to underlie the functional observation of Ca\(^{2+}\) sparks (M. B. Cannell et al., 1994), producing at a given site (and in a given state of the cell) fairly stereotypic microscopic Ca\(^{2+}\) release events (Bridge, Ershler, & Cannell, 1999); an idea that has been refined in the theory of local control (M. B. Cannell & Kong, 2012). RyR clusters are seen as punctate labelling densities in confocal immunofluorescence images of rat (Chen-Izu et al., 2006; Scriven et al., 2000; C. Soeller et al., 2007) and mouse (T. Hayashi et al., 2009) cardiac myocytes. Spread of regenerative Ca\(^{2+}\) waves is thought to correlate with elevated luminal Ca\(^{2+}\) levels in the SR (Diaz, Trafford, O’Neill, & Eisner, 1997; Lukyanenko, Viatchenko-Karpinski, Smirnov, Wiesner, & Gyorke, 2001) and the probability of such regenerative propagation may be facilitated by the effective shortening of the longitudinal distances between RyR clusters resulting from the helical dislocations of the z-lines (D. Baddeley, Jayasinghe, Cremer, et al., 2009; I. D. Jayasinghe et al., 2010). The relatively large inter-cluster distances seen in confocal data (mean of 0.6-1 µm, (Chen-Izu et al., 2006; C. Soeller et al., 2007)) were thought to serve to reduce the propensity of spontaneous propagation of Ca\(^{2+}\) release in myocytes (Izu, Wier, & Balke, 2001; Ramay, Jafri, Lederer, & Sobie, 2010) as recently reviewed (Izu, Xie, Sato, Banyasz, & Chen-Izu, 2013).

The understanding of excitation-contraction (E-C) coupling and its complex interplay with nanoscale subcellular structure has evolved with more recent volume electron-microscopy (EM) and other high resolution images of RyRs within ventricular myocytes. Super-resolution microscopy based on single molecule localisation (e.g. dSTORM) has allowed us to study RyR distributions within striated muscle at nanometre-scale resolution, with the larger fields of view typical for light microscopies and the high molecular specificity of immunofluorescence imaging (D. Baddeley, Jayasinghe, Cremer, et al., 2009; D. Baddeley, Jayasinghe, Lam, et al., 2009; I. D. Jayasinghe, Munro, Baddeley, Launikonis, & Soeller, 2014). EM studies have suggested a less rigid or static arrangement of receptors within each cluster (Asghari et al.,...
2014), which may reside within nanometre distances from their neighbours (T. Hayashi et al., 2009). The wide distribution of cluster sizes within peripheral couplings, many containing only a few receptors (D. Baddeley, Jayasinghe, Lam, et al., 2009), has raised several fundamental questions: (a) Is a similar distribution observed in the dyadic couplings (in the myocyte interior) that account for a large fraction of the cytosolic Ca\textsuperscript{2+} transient? (b) Are these clusters functionally organised in local ‘superclusters’? (c) and if so, how close are clusters (or superclusters) to each other, specifically are they close enough to support the spread of regenerative Ca\textsuperscript{2+} waves via Ca\textsuperscript{2+} induced Ca\textsuperscript{2+} release (CICR) across the Z disc? An additional focus on RyR cluster formation and maintenance is warranted based on the recently reported effects of overexpression of the junctional protein junctophilin-2 (JPH2) on RyR arrangement and dyad area (Ang Guo et al., 2014) highlighting a need for a more explicit investigation of the spatial relationship between JPH2 and RyR2 in the normal myocardium.

In this study we have combined super-resolution imaging with immunohistochemistry to visualise the Ca\textsuperscript{2+} release sites within extended regions of rat ventricular myocytes at near single receptor resolution. We find that throughout the cross-section of each myocyte RyR clusters are lining a consistent fraction of the myofibril perimeter, at much smaller edge-to-edge spacing than previously suggested by confocal data. The mean cluster size is substantially larger in dyadic couplings than in peripheral couplings. Multicolour dSTORM images also provide evidence that the supercluster grouping of RyR clusters reveals surprisingly large clusters that are strongly aligned with the local t-tubule geometry. With a near-perfect co-localisation with RyR, super-resolution images of JPH2 distribution underline a putative role in the assembly and modulation of RyR cluster function. We also present an analysis to reconcile the data from previous confocal studies with the new super-resolution data presented here. The new data provides a mechanistic basis for detailed models of EC coupling in myocytes, with a particular relevance for the transition to regenerative Ca\textsuperscript{2+} wave spread which is of great importance to understand the development of arrhythmogenic behaviour.
Methods

Sample preparation and Immuno-staining

Adult wistar rats weighing 250-300g were killed using an intraperitoneal injection of 100 mg/kg sodium pentobarbitone according to a protocol approved by the University of Auckland animal ethics committee. The heart was quickly dissected, cannulated at the aorta, and retrogradely perfused for 10 minutes with 2% (w/v) paraformaldehyde (Sigma-Aldrich; St. Louis, MO)) dissolved in phosphate buffered saline (PBS) at room temperature using a Langendorf perfusion system. The ventricles of the fixed heart were excised and immersed in fresh PBS to wash off excess fixative. Ventricular tissue was diced and cryo-protected in PBS containing 30% sucrose (w/v; Sigma-Aldrich) prior to freezing in methylbutane (Sigma-Aldrich) cooled in liquid nitrogen. 10µm-thick cryo-sections were cut using a CM 1900 cryostat (Leica, Germany) at -25°C. Sections were mounted onto No. 1.5 glass coverslips (Menzel-Gläser, Germany) coated with 0.05% poly-L-lysine (Sigma-Aldrich). Cryo-sections were hydrated in PBS and further permeabilised with 1% Triton X-100 (v/v; Sigma-Aldrich) in PBS for 15 minutes at room temperature and then washed in fresh PBS for further 15 minutes. Sections were then blocked in Image-iT FX signal Enhancer (Life Technologies) at room temperature. Primary and secondary antibodies were dissolved in an incubation buffer (see below) and applied to the sample overnight at 4°C and for 2 hours at room temperature respectively. Following each of these steps, sections were washed three times in PBS at room temperature. Samples were then mounted within the dSTORM imaging buffer (described below) and the coverslip was sealed onto a glass microscope slide.

dSTORM imaging

Slides containing the samples were clamped onto the stage of a modified Nikon TE2000 inverted fluorescence microscope. A custom objective holder and stage bracket was used to minimize drift, with focusing controlled by a piezo focuser (P-725, Physik Instrumente, Germany). The laser beam from a solid-state 671 nm laser (Viasho, China) was focused onto the sample via a 60x 1.49NA oil-immersion TIRF objective (Nikon) in a highly inclined light sheet (Tokunaga et al., 2008) to achieve an ~10⁹ W/m² non-TIRF illumination within a 20 µm wide area up to several microns deep within the sample. Emission light was passed through a Q680LP dichroic mirror (Chroma Technology) and an XF3104-690ALP emission filter.
(Omega optical) prior to being split into two spectral channels using a custom-built splitter device built as described previously (D. Baddeley et al., 2011). Transverse 2D dSTORM optical sections through ventricular myocytes (tissue sectioned parallel to Z discs) were acquired from regions where the local z-line plane was in focus. Shallow z-stacks of such 2D dSTORM images were constructed by stepping the focus repeatedly through a 1000 nm z-range at 150 nm steps. The emission light in the two channels were recorded onto the two halves of the cooled EM-CCD chip of an IXon DV887DCS-BV camera (Andor Technology, Belfast) in sequences of 20,000-40,000 frames at 20 frames/s.

**dSTORM Image analysis**

Single molecule events were localised at a typical mean localisation precision of ~13 nm (Alexa 680) and ~17 nm (Alexa 750) (see Supplementary Fig. S1) and spectrally separated into fluorochrome identities using custom-written algorithms implemented in Python and described previously (D. Baddeley et al., 2011). Residual drift during acquisition was corrected by using a correlation method where several images from events in subsets of the total series of frames were generated and spatially correlated. This allowed drift correction to better than ~5 nm with drift of several hundred nanometres (Mlodzianoski et al., 2011). To minimize the potential for drift induced errors we excluded series that exhibited a total drift exceeding 150 nm. The computed drift time course was used to correct the position of events and generate essentially drift-free coordinates for further processing.

As described above shallow image stacks were acquired by stepping the focus slightly around the estimated location of the Z disc (determined by wide-field focusing of the image prior to dSTORM acquisition). This was done to ensure capturing the z-lines at optimal focus. The data was rendered as a sequence of 2D dSTORM images in which the nominal focus changed by 200 nm between adjacent dSTORM images. We then selected the 2D dSTORM image which captured the Z disc distribution of RyRs with the highest number of in-focus events (out-of-focus events where rejected based on their Gaussian best fit diameter). Point data were rendered into a 2D greyscale TIF image with a 5 nm x 5 nm pixel size for further analysis using a protocol based on a jittered triangulation as described previously (D. Baddeley et al., 2010). The axial resolution in the resulting 2D dSTORM image was ~600 nm (diffraction-limited).
For analysis of RyR cluster sizes and co-localisation with other proteins, binary masks of the regions of positive labelling were calculated by thresholding the image so that the masked area contained 80% of the total labelling intensity. This choice is consistent with the event densities observed in our experiments and in test simulations correctly extracted cluster extents (Fig. S2). The number of RyR channels within each region of labelling was calculated assuming an isotropic 30 nm centre-to-centre receptor packing density (Yin & Lai, 2000). To convert this into a concentration value, area densities of RyRs at the z-line (assumed to be the vast majority of RyR found within the cell) determined from our data were divided by the normal sarcomere length 1.8 µm to obtain total RyR per unit volume. The binary masks were also used for a colocalisation analysis according to a protocol described by Jayasinghe and others (I. D. Jayasinghe et al., 2014). This analysis generates histograms of the percentage of labelling of protein A as a function of the Euclidean distance (the Euclidean distance being the straight line distance between two objects/points on a Cartesian plane) to the edge of the nearest region of labelling of protein B. The shaded bars in these histograms illustrate the percentage of protein A determined to be co-localised within the mask of protein B.

**Solutions and antibodies**

Primary and secondary antibodies were incubated in PBS containing 0.05% NaN₃ (w/v), 2% bovine serum albumin (w/v), 2% normal goat serum (v/v) and 0.05% Triton X100 (v/v). The imaging buffer for dSTORM contained 90% Glycerol (v/v), catalase (0.005mg/mL; Sigma-Aldrich), glucose oxidase (0.05mg/mL; Sigma-Aldrich) and 5 mM β-mercaptoethylamine (Sigma-Aldrich) freshly dissolved in PBS and adjusted to pH 7 (D. Baddeley et al., 2011). RyR2 were labelled using either a mouse monoclonal IgG1 anti-RyR2 (Cat# MA3-916; Thermo Scientific) or a rabbit polyclonal IgG anti-RyR2 (Cat# HPA016697; Sigma-Aldrich) at dilutions of 1:100. Myofibrillar Z discs were labelled using a mouse monoclonal IgG anti-α-actinin (Cat# A7811, Sigma-Aldrich) at a dilution of 1:200. A rabbit polyclonal IgG anti-JPH2 gifted by Xander Wehrens was diluted at 1:200 in double labelling experiments investigating the dyadic distributions of JPH2 and RyR2. T-tubules were stained by a combination of a mouse monoclonal IgG anti-caveolin-3 (Cat# 610420; BD Transduction) and a mouse monoclonal IgG anti-Na⁺-Ca²⁺ exchanger (Cat# R3F1; SWANT, Switzerland) both applied simultaneously at dilutions of 1:100 (see also (I. Jayasinghe et al., 2012)). This approach for visualising T-
tubules was adopted following our previous results that demonstrate that the combination of these two antibodies provides a more complete and reliable image of the rat ventricular t-system (I. Jayasinghe et al., 2012; I. D. Jayasinghe et al., 2009). Double labelling experiments for imaging the t-tubule and dyad distributions were performed with this antibody combination together with the rabbit polyclonal anti RyR2 antibody. A goat anti-mouse IgG Alexa 680 conjugate (Cat# A31563; Life Technologies) was used at a dilution of 1:200 as the preferred secondary antibody for single-colour imaging experiments. In double labelling experiments, Alexa 680- and Alexa-750 conjugated goat anti-mouse IgG and goat anti-rabbit IgG antibodies (Life Technologies) were used at the same dilution.

**Statistical comparisons**
Means are generally reported with the standard error of the mean (SEM) as uncertainty. Statistical tests were conducted using SigmaPlot v11.0.

**Results**

**Quantitative super-resolution imaging of RyR distribution**

dSTORM images of rat ventricular tissue, sectioned such that myocytes could be imaged transversely, and immuno-labelled for RyR revealed a dense organisation of RyR clusters throughout the interior of myocytes where the z-lines were in focus (Fig 1A). This method of sectioning enabled the single layer approximation as the relatively lower resolution in the axial plane leads to overlapping clusters in longitudinal arrangement. Staining was characteristically absent in spaces presumably occupied by myofibrils, mitochondria and nuclei, a labelling pattern qualitatively similar to diffraction-limited transverse images of RyR immunofluorescence staining in rat ventricular myocytes in previous studies (Chen-Izu et al., 2006; Scriven, Asghari, Schulson, & Moore, 2010; C. Soeller et al., 2007). Upon close examination of super-resolution images, we observed a wide range of sizes of RyR clusters some of which exhibited highly elongated or other, more complex shapes (Fig 1B). We analysed the cluster sizes by normalizing with the 2D area occupied by a single RyR in a crystalline array (~ 900 nm²) as used previously (D. Baddeley, Jayasinghe, Lam, et al., 2009), an approximation that we discuss below. The resulting frequency histogram has an approximately exponential shape over a wide range of sizes and revealed cluster sizes
ranging from a single receptor to ~800 receptors (Fig 1C). The analysis shows that single (isolated) receptors account for ~25% of all clusters, and >80% of the clusters contain <100 receptors (see also cumulative histogram in Fig 1C). The mean cluster size was ~63 receptors, although a few (<0.5%) of the clusters contained >500 receptors, sufficiently large to be partially resolved by conventional fluorescence microscopy modalities. From this data

**Figure 1**: RyR distribution in rat ventricular myocytes. (A) Pseudocolour transverse dSTORM image at the z-lines of a myocyte. Note the dense, clustered organisation across the myocyte width and the exclusion of receptors in a nuclear region. The inset is a schematic of the overall cell orientation (green pseudo-coloured) achieved in relation to the image plane (white outline) of the optical sectioning achieved with our imaging and sectioning approach. (N). (B) Magnified view of the box region in pane A illustrates closely arranged clusters of varying sizes. (C) The frequency histogram of cluster sizes (notice log scale) exhibits a near-exponential size distribution with a mean size of 62.8 ± 2.98 RyRs (mean ± SEM) and a few notable areas containing >500 receptors. A cumulative percentage histogram (inset) is shown as function of cluster size, illustrating that ~25% of the resolved binary patches correspond to solitary RyRs. (D) Frequency histogram (main panel) and the cumulative
percentage histogram (inset) shown as a function of the edge-to-edge nearest-neighbour distance. >60% of clusters located at distances shorter than the mean inter-cluster distance of 139.5 ± 8.5 nm. Scale bars, A: 3 µm, B: 1 µm.

we also determined the apparent area density at z-lines and converted this to a measured RyR density per volume by using the z-line spacing of ~1.8 µm. From this calculation we obtained a mean density of 134 ± 7 RyRs/µm³ in rat ventricular myocytes. We also determined the distribution of nearest neighbour edge-to-edge distances from the RyR cluster masks (Fig. 1D) which has a mean near 140 nm (see also Table 1). The cumulative histogram (Fig 1D inset) showed that ~56% of the clusters were detected at an edge spacing of 100 nm or less, an inter-cluster separation typically unresolvable with diffraction-limited imaging approaches. In addition, the data was used to obtain a cluster density per unit cell volume which had a mean value of ~2.2 clusters/µm³ (Table 1).

**Analysis of RyR superclusters**

We had previously introduced the notion of RyR superclusters (D. Baddeley, Jayasinghe, Lam, et al., 2009) and therefore analysed the inter-cluster distances using binary masks of the dSTORM data by performing a Euclidean distance analysis of the surrounding regions of each cluster. On the basis that RyRs within a 100 nm distance may co-activate at > 10 µM local [Ca²⁺], (Sobie et al., 2006), we examined the dSTORM images for clusters that were separated by distances ≤ 100 nm which were identified by surrounding clusters with a 50 nm wide band (Fig. 2B), and found that RyR ‘superclusters’ typically consisted of 2 – 6 sub clusters (3.4 sub-clusters per supercluster on average, Table 1, see also Supplementary Fig S3). Notably, 43.7% of the RyR clusters did not coalesce into superclusters (seen in Fig 2B as unlined regions). Coalescing clusters into superclusters (and including trivial superclusters, i.e. a large cluster with associated single receptors, see Fig. S3), we compared the frequency histograms of the resulting cluster sizes (Fig 2C, main panel) to the original size distribution which ignored this “functional grouping”. The supercluster grouping reduces slightly the proportion of smaller clusters and extended the tail of very large clusters which now approaches 1900 RyRs. Analysis of the fraction of RyRs in clusters of a specific size (Fig 2C, inset) shows a similar change upon grouping into superclusters. The supercluster units contained on average ~103 RyRs compared to ~63 RyRs when considering individual clusters (Fig 1C). The modes of the fractional cluster size occupancy histograms (inset in Fig 2C) also
indicate that the majority of the cell’s RyRs are found within clusters containing less than 500 RyRs, the frequency maximum is between 100-200 RyRs in both the individual cluster and supercluster distributions. Fig 2D shows an overlay of a super-resolution RyR image with contours indicating 250 nm of Euclidean distance from the nearest RyR cluster edge. The fact that these contours contain virtually all clusters shows that almost all clusters are within edge distances of <500 nm. An example overview of the 250 nm distance contours in a whole cell transverse view is shown in supplementary Fig. S4.

Figure 2: Analysis of RyR cluster spacing in dSTORM images. (A) Binary mask of a transverse dSTORM image of RyR distribution in a rat ventricular myocyte. (B) Magnified view of the box region illustrates grouping of RyR clusters into superclusters in which member clusters have edge-to-edge distances <100nm (shown with surrounding distance bands of 50 nm. When these touch or overlap clusters are <= 100 nm apart; coloured to illustrate distinct superclusters). (C) Comparison of the frequency histograms of RyR supercluster sizes (blue) and the individual clusters (red). With a mean supercluster size of 102.7 ± 3.5 RyR compared to the mean individual cluster size of 62.8 ± 2.98 RyR, a reduction in the frequency of solitary receptor clusters and more clusters with > 1500 RyR are notable.
Percentage histograms (inset) comparing the fractional RyR content as a function of cluster size show that many RyRs are found in clusters containing 100-200 RyRs (red line) and similarly on the basis of supercluster grouping (blue) (17.2% and 9.2% respectively). An overlay of a transverse dSTORM image of RyR labelling within a myocyte with a 250 nm distance contour measured from cluster edges (white lines) shows that nearly all clusters were within <500 nm distance of each other. Note that grey areas indicate distances >250 nm. Scale bars: A, B: 2 µm, D: 1 µm.

**Relationship of RyR clusters to other myocyte structures**

We investigated the organisation of RyR clusters in relation to other cellular components such as myofibrils (MF) and T-tubules using dual colour dSTORM data. In transverse view, the MF z-disc protein α-actinin was detected at very high marker density throughout the MF cross-section (Fig 3A, red) as observed in previous dSTORM experiments (Y. Hou et al., 2014a). RyR clusters (Fig 3A, green) were observed occupying the inter-myofibrillar spaces and positioned at the edges of regions with α-actinin labelling. We utilised the strong contrast of the α-actinin labelling in these images to trace the perimeter of myofibrils and measure the perimeter fraction occupied by RyR clusters. A scatter plot exhibited an approximately linear relationship (Fig 3C) indicating that ~57% of the outline of myofibrils at the z-line is lined by RyR labelling ($r^2 = 0.823$).

![Figure 3](image)

**Figure 3**: RyR cluster localisation in relation to myofibrils and T-tubules. (A) Overlay of multicolour transverse dSTORM images of myofibril marker α-actinin (red) and RyR (green) at the z-line planes of a rat ventricular myocyte. (B) Magnified view of the box region in
panel A illustrating the RyR clusters closely lining the myofibrillar spaces. (C) Scatter plot of the total perimeter length of myofibrils lined by junctions (traced similar to blue schematic line in inset) against the total myofibrillar perimeter (yellow line). The strong linear relationship ($r^2 = 0.823$) with $57.18 \pm 8.58\%$ (n = 65) of the myofibril perimeter was lined by RyR. (D) Overlaid dSTORM images of RyR (green) and T-tubules labelled with a combination of anti-CAV3 and anti-NCX antibodies (red). (E) A magnified region illustrates the strong co-registration between the elongated axes of the RyR clusters and the local t-tubule geometry. Note large (>500 nm long) clusters co-locating with t-tubule branch points. (F) The RyR superclusters within the same region in panel E overlaid with 50 nm Euclidean contours to illustrates that supercluster grouping often grouped individual clusters that lined up along the same T-tubular connections. Scale bars: A, D 2 µm, B, E, & F: 0.5 µm.

The geometry of the t-system in relation to the RyR clusters was visualised using a combination of anti-CAV3 and anti-NCX antibodies. Fig 3D shows the t-system and RyR in a transverse dSTORM image of a rat ventricular myocyte. T-tubules appear to connect most RyR clusters across the cross-section of the myocyte, qualitatively similar to previous confocal data (I. D. Jayasinghe et al., 2009). Closer examination showed that branch points of the T-tubules were often occupied by larger RyR clusters (Fig. 3E). This data also suggested that large RyR superclusters are often aligned with the same or closely connected segments of T-tubules (Fig. 3F).

**Co-localisation of junctophilin-2 within dyads**

The diverse shapes and sizes of RyR clusters observed throughout the interior of rat ventricular myocytes led us to investigate the nanoscale distributions of JPH2 which is thought to play a central role in maintaining the dyadic coupling between the SR and T-tubules. Fig 4A shows a typical transverse image in the interior of a rat ventricular myocyte where JPH2 (magenta) and RyR (green) show strong overlap and both channels exhibit distinct cluster shapes that are very similar indicating the presence of both RyR and JPH throughout junctional clusters (Fig 4B & C). Some regions contained either only JPH2 or RyR, indicating that the overlap between these two proteins is not perfect. We quantified and compared the spatial association of RyR with JPH2 on the nanometre scale using a distance based co-localisation analysis and the resulting distance histograms are shown in Fig 4D (where negative distances denote regions of labelling of RyR that are within areas occupied by JPH2). A mean of $\sim 61\%$ of the RyR signal directly overlaps with the JPH2 positive regions. Similarly, $\sim 60\%$ of the JPH2 labelling is directly co-localised with RyR. We estimated the
The error involved in determining the RyR cluster edges using dSTORM is at least 30 nm, i.e. on the order of our spatial resolution (D. Baddeley, Jayasinghe, Cremer, et al., 2009) and similar to the uncertainty arising from the detection of a single RyR. On this basis we also calculate the labelling fractions up to a Euclidean distance of 30 nm outside of the mask which increases the above percentages to ~77.9% and ~77.3%, respectively (i.e. densely and lightly shaded bars of this histograms in Fig. 4D).

Figure 4: JPH2 distribution in relation to RyR. (A) Overlaid transverse dSTORM images of JPH2 (magenta) and RyR (green) within a rat ventricular myocyte. Magnified regions of the (B) JPH and (C) RyR images report strong overlap between the two proteins and the similarities in the shapes and sizes of the corresponding clusters. Minor regions of poor overlap were also observed (arrowheads). (D) Comparison of the histograms of the percentage of RyR staining as a function of the distance to the edge of the nearest region of JPH2 staining (front) and percentage of JPH against the distance to the nearest RyR cluster (back) show 61.22 % and 60.51% of RyRs co-localizing with JPH1 respectively (strongly shaded regions of the histogram). (E) Overlaid transverse dSTORM images of a rat myocyte double-labelled with a mouse anti-RyR (red; also panel F) and a rabbit anti-RyR (red; also panel G) antibody. (H) Both percentage histograms of r-RyR distribution in relation to the edge of m-RyR (front) and the reciprocal analysis illustrate 70.24 % and 72.40% of r-RyR and m-RyR co-localising with each other. Note the lightly shaded regions of the histograms in panels D & H indicate the co-localising percentages of labelling detected in Euclidean distances of up to 30 nm outside of the binary mask. Scale bars: A: 2 µm, B,C, E, F & G: 0.5 µm.

The co-localisation observed in the JPH2 and RyR double labelled samples was not complete which is not unexpected, both due to stochastic antibody labelling and SM detection effects. To investigate the maximal co-localisation that can be expected between completely co-
located targets, we performed double labelling of RyRs using two distinct mouse and rabbit origin anti-RyR2 primary antibodies. Fig 4E-G shows dSTORM images of mouse anti-RyR2 (m-RyR) and rabbit anti-RyR2 (r-RyR) labelling within a rat ventricular myocyte. The labelling patterns in the two labelling channels show strong similarities in location, shape and size although there were also regions with incomplete agreement (e.g. arrowheads in Figs 4F & G). The histogram of the percentages of r-RyR labelling as a function of distance from the cluster edge exhibits a large fraction (~72%) of directly overlapping r-RyR component (Fig 4H front). A similar fraction (~70%) of m-RyR was observed in direct co-localisation with r-RyR (Fig 4H back). When additionally labelling up to a distance of 30 nm surrounding each region in the binary mask (lightly shaded bars of the histograms in Fig. 4H) was included, these fractions increased to 84% and 83%, respectively (Table 2).

Discussion

Super-resolution imaging of internal RyR clusters in transverse sections

In this study we have imaged RyR clusters (and several closely associated structures) in transverse tissue sections through ventricular myocytes of the rat heart. This geometry clarifies the arrangement of RyR clusters at the z-line and also clearly reveals the pattern of myofibrils and the t-system which in small rodents is a complex meshwork located almost entirely at z-lines (C. Soeller & Cannell, 1999). The use of the transverse geometry is advantageous as the high lateral resolution is essentially aligned with Z discs, as we have exploited before (Chen-Izu et al., 2006; C. Soeller et al., 2007). To achieve this we used cryo-sectioned tissue slices rather than the ‘agar sausages’ employed before (Chen-Izu et al., 2006; C. Soeller et al., 2007) since background fluorescence was too strong in this approach (data not shown) which greatly reduced the contrast for single molecule fluorescence detection (i.e. the basis of the dSTORM method). Using thin transverse ventricular tissue sections (~5-10 µm) dSTORM images exhibited high contrast and the high resolution (~30 nm) associated with this super-resolution modality (D. Baddeley et al., 2011).

RyR cluster shapes and sizes in internal couplings

We have previously determined the morphology and size distribution of RyR clusters in peripheral couplings with the surface membrane (D. Baddeley, Jayasinghe, Lam, et al., 2009)
where we found an unexpectedly wide size distribution that had a near exponential shape over a wide range. RyR clusters in internal couplings between terminal SR and the t-system exhibit a similar distribution (Fig. 1) indicating that the majority of clusters are relatively small. The extent of some clusters, however, is surprisingly extended and these larger clusters dominate the appearance of transverse images at the z-line since they occupy a large fraction of the total space taken up by RyR clusters, consistent with the fact that >80% of all RyRs are in clusters with 100 or more members (>90% when superclustering is considered, Fig. 2C). Internal couplings contained ~63 RyRs on average which is a large increase as compared to peripheral couplings (~14, (D. Baddeley, Jayasinghe, Lam, et al., 2009)) and broadly similar to a value of ~43 RyR capacity in mouse dyadic couplings that was determined recently (Das & Hoshijima, 2013). The approximate exponential distribution furthermore did not fit well at smaller cluster sizes. This again was seen in peripheral clusters previously and complex fitting carried out within those analysis (D. Baddeley, Jayasinghe, Lam, et al., 2009) has suggested that perhaps this may be caused by a nucleation threshold.

These findings use the size calibration approach that we introduced for peripheral couplings (D. Baddeley, Jayasinghe, Lam, et al., 2009) and recently employed in quantifying RyRs in fast and slow-twitch skeletal muscle (I. D. Jayasinghe et al., 2014). Here the area occupied by a single RyR (estimated at ~30 nm x 30 nm, (Yin & Lai, 2000)) is used to obtain an estimate from the total area occupied by the cluster in the dSTORM data. In the current investigation this provides a lower bound on the number of RyRs as we effectively see 2D outlines (or projections due to the diffraction-limited axial resolution the data) of 3D clusters which may have different orientation with respect to the imaging plane as they wrap around a t-tubule as previously discussed (C. Soeller et al., 2007). In some cases the cluster may be on both sides of a tubule when seen in transverse sections which could result in an underestimate by a factor of ~2. In principle, this ambiguity may be resolved by adopting a super-resolution strategy that provides adequate resolution in all three dimensions. However, with the background fluorescence in our optically thick sections 3D dSTORM imaging with either astigmatism (Huang et al., 2008) or phase ramp imaging (David Baddeley et al., 2011) resulted in a depth resolution (i.e. the ability to distinguish two axially nearby objects) of ~130 nm, insufficient to fully resolve cluster orientation. In addition, the lateral resolution
was also significantly reduced due to the lower contrast when using these PSF modifications in thick samples. We therefore resorted to lateral super-resolution which resulted in high contrast dSTORM images. In close analogy to arguments presented before (C. Soeller et al., 2007) a conversion of our estimates to values taking cluster orientation into account may be achieved by adopting a correction factor that corrects the average underestimate resulting from the limited axial resolution. The situation is improved by the observation that two other effects may offset a potential underestimate. Firstly, not all clusters may be fully close-packed and arrays may vary locally in packing density (Asghari et al., 2014) or have small gaps of “missing” receptors (T. Hayashi et al., 2009) that may be difficult to capture even with the higher resolution available in dSTORM. We suggest that the combination of factors may largely cancel their effect on quantitative estimates and result in a correction factor not very different from unity.

To test this idea we compared the density of RyRs calculated on the basis of our measurements with biochemical measurements that estimate the number of ryanodine binding sites in rat ventricular tissue. Using a sarcomere spacing of 1.8 µm, we calculated a cytosolic RyR density of \( \sim 134 \text{ RyR/µm}^3 \), an estimate \( \sim 27\% \) smaller than our previous measurement (183.82 RyR/µm\(^3\)) based on confocal data of rat ventricular couplons (C. Soeller et al., 2007). Bers ((D.M. Bers, 2001), page 192) derives the RyR protein density in rat ventricular homogenate to be 833 fmol/mg of homogenate protein based on ryanodine binding experiments by Bers & Stiffel (D. M. Bers & Stiffel, 1993). This value can be converted to a RyR concentration of \( \sim 95 \text{ RyR/µm}^3 \) of cell volume (using a protein wet density in ventricular homogenate of 292 g/L cytosol and 0.65 L cytosol/ L of cell volume (D.M. Bers, 2001)), a value slightly smaller but in general agreement with our estimate based on super-resolution imaging. This calculation suggests that our area based calibration provides a reasonable normalisation of our data and argues against a significant underestimate. In any case, if more detailed information became available in the future, our results may be rescaled by using an improved estimate of the required correction factor.

**Small and Large RyR clusters**

A hallmark of both our previous analysis of peripheral couplings (D. Baddeley, Jayasinghe, Lam, et al., 2009) and the data from internal RyR clusters presented here is the large number of very small clusters containing one or only a few RyRs. We have previously
estimated that 1-4 secondary antibodies occupy each RyR (D. Baddeley, Jayasinghe, Lam, et al., 2009; C. Soeller et al., 2007). Each antibody is expected to have multiple attached dye molecules (4-5 in the antibody batches used) and in this study we detected ~8 events per RyR (Fig. S7) which is consistent with this estimate. Using our ability to visualize the RyR cluster distribution in relationship to the contractile apparatus (Fig. 3), we confirmed by inspection that the majority of small clusters are located in regions in immediate vicinity to the outline of myofibrils (Fig. S5) where they would be expected to be close to T-tubules and in a position so that released Ca\(^{2+}\) rapidly diffuses to myofibrillar proteins. The actual importance of these clusters in contributing to total release is still unclear. It may be tempting to explain the reported observation of “nonspark” related release (Santiago et al., 2010) with release from very small clusters but it may also be explicable by very brief (“aborted”) release from larger clusters. Finally, we cannot exclude the possibility that some of the very small clusters are part of a trafficking pool of RyR which is not functionally involved in Ca\(^{2+}\) signalling.

At the other end of the size range there are very large, extended clusters in which the RyR labeling appears continuous and these clusters are often aligned with segments of T-tubules (Fig. 3F). Adopting our concept of superclusters (D. Baddeley, Jayasinghe, Lam, et al., 2009) which is based on the idea that very closely spaced clusters may be strongly coupled in their activation via CICR, the largest superclusters occupy an area harbouring up to ~1800 RyRs. This raises the question if spontaneous release starting anywhere along such a cluster may lead to propagated release across the whole extended cluster, in some cases extending around a complete myofibril (e.g. Fig. 3F). Such a behaviour should be observable using fast confocal microscopy of cytosolic Ca\(^{2+}\) (Cleemann, Wang, & Morad, 1998) but for best contrast may require myocytes “standing on end”.

In connection with our observations we note that in EM tomograms many adjacent couplings were observed along T-tubules in mouse myocytes (Das & Hoshijima, 2013; T. Hayashi et al., 2009). It is possible that the resolution in super-resolution images is still insufficient to resolve very small gaps and that at even higher spatial resolution several adjacent but distinct sub-clusters emerge. Even if that were the case we would still expect these to be strongly coupled via CICR unless another mechanism could effectively decouple
clusters within nanometre proximity (e.g. negative coupling via local SR based depletion which could be communicated via diffusion to neighbouring termini).

In addition to the super-cluster concept which identifies clusters in very close proximity we also noted that virtually all clusters across a Z disc are contained within a common distance band of ~500 nm. In a fire-diffuse-fire scenario of Ca\(^{2+}\) release propagating by CICR (Dawson et al., 1999) activation could rapidly spread across a whole Z disc provided a critical distance of ~500 nm were sufficient to activate a cluster from another firing cluster with high probability.

**Super-resolution versus diffraction-limited imaging**

Two of the prominent features observed in the super-resolution data presented here are the large population of small RyR clusters and the comparatively small nearest neighbour edge-to-edge distances which are considerably smaller than cluster to cluster distances based on confocal fluorescence images (Chen-Izu et al., 2006; C. Soeller et al., 2007). We therefore sought to clarify the relationship and compatibility of previous data with the newer higher resolution data. We compared a super-resolution image (Fig 5A) with the equivalent confocal data set (Fig 5B) which can be “generated” from the super-resolution marker position data (see Supplementary Methods). The resulting simulated diffraction-limited data exhibited the characteristic punctate labelling pattern throughout the transverse view of the myocyte as observed previously (C. Soeller et al., 2007). The diffraction-limited punctate RyR pattern was often larger than the corresponding super-resolved clusters where groups of small clusters located in close proximity appeared as single extended puncta in the confocal image (Fig 5C,D). We analysed the RyR labelling distribution in the simulated confocal data using a matched filter centroid detection algorithm as previously described (C. Soeller et al., 2007). The calculated centroids in the confocal image appeared in reasonable agreement with the local labelling densities within the punctate RyR pattern. However, the positions and number of centroids correlated poorly with the super-resolved RyR cluster in several regions. Over a larger image area a considerable number of super-resolved clusters were missed in the diffraction-limited centroid analysis (circled areas in Fig 5E). The detection of a cluster in the confocal data depended both on the size of the cluster (small ones were often missed) but also on the proximity to other clusters (“merging”). Clearly, the unreliable detection of small clusters
will bias a cluster size histogram based on diffraction-limited data. To qualitatively consider the detection bias we made the simplifying assumption that the diffraction-limited “apparent” size distribution can be obtained from the more detailed super-resolution information by multiplying our histogram (Fig. 1C) with a detection function. We chose a detection probability function that rises from near zero for small clusters to virtually certain detection for large clusters as shown in the inset in Fig. 5F (neglecting a second order effect that detectability also depends on proximity to other clusters). The resulting “biased” histogram had a mode at ~100 RyR/cluster (Fig 5F) and a mean cluster size of ~165 RyRs, broadly similar to previous confocal based findings in rat (C. Soeller et al., 2007) and in reasonable agreement with EM based measurements in rat (~260 (C. Franzini-Armstrong, F. Protasi, & V. Ramesh, 1999), an estimate which assumed that RyR patches are round). In addition, the large number of small RyR clusters detected with super-resolution fluorescence imaging is the principle reason for the about doubled cluster density estimate (~2.2 μm⁻³) as compared to previous measurements (C. Soeller et al., 2007).

This qualitative comparison strongly suggests that the previous diffraction-limited data can be fully reconciled with the newer higher resolution data sets and emphasizes the bias introduced by diffraction-limited analysis. In addition, our comparison confirms that diffraction-limited data cannot just be interpreted as showing the centroids of actual superclusters (as revealed by super-resolution imaging). The relationship between the data at different resolution scales is complex and one needs to critically question if a given set of, say, confocal data is suitable for a specific quantitative analysis. This also applies to cluster distances which are affected by the often incorrect determination of centroid locations. More importantly, the CICR induced cross-talk between neighbouring clusters is more likely a function of edge-to-edge distances, rather than centroid distances. Our analysis shows that these edge-to-edge distances are substantially smaller than previously determined centroid distances (Chen-Izu et al., 2006; C. Soeller et al., 2007) and are compatible with the idea that in the regenerative regime Ca²⁺ waves can rapidly spread across the cross-section of a cardiac myocyte.
Figure 5: Simulation of confocal RyR images with the super-resolved RyR staining in rat ventricular myocytes. (A) A transverse dSTORM image focused near the z-lines of a myocyte. (B) The equivalent confocal image was generated by convolving the localised marker positions with a confocal point spread function. Close examination of magnified (C) dSTORM and (D) confocal images revealed the lack of notable fluorescence from the small RyR clusters (arrowheads). Centroids of the punctate RyR labelling in confocal images (overlaid
in panel D) had comparatively poor correlation with the number and shapes of some RyR clusters (e.g. region indicated by dashed lines). (E) Shown, is a larger overview of the super-resolved RyR image overlaid with the centroids of the detected puncta from the confocal data to illustrate the many clusters that were not detectable in the diffraction-limited approach. (F) The “apparent” RyR cluster size frequency histogram obtained by considering a detection function (inset) resulting in a mode at ~100 RyR with a mean apparent cluster size of ~165 RyRs. Scale bars: A, B 1µm; C-E 0.5 µm.

**RyR clusters and JPH**

JPH has been previously implicated in the assembly and maintenance of RyR clusters (Takeshima et al., 2000) and its expression level has significant effects on the architecture of the transverse tubular system (Wei et al., 2010), including an important role during early t-system development (Reynolds et al., 2013). It was therefore of interest to quantify the degree of co-localisation between JPH and RyR. Here we used distance based measures as reported before (I. D. Jayasinghe et al., 2012; I. D. Jayasinghe et al., 2009; I. D. Jayasinghe et al., 2014; Wong et al., 2013) to test if these proteins co-cluster into similar local aggregates based on the cluster masks generated from the high-contrast data (Fig. S2).

The use of co-localisation is well established in diffraction-limited imaging (e.g. (I. D. Jayasinghe et al., 2012; I. D. Jayasinghe et al., 2009; Scriven et al., 2000)). It has been observed before that targets that should perfectly co-localise (e.g. two epitopes on the same protein) exhibit lower effective overlap in experiments (Scriven et al., 2000) due to a number of effects, such as steric hindrance, incomplete labelling etc. In principle, super-resolution imaging improves on diffraction-limited co-localisation due to the improved spatial resolution which helps reduce artifactual overlap as a consequence of strong optical blurring. We have demonstrated previously that the Euclidean distance-based co-localisation analysis with dual-colour dSTORM images provides a robust method for quantifying the nanoscale overlap of junctional proteins (see supplementary data in (I. D. Jayasinghe et al., 2012)). Such analyses are limited by resolution and labelling density, in our experimental conditions, to an uncertainty between 30 to 40 nm (the respective resolution in the two data channels, Fig. S1). The limited resolution may reduce or increase the observed overlap (versus actual overlap), depending on the properties of the distribution of the two targets. To address this uncertainty and provide an upper bound on the likely overlap fraction, we additionally analyse the labelling fractions by including a 30 nm
distance band immediately outside of the binary mask areas to calculate a conservative upper bound estimate on the co-localising fractions.

To establish the amount of co-localisation observed for expected “perfect” co-localisation we conducted a super-resolution validation experiment with two antibodies against the same RyR target. The theoretically complete co-localisation scenario (RyR vs RyR) resulted in co-localisation values of 70-80%, due to stochastic effects of dSTORM imaging and also target labelling by antibodies (which is supported by a simulation, see Supplementary Methods and Fig. S6, that only considered the effects of stochastic switching in dSTORM and resulted in slightly higher co-localising fractions of 78-90%).

On this basis, we found a very strong degree of co-localisation between RyR and JPH in internal dyadic couplings (Fig. 4 and Table 2), similar to previous results in peripheral couplings of rat ventricular myocytes (I. D. Jayasinghe et al., 2012). Notably, JPH forms clusters just as RyR does and these two cluster populations strongly overlap, indicated by their comparatively high co-locating fractions (between 60-78%). While the co-localisation fractions of RyR labelled with two separate primaries (the model of “perfect” co-localisation) were broadly comparable to those between RyR and JPH, the RyR-RyR values were consistently somewhat higher (Table 2). We compared the measurements statistically using a pairwise Bonferroni t-test which indicated a statistically significant difference between the pairs RyR vs JPH2 and m-RyR vs r-RyR evaluated at 0 nm distance (but no difference between JPH2 vs RyR and m-RyR vs r-RyR). The same statistical test conducted with data up to and including a 30 nm distance (as an upper bound estimate) showed a statistical difference between the pair JPH2 vs RyR and the validation pair m-RyR vs r-RyR (but now none between the pairs RyR vs JPH2 and m-RyR vs r-RyR). We interpret this result as showing that there is a subtle difference between the distribution patterns of RyRs and JPH2 but that this difference is not large and is difficult to distinguish from “perfect” co-localisation.

Taken together this data suggests that JPH2 is present throughout the extent of RyR clusters and is in a position which is at least compatible with JPH2 binding to RyRs - an interaction which has been suggested to be a modulator of RyR function (van Oort et al., 2011). In addition, this is as a strong indication that the vast majority of the identified interior RyR
clusters analysed here are in dyadic clusters, compatible with the role of JPH2 to help
maintain the close apposition between t-tubule and terminal SR membranes (Takeshima et
al., 2000).

RyRs and contractile machinery
We observed a fairly tight relationship between the perimeter of myofibrils and the total
length of the perimeter lined by RyR clusters. This observation is a refinement of the
previous measurement of a mean distance between RyR clusters along the perimeter of
myofibrils (C. Soeller et al., 2007) made possible due to the much improved resolution that
was important both for the clear distinction of myofibrillar boundaries (Yufeng Hou et al.),
and for revealing the extended nature of RyR clusters. It seems reasonable that the cell
ensures a sufficient density of dyadic release sites to efficiently activate the contractile
machinery and raises the question if this relationship may be altered in some pathological
states of cell remodelling (D. J. Crossman, P. R. Ruygrok, C. Soeller, & M. B. Cannell, 2011;
Ang Guo et al., 2014; Louch et al., 2006; Pinali, Bennett, Davenport, Trafford, & Kitmitto,
2013; Song et al., 2006). The methods presented here should allow such investigations in
the future.

Limitations
Our analysis has highlighted the effects of limited spatial resolution and while current super-
resolution methods clearly improve measurements substantially it will be important to
further improve 3D resolution in these optically challenging samples for further
refinements. The functional role of some clusters as well as the role of cluster size is still
unclear. For a more complete understanding of EC coupling the intricate nature of the SR
itself (Pinali et al., 2013) may need to be considered in addition to the distribution of RyRs.

Conclusions
Using single molecule localisation based super-resolution immunofluorescence images, we
have characterised the spatial distribution of RyRs in the internal couplons of adult rat
ventricular myocytes. Similar to previous findings in peripheral couplons, regions of labelling
corresponding to solitary receptors as well as very large clusters were observed in high co-
localisation with JPH2. Groups of RyR clusters in close proximity (≤100 nm) that formed
superclusters were typically found in alignment with the same T-tubular connections, supporting the possibility that member sub-clusters may all reside within the same or closely aligned physical junctions. Additionally, the presence of some receptors which were possibly not in close association with t-tubules are identified, however, the occurrence of this appeared to be rare. The largest of these superclusters occupy areas that may contain nearly 2000 RyRs, raising the question if these regions are tightly coupled during spontaneous release. The new observations are compatible with previous data on the cell-wide RyR distribution based on confocal micrographs but the ~10-fold improvement in resolution and the greater sensitivity for small protein clusters provide substantive new information previously inaccessible with conventional light microscopy. The data presented here and its associated analysis will be useful for the development of more detailed computer models of cardiac EC coupling that capture both the variability in cluster size as well as the proximity between adjacent clusters.

Acknowledgements

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# Tables

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Mean ± SEM</th>
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<tr>
<td>RyR cluster size (RyR), (4492 clusters measured)</td>
<td>62.8 ± 2.98 (13/3)</td>
</tr>
<tr>
<td>Cluster nearest-neighbour edge-to-edge transverse distance (nm), (4492 clusters measured)</td>
<td>139.53 ± 8.51 (13/3)</td>
</tr>
<tr>
<td>RyR density (RyR/µm³)</td>
<td>133.86 ± 7.02 (6/3)</td>
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<td>RyRs per supercluster</td>
<td>102.72 ± 3.54 (6/3)</td>
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<tr>
<td>Sub-clusters per supercluster</td>
<td>3.40 ± 0.73 (6/3)</td>
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<td>RyR cluster density/µm³, (n=5 cells)</td>
<td>2.21 +/- 0.30</td>
</tr>
<tr>
<td>Percentage of MF perimeter lined by RyR (%), (65 myofibrils, n=3 cells)</td>
<td>57.18 ± 8.58</td>
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</table>

### Table 1: Summary of measurements made from transverse dSTORM images of RyR labelling in rat ventricular tissue sections.

<table>
<thead>
<tr>
<th>Label A</th>
<th>Label B</th>
<th>% of A colocalising with B</th>
<th>% of B colocalising with A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>At 0 nm</td>
<td>At + 30 nm</td>
</tr>
<tr>
<td>JPH2</td>
<td>RyR</td>
<td>60.51 ± 1.47 (10)</td>
<td>75.34 ± 4.95 (10)</td>
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<tr>
<td>Mouse anti-RyR</td>
<td>Rabbit anti-RyR</td>
<td>72.40 ± 1.48 (5)</td>
<td>83.61 ± 1.80 (5)</td>
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<tr>
<td>Simulated m-RyR</td>
<td>Simulated r-RyR</td>
<td>78.10</td>
<td>86.39</td>
</tr>
</tbody>
</table>

### Table 2: Co-localising fractions from dual colour dSTORM images of JPH2 and RyR, mouse anti-RyR and rabbit anti-RyR and Simulated dSTORM images of RyR cluster labelling with Alexa 680 and Alexa 750. Stated values correspond to mean % ± SEM (number of cells analysed). For each analysis, the absolute overlap between the labelling of the two proteins (at 0 nm) and the overlap with the surrounding 30 nm band of a given region of the second protein (at 30 nm) are shown.
Supplementary Methods

Simulated Data

Two construct simulated dSTORM images a 2D binary mask of RyR clusters was generated by segmenting experimental transverse 2D images of RyR staining in a rat ventricular tissue section. Simulated dSTORM images were constructed from this mask by adopting a labelling density of 0.05 fluorophores per mask pixel (2x2 nm area). Based on experimental measurements, the mean background photon counts were set to 300 per pixel (since we are working in tissue with higher background counts) and 1300 photons per localised fluorescence event, each repeating an average of 1.5 times. These parameters recreate the photo-physics, time course and localisation accuracy of single molecule events observed in typical experimental data series acquired from tissue sections stained for RyR. The coordinates of the events detected from simulated data were rendered into a greyscale image using jittered triangulation rendering as described previously (D. Baddeley et al., 2010) and the images used for further analysis of co-localisation as described.

Generation of diffraction-limited images

Pseudo-confocal images were generated from super-resolution images by rendering each detected single molecule events as a Gaussian using the width and position parameters extracted from the fit (i.e. replacing each molecule with its fitted representation) and summing the images of all fitted molecules. The super-resolution data to generate simulated confocal stacks were obtained by modulating the z-position during acquisition of super-resolution sequences. We note that 2D super-resolution imaging has axial resolution similar to confocal due to the rapid decline of detection probability for single events with increasing defocus. We also filter detected molecules based on their fitted width, further adding to the sectioning ability.
Supplementary Figures

Figure S1: Binarisation of dSTORM super-resolution images. (A) A binary mask generated from a transverse dSTORM image of a myocyte within a rat ventricular tissue section stained for RyR. The mask was used as the assumed “true” cluster distribution from which a simulated dSTORM image was generated. (B) A simulated dSTORM image using an average marker density similar as in our experiments was used to generate events in positive mask areas. In addition, a nonspecific labelling fraction of 5% was added in the simulation to evaluate the effect of a weak non-specific background. (C) An intensity threshold (80% threshold, see Methods) was applied to the simulated dSTORM image. The threshold image robustly reproduced and captured the area and shape of the clusters underlying the simulation. Scale bar: 0.5 µm.

Figure S2: Histogram of the number of individual clusters in a RyR supercluster. For this analysis only non-trivial super-clusters were considered, i.e. super-clusters containing at least two sub-clusters. Fractions are given as percentage of all non-trivial superclusters.
Based on this data a non-trivial super-cluster contains on average 3.4 sub-clusters (see also Table 1, main text). Error bars indicate SEM (n = 8 cells).

Figure S3: Single receptor RyR clusters in rat ventricular myocytes. (A) Transverse dSTORM image of a myocyte within a rat ventricular tissue section stained for RyR. (B) Magnified view of the region indicated by the box in panel A illustrates RyR clusters that are >500 µm
in extent as well as regions of labelling corresponding to a single RyR (circled). (C) Overlay of transverse dSTORM images with a mask of α-actinin reveals that both the large clusters and most solitary receptors either line or are close to the edge of the myofibrillar (MF) and/or putative mitochondrial (MIT) spaces. Scale bars: A: 2 µm, B&C: 0.5 µm.

**Figure S4:** Simulation to investigate the effect of stochastic antibody binding and photoblinking of Alexa 680 and Alexa 750 fluorophores on co-localisation. A binary mask with an RyR-like cluster pattern was used to generate two independent sets of simulated images with foreground and background fluorophore densities typically observed in experimental Alexa 680 and Alexa 750 images. The cluster patterns in the simulated channels (A & B) are quite similar, as expected, but differ in small details (e.g. see arrow heads). C. The distance histograms show similar fractions of Alexa 680 and 750 labels overlapping with the other channel (78% and 80%, respectively, see Table 2 in the main text). Including the labelling in the surrounding 30 nm zones (light green shaded areas) improved the apparent co-localisation to 86-90%. 
Chapter 3

Nanoscale distribution of calcium release unit in rats, normal human and diseased human cardiac myocytes
**Introduction**

The understanding of cardiac disease function acts as a central driver in cardiovascular research. Although animal models have been extensively used to isolate and identify the various causal agents in disease manifestation, it is still however important to appreciate the at times significant differences in normal physiology and time frame of disease exist between animals and humans. To this end, use of human tissues directly provides the best understanding of human disease progression.

Due to the recent development of super-resolution imaging modalities, there has as of yet not been extensive studies into disease formation in human hearts using this technique. The current use of dSTORM and other super resolution setups have already revealed additional detail concerning the nanoscale features of calcium handling structures within rodent models (Y. Hou et al., 2014b; Y. Hou et al., 2015; I. D. Jayasinghe et al., 2012; I. D. Jayasinghe et al., 2014; C. Soeller & Baddeley, 2013; Wong et al., 2013). This is of particular importance as it has been shown that the nanometre detail of calcium regulating proteins is essential for correct cell function (M. B. Cannell et al., 2006).

This chapter therefore aims to use dSTORM techniques as demonstrated previously (Y. Hou et al., 2014b; Y. Hou et al., 2015) on human normal and diseased samples in order to identify possible nanoscale changes to the calcium handling proteins which may impact cell function. Specifically, the RyR protein will be examined in depth and comparisons with previous rodent data will be made to identify possible species variation in CRU organisation.

**Use of animal models for disease**

Important in evaluating the use of animal models for the understanding of normal and diseased cardiac function within humans is the need for an appreciation of the differences and similarities in physiology between the numerous animal models and humans.

**Benefit of animal models**

Animal models are currently one of the go to methods for studies into cardiac disease mechanisms. They possess numerous advantages when compared to human subjects. Other than ease of obtaining tissue, the ability to create genetic models and specific disease models allow for the close inspection of disease progression. Within cardiac science, animal models are currently being extensively used in the study of a plethora of different
pathologies including; ischemia reperfusion injury (Pedrozo et al., 2010; Zweier, Flaherty, & Weisfeldt, 1987), hypertrophic cardiomyopathy(Geisterfer-Lowrance et al., 1996), and dilated cardiomyopathy(Matsumori & Kawai, 1982). These models have significantly advanced understanding of different aspects of disease formation such as the progressive degeneration of the T-tubular system (Balijepalli et al., 2003; Lyon et al., 2009), and the disruption of calcium release leading to reduction in heart performance (D. M. Bers, 2008; van Oort et al., 2011).

Central to animal models have been the use of murine species. Both rats and mice are the predominant animal models in use today due to their readily available genetic information, short time to maturation, ease in creating genetic mutants, and while showing differences, being relatively similar in physiology due to their mammalian nature (Hasenfuss, 1998). The versatility means that murine models are typically the go-to models for disease progression and experimental drug/therapy intervention approaches (Sakai et al., 1996). The ability to create transgenic strains also makes them particularly useful for single gene knock-in or knock-out experiments typically with the goal to reveal the impact of single proteins on heart function (van Oort et al., 2011). Important of note is the relatively simplistic nature of many of these transgenic lines, where only single genes/protein expressions are typically altered.

The mammalian nature of the murine samples means that the majority of cardiac systems remain essentially the same in humans and murine models. Murine models also possess a similar four chamber arrangement of the heart with distinct separation of the atria and ventricles. Both species additionally show the presence of extensive conduction systems throughout the chambers (Miquerol et al., 2004). At the cellular level key proteins necessary in CICR remain functionally the same with major subcellular structures (such as the T-tubular system) also being present. Localisations of proteins also remain similar with the key CICR associated organelles such as the sarcoplasmic reticulum, and T-tubules localising along the z disc of the sarcomere (Forssmann & Girardier, 1970; Isuru D. Jayasinghe et al., 2014). Intriguingly, the similarities are present to a point where human stem cells can be grafted into rat infarcts to assist healing (Laflamme et al., 2005).
While many similarities exist between murine and human tissue, it is important to note that there are nonetheless many factors which differ between the species. Both physiologically and structurally there are differences which need to be accounted for in models of disease as possible confounding factors. Some of these factors are roughly outlined in the following sections.

Problems associated with animal models
Related to the differences between human and rats are the differences in lifespan and rate of disease progression that occur between species. Current animal disease models often employ genetically knocked in or knocked out models that have rapid disease onset (Hasenfuss, 1998; Pinto, Paul, & Ganten, 1998). While allowing more rapid experiment, this may not accurately mimic human disease which can take many years to fully develop. These models are typically used to model CPVT and associated genetic diseases, however even more heart failure simulating models such as trans-aortic constrictions typically have much faster disease onset than what is expected for humans. The typically late in life onset of human cardiac disease means that there are the additional confounding factors of decades of tissue wear, nutritional differences, and any prolonged medical interventions which are not present in rats.

Cellular variation between human and rat cardiomyocyte

T-tubular difference
The T-tubular system is a complex extension of the myocyte surface membrane which projects to the core of the cell to assist in conducting the action potential deep into the cell. This enables increased synchronisation of excitation contraction coupling (EC coupling) and calcium induced calcium release (CICR.)

There exists a wide variety of T-tubular morphologies. For example, we have shown a highly branched and thin T-tubular arrangement in murine models whereas confocal imaging of humans, pigs and horse myocytes have shown a comparatively simpler network yet much larger individual tubular dimensions (Y. Hou et al., 2015; Isuru D. Jayasinghe et al., 2014). Currently, there is extensive details concerning alteration in T-tubular morphology in in disease progression (Balijepalli et al., 2003; Heinzel et al., 2008; Louch et al., 2006; Lyon et al., 2009), with additional detail regarding variations across different species (I. Jayasinghe
et al., 2012; Isuru D. Jayasinghe et al., 2014). However, less detail is known regarding the precise functional effects of altered t-tubule arrangement in between species. In atrial cells, data have suggested an overall reduction in T-tubular network when compared with ventricular cells, with more recent evidence pointing to the presence of moderately extended networks in different species (Smyrnias et al., 2010). In ventricular cells development of T-tubules occurs late in the gestation period, or in the case of rats, in the postnatal period (Brette & Orchard, 2003).

**Ryanodine receptor cluster sizes**

In terms of raw numbers, Franzini-Armstrong et al provides the closest comparison of release cluster sizes through the use of thin section electron microscopy and the counting of feet observed within dyads (Clara Franzini-Armstrong et al., 1999). What they found when comparing different animals is that, in general, the sizes and density of rat CRU (~188 RyRs) tends to be considerably larger than what is observed in larger mammals such as dogs (~45 RyRs). Unfortunately, this study did not include human samples and relied on estimates on cluster geometry (assumed a circular dyad). Additionally, the small sample size of measurements (n = 30 clusters for rats) leaves considerable uncertainty. More recent estimates of mice couplon sizes (T. Hayashi et al., 2009) suggest ~43 RyR on average per cluster. From light microscopy samples, estimates of RyR cluster size in rats sits typically around ~74 RyR (Chen-Izu et al., 2006) with confocal microscopy and more recently we’ve estimated ~63 RyR using super resolution microscopy (Y. Hou et al., 2015).

Precise cluster measurements of human tissue is still lacking, it is expected that for humans a similar trend should be observed with larger mammals such as dog and rabbit. This is because the T-tubular and calcium release dynamics of the cells much more closely resemble these species (Hasenfuss, 1998; Isuru D. Jayasinghe et al., 2014). Confocal microscopy work on human tissues has shown that this is likely to be the case with greater RyR nearest neighbour separation in human tissue as compared with rat (C. Soeller et al., 2007). This results in an approximate 30% decrease in RyR labelling seen in humans as compared with rats.
Differences in calcium handling

In terms of overall cell calcium transient, there are considerable changes between the rodent species as opposed to human and other larger mammals. Alongside the T-tubular and release cluster changes, the overall calcium flux balance between external and internal calcium stores are different (D. M. Bers, 2000). In rat isolated cell experiments, it has been shown that approximately 8% of cell calcium flux occurs across the sarcolemmal or T-tubular membrane; leaving the remaining 92% of calcium from the internal sarcoplasmic reticulum stores (Bassani et al., 1994). This is in contrast to the larger mammals (in this example rabbit) which show approximately 70% from the SR and 28% from the NCX systems. Humans have similar flux balances to rabbits, though with even more bias towards the NCX pathway of 1:1 extracellular to intracellular (Pieske et al., 1999).

This difference appears to be related to an increased SERCA activity in rodent samples which is matched by a comparative decreased LTCC influx during activation. Influx and efflux must remain balanced to prevent net gain or loss of calcium during the contraction-relaxation cycle and there is evidence showing a greater $I_{Ca}$ for rabbit as compared with rats (Yuan, Ginsburg, & Bers, 1996). The origin of this increased SERCA activity is not clear, however, it may be due to an increase in protein levels thereby increasing efflux rate (D. M. Bers, 2000). For rats, the resting SR load is high which occurs due to a slowdown of NCX because of high cellular $I_{Na}$ (Shattock & Bers, 1989). When contraction resumes this leads to a larger initial contraction which slowly diminishes forming a negative treppe (staircase) response at low stimulation rates. At faster stimulation rates normal treppe response occurs.

Changes to calcium handling systems in diseased hearts

Dilated cardiomyopathy

Dilated cardiomyopathy (DCM) manifests as a dilation of a pumping ventricle (typically the left) leading to reduced cardiac function. It may lead to subsequent heart failure as the mechanical performance of the heart deteriorates (Roberts, Siegel, & McManus, 1987). The idiopathic form of DCM (IDC) has no clearly discernible cause and presents as a hypertrophy of the heart typically with both ventricular cavities expanded in volume. Progression occurs in a futile cycle where the mechanical stresses upon the heart from the enlarged chamber as a consequence of Laplace’s law leads to further dilation as the heart attempts to
compensate (Douglas, Morrow, Ioli, & Reichek, 1989). This dilation further increases wall stress and deterioration. While interventions such as beta blockers have been shown to increase function of the dilated heart (Bristow et al., 1994; Gilbert et al., 1990; Waagstein et al., 1993), it is often not enough to completely halt the progression of disease and heart transplants are often the only viable treatment.

Histological and biochemical studies have shown numerous changes which occur in producing IDC pathology (Brillantes, Allen, Takahashi, Izumo, & Marks, 1992; D. J. Crossman et al., 2011; Lyon et al., 2009). Typically different regions of the chamber will show different extents of functional decline (as measured in terms of shortening.) This feature of IDC has been well documented in many whole heart imaging studies such as echocardiogram (Bach, Beanlands, Schwaiger, & Armstrong, 1995), and tagged MRI imaging (A. A. Young et al., 2001). At the cellular level, changes to both T-tubular arrangement and calcium handling dynamics have been shown to be central in the eventual decline in cellular function (Balijepalli et al., 2003; Ibrahim et al., 2011; Lyon et al., 2009; Wei et al., 2010).

**T-tubular changes**

Changes to the T-tubular layout have been extensively documented in ICM and other cardiac diseases (Balijepalli et al., 2003; M. B. Cannell et al., 2006; A. Guo et al., 2013; Heinzel et al., 2008; Ibrahim et al., 2011; Lyon et al., 2009; Wei et al., 2010). Disruption of T-tubules has direct follow-on effects on calcium handling by changing the dyadic interface between the T-tubular membrane and the SR membrane where coupling between cell excitation and subsequent contraction occurs (He et al., 2001). Under normal circumstances these membranes are held at nanometre precision to enable the rapid coupling between calcium release from the l-type calcium channel (LTCC) and the calcium induced calcium release from SR via Ryanodine receptor channels (RyR) (M. B. Cannell et al., 2006; D. J. Crossman et al., 2011). The changes in T-tubular membrane structure include an increase in the overall width of the T-tubules, loss of T-tubular features within cells, and loss of T-tubular organisation. These changes may lead to alterations of RyR spatial arrangement and the overall cell calcium transient.

Evidence for the relationship between T-tubular organisation and corresponding shape of the calcium transients from within the cell have been tied in with numerous experiments
(Balijepalli et al., 2003; Beuckelmann, Nabauer, & Erdmann, 1992; Brette & Orchard, 2003; M. B. Cannell et al., 2006; Heizel et al., 2008; Sobie et al., 2006) showing that the close coupling of the T-tubular and SR membrane play an essential role in the control of calcium release within the cell. These changes have been identified as a reduction in peak transient flux, an increased duration of the transient, and an overall reduction in transient decay rate (Heinzel et al., 2008). This is likely to be associated directly with a corresponding decoupling of the RyR release sites from the T-tubular system leading to these receptors being reliant on calcium diffused from coupled sites for release by a mechanism known as fire-diffuse-fire propagation (Dawson et al., 1999) which also underlies the propagation of waves.

A method that has been widely used in the measurement of T-tubular regularity has been the application of Fourier transforms to quantify the loss of axial regularity of tubules (Wei et al., 2010). This method, coupled with direct imaging methods have enabled the characterisation of the extent of T-tubular loss and disorganisation. Within dilated cardiomyopathy, the progressive loss of T-tubules is correlated directly with the corresponding loss of heart function (D. J. Crossman et al., 2011).

The precise cause for the loss of T-tubular system is not well understood, however, Junctophilin (JPH) is implicated as a key protein associated with T-tubular loss. JPH is a membrane protein responsible for the close linking of the SR to the T-tubular membrane by directly bridging across the dyadic cleft (Takeshima et al., 2000). Experiments knocking down the expression of JPH in mice have produced results similar to what occurs during heart failure – an overall reduction in expression of T-tubules and a loss of RyR clustering within the cell (Garbino & Wehrens, 2010). Within human diseased samples, results tend to suggest a decrease in JPH2 protein expression (Landstrom et al., 2007; Minamisawa et al., 2004). There is in addition conjecture regarding the possible cleaving of JPH protein (C. Y. Wu et al., 2014). While this has yet to be tied in with disease models other than acute reperfusion, it may be possible that the cleaving of JPH leads to deregulation of SR – t-tubule linkage further hampering the calcium transient.

**Storage and release changes**

Occurring in parallel to the changes in T-tubular structure is a corresponding alteration in RyR function and SR calcium load. Hyper-phosphorylation of the RyR protein through
chronic beta-adrenergic activation has been documented in heart failure (Ai et al., 2005). The hyper-phosphorylated RyR is shown to be more calcium sensitive (Witcher, Kovacs, Schulman, Cefali, & Jones, 1991) thereby increasing the open probability at rest. This change then results in an increase of diastolic leak from the SR draining the SR and contributing to reduced cellular contractility (Shan et al., 2010).

Structurally, the dyadic clusters formed by RyR on the SR membrane have been shown to change with regards to its association with the T-tubular membrane (Song et al., 2006). One of the central observations is the maintenance of regular RyR arrangement (both longitudinally and transversely) while the T-tubular arrangement becomes disrupted. With the loss of regular T-tubular spacing, the arranged RyR channels become isolated and must function based on a fire-diffuse-fire scenario (Dawson et al., 1999).

Within the SR network, during IDC there are no significant shifts in SERCA expression (Schwinger et al., 1995). However, there is an overall decreased rate of reuptake. The origin of this appears to be in the change of ratio of phosphorylated and unphosphorylated forms of the regulator protein phospholamban leading to greater suppression of SERCA activity (Schwinger et al., 1999). Reduced SERCA2a activity then further exacerbates the already reduced SR load from RyR phosphorylation leading to continued reduction in SR calcium release during systole.

Unanswered questions

While there has been extensive use of rat models in investigating the progression of cardiac disease, less is known about the similarities and differences between subcellular protein arrangements when compared with humans. It has been implicated that subtle changes in cluster arrangement will result in alterations in cell calcium dynamics with smaller dyadic separation allowing better coupling of membrane depolarisation and SR calcium release (M. B. Cannell et al., 2006). As rats and humans possess different dependencies on SR and extracellular calcium sources (D. M. Bers, 2000; Pieske et al., 1999; Shattock & Bers, 1989), as well as extensively different T-tubular arrangements (Isuru D. Jayasinghe et al., 2014), there could likely be differences in nanoscale organisation of RyR into calcium release units.

While extensive research has been documented on the changes in regulation and phosphorylation of the ryanodine receptor protein as a consequence of heart failure (Ai et
al., 2005; Sobie et al., 2006; Witcher et al., 1991), structurally, less detail is known. Part of this gap in knowledge originates from the lack of nanoscale structure and which may nevertheless have significant impact on the overall calcium transient (M. B. Cannell et al., 2006). It is difficult to visualise these structures using conventional imaging methods. EM is able to provide high resolution images, however, the ability to label specific protein targets is limited (Clara Franzini-Armstrong et al., 1999). Confocal microscopy has been the main conventional method of imaging proteins (Balijepalli et al., 2003; Chen-Izu et al., 2006; C. Soeller et al., 2007). In the case of cardiac dyads however, it has been shown that the Abbe resolution limit produces images of clusters which lack detail (T. Hayashi et al., 2009).

The advent of super resolution microscopy with resolution beyond the diffraction limit down to single receptor channels provides a means of filling this gap in knowledge. Key factors such as changes in RyR – JPH relationship which have been shown to cause alterations in calcium release (Garbino & Wehrens, 2010; van Oort et al., 2011), and arrangement of the RyR both in terms of distance to adjacent clusters and raw cluster dimensions can also be explored in detail. Use of super-resolution microscopy has already been demonstrated in isolated cells of rat and mice, and was able to reveal the similarities between JPH and RyR distribution, as well as detailed cluster arrangements (Isuru D. Jayasinghe et al., 2014).

**Section aims**

This section aims to continue work from previous sections through the employment of the dSTORM imaging modality to investigate human healthy and diseased tissue to attempt to identify nanoscale structural changes in CRU arrangement. This section will also aim to further elucidate the relationship between JPH and RyRs in the context of human heart failure in order to understand if changes in JPH arrangement will impact the arrangement of CRU clusters.
Methods

Tissue preparation and extraction

All work on human tissues received prior ethics approval from the New Zealand Health and Disabilities Ethics Committee (Code: NTY/05/08/050) as well as the express permission of the donors and family. LV tissue from idiopathic dilated cardiomyopathy patients was obtained during cardiac transplantation surgery. Non-failing tissue was obtained from organ donor hearts that were deemed unsuitable for potential heart transplant recipients. Left ventricle samples were transferred to ice-cold cardioplegic solution and transported to the laboratory (~10 min) for processing. Tissue preparation and extraction of normal and diseased human heart samples from donors were carried out by David Crossman and Michelle Munroe.

Cut chunks are fixed in 2 % PFA for ~1hr at 4 °C before being cryoprotected through 10% sucrose grades until 30 % sucrose solution incubated overnight. Cryoprotected samples are then rapidly frozen in cryotubes placed in liquid nitrogen cooled isopentane to enable rapid sample freezing. The samples are stored at -80 °C until used for sectioning. Tissues are then cryosectioned in a Leica CM3050 cryomicrotome. Sections are imbedded in Tissue-Tek® O.C.T. Compound frozen to -25 °C and cut to a thickness of 10 micron ± 2 micron before being adhered to the surface of prepreared poly-l-lysine coated coverslips. Cut coverslip sections were stored in a slidebox within a -80 °C freezer until use in immunohistochemistry.

Immunolabelling

All procedures are carried out at room temperature within a humidified chamber unless otherwise specified. Slides are first hydrated for 15 minutes using 200 µL PBS before permeabilisation with 100 µL 1% triton x-100 in PBS for 15 minutes in order to expose additional antigen binding sites and increase the ability of primary and secondary antibodies in accessing epitopes. The triton solution is then washed off with 200 µL PBS and subsequently excess PBS was removed. Blocking was carried out through one drop of Invitrogen Image IT FX signal enhancer on the coverslip incubated for 1 hour. The blocked coverslips are washed with 200 µL PBS and incubated for 20 minutes before having excess removed and primary antibodies applied.
Primary antibody concentration varied depending on protein target for labelling. All primary antibody stocks are prediluted 1:1 with glycerol to prevent freeze thaw cycles. Ryanodine receptor antibodies (Thermofisher MA3-916) were diluted at 1 in 100 with bovine serum albumin (BSA) solution composed of 1% BSA, 0.05% Triton X-100, and 0.05% NaN₃, Junctophilin antibody (Yenzym YZ2635, YZ2636 custom made) was diluted at 1 in 100. T-tubule cocktail labelling comprised of 1 in 100 Caveolin-3 (BD Transduction 610420) and 1 in 100 Sodium calcium exchanger (Swant R3F1) antibodies. 70µL of diluted primary antibody solution was added to the sample and incubated overnight at 4°C. Samples then had excess primary antibody solution removed and were washed 3 times with 200 µL PBS incubating each time for 20 minutes. For dual labelling of samples of RyR with JPH, both antibodies are diluted and added together.

Secondary antibodies are then applied at 1 in 200 dilution. In dSTORM imaging, Alexa Fluor 680 conjugate (mouse; Invitrogen A21058 rabbit; Invitrogen A21109) is used for the primary colour channel and Alexa Fluor750 (mouse; Invitrogen A21037 rabbit; Invitrogen A21039) is used for the second channel when required. In dSTORM samples, phalloidin with Alexa Fluor 488 conjugate (Invitrogen A12397) is added as a third colour. This is for identification of targets and tissue orientation by eye as the far red colours are barely visible to the human eye. Excess PBS was removed and 70μL of diluted secondary antibodies were added to the coverslip. Samples were then incubated for 2 hours and washed three times with 200µL PBS for 20 minutes. After the final wash, samples are stored at 4°C until mounting and imaging.

**Mounting**

Samples are mounted in specialised dSTORM mounting media made on the day of imaging comprised of 90 % glucose solution mixed with 10 % 100mM Mercaptoethanol (MEA) in 10X PBS for a reducing mountant needed for switching. Due to the viscosity of the 90 % Glycerol solution, the MEA solution is mixed on a rocker table for 1 hour. Excess PBS is first removed from the slide to be imaged, before applying 7 µL of mixed mountant on top of the tissue. The slide is then placed on top to spread the mountant between it and the coverslip making sure air bubbles are not present. The slide is sealed with nail polish and imaged.
Imaging

The dSTORM microscope is a customised Nikon Ti Eclipse TE 2000 inverted TIRF fluorescence microscope. In order to accommodate for the higher level of precision required in super resolution imaging, the objective turret was replaced by a high precision piezoelectric focusing motor with a motion range of 400µm in the lateral axis. Stage movement is controlled by dual axis electronic stepper motors under joystick control. Imaging objective used was a 63X oil immersion NA 1.49. A pressurised air table is used to reduce vibration and drift. Processing of acquired images and control of camera frame rates and acquisition settings were done via the Python Microscopy Software suite by David Baddeley (https://bitbucket.org/david_baddeley/python-microscopy). The acquisition camera system is an Andor iXon (DV887DCS-BV) cooled EMCCD 512X512 pixel camera. For dual colour imaging, a splitter system is in place before the imaging camera; breaking the imaging field to two equal 256 X 512 pixel fields. This is a much simpler design when compared with dual camera systems as complexities associated with alignment of focus, region of interest, and detection gain between cameras can be avoided, instead mismatch is typically small and is corrected through the imaging of a calibration bead sample, as detailed below.

A shift field is acquired prior to the imaging procedure for the matching of the two sides of the splitter system, and consisted of a fluorescent bead slide illuminated evenly across the field of view. The bead density of the slide is such that they are still identifiable as distinct individual beads. While imaging, the slide is moved at set amounts around the field of view so that in the final stack, there is a high density of beads at different positions imaged across the sequence. The beads are then correlated between the upper and lower halves of the split sensor and localised. The mismatch of localisation between the upper and lower halves of the splitter is then used to create a residual map of the shift between the different halves of the imaging field. This is then applied to images to match up labelling from the two halves of the splitter.

To inspect sample location and orientation, a mercury arc lamp filtered fluorescent illumination is used. Filters include 488, 568/594, 680 and 750 excitation wavelengths. Switching between filters is facilitated by a rotating filter wheel. For imaging, a 671nm Viasho 800 mW laser is used for primary illumination. Laser intensity at the start of imaging...
is tested and should be within the range of 250-300mW at the entry to the illumination light path. Some attenuation is expected by the numerous filters present before being focused onto the sample.

To acquire an image, a suitable region within the sample was first located using the same criteria as outlined in the confocal section. The laser illumination however, is not uniform across the entire 256 x 512 pixel imaging field (due to the Gaussian beam profile that can in principle be eliminated by over-expanding the beam; significant over-expansion, however, would have reduced the illumination intensity too much) and so a smaller region of interest inclusive of only the higher intensity laser illumination area is selected. A typical ROI contains an approximate imaged region of 12 X 12 µm encompassing the majority of a single myocyte in cross-section. Individual frames of the acquisition sequence had typically a 50 ms integration time. The imaging protocol involves an initial bleaching period of ~6 seconds where the majority of fluorophores on the sample is pushed to their dark states. During this procedure, the electron-multiplication of the camera is switched off to minimize EMCCD gain aging (Sharma, 2006). After the bleaching period, the gain is switched to the standard electron multiplication factor (~30x) and sequence acquisition begins.

Cells are selected based on the presence of T-tubules and the transverse nature of their arrangement. Focus of the stage is shifted through the focal range to determine if the alignment is near perfectly transverse. After a cell is selected the focus is then moved through until the T-tubules are in focus.

Typical acquired sequences range between 20000-40000 individual frames lasting between 10-20 minutes in total. Images sequences are first stored in raw form as an HDF5 file containing all individual frame data plus additional acquisition metadata. The images are then analysed by the PYME software to localise the individual events. Each individual localisation contains information relating to the single event that it was localised from including: amount of photons released, duration of event, dimensions of the fluorescent blink (based on the fitted Gaussian) and the localisation precision in both X and Y directions. From this information, additional filtering is done on the points so that events that do not fit the expected output criteria are eliminated.
To compensate for drift accumulated in the 20 minute imaging duration, post imaging drift correction is applied through the use of 5 point piece wise linear fitting of a small region within the imaging data. Images with excessive drift which could not be adequately compensated for are eliminated from subsequent analysis.

**Image processing**

Image processing is carried out primarily through the PYME acquisition software and ImageJ. Additional processing was performed with custom written Python scripts using the numpy and SciPY Python modules available for data analysis.

Raw images are first rendered though PYME into event density based images (D. Baddeley et al., 2010). These images are then used for subsequent analysis. Cluster size data is obtained through ImageJ’s particle analysis software. As dSTORM data may contain spurious density peaks from nonspecific labelling, a threshold on minimum cluster size was placed to prevent analysis of these features. In the images taken, the threshold was set as the area of 4RyR molecules (3600 nm²). Cluster size data is output as a text file and read via excel for further processing. Cluster sizes are binned and statistical analysis (T-test unequal variance) and plotting was then carried out for human samples vs rat samples and human normal vs human disease samples.

Nearest neighbour analysis is carried with custom written Python scripts. Clusters are first identified by the script before region growing though Euclidean distance mapping of the non-cluster region. This then enables the establishment of distances to adjacent labels with the nearest cluster being identified and the distance to it measured. These distances are then compiled for all clusters within an image and analysed.

Colocalisation is carried out through the colocalisation feature from the PYME microscopy suite. A mask is created of one channel and the distance of labelling of the opposing channel which fits into the mask is calculated. This data is then binned and presented as a frequency histogram. Overall colocalisation fraction is presented as the percentage of labelling which is within the mask. A reverse colocalisation is also carried out where the role of the mask and label between the two channels are reversed. This analysis was done on human normal, human diseased, and rat samples and compared.
Measurement of t-tubule data was carried out in ImageJ. Line plots were made along random segments of T-tubules. The plotted intensity at the given point of a line is calculated as the average of a line of 50 nm wide. The resulting measurements of the FWHM are compiled and analysed for changes between normal, rat and diseased samples.
Results

Comparison of Rat and human samples

Transverse imaging
Images presented in these results were obtained in a transversely oriented position where the focal plane is perpendicular to the long axis of the cell. Transverse sections provides a full in focus view of the Z line visualising the majority of RyR release clusters (which localise to the Z disc.) This enables the higher lateral resolution to show the tight separation between transverse clusters as well as the arrangement of the T-tubular system which show the highest density around the Z disc.

T-tubular morphology

![Figure 1: Comparison of t-tubule dimension in humans and rats. (A) Show a t-tubule cocktail (combination of Caveolin 3 and NCX antibody labels) labelling of human cardiac myocytes in transverse orientation. (B) Equivalent labelling in in rat myocytes. (C) Numeric comparison of t-tubule diameters between human and rat samples. Human samples show a higher mean diameter compared with rat samples whose diameter falls below the diffraction limit. Scale bars 2µm.](image)

As expected, the greatest difference was observed in T-tubular structure (Figure 1) where rats typically showed extensive network lattice of T-tubules whereas the human samples showed a more spoke like pattern radiating towards the edges. Compartment wise, the rat T-tubular system’s network appears to associate around the negatively labelled myofibrillar bundles (Figure 1B).

T-tubule dimensions also appeared to differ significantly; human tubules typically appear thick with a mean diameter of 373 nm ± 27 nm (Table 1, Figure 3C). Rat tubules show reduced diameter compared with human samples with a mean thickness of 82nm ± 6nm.
(Table 1, Figure 1C). Interestingly, due to the size of the human T-tubular network, luminal details could be observed with distinct separation of the two opposing sides of the tubule being visible (Figure 1A). Important to note is that junctional regions as seen in rat samples appear to broaden to accommodate for the dyad link with the SR that is around 200 nm (Figure 3B). This feature was not observed in human samples.

**Figure 2:** Comparison of human and rat T-tubular thicknesses in junctional and non-junctional regions. (A) Shows t-tubule cocktail (green) (non-junctional) and JPH (red) (junctional) labelling of transverse cardiac myocytes. (B) Comparative image of A in rat cardiac myocytes. (C) Bar graph of label thickness as FWHM between T-tubular and junctional regions in human cardiac myocyte. (D) Comparative bar graph for rat cardiac myocytes showing a greater difference in thickness between regions.
Interestingly, there appears to be regional variation in relative T-tubular thickness which is different between human and rat samples. Most prominently within rats, (Figure 2B) regions showing junctional labelling (in this case JPH2) tended to be thicker (mean 162 nm ± 18 nm n = 15 measured sites) than the comparative non junctional region (mean 74 nm ± 7 nm n = 15 measured sites). This change was further statistically calculated to be significant (p = 0.0001.) This alteration is not found in human samples (Figure 2C), where junctional regions showed (mean 382 nm ± 30 nm n = 11 measured sites) and non-junctional regions showed (mean 368nm ± 29nm n = 11 measured sites.) In terms of labelling, both species were labelled with a labelling cocktail of antibodies against NCX and Caveolin 3 and junctional studies were labelled with the JPH2 protein.

**RyR Cluster differences**

Mean RyR cluster sizes are striking similar in both rat and control human cardiac myocytes with a mean of 63 ± 3 (Y. Hou et al., 2015) and 59 ± 7 RyR per cluster respectively. Within the distribution there is little variation between the two species with no range showing significant variation compared with the other. Distribution shape also was similar with both showing an approximately exponential distribution exhibiting a majority of smaller cluster sizes (Fig 1 C,D). The range of values remained also approximately the same for both species with between 1 and ~800 RyR per cluster.

In the nearest neighbour distance measurements, again both species showed a very high level of similarity (Fig 1E, F). Mean neighbour distances were near identical with a mean of 139 nm ± 15 nm for both rat and human samples (n = 6 cells from 2 hearts for humans n = 13 cells from 3 hearts for rats) (Table 1) – a value below the diffraction limit for standard confocal microscopes. The distribution for both species again remained similar with the modal distance around the 50 nm bin. This distribution then tapers off as the distance bins increased with the vast majority (90%+) of all clusters sitting within 500 nm or less of the closest adjacent cluster.
Figure 3: RyR cluster distribution in Human and Rat cardiac myocytes. A dSTORM image of healthy human donor myocytes labelled for RyR in transverse orientation.. (B) RyR labelling in transversely oriented rat cardiac myocytes. (C) Shows the human cluster size distribution as a frequency histogram and cumulative frequency histogram (insert). (D) shows the cluster size distribution.
distribution in a frequency histogram and cumulative frequency histogram (insert) for rat cardiac myocytes. (E) Shows the nearest neighbour distance frequency histogram for human cardiac myocytes. (F) Nearest neighbour distances plot for rat cardiac myocytes. Scale bars - 2µm insert – 0.5µm

In the observations of release cluster morphology (RyR labelling), we found the labelling to be broadly comparable between rats and humans. In the rat samples, clusters tended to distribute similarly and homogenously around the myofibrils. Within human samples, there appear to be two distinct groupings of the calcium release clusters split between coupled sites where the cluster borders the T-tubular system (double flanking seen in figure 3A inset) and apparent decoupled sites where the cluster appears to sit away from a T-tubular membrane (region below inset box Figure 3A).

**Colocalisation**

Junctophilin labelling was comparatively more variable in human samples compared to rat. Rat JPH typically followed very closely the cluster arrangements of the RyR with both labels appearing in close proximity (figure 4B). Within humans, JPH distribution is focused primarily around the T-tubules and sarcolemma, showing comparably higher event rates and density in relation to RyR (figure 4A, table 1). Within the interior and non T-tubular sections, the comparative JPH labelling is decreased.

More apparent differences are present when comparing colocalisation of RyR and JPH with the rat samples in general showing higher colocalisation rates when compared with human samples. For percentage RyR on Junctophilin the results showed an average of 42.3% ± 1.86% for human samples and 61.2% ± 1.58% for rat samples (table 1). For percentage JPH on RyR we found and average of 34.3% ± 1.45 for human samples and 60.5% ± 1.47% for rat samples(figure 4 E,F). This difference is most apparent in the colocalisation distance distribution plots (figure 4 C,D) where rat samples show a sharp fall off from the 0nm distance to the larger ranges while the human samples demonstrate a much longer and gradual positive tail for labels.
Figure 4: Analysis of colocalisation between RyR and JPH in human and rat cardiac myocytes. (A) dSTorm image of RyR (red) and JPH (Green) in transversely orientated healthy donor myocyte (B) dSTORM image of JPH (green) and RyR (red) in transversely orientated rat cardiac myocyte. (C) colocalisation of JPH on RYR plotted as a frequency histogram showing distance of label from the RyR mask for rat (orange) and human (blue) cardiac myocytes. (D) JPH – RyR colocalisation plot for rat (orange) and human (blue) cardiac myocytes.
myocytes. (E) Bar graph showing changes in JPH on RyR colocalisation between human and rat samples. (F) Corresponding bar graph of RyR on JPH colocalisation between human and rat sample. Scale bars: 2µm

Comparison of Human healthy and diseased samples
Following comparisons between rat and human myocytes, focus was placed on investigating the changes associated in normal functioning human myocardium and heart failure myocardium.

Cluster morphology changes
Subtle cluster arrangement differences were observed between normal and diseased samples. Most notable is the apparent reduction seen in RyR event density - in the data presented in (figure 7) as normalised against JPH labelling (panel E) which western blots suggest remain unchanged - resulting in reduced event density of super resolution imaging (table 1). Because of the loss of T-tubular coverage of the cell, it also appears that there are less coupled receptors in relation to the uncoupled receptors found between T-tubules as evident in the changes in colocalisation presented in (Figure 5). There are additionally no readily apparent changes seen in surface labelling.

Measurements of release clusters centred on the same parameters as undertaken in the previous section. Summary of this data is shown in Figure 5 and Table 1. In terms of mean cluster size surprisingly there is very little change. A mean shift of approximately 8 RyR from normal (59 ± 7 for normal vs 51 ± 8 for diseased) is observed. When viewed as a complete cumulative distribution of sizes, there is again little difference (Figure 5 C,D). The 15% difference observed in the raw average is not evidently clear. Both samples follow the same roughly linear logarithmic distribution with slight weighting on the smaller cluster sizes. Intriguingly, in the diseased sample there is a slight decrease at the extremities with proportionately lower amounts of very small (<20RyR) and very large (>500RyR) clusters. When this is viewed as a cumulative line plot, the results show that there is a faster accumulation yet slightly slower levelling off of the normal samples as compared with diseased samples (Figure 5 C,D inset). The crossover occurred at approximately 140 RyR sized clusters. This indicates that there is a higher proportion of midrange clusters in diseased samples.
Figure 5: Comparison of Normal human and diseased human RyR cluster distribution in transversely oriented cardiac myocytes. (A) Healthy donor human myocytes showing the typical distribution of clusters around possible t-tubule structures and myofilaments. Arrows point to sites of single receptor clusters. (B) Diseased sample distribution of RyR arrows pointing to single receptor clusters. (C) Cluster distribution as a frequency histogram and cumulative frequency histogram (insert) of RyR cluster size. (D) Comparative size distribution in diseased cardiac myocytes. (E) Shows the nearest neighbour distribution for
normal samples. (F) Shows the comparative distribution in diseased samples. Scale bars - 2µm insert – 0.5µm

The density of clusters is deduced from the amount of clusters over the total cell area. From normal and diseased tissue, the results showed an overall reduction in the diseased samples cluster density (2.36 clusters/µm ± 0.38 in healthy vs 1.82 ± 0.24 clusters/µm in diseased; n = 2 heart; 6 cell each.) To ensure that this does not simply mean cluster sizes were enlarged in the diseased samples the overall RyR coverage of the Z disc was also assessed by dividing the area of RyR label with total cell area. The results showed that in this case there was again a significant difference (P = 0.02) between normal and diseased with normal samples showing an average of 13% against 7% for diseased samples (table 1).

After identification of the changes in size distribution, nearest neighbour measurements were taken. Again, there was a significant difference between normal and diseased samples with normal cells showing on average closer association between identified clusters (139nm for normal cells against 201 nm for diseased cells; P = 0.017; n = 2 heart, 6 cells each) (figure 5E,F). In the distribution of nearest neighbours the results found that while both samples remained heavily biased towards close edge to edge distances, with modal values for both samples being the 25 nm bins, there is a comparatively reduced fraction of diseased cells that falls in this range. The normal samples show an increased fraction of the lowest 5 bins when compared with diseased cells with the difference being more prominent as the distances got smaller (at the lowest 25 nm bin the difference is 16% in normal against 12% in diseased)(figure 5 E,F). After the 150 nm bin, the bins begin to favour diseased samples with higher fraction of diseased compared with normal for all but 2 of the remaining bins. This finding coupled with the previous density findings in table 1 to suggest there is an overall reduction in RyR 2 expression in diseased when compared with normal.

**Colocalisation changes**

JPH disruption in comparison with RyR disruption is much more extensive. Though some clustering at focal points is seen, in general because of the quantity of non-focal labelling, they appear to be more diffuse. This loss of JPH clustering is reflected in the amount of overlap with the RyR label. Comparatively, less association between the labels seems to occur (figure 6 A,B). Whereas in normal samples focal points of JPH are mirrored by focal
points of RyR labelling along the T-tubular wall (i.e. co-clustering), appears reduced in diseased samples.

**Figure 6:** RyR and JPH colocalisation in normal donor and failing human cardiac myocytes. (A) Shows dual colour image of RyR (red) and JPH (Green) labelling in normal donor cardiac myocyte. (B) Shows the corresponding labelling in diseased myocyte. (C) Colocalisation plot of JPH on RyR labelling for normal human (orange) and diseased (blue) samples. (D)
We found that there were subtle changes to colocalisation between normal and diseased samples. Interestingly, in terms of RyR colocalising with JPH diseased samples showed a very slight increase in colocalisation fraction (46.3% ± 1.33% in diseased vs 42.3% ± 1.86% in normal cells. n = 3 images each) JPH colocalising with RyR however showed a decreased colocalisation fraction in diseased as compared with normal cells (34.3% ± 1.45% in normal vs 27.3% ± 4.26% in diseased n = 3 hearts; 3 images per heart each). This data seems to broadly agree with the observation of decreased clustering of JPH labelling in favour of greater labelling uniformity. When viewed as a distribution, the differences are primarily seen as an extended tail for non colocalised bins. Overall for both samples, RyR colocalising with JPH tended to show higher fractions than JPH on RyR, and this can be seen reflected on the colocalisation plots where RyR colocalisation graphs show a tighter clustering around the zero point as compared with

T-tubular changes

Qualitatively, there were apparent changes in the overall morphology of myocytes. There results show a possibility of small changes in T-tubular morphology, specifically possibly greater overall thickness in diseased cells (Figure 7 A,B). This finding however is not overtly clear and statistically is not significant from the limited data set used. Other papers concerning this however also show similar changes in T-tubular morphology from normal to diseased cells (A. Guo et al., 2013; H.-B. Zhang et al., 2013). Transversely, particularly severely affected cells show nearly no presence of T-tubular systems with those that do showing highly irregular T-tubules with variable lumen sizes and complex turns. However, the specific selection of cells for imaging targeting those with prominent T-tubules for visualisation often excluded such cells from the analysis.

Most prominent in the observed T-tubular changes is the variation in T-tubular thickness. Whereas normal tissue remains quite uniform across all hearts with T-tubular dimensions of 373nm ± 27nm (n = 3 images; 27 sample points) in diameter, correspondingly, the diseased sample show and average diameter of 414nm ± 41nm (n = 3 images; 27 sample points) (Figure 7C, Table 1). In terms of labelling, the T-tubular cocktail again remained effective for
both sample types as well as WGA. Sarcolemma intensity also for the most part remained the same though the cocktail labelling of the ECM appeared somewhat increased in diseased samples (Figure 7). The most noticeable change observed between normal and diseased however is the increase in background labelling density.

**Figure 7**: Showing T-tubular distribution differences between normal and diseased samples. (A) Shows the T-tubular arrangement in normal cells labelled using t-tubule cocktail (combination of NCX and Cav3 antibodies.) (B) Corresponding image of (A) for diseased cells. There is an increase in background labelling, and increased turning and twisting of the T-tubules. The tubular diameters appear to be more varied with fluctuations in thickness along the length of a tubule. (C) Plot showing the differences in T-tubular diameter between normal and diseased samples. There is a trend towards thicker tubules in diseased samples along with an increase in thickness variation. Scale bars 2µm

**Numeric Results**

Finally, in relation to the observation of changes in the RyR and JPH dynamic, a semi-quantitative method was used involving identified single molecule events in the super-resolution data to determine changes in relative labelling of RyR and JPH (Figure 7) which we would expect to reflect changes in protein expression levels. By utilising the total amount of RyR labelling and expressing it as a fraction of the amount of JPH labelling, images were standardised to show the relative expression levels. The results showed a significant reduction in expression of RyR relative to JPH of 0.13 for diseased versus 0.22 for normal samples (n = 3 hearts each, 9 images; p = 0.04) further supporting a likely reduction in RyR expression in diseased cells.

Western blotting results provided by David Crossman show a similar trend to RyR JPH event data ratios. JPH showed relatively little change in fluorescence value normalised to total cell protein between normal and diseased cells (Figure 8). RyR labelling showed a significant decrease in protein levels in diseased cells as compared with normal cells (P = 0.005).
Figure 8: Showing quantitative analysis of western blot and event data. (A) RyR fluorescent Western blot run by Xin Shen and David Crossman labelled with Anti RyR antibody. Bands from left to right show ladder, 3 normal samples and 8 diseased samples. (B) JPH fluorescent Western blot run by Xin Shen and David Crossman labelled with Anti JPH2 antibody. Bands from left to right show ladder, 6 normal samples and 6 diseased samples. (C) Close up of filtered events in high density labelling region of JPH (red dots) and RyR (green dots) for normal human tissue and diseased human tissue. (D) Bar graph of relative protein fluorescence for RyR fluorescence western blots in normal and diseased human samples.
Fluorescent values are normalised to total protein content. (E) Bar graph of relative protein fluorescence for JPH fluorescence western blots in normal and diseased human samples. Fluorescent values are normalised to total protein content. (F) Bar graph of event count ratio for normal and diseased samples. Event counts are calculated though Total cell RyR events/Total cell JPH events. Scale bars: 50nm

Table 1: Numeric data comparing key T-tubular and RyR release cluster parameters between normal rat, human, and diseased human samples.

<table>
<thead>
<tr>
<th></th>
<th>Humans</th>
<th>Rats</th>
<th>Human Diseased</th>
</tr>
</thead>
<tbody>
<tr>
<td>TT Diameter</td>
<td>373nm ± 27nm</td>
<td>82nm ± 6nm</td>
<td>413.6nm ± 40.7nm</td>
</tr>
<tr>
<td>TT Branching extent</td>
<td>Low</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>RyR Coverage at Z disk</td>
<td>13.07% ± 3.50%</td>
<td>7.88% ± 1.00%</td>
<td></td>
</tr>
<tr>
<td>RyR Cluster density at Z disk</td>
<td>2.36 ± 0.38 µm⁻²</td>
<td>7.88% ± 1.00%</td>
<td></td>
</tr>
<tr>
<td>RyR Cluster mean size</td>
<td>59.05 ± 7 (SEM) RyRs (6/2)</td>
<td>62.8 ± 2.98 (SEM) RyRs (13/3)</td>
<td>51.95 ± 8.42 (SEM) RyRs (6/2)</td>
</tr>
<tr>
<td>RyR nearest neighbour</td>
<td>139.15nm ± 14.63nm (6/2)</td>
<td>139.53nm ± 8.51nm (13/3)</td>
<td>201.90nm ± 20.60nm (6/2)</td>
</tr>
<tr>
<td>JPH on RyR Co-localisation</td>
<td>34.33% ± 1.45% (n = 3)</td>
<td>60.51% ± 1.47% (n = 10)</td>
<td>27.33% ± 4.26% (n = 3)</td>
</tr>
<tr>
<td>RyR on JPH Co-localisation</td>
<td>42.33% ± 1.86% (n = 3)</td>
<td>61.22% ± 1.58% (n = 10)</td>
<td>46.33% ± 1.33% (n = 3)</td>
</tr>
<tr>
<td>Ratio of JPH labelling to RyR</td>
<td>0.223 ± 0.030</td>
<td>0.129 ± 0.029</td>
<td></td>
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</tbody>
</table>
Discussion

Comparison of human and rat cardiac myocytes

Calcium release clusters

Our results showed that there is very little difference in the size and cluster distances between rat and human samples. The highly similar distribution shape as well as the relative frequency at each binned size suggests that in terms of function there is little change. It is however well known that there are distinct differences in calcium currents between rat and human myocytes (D. M. Bers, 2008), specifically a greater contribution of the extracellular calcium pool in humans as compared with rats. In addition to this, the exponential distribution of release cluster sizes has previously been suggested as being indicative of non-targeted association into clusters (D. Baddeley, Jayasinghe, Lam, et al., 2009). Interestingly, additional complexity of the dyadic spacing has been shown to exist in rat cardiac myocytes with complex folding of membranes present (T. Hayashi et al., 2009). Such membrane folds could not be seen within our dSTORM images which may result in underestimating sizes of rat release clusters.

There are also similar distributions in the nearest adjacent neighbour distance. Both rat and humans samples show a net separation of approximately 130nm with distributions also highly similar. As previously discussed (D. Baddeley, Jayasinghe, Lam, et al., 2009; Y. Hou et al., 2015), clusters which reside in close proximity to each other may exhibit functional coupling where activation of both clusters occur when one is stimulated. The similar neighbour distances between species suggests this level of functional coupling may be preserved between species. >90% of all clusters for both species additionally sit within 500nm of the nearest adjacent neighbour (Figure 1 C,D).

Previous work has identified within diseased hearts the presence of “orphaned” RyRs which are decoupled from T-tubular activation (Song et al., 2006). Our results have shown that non T-tubular coupled receptors within human are also a feature of healthy cells (Figure 1A). As these clusters are not associated with the T-tubules directly, their activation during excitation contraction would rely purely on fire diffuse fire scenarios (Dawson et al., 1999). In such instances their activation will become heavily dependent on the distance to adjacent clusters.
The increased reliance of human cells on fire-diffuse-fire mechanism of calcium release is likely then associated with the apparent upper limit for nearest adjacent cluster separation of 500nm. It may indicate the maximal range of separation between clusters before the fire diffuse fire mechanism of activation is no longer possible or occurs at too long of a time frame (Dawson et al., 1999). This feature however is shown to also be present in rat myocytes where the extensive t-tubule layout should mean reduced dependence on fire-diffuse-fire mechanisms. Therefore there may be further unidentified physiological constraints on cluster spacing.

**JPH colocalisation**

In addition to viewing the CRU in terms of RyR release sites, the relationship between JPH and RyR was also examined. JPH acts as the primary structural linker of the t-tubule to the SR membrane (Takeshima et al., 2000) and as such, its presence within RyR clusters assists in maintaining the nanometer separation between the T-tubular and SR membrane. As small alterations to the dyadic cleft will result in significant changes to the overall CICR performance (M. B. Cannell et al., 2006), it is important to understand the relationship between JPH and RyR. To study this relationship, our experiment employed the use of colocalisation analysis to identify how closely these proteins associate. Colocalisation is already widely used in diffraction limited studies (D. J. Crossman et al., 2011) and is also relied upon for this study where it was expected that the increased resolution of dSTORM will enable the detection of more subtle changes in the spatial relationship of these two proteins.

Within rats and other rodents, JPH shows a high level of colocalisation with RyR. In our rat sample analysis we found that there was a level of colocalisation between the two labels of 60%. In human samples, the expression of JPH in relation to RyR is different. The overall cell wide colocalisation is significantly lower than what is seen in the rat at 42% for RyR on JPH and 32% for JPH on RyR. As both rat and human samples were from healthy myocytes, the differences in JPH colocalisation are related to species variation rather than pathology.

Little literature exist documenting JPH RyR association difference between human and rat samples seen in this study. However, the understanding of these differences is important to appreciate when using animal models. What literature is available typically is oriented
towards disease states where it has been found that loss of JPH is both shown to occur in cases of hypertrophic and dilated cardiomyopathy (Landstrom et al., 2007; Minamisawa et al., 2004) for humans and cellular hypertrophy in rats (Garbino & Wehrens, 2010; Landstrom et al., 2011; C. Y. Wu et al., 2014). From this evidence, we conclude that JPH regulation is essential to normal cellular function. It is possible that JPH association with RyR is reduced in humans due to the decreased quantity of T-tubular associated RyR clusters. One way of testing this hypothesis would be to segment T-tubular regions and non T-tubular regions and analyse these separately. This additional analysis has yet to be completed, but is planned for future work.

T-tubular Structure

The T-tubules play a central part in overall excitation contraction coupling and have been implicated as a major point of remodelling in the disease process (Isuru D. Jayasinghe et al., 2014). Our results show that across species there is considerable variation in T-tubular structure with rodents such as mice and rats possessing much denser network like arrays of T-tubules as opposed to the sparser but thicker human tubules. Previous rat t-tubule estimates suggest a range of between 20 and 450nm thicknesses for rats (Brette & Orchard, 2003; C. Soeller & Cannell, 1999) however, recent findings in mice suggests that the thickness of the T-tubules vary depending on whether it is at the junctional interface with the SR or in the non-junctional regions (Wong et al., 2013) with junctional regions being comparatively thicker than non-junctional regions. This is also apparent in rat samples we’ve observed previously (Y. Hou et al., 2015). This is in line with our results obtained showing an average of roughly 80nm for cav-3 NCX combination labelled rat T-tubules.

Human T-tubules are comparatively thicker than rat T-tubules (Isuru D. Jayasinghe et al., 2014) and are more akin to those in cells from other large mammals such as sheep and rabbits. The relationship between different species in terms of T-tubular arrangement and EC coupling is still not fully understood. However, there is a correlation between T-tubular arrangements and cell calcium dynamics. Mice and rats typically show a reduced dependence on extracellular calcium with NCX efflux of calcium accounting for ~8% of total calcium whereas humans and rabbits typically show greater levels of dependence ~28% of NCX efflux (D. M. Bers, 2000). It is possible that the larger lumen of the rabbit and human T-
tubules may play a role in the greater NCX efflux alongside protein expression, however, the link between these is still not readily clear.

Interestingly, the variation in T-tubular size between humans and rodents results in a situation where the resolution of human T-tubular networks are easily possible in confocal imaging while in rats, their thinner 50-100nm tubules require d-STORM imaging to resolve (Isuru D. Jayasinghe et al., 2014). The resolution improvement offered by d-STORM however is still useful for human T-tubular structure as it is capable of separating out the T-tubular lumen from the walls allowing for the visualisation of luminal structures (Crossman, Hou, Jayasinghe, Baddeley, & Soeller, 2015).

**Human myocytes from normal and diseased hearts**

After analysis of species variation, experiments then focused on the changes that are apparent in disease with samples obtained for these experiments from patients in end stage dilated heart failure where the failing heart has been explanted during transplant surgery. As these were end stage failure hearts, it was expected that significant changes would be present in structural and protein organisation of the cell.

**Membrane changes**

T-tubular changes in disease are now among the most well characterised changes associated with chronic heart disease (A. Guo et al., 2013). Currently, there is extensive literature documenting the various changes in T-tubular structure across a range of animals and diseases (Balijepalli et al., 2003; D. J. Crossman et al., 2011; A. Guo et al., 2013; Heinzel et al., 2008; Lyon et al., 2009; Wei et al., 2010; H.-B. Zhang et al., 2013). From the results in this study, we show that the changes in T-tubular arrangement towards end stage heart failure are extensive at the cell wide level. Our structural results tend to agree with findings documenting a marked decline in the quantity of T-tubules, dilation of the lumen, and overall loss of structural regulation leading to greater variation in lumen size. In order to identify alterations in diseased T-tubules, there is an intrinsic bias in cell selection where only cells with clear evidence of T-tubules are selected for. It is however noted that in possibly the most severe sample, we found the majority of the tissue to be devoid of T-tubular labelling. This loss of t-tubular density and presence within cells has been
researched using confocal microscopy, which enabled a larger overview of cell distribution within the tissue as a whole (Crossman et al., 2015; D. J. Crossman et al., 2011).

T-tubular loss has been implicated in the decline of CICR regulation (Heinzel et al., 2008). This is seen in calcium transient measurements where the overall transient shows a reduced peak and increased duration suggesting a much slower and less co-ordinated release from the SR. The dysregulation of the T-tubules likely resulted in the loss of coupling between the SR membrane and T-tubular membrane reducing the effectiveness of local control (M. B. Cannell et al., 2006). The reduction in T-tubules also increases the relative quantity of non-coupled receptor clusters which relies on the fire-diffuse-fire method of activation (Dawson et al., 1999). Alongside our finding of increased cluster separation for calcium release clusters (figure 4 E,F), this further decreases the synchrony of the calcium transient.

Changes in RyR labelling density

We then focused on analysing the changes observed in the distribution of CRUs within the imaged cells. Previously it was already shown that there was little change in cluster sizes or spatial distribution between rats and humans, however, in terms of normal and diseased samples past studies have shown that calcium release alteration and T-tubular changes occur side by side (D. J. Crossman et al., 2011; Heinzel et al., 2008).

One of the key observations made in our studies was an apparent reduction in RyR labelling in late stage heart failure. From a qualitative observation we noticed that dSTORM images of RyR clusters appear patchier when imaging diseased samples when compared with normal samples. This observation was extracted based on calculating the ratio of the quantity of events for JPH and RyR. A ratio is used in comparing labels as opposed to raw event numbers due to the different fluorophore sensitivities to the excitation laser used in the microscope. The comparatively bright 680nm channel tends to produce more events in comparison to the 750nm channel.

The results showed a general reduction in ratio between RyR and JPH in diseased samples suggesting that there was relatively less RyR present in the labelled samples as compared with JPH. This result from 6 images each for diseased and normal samples was statistically significant (p = 0.037) and indicated a ratio reduction of nearly 50%. Though this shows changes in the relationship between these two proteins, it does not show whether it is the
RyR which has declined or whether JPH has increased in expression. To confirm this, western blots were also carried out (figure 7) showing that there was no change in JPH labelling while an approximate halving of the RyR protein content was observed ($p = 0.005$). This along with the image analysis provides strong evidence that there was an observed reduction in RyR protein content in samples.

Within current literature there is little detail concerning JPH and RyR association outside of animal models. Minamisawa suggest based on their western blot experiments that there should be a down regulation of JPH2 in diseased sample (Minamisawa et al., 2004). In terms of RyR, the opposite is true where there is anticipated little to no change in expression. Though other mRNA RyR expression evidence also suggest a possible decrease of $\sim 30\%$ (Brillantes et al., 1992) making this topic still a point of contention. However, a possible reason for this apparent discrepancy likely lies in the methods for characterising the expression of RyR. In the previous studies (Arai, Alpert, MacLennan, Barton, & Periasamy, 1993; Brillantes et al., 1992) the focus has been on the expression of RyR mRNA through northern blots. The size of the RyR molecule ($>2MDa \sim 400KDa$ monomer) (Lanner et al., 2010) requires carrying out western blots and protein gels on cell homogenates in a way adapted to separating large proteins. More recently, with further development of the technique, the use of western blotting on RyR protein has become more common (Ai et al., 2005) however; data especially of human RyR2 expression levels in heart failure are still sparse. mRNA alone however does not indicate the final functional protein content in the cell and so a discrepancy is possible where RyR is decomposed rapidly after formation. Evidence for RyR degradation is seen in ischemic reperfusion (Pedrozo et al., 2010) through calpain mediated proteolysis, however, research into more chronic forms of heart disease is lacking.

**RyR size distribution**

In comparison between normal and diseased hearts, there was an apparent decrease in overall labelling density in diseased cells. This is also reflected as a possible reduction in overall cluster count and size presented in table 1 as a percentage label coverage (label area/cell area *100). Interestingly, while there was an observed change in the overall labelling intensity, there was comparatively little change in the overall cluster size distribution. In terms of mean there was a small shift of approximately 15% reduction which
did not meet significance in diseased samples as compared with normal samples. This slight decrease is not enough to result in a ~50% reduction in overall protein expression. From this, there is a gap between the expected protein density from images and actual protein changes as seen from western blots (figure 7). The cause of this may be related to methods of thresholding. Currently, a simple percentage signal fraction is employed in the analysis of data; however, this does not take into account the changes in event rates seen between images. Using event rates for thresholding is difficult, as the day to day variation in mountant effects, labelling efficiency, and extent of tissue fixation act as confounding factors in being able to relate the event count to the actual protein content. Because of this, the final size measurements for the individual clusters may be an overestimation of the final sizes.

The size distribution becomes even more similar when separated into bins. The results showed that normal tissue tended to exhibit greater quantities of clusters at the extremities (very small and very large) when compared with the diseased samples. This difference however is very small and not statistically significant.

As these distributions are representative of a stochastically associating population of protein (D. Baddeley, Jayasinghe, Lam, et al., 2009), any changes in the distribution layout hints at possible changes to the binding affinity between RyR molecules. In this case, as there is little change observed, it can be appreciated that there is only small, if any change to the overall RyR binding affinity. The subtle changes that were observed may well be related to alterations in expression of RyR and the ratio of RyR JPH labelling affecting the overall cluster layout. Within mice samples it has already been shown that alterations in JPH expression while maintaining RyR expression led to changes in the overall RyR cluster formation dynamics (van Oort et al., 2011).

**RYR cluster nearest neighbour distance**

A much greater change was observed in the measurements for the nearest neighbour properties. Qualitatively, it was noted that diseased samples tended to have more disorganised cluster arrangements and scarcer labelling. This, combined with the overall reduction in event rates (and JPH RyR ratio) further contributes to an apparent reduction in RyR labelling. From normal to diseased cells the mean nearest neighbour distance shifted
from 135nm to 200nm ($P = 0.047$) (table 2). This difference in means likely reflect the observed change in overall protein content. As previously mentioned, this affects the fire-diffuse-fire mechanism of cluster activation (Dawson et al., 1999) slowing the development of the calcium transient. This outcome is experimentally shown in ischemic cardiomyopathies by Heinzel et al where the overall calcium transients from isolated cells were extended and flattened in diseased as compared with normal samples (Heinzel et al., 2008).

In terms of the distribution shift observed, there was a large decrease in the lower distance bin and an increase in the bins larger than 150nm. In addition, there was a near 2 fold increase in the amount of clusters further than 500nm from the nearest neighbour. From previously presented rat data and human data, it was noted that the vast majority of clusters sit within the 500nm range limit, and it is possible that this 500nm limit is present to enable the reliable activation of this cluster by adjacent cluster activation (Dawson et al., 1999). The increase in these clusters highly separated from other clusters would then mean both decreased synchrony of calcium release as trigger fluxes must diffuse further away to activate adjacent clusters, and reduced reliability of opening of cluster due to reduced calcium concentration reaching these receptors (M. B. Cannell et al., 2006). Though these points are only valid if clusters are no longer membrane coupled (as membrane coupling allows synchronisation with the action potential), evidence highlighting the associated decrease in overall membrane (sarcolemmal and T-tubular) coupling supports the likelihood of membrane decoupling occurring (Balijepalli et al., 2003; A. Guo et al., 2013; Lyon et al., 2009). Further evidence regarding this mechanism relates measured changes of calcium transients as the T-tubular structure changed. A reduction in peak, extension of the overall transient, and loss of synchrony measured by (Heinzel et al., 2008) can be at least partially explained by this change in RyR cluster dynamic. To further validate this theory, modelling by (M. B. Cannell et al., 2006) have shown a similar change in calcium transient dynamic when junctional distances are altered.

In addition, as previously suggested by (D. Baddeley, Jayasinghe, Lam, et al., 2009), clusters which lie below 50nm of the nearest neighbour due to the proximity will likely functionally couple - where the activation of one of the receptor groups nearly certainly will trigger the activation of this immediate neighbour. Evidence for such linking has been provided by
modelling exercises which has shown that free calcium levels will likely within this radius be sufficiently high to facilitate activation of a neighbouring RyR (Sobie et al., 2002; Sobie et al., 2006). This coupling would therefore make grouped systems behave as a larger functional cluster. An increase in the mean neighbour to neighbour distance therefore reduces the amount of functional coupling present.

Spark and wave parameters of a failing heart.

Of direct consequence in alteration to t-tubular and calcium management system changes in the failing heart on function have been the changes associated with spark and wave propagation. Studies into the failing human myocardium show that overall there is typically a reduction in calcium spark rates, as well as calcium waves (Lindner et al., 2002). Elevation of extracellular calcium to 10mM that would typically trigger spontaneous wave formation in normal cells was found to not create the same effect in failing cells. From our data, this is a plausible finding and corresponds well to the reduction in overall RyR protein expression, and reduction in overall t-tubular coupled receptors (due to reduction in the t-tubules.) Changes to spontaneous calcium wave formation can also be consolidated with our data in the form of greater cluster to cluster separation thereby requiring a higher calcium release from clusters to enable propagation through a fire-diffuse-fire mechanism.

**JPH and RyR relationship**

Whereas in normal samples JPH labelling typically forms distinct loci along the edge of the T-tubules interfacing with the corresponding RyR, in diseased samples there is a tendency for a more distributed appearance. The foci while still present often appear diffuse and less frequent (Figure 5A, B). Instead, the diffuse distribution is found in more severe samples to spread throughout the tissue being in locations where SR is likely to run but not interacting with the T-tubular systems. This change initially was thought to be possibly caused by a large up regulation of JPH leading to increased expression. However, based on western blots this was unlikely to be the case (Figure 7). Evidence exists of a possible cleavage of the JPH target however with the cleaving resulting in a non-functioning protein (C. Y. Wu et al., 2014). This cleavage end product is able to diffuse throughout the SR leading to the pattern of labelling observed. Our western blot results did not show a double or triple band as expected from this (Figure 7). While this may be seen as evidence against JPH cleaving, the
location of cleavage is not well known and it is uncertain if our antibody target sites will include the cleavage point.

In terms of the relationship between JPH and RyR in these samples, the results of colocalisation measurements show a minor reduction in colocalisation of JPH labelling with the RyR mask. Interestingly, in the reverse, with RyR labelling on JPH mask colocalisation, there was a small increase (not statistically significant). These results are likely a by-product from changes in relative JPH to RyR protein expression levels, where less RyR labelling is present (reducing mask size) for JPH label to colocalise within. The slight increase may indicate that the removed RyR is preferentially from non T-tubular associated clusters, however, just as likely is the increased JPH mask size from more diffuse labelling previously highlighted encompassing more of the RyR label.

Unfortunately, data regarding RyR-JPH spatial interaction and its role in disease is scarce. What data is present typically highlight changes in protein content (Landstrom et al., 2011; Minamisawa et al., 2004; van Oort et al., 2011) without emphasis on spatial distribution. For the junctional coupling of JPH2 to affect CICR, the protein must be in tight association with RyR. From our results, there are indications that this association changes in disease alongside protein changes. The impact of this on overall CICR can be seen in modelling studies (M. B. Cannell et al., 2006) emphasising the importance of close linkage between the SR and T-tubular membranes. Also important to note from our data however is the relatively small sample size, which may not fully reveal the extent of variation in colocalisation between cells.

Implications for heart failure

These experiments have shown through a combination of image data extraction and western blot that similar to some previous mRNA measurements (Arai et al., 1993; Brillantes et al., 1992), the RyR protein content likely decreases throughout the cell. This decrease also causes significant changes in RyR cluster separation distances. Coupled with the well documented changes in T-tubular system (A. Guo et al., 2013; Lyon et al., 2009) resulting in the overall activation of clusters becoming more dependent on fire-diffuse-fire mechanisms (as opposed to action potential activation via LTCC), there is likely a decreased synchrony of calcium release resulting in the observed changes to isolated cell calcium transients. These
changes then carry over to the whole heart situation where the already stressed chamber (due to Laplace’s law) has further reduced contractile function leading to additional decline in performance.

**Limitations and Outlook**

Whilst the current results have allowed the extraction of important data in understanding of structural changes associated with heart failure, there is still none the less many questions which remain unanswered. While these experiments have focused on the understanding of late stage heart failure, it is important to investigate the changes at varying points in the pathological progression. Ethics limitations however make obtaining earlier time point data from humans difficult and likely a similar animal model may be used. The use of animal models however needs to consider the physiological differences present between species. By understanding the time course in heart failure pathology, it should become possible to identify the sequence of changes that occur from normal to diseased providing better targets for treatment and markers for the diagnosis of pathological progression.

An interesting point associated is the implications of different t-tubular geometries on not just calcium fluxes, but also overall cell ion dynamics. Evidence currently point to at least within the thin tubules of rats, local areas known as fuzzy space which can be considered distinct from the extracellular matrix exterior to the tubular lumens (Scriven et al., 2000). Whether the wider human t-tubules show similar local environment dependence is still uncertain, but with additional data on dimensions and changes in disease, this may become easier to elucidate. An additional effect of the changes in t-tubular geometry and dynamics may be the likely alteration in conduction properties of the cell. Conduction performance is a complex system involving both the relative conductance of ions, and their electrochemical gradients. It is known that in heart failure, a general change in ionic fluxes leads to a change in action potential morphology (Nattel, Maguy, Le Bouter, & Yeh, 2007). Additionally, models of heart failure in rabbit and dogs show that changes to conduction velocity can be attributed to an overall increase in cell diameter with experiments showing an increase in conduction velocity (Akar et al., 2007; Wiegerinck et al., 2006). Unfortunately, detailed studies of how the t-tubular system contributes to these changes is unclear, most likely due to the difficulty in isolating t-tubule presence as a single variable separate from changes in ionic composition. Within single myocytes, t-tubule diameter changes may influence AP
propagation throughout the cell but to our knowledge no detailed measurements have been conducted as yet.

**Conclusions**

Within this chapter, an assessment of differences in species expression of CRU proteins were analysed as well as the changes in CRU protein expression in failing human hearts. Our results showed considerable differences between human and rat ventricular myocyte CRU structure which is an important consideration when deciding the appropriateness of the rat model as a model of human disease. The decision of whether the rat is an appropriate model however was not conclusively stated as various other factors not necessarily associated with the underlying physiology also needs to be considered (n numbers for statistics, ability of genetic alterations in proteins, ethical issues, etc.)

In between normal and failing hearts we find that there was an overall likely decrease in RyR expression in failing hearts reflective of some mRNA studies into this topic. This also showed an ability for the quantitative processing of data available to the dSTORM setup. Furthermore, our data suggested that there was likely an increase in corbular non t-tubular associated calcium release unit distribution that may indicate a greater reliance on fire-diffuse-dire mechanisms of calcium induced calcium release propagation. Finally, this data appears to consolidate well with functional experiments on spark and wave parameters in the failing heart with a general reduction in spark and wave frequencies seen.
Discussion

The studies presented showed the usage of super resolution imaging in the analysis of organelles and proteins structures essential in CICR. This was first demonstrated in the analysis of z disc features where small separations between myofibillar clusters were identified (Chapter 1 figure 4.) Furthermore the ability of dSTORM in resolving structures especially the smaller RyR clusters as shown in (Chapter 2 Figure 5) revealed the exponential RyR cluster size distribution previously identified in peripheral couplons. Important to consider in these findings is the impact of nanometer changes on the overall physiology and calcium dynamics of the cell both in health and disease situations. Therefore, the primary focus of analysis has been on the aspects of super resolution imaging and its application in visualising small EC coupling structures as well as how the structural changes and differences seen in the experiments will impact overall cellular functions.

Methodological Considerations

Due to the still relatively new development of dSTORM microscopy and localisation microscopy as a whole, it was important to first appreciate the differences between dSTORM and the more established confocal methodologies. Central to this is the appreciation of both the different means of image acquisition such as the use of transverse tissue sections and the ability to identify nanoscale structures and the impacts it has on the final analysis of obtained data.

Use of dSTORM for tissue imaging

Initial focus was placed on validating the usage of super-resolution dSTORM imaging on tissue samples. The main reason for this approach was to confirm its ability to be used successfully in tissue sections which are known for causing higher intrinsic background fluorescence than isolated and cultured cells. This background can disrupt the overall point resolution and point detection abilities of the dSTORM technique as explained in the initial models of single molecule localisation(Chao et al., 2012). At the time of experimentation, there was little documented research in the application of dSTORM in thick tissue sections with the majority of work involving the approach being used in isolated cell samples (D.
Baddeley, Jayasinghe, Cremer, et al., 2009; D. Baddeley, Jayasinghe, Lam, et al., 2009; Rust et al., 2006). Previously our laboratory attempted imaging using cut agar sections but was unsuccessful due to background fluorescence by the agar media. The validation of dSTORM is therefore an essential component before more extensive usage of the modality for analysis of rat and human samples as it enabled the identification of problems which needed to be addressed.

From our results we found that there were effects of tissue section thickness on the overall image quality. This effect was seen primarily as a reduction in event detection rates in thicker sections but surprisingly not strongly reflected in the localisation accuracy. This is possibly due to the detection threshold for the individual events, as highlighted by (Ha & Tinnefeld, 2012) where greater background fluorescence reduces the contrast between events and background intensity fluctuations increasing the difficulty in separating events from background. From this information we found that sections thicker than 15 µm were generally not conductive to dSTORM imaging. While a stronger excitation was considered a possible solution to this, increased intensity of excitation often leads to a decreased blink cycle time and limited improvements to overall blink intensity (in terms of photon count)(Ober et al., 2004; Thompson et al., 2002).

The localisation precision was approximately normally distributed with an extended tail towards lower precision. The modal precision was approximately 10-15 nm with the main localisation plateau between 10 and 20 nm. This precision is somewhat lower than the optimal predicted precision for this setup of 5-10nm (D. Baddeley, Jayasinghe, Cremer, et al., 2009) and may be attributable to the increased background from tissue sections, reduced fluorescent intensity of individual events, and refractive index mismatches due to dilution of glycerol solution by the active components of the mountant (a PBS based solution) (Ober et al., 2004). These resolution values are for the most part consistent with typical dSTORM applications (Deschout et al., 2014; Rust et al., 2006).

In our initial tissue experiments, the Z disc protein α actinin was used to test imaging capabilities. The choice of α actinin as a staining target was decided based upon; the high quantity of epitope expression, the availability of a known reliable antibody, the presence of both large as well as small overall structural features in the pattern of expression, and its
primarily structural role which enabled comparison to more structurally oriented imaging modalities such as electron microscopy. There is already a considerable body of data concerning alpha actinin and the cardiac myofibril (E. Lazarides, 1976; Lu et al., 1992; Ribeiro Ede et al., 2014; Sjoblom et al., 2008) and as such provides a base from which comparisons could be drawn. In addition, being a marker of the z disk, alpha actinin labelling reflects the site of alignment for the majority of proteins and structures responsible in excitation contraction coupling (Frank et al., 2006).

We observed that there was a significant amount of intensity fluctuation across a region of expected uniform labelling. It is believed that these features are indicative of stochastic changes in reswitching of the label where individual fluorophores vary in the amount of reactivations before being permanently bleached (Deschout et al., 2014). This is a feature of stochastic super-resolution methods and can be seen in other imaging studies employing the use of super resolution (Huang et al., 2008; Rust et al., 2006; van de Linde et al., 2011). Interestingly, STED microscopy which does not rely on stochastic switching also shows signs of intensity fluctuation (Hein et al., 2008). Because of this, stochastic variation may not account for the entirety of this observation. It is possible that at the resolution at or below 30 nm, individual antibody complexes are detected, and this represents actual stochastic variation in the presence of markers.

Unfortunately, these fluctuations meant measurements of thickness and diameter was complicated as they resulted in large random spikes and peaks in profile plots. Additionally, this made attempting to separate smaller gaps in between regions of labelling difficult. It was possible to partially reduce this effect by filtering images (e.g. Gaussian and median) however this came at a cost of reducing image resolution.

From our results the overall resolution of dSTORM imaging under these conditions was found to be approximately 30-50 nm. This measurement is based upon the smallest detectable gap in labelling. Practical resolution however may be slightly worse than this due to imaging drift, variation in labelling density, and the aforementioned stochastic fluctuations (Deschout et al., 2014). This value is roughly consistent with systems used in other studies. However, this resolution is reduced when comparing to the imaging with essentially flat samples, e.g. (Huang et al., 2008; Rust et al., 2006; van de Linde et al., 2011).
Comparison of dSTORM with other imaging modalities

Electron microscopic images showed the greatest overall resolution clearly resolving the zigzagged nature of the z disk as well as associated membrane structures such as the sarcoplasmic reticulum and T-tubules. This is unsurprising as depending on the intensity of the electron beam, and microscope build, individual proteins can be resolved (Huxley, 1963). Electron microscopy is however a primarily structural based imaging modality. Diffusely distributed proteins or specific targets cannot be easily specifically labelled. Immunogold labelling remains a primary method for imaging specific protein targets (Amiry-Moghaddam & Ottersen, 2013).

Confocal microscopy is a second imaging mainstay used for visualising cardiac myocyte structure and function. While possessing improved subject detection by enabling specific targeting to proteins or structures of interest with reduced complexity when compared with immunogold, the diffraction limited nature of the optical approach makes resolution of nanoscale detail difficult or even impossible. In our studies, the use of confocal microscopy for whole cell and local tissue overview images were its main advantage. Deconvolution enabled a cleaner image, however, the improvement in overall resolution was limited (McNally et al., 1999). In comparison with dSTORM imaging, we found that larger structures such as the myofibrils showed similar patterns. However, smaller structures such as calcium release related structures show a marked improvement when using dSTORM instead of confocal microscopy.

These results highlight the importance of using the correct methodology for the task at hand. For example, imaging of nanoscale features of membrane systems is still squarely in the domain of electron microscopy where the sheer resolution advantage is often key for analysis. In contrast, confocal microscopy can be used to produce detailed images of an entire tissue block (Sands et al., 2005) to provide context to the overall images. Finally, dSTORM can be used for structures which require the specificity of immunolabelling while also requiring resolution higher than what is available via diffraction limited methods (D. Baddeley, Jayasinghe, Lam, et al., 2009; Y. Hou et al., 2014b; Y. Hou et al., 2015).
Analysis of dSTORM data

One of the first problems encountered when using the dSTORM modality is the suitability of methods by which the dataset can be analysed. Though often compared to confocal in terms of their fluorescent and target specific imaging modalities, dSTORM and confocal imaging nevertheless acquire and reconstruct images fundamentally through differing methods (Sands et al., 2005; Thompson et al., 2002). Where confocal imaging relies on imaging the combined fluorescent output of many fluorophores producing a smooth intensity based output, dSTORM generates localised point-like measurements (effectively binary on or off) more akin to pointillism paintings (D. Baddeley, Jayasinghe, Lam, et al., 2009; Ober et al., 2004; Rust et al., 2006). Because of this, the image processing techniques used in confocal imaging are often not directly applicable to processing using dSTORM.

Whereas outputs from the confocal modality are directly obtained as “pixelised” images barring adjustments of contrast and exposure, the rendering for dSTORM images is comparatively more complex. Rendering used in the dSTORM modality is achieved through Delaunay triangulation of the individual points (D. Baddeley, Jayasinghe, Cremer, et al., 2009). This results in a density based intensity profile across the image which visually appears similar to a higher resolution confocal image.

These differences between images require different approaches in image analysis. Firstly, the density based rendering can be considered to be semi quantitative. Each individual point reflects a single fluorescent event. Knowing the average re-switching rate and duration of events then allows for a quantitative approximation of protein density (Y. Hou et al., 2015). In our work this was used in the comparison of human normal and diseases event ratio of JPH and RyR to support a reduction in RyR expression. In contrast, confocal methods are dependent on many other factors such as the microscopes point spread function, background fluorescence, and the overall reduced resolving power makes quantification difficult (McNally et al., 1999).

Arguably dSTORM imaging is affected by the same factors. However, dSTORM allows removal of intensity variation as one counts molecules rather than measuring fluorescence intensity per se that can be hard to discriminate from background. With
dSTORM the shape of fluorescent blink events is reasonably obvious and filterable hence resulting in improved signal to background ratio.

A second difference arises from methods of identifying labelling for image segmentation. Typically intensity based thresholding is used to identify clusters of interest. Within confocal microscopy there is an extensive suite of methods and calculations that reduce the need for observer input in automating thresholding (Velasco, 1979). We found in our study that isodata thresholding was unable to threshold signal reliably. Likely this deviation is caused by the intrinsic approximation of background fluorescence present in confocal images which is not present in dSTORM images (for examples see (D. J. Crossman et al., 2011; Peng et al., 2012)). dSTORM intrinsically exhibits very low background due to filtering of fluorescent events which therefore does not need to be subtracted from the image (D. Baddeley, Jayasinghe, Lam, et al., 2009; Huang et al., 2008). What remaining background is present exists as points of high density caused by either single molecule event clusters or non-specifically bound fluorophores.

The resulting threshold method used in this study relied on selecting fractional image intensity based thresholds from simulations of the expected sample labelling and accepting thresholds that closely match the initial data used in generating the simulated dSTORM data. While this method is reliable, it as yet lacks a fully automated implementation, and requires a reliable estimate of the expected labelling. These caveats were valid for less critical for imaging of RyRs as they are already characterised to some extent by confocal methods (D. J. Crossman et al., 2011), but for truly unknown samples, there is no effective way of determining proper label from background.

**Impact of findings for cardiac biophysics and physiology**

After confirming the performance of dSTORM within tissue samples in relation to other commonly used modalities, the focus then shifted to possible impact of the nanoscale changes and features observed in the Z disc and CRU on overall cell performance. To do so, the newly acquired data needed to be consolidated with previous findings via imaging and modelling in order to determine how these features may apply to overall cell physiology.
**Alpha actinin and myofibrillar distribution**

An initial protein of interest in this study was the z disk localised myofibrillar protein α actinin. α actinin was selected as a suitable target to validate our method capabilities and determine suitable methods of analysis for the dSTORM modality. However, in the process of imaging we also identified additional details concerning its nanoscale distribution.

From the EM data obtained, there appeared to be distinct subdivisions of the myofibrils which were not clearly evident in confocal images. The use of dSTORM showed that these subdivisions are present with potentially elements of SR passing through them. From the dSTORM data, the diffusion distances were also extracted showing that the maximal distance tended to stay below 500 nm. Interestingly, this 500 nm distance appears to be quite significant in the overall cell function as this distance is also the near maximal distance for nearest adjacent cluster separation.

When 3d-dSTORM was used, it became possible to resolve the 3d nature of the z disk. Our results estimated the overall thickness of the z disk to be approximately 100nm thick. Although at the limit of axial resolution for the current setup, this value agrees with EM images which show an electron dense patch of approximately 100nm thick. Ferritin based electron microscopy immune assays also support this estimation of thickness correlating well with EM z disc protein densities (Lemanski et al., 1985).

Being a central location for numerous signalling pathways, changes to z disk topology are implicated in a range of cardiomyopathies. Key proteins such as Muscle LIM protein, Actin associated LIM protein and the enigma protein family’s disruption is directly related to alterations in cell morphology (Arber et al., 1997; Cheng et al., 2010; Pashmforoush et al., 2001). The demonstration of super-resolution microscopy in being able to resolve nanoscale alterations in z disc structures allows the detection of subtle changes in the distribution of these proteins and interaction and distribution within the myocyte that may impact on cell function.

**Effects of RyR Ca^{2+} release cluster distribution on cell wide calcium dynamics**

One of the main points of interest in the imaging of calcium release units within rat cardiac myocytes was the highly interconnected nature of the clusters. In the analysis of nearest
adjacent neighbour distances, there was a heavy bias towards closer distances. In addition, the distribution of cluster sizes showed the presence of very large extended clusters spanning up to 2 µm and containing in excess of 800 individual RyR channels. This raises questions regarding the grading of the calcium release transient by the DHPR as the closeness of these clusters within 100 nm is expected to give rise to functional coupling between release sites (D. Baddeley, Jayasinghe, Lam, et al., 2009; Sobie et al., 2006).

Additionally, the opening of receptors within one of the larger clusters could feasibly trigger the activation of a wave and cause cell wide propagated calcium release. This would result in overall reduced cell wide calcium stability leading to increase spontaneous calcium releases. Confocal imaging studies focusing on cluster distribution tended to favour a cluster centroid to centroid measure biased to larger distances due to the limited resolution (Chen-Izu et al., 2006; C. Soeller et al., 2007). While the resultant distribution is similar in shape, comparison with our results shows a detection limit at smaller sizes and a tendency for multiple maximal intensities to occur on larger cluster resulting in their fragmentation. This may explain the reason these extended clusters were not earlier detected.

When comparing between species, there were many factors which were preserved. One of the most prominent features observed is that the nearest neighbours for both rat and human samples remained very similar. In addition, the distributions show nearly all clusters for both species reside within 500 nm of each other. This is intriguingly correlated with the myofibrillar imaging where a similar maximal diffusion distance into the myofibril of 500nm is also observed. Although these experiments do not explicitly reveal the underlying mechanism behind this phenomenon, it can be speculated that this marks as an upper limit for diffusion in the case of a fire diffuse fire system (Dawson et al., 1999). It is possible that beyond 500 nm, the diffusion of calcium is either insufficient to trigger activation or activation occurs too slowly.

It is interesting to observe that within diseased samples there is a disruption of this apparent 500 nm distance limit with more clusters appearing at greater distances apart. This disorganisation coupled with alterations in T-tubular morphology, both shown here and in previous studies (D. J. Crossman et al., 2011; A. Guo et al., 2013; Lyon et al., 2009), likely act as contributors to the overall pathological cell calcium dynamics. (Beuckelmann et al., 1992)
showed that there was an extending and flattening of the overall calcium transient in isolated cells from DCM patient hearts. This is consistent with a fire diffuse fire model of activation which is likely to be the main activation method for cells with compromised T-tubular systems (Dawson et al., 1999).

The greater apparent separation between clusters is correlated with a reduction in overall cluster density and cellular RyR content and offers a means of providing a possible mechanism for linking changes in protein content and function. Additionally, evidence from previous confocal imaging studies has alluded to this possibility with a detected small reduction in overall cluster quantity (D. J. Crossman et al., 2011) with the additional resolution from dSTORM based imaging further clarifying this observation and demonstrating a greater change in cluster density (table 1).

Whilst the data presented showed the subtle changes to cluster morphology as well as possible mechanism for changes in overall cell calcium handing dynamics related to these structural changes, the DHPR distribution has not been investigated in this study. Investigation of the DHPR distribution in this study was hindered by the lack of reliable primary antibodies for the DHPR protein. The importance of DHPR and RyR coupling is central in the overall gain of CICR as well as the time frame required for CICR to occur (D. M. Bers, 2008). It has been reported that there is a small change in DHPR RyR relationship (in terms of colocalisation) (D. J. Crossman et al., 2011) however, the confocal nature of imaging means smaller changes were not detectable. The future use of dSTORM imaging on DHPR and RyR dual labels should elucidate details the nature of their relationship and whether changes observed between healthy and diseased samples would significantly impact the overall calcium release characteristics of the cell.

**Relationship between RyR and JPH distribution – impact on cardiac function**

One of the relationships explored in depth by this study has been the positional relationship between RyR and JPH proteins. JPH acts as a linker between the sarcoplasmic membrane and T-tubular membrane important for maintaining a narrow dyadic cleft (Takeshima et al., 2000). Within animal models, JPH loss is correlated with manifestations of heart failure like pathology (A. Guo et al., 2013; Landstrom et al., 2011). We found that across both species
there was a very similar distribution between the proteins. Rat samples showed a higher amount of signal colocalisation (see chap 3 table 1) when compared with human samples. This difference is likely related to the difference in T-tubular structure between species. The dense and network like t-tubule system from rat cells implies that a majority of receptors are adjacent to T-tubules which requires JPH to bring the membranes in close contact. In humans, the thicker T-tubules are more sparsely distributed with many clusters not directly coupled and distal to T-tubules.

The differences between rat samples and human samples are of particular importance for the understanding of disease progression. The majority of current data attempting to relate changes in JPH and changes in T-tubular models involve the use of animal models (Garbino & Wehrens, 2010; Landstrom et al., 2011; van Oort et al., 2011). Reduction in JPH2 expression within mice and rats has shown a corresponding alteration in T-tubular structure as well as alteration in formation of calcium release units. The cells additionally exhibit features akin to hypertrophic heart failure pathology such as an increase in volume and progressive loss of T-tubules (Landstrom et al., 2011). However, because of species differences as well as the single protein target nature of the experiments elucidating JPH effects, changes in JPH expression need to also be correlated with human samples.

From our studies of the JPH RyR relationship in healthy and diseased cells we found little change in the overall relationship between RyR and JPH. There was a subtle reduction in colocalisation of RyR with JPH which failed to meet significance. This reduction appears related to the possible reduction in RyR quantity rather than changes to the interaction between JPH and RyR. This is supported by western blots carried out within our group showing no change in amount of JPH. Other literature however reports different findings where JPH2 is down regulated in heart failure pathologies (Minamisawa et al., 2004). Important to note, however, is that the majority of these studies were undertaken in rat and mice. While data on human samples is sparse, the available literature suggests that there is a reduction in JPH expression in hypertrophic cardiac myopathy (Landstrom et al., 2007) and dilated cardiac myopathy (H.-B. Zhang et al., 2013). This is in contrast to our data showing no change in dilated cardiac myopathies. Possible causes for this difference may be differences in progression of pathology leading to differences in protein distribution in addition to intrinsically small sample sizes due to difficulties in obtaining donor samples.
A second hypothesis regarding changes to JPH colocalisation was the possibility of enzymatic cleavage of protein. Evidence exists demonstrating the calpain dependent cleaving of JPH2 within mice models of heart failure (C. Y. Wu et al., 2014). Our western blots however do not show strong evidence for this (Chapter 3 Figure 7). However, this may merely be due to antibody target sequence being part of the same fragment as discussed within the chapter.

**Relevance of animal models for release**

In the comparison of human and rat models, the results showed that while there were differences in T-tubular arrangement, in terms of spatial distribution of RyR clusters, there was little change between the species. The nearest neighbour distances were also unchanged between species. This conservation makes rat models more relevant to human disease. However, there is nonetheless a significant difference in T-tubular networking between rat and human models as emphasised within chapter 3. While this study did not explore the effects of the difference in rat T-tubular network when compared with humans, it can be speculated that these differences reflect different physiological requirement of different species such as organ size, metabolism, and resting heart rates.

**Future directions**

There still remain many unanswered questions in regards to EC coupling and the changes that take place in the progression from health to disease. While the studies presented has enabled better clarity to some of these, such as detailed changes to cluster distribution and JPH RyR interactions in Human myocytes, the additional understanding has inevitably lead to further unanswered questions.

A key component of the presented research has been the wide application of dSTORM imaging of tissue samples. dSTORM and super resolution imaging are still relatively new and still require considerable development especially in the field of image processing and analysis. Specifically, dSTORM has already demonstrated the possibility for molecular level quantification of proteins both within the presented work as well as previously shown by (D. Baddeley, Jayasinghe, Lam, et al., 2009).
In the analysis of rat and human samples, the identification of a broad range of cluster sizes needs to be explored in more depth. A big problem arising from the current analysis technique has been assuming a flat plane for release cluster geometry (D. Baddeley, Jayasinghe, Lam, et al., 2009; Y. Hou et al., 2015). 3D super-resolution technology can be seen as a next step in characterising release cluster dimensions with latest proofs of concept showing axial resolution down to 50 nm being possible in favourable circumstances (Olivier, Keller, Gönczy, & Manley, 2013). Especially interesting in the imaging of release clusters is the possible reason for an increase in t-tubule diameter at the release sites. Electron microscopy has shown that the membranes undergo possible complex folding which contain RyR proteins (T. Hayashi et al., 2009). If so then the assumption of flat plane geometry may be incorrect and an underestimation of the actual cluster size.

While interactions of RyR and JPH were considered in this study, there are still a large number of unexplored proteins which directly impact RyR function. Central to RyR activation is the DHPR protein which currently has not been extensively imaged at high resolution. While some confocal images of DHPR and RyR have been produced, the use of confocal imaging resulted in poorly defined cluster regions (D. J. Crossman et al., 2011). The use of super-resolution on an appropriate dual labeled DHPR RyR samples would be able to shed light on the positional relationship of these release units.

In the study of human normal and diseased samples there were significant changes to the T-tubular distribution as well as cluster spacing. These changes appeared broad and extensive. While some conclusions were made on the changes, the mechanism of causality could not be identified due to the late stage nature of the disease. Unfortunately, acquisition of a range of hearts from different stages of DCM is nearly impossible due to limited tissue availability. Instead, further comparison of late stage animal models can be correlated with late stage DCM and used as a model for dSTORM imaging during different phases of disease manifestation to better explore the changes that occur from healthy to diseased states.

**Conclusion**

Our results from the experiments shown demonstrated the ability of dSTORM microscopy in the context of tissue section imaging which have not previously been extensively studied. It
was first demonstrated using the myofibrillar Z disc, that dSTORM was applicable and capable of identifying structural features nearly on par with electron microscopy. When used in the examination of the calcium release units, we demonstrated that the exponential distribution of cluster sizes as shown in previous studies on peripheral clusters were also present in the central clusters. Finally in imaging human samples, a similar distribution is seen in healthy cells, but with IDC cells, there is an increase in cluster separation which may be implicated in changes to CICR through the increase in diffusion distances.


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