Spectral Phasor Characterisation of Sodium Microenvironments in Live Myoblast Stem Cells

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Statement of Originality

This is to confirm that to the best of my knowledge and belief, the contents of this thesis is original, unless acknowledged in the text. I hereby declare that the work presented here has not been submitted previously, either in full or in part, for another degree or institution.

Hamid Sediqi

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<tr>
<td>λ</td>
<td>Lamda max</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
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<tr>
<td>μL</td>
<td>Microlitre</td>
</tr>
<tr>
<td>μM</td>
<td>Micro Molar</td>
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<tr>
<td>CICR</td>
<td>Calcium Induced Calcium Release</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon Dioxide</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Media</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal Bovine Serum</td>
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<tr>
<td>Hz</td>
<td>Hertz</td>
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<tr>
<td>K⁺</td>
<td>Potassium Ion</td>
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<tr>
<td>Leica TCS – SP5</td>
<td>Leica True Confocal Scanner      Spectro-Photometer 5</td>
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<tr>
<td>min</td>
<td>Minute</td>
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<tr>
<td>Na⁺</td>
<td>Sodium Ion</td>
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<tr>
<td>NCX</td>
<td>Sodium Calcium Exchanger</td>
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<tr>
<td>NHE</td>
<td>Sodium Proton Exchanger</td>
</tr>
<tr>
<td>NKA</td>
<td>Sodium Potassium Antiporter</td>
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<tr>
<td>nm</td>
<td>nanometre</td>
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<tr>
<td>PBS</td>
<td>Phosphor Buffer Saline</td>
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<tr>
<td>PMT</td>
<td>Photon Multiplier Tube</td>
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<tr>
<td>siRNA</td>
<td>Short Interfering Ribonucleic Acid</td>
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<td>SPA</td>
<td>Spectral Phasor Analysis</td>
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<td>SR</td>
<td>Sarcoplasmic Reticulum</td>
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Abstract

Sodium is implicated in many cellular processes including, muscle contraction, proliferation, and differentiation. To date, studies investigating the role of sodium primarily focus on cell-wide changes or specific membrane pores. Characterisation of sodium microenvironments within live cells is expected to lead to a better understanding of the role of sodium in cellular processes including, dynamic interactions with proteins, DNA and RNA in stem cell differentiation. The advent of cell permeant dyes that fluoresce when bound to specific ions provides one element in which to investigate sodium microenvironments in live cells. In order to characterise the spectral properties of sodium bound dye, there needs to be an approach that can acquire data, from live cells, in real time. More recently, Spectral Phasor, a spectral imaging analysis technique that overcomes the need for complex analysis algorithms has been used to demonstrate microenvironment changes with fluorescent proteins. The aim of this thesis was to determine the applicability and potential development of the Spectral Phasor Approach (SPA) for the study of sodium microenvironments in live cells.

This thesis reports the application of the sodium ion dye CoroNa Green, and initial modifications of Spectral Phasor, particularly in the context of characterising microenvironment changes in live progenitor myoblast stem cells undergoing differentiation. The SPA was developed by isolating regions of the spectrum exhibiting the largest spectral shifts for spectral acquisition. Acquiring data in the 520 – 550 nm range enabled the identification of distinct emissions in differentiating cells versus undifferentiated cells. Acquiring spectral data in this range also reduced the acquisition time which consequently appeared to enable minute-interval spectral analysis, and three-dimensional spectral characterisation of live cells.

Initial experiments utilising full range scans of the spectrum revealed most significant spectral shifts at 10 minutes. Utilising the more ‘optimised’ parameters
made apparent that the largest spectral shifts occurred at 3 minutes post induction of differentiation, but increasingly became similar overtime. These data confirms previous research which demonstrated sodium influxes as an early antecedent event to proliferation, DNA synthesis and differentiation, and in vitro research which reports the involvement of sodium ions in regulating gene expressions. Moreover, spectral shifts reported here appear to match the initial temporal wave of gene expression reportedly involved in differentiation. This indicates a potential correlation between the early sodium microenvironment changes and the expression of genes involved in the early stages of differentiation. Future research may focus on perturbing sodium influxes to determine the role of sodium microenvironments in the expression of the initial wave of gene expression. This study demonstrates the utility of the Spectral Phasor approach in characterising sodium microenvironments in live cells.
Literature Review

1.1 - Role of Sodium in Cellular Processes

1.1.1 - Sodium in Cellular Proliferation

Analysis of sodium in live cells is expected to provide critical information about its contribution to processes including cellular proliferation, differentiation, and muscle contraction. In various cell lines, sodium influxes have been shown to be one of the earliest antecedent events to DNA synthesis (Koch and Leffert, 1979, Rozengurt et al., 1981). Evidence for this role comes from experiments which demonstrated that treatment with pore forming molecules such as Amphotericin B, an antibiotic which induces the influx of sodium ions, ultimately initiates DNA synthesis in quiescent cells (Koch and Leffert, 1979, Rozengurt et al., 1981). Additionally, molecules which stimulate proliferation such as 12-O-Tetradecanoylphorbol-13-acetate and vasopressin were observed to induce sodium influxes prior to downstream DNA synthesis (Koch and Leffert, 1979, Rozengurt et al., 1981).

Sodium has also been reported to regulate cellular proliferation by controlling the cell cycle. The sodium-proton exchanger (NHE) has specifically been implicated in this process by regulating intracellular sodium concentrations and pH levels (Putney and Barber, 2003). Experiments in which NHE activity or expression were inhibited in pulmonary artery smooth muscle cells by amiloride and short-interfering RNA (siRNA), demonstrated an attenuation of proliferation, hypertrophy and cellular migration. In fibroblast cells, Putney and Barber (2003) demonstrated that blocking the activity of NHE1 led to the inhibition of cellular proliferation, otherwise stimulated by growth factors. Furthermore, it was found that NHE1 exhibited a transient activation just prior to G2/M phase, and mutant cells lacking functioning
NHE1 displayed attenuation in intracellular alkalinisation, delayed S-phase, and hindrance in the progression of G2/M phase (Putney and Barber, 2003).

1.1.2 - Sodium in Cellular Differentiation

The regulation of intracellular sodium concentrations and pH levels not only facilitates proliferation but is also implicated in playing a direct role in the differentiation process (Yu and Hales, 2011, Wang et al., 1997). Experiments by Wang et al. (1997) demonstrated that P19 embryonal carcinoma cells treated with NHE inhibitor (Hoe 694) exhibited elimination of retinoic acid-induced differentiation. Knockout NHE1 P19 cells also lacked the ability to undergo differentiation; however, this ability was restored when NHE was reintroduced (Wang et al., 1997). Furthermore, a number of studies reported a significant increase in NHE1 mRNA levels in cells induced to differentiate (Krapf et al., 1991, Horie et al., 1992, Rao et al., 1992). For example, rat myoblast stem cells (L6), 6 days post induction of differentiation demonstrated a 2-3 fold increase in NHE1 mRNA levels (Yang et al., 1996), human leukemic cells demonstrated an 18-fold increase in NHE1 mRNA levels (Rao et al., 1992, Ladoux et al., 1988), and mice P19 cells displayed a 10-fold NHE mRNA levels (Dyck and Fliegel, 1995). Despite evidence of sodium’s involvement in both proliferation and differentiation, the exact mechanism by which these downstream effects are induced remains to be elucidated.

1.1.3 - Sodium in Muscle Contraction

Sodium plays a critical role in muscle contraction by linking the initial depolarisation moment to calcium influxes, in a process known as excitation-contraction coupling (Aronsen et al., 2013). In this process, the initial depolarisation of the membrane triggers the influx of sodium ions through the voltage gated sodium channels, consequently causing further depolarisation of the membrane in a feedforward
amplificatory manner (Aronsen et al., 2013, Verdonck et al., 2004). As a result, L-type voltage-gated calcium channels open to permit the influx of calcium ions which then binds to Ryanodine receptors (RyRs) located in the membrane of the sarcoplasmic reticulum (SR) (Aronsen et al., 2013, Verdonck et al., 2004). This process, in which an initially small quantity of calcium ions triggers the release of a larger pool of calcium ions from the SR through RyRs, is referred to as calcium-induced calcium release (CICR) (Aronsen et al., 2013, Verdonck et al., 2004). Muscle contraction is achieved when this release of calcium from the SR binds to troponin causing a conformational change and exposing tropomyosin to allow cross bridge formation with actin filaments (Figure 1.01) (Aronsen et al., 2013, Verdonck et al., 2004). The intracellular concentration of sodium is critical in the regulation of cellular contractility, whereby even minute modifications significantly alters contractility (Lee and Dagostino, 1982, Pieske et al., 2002). The tight regulation of intracellular sodium concentrations enables cells to establish an electrochemical gradient which subsequently can be used as the basis for the active transport of various ions such as protons and calcium, thereby linking sodium to both pH regulation and EC-coupling (Aronsen et al., 2013, Verdonck et al., 2004).
1.1.4 - Sodium Microenvironments

Independent of the voltage-gated L-type calcium channel, sodium influxes also induce CICR by influencing the activity of the sodium-calcium exchanger (NCX) (Aronsen et al., 2013, Leblanc and Hume, 1990). Leblanc and Hume (1990) initially reported that sodium influxes through the voltage-gated sodium channels triggered CICR by inducing the reverse mode activation of NCX. Normally this exchanger extrudes calcium out of the cell and translocates sodium into the cell (Leblanc and Hume, 1990). However, when a sufficient local intracellular concentration of sodium is established, this transporter works in reverse whereby sodium is extruded out, and calcium is translocated into the cell. This calcium subsequently binds to RyRs in the SR membrane to trigger further calcium release via CICR (Aronsen et al., 2013, Leblanc and Hume, 1990, Verdonck et al., 2004).

Following this initially discovery, Lederer et al. (1990) demonstrated that sodium influxes from the voltage-gated sodium channels were not sufficient in establishing local concentrations required to induce the reverse mode of NCX. As a consequence, the sodium microenvironment (initially referred to as sodium ‘fuzzy space’ by Lederer et al. (1990)) was proposed as a mechanism by which sufficient local sodium concentrations could be established (Lederer et al., 1990). These sodium microenvironments were reasoned to have specific qualities such as the ability to slow the diffusion of sodium in the short term but also enable equilibration over longer time periods (Lederer et al., 1990). Furthermore, the regulation of these microenvironments was proposed to occur through the action of different sodium transporters such as NCX, sodium-potassium antiporter (NKA), and NHE (Aronsen et al., 2013). Further regulation may also occur through the coordination of these transporters such as NKA and NCX which have been shown to co-localise through interaction with Ankyrin B (Figure 1.01) (Mohler et al., 2005, Mohler et al., 2003). Transporters which extrude sodium such as NKA would cause a depletion of sodium concentrations in the local microenvironment, and hence are referred to as ‘cold-
spots’ (Aronsen et al., 2013). Conversely, transporters such as the NCX which translocates sodium into the cell would increase the local sodium concentration, and hence are referred to as ‘hot-spots’ (Figure 1.01) (Verdonck et al., 2004). It is conceivable that within each sodium microenvironment, sodium interacts with specific molecules to induce downstream events such as proliferation and differentiation.

Since the initial suggestion, further research aimed to provide evidence for the existence and regulatory function of these sodium microenvironments (Aronsen et al., 2013). For example, electrophysiological experiments demonstrated significantly higher subsarcolemal sodium concentration relative to the bulk cytosolic concentration in cardiac muscle cells (Figure 1.01) (Aronsen et al., 2013, Verdonck et al., 2004). Moreover, influxes through various sodium transporters were observed to alter cellular contractility without inducing bulk cytosolic changes in sodium concentrations (Lee and Dagostino, 1982, Pieske et al., 2002). These microenvironments may in fact exist as standing gradients as reported by Silverman et al. (2003), who demonstrated the persistent existence of higher sodium concentration during systole and diastole in contracting cells. However, considering the rapid diffusion of sodium ions (millisecond scale), it is also conceivable that these microenvironments are transient in nature (Aronsen et al., 2013, Verdonck et al., 2004). Since the initially proposal by Lederer et al. (1990), the existence of sodium microenvironments have not yet been conclusively demonstrated, nor has their molecular anatomy been fully characterised.

1.1.5 - Sodium Microenvironments in the Nucleus

Research over the past decades has provided evidence for the involvement of sodium in nuclear events such as DNA synthesis and gene expression. For example, Spicer et al. (1968), while using pyroantimonate found a heterogeneous distribution of sodium

5
within the nuclei of several cell lines. Moreover, it was found that sodium predominantly co-localized with heterochromatin towards the periphery of the nucleus, indicating a potential role in the regulation of gene expression (Spicer et al., 1968). More recently, Garner (2002) demonstrated that the nuclear pore complexes were not as liberally permeable to cations such as sodium and potassium as once thought. Furthermore, Garner (2002) reported the localisation of the sodium-potassium ATPase in the inner membrane of the nuclear envelope, and consequently implicated this transporter in the establishment of Na⁺/K⁺ gradients observed in the nucleus.

Building on the observations of Garner (2002), Galva et al. (2012) reported the localisation of NCX in the inner nuclear envelope, and demonstrated the co-localisation of this transporter with NKA. It was proposed that NKA works to establish sufficient local concentrations of sodium in the lumen of the nuclear envelope which consequently would induce the reverse mode of NCX (Garner, 2002). As a result, calcium would be extruded out of the nucleoplasm, while sodium would be translocated into the nucleoplasm (Galva et al., 2012). These two transporters are proposed to work in conjunction to regulate both nucleoplasmic sodium and calcium concentrations, and consequently influence DNA synthesis and gene expression (Galva et al., 2012). However, there is a lack of in vivo experiments which demonstrate sodium’s involvement in these nuclear events.

Despite the paucity of in vivo evidence, there are nonetheless convincing in vitro studies which provide support for the role of sodium in these processes. For example, experiments (Blackburn, 2006, Garner, 2002, Kettani et al., 1998) have demonstrated that since DNA has a high axial charge, it requires stabilization through electrostatic interactions with counterions in the nucleus. Consequently DNA functions as a cation exchanger, whereby counterions such as potassium, ammonia, lithium, caesium, calcium and sodium, compete against each other to bind
electrostatically with nucleic acids (Blackburn, 2006, Kettani et al., 1998). In this counter-ion condensation model, ions such as sodium are restricted to within a few angstroms of the DNA surface, in other words, they are still free to move along the helix structure, forming a ‘cloud’ around the nucleic acids (Blackburn, 2006, Kettani et al., 1998). Counterion condensation of monovalent cations primarily influences the minor-groove structure of DNA by reducing the effective charge of the nucleic acids (Blackburn, 2006, Kettani et al., 1998). As a consequence, this significantly affects nucleic acid structure (Blackburn, 2006, Kettani et al., 1998), binding interactions (Fogolari et al., 1997, Ozers et al., 1997), stability (Blackburn, 2006, Kettani et al., 1998), and solution properties (Blackburn, 2006).

Considering the co-localisation of NKA and NCX in the nuclear envelope, and the fact that in vitro studies (Fogolari et al., 1997, Kettani et al., 1998, Ozers et al., 1997) demonstrated sodium playing a role in modulating DNA structure and DNA-protein interactions, it is conceivable that, analogous to the cytoplasm, the nucleus may also possess sodium microenvironments. These microenvironments may exhibit varying concentrations of sodium (‘cold spots’ & ‘hot spots’), which consequently may affect DNA structure and protein-binding affinity in order to regulate gene expression (Figure 1.01). Furthermore, these microenvironments in the nucleus can be a function of sodium interacting with different species of molecules (proteins and other macromolecules) involved in regulating gene expression and DNA synthesis. The study of these microenvironments, however, has not yet been reported in the literature and necessitates further investigation to determine their existence and contribution to DNA synthesis and gene expression.

Based on the literature, the model that appears to be developing for the role of sodium has been summarised schematically in Figure 1.01. Based on this model, the composition of sodium microenvironments found in the cell is not only contingent spatially (nucleus vs cytoplasm), but also temporally (for example,
microenvironmental changes during early stages of differentiation). These factors require consideration when attempting to characterise sodium microenvironments.

Figure 1.01 – Sodium Activity in Cells as Reported in the Literature. The subsarcolemmal space has been reported to exhibit higher concentrations of sodium relative to the bulk cytosol (indicated by red colour). Sodium influxes induce various cellular responses including proliferation, DNA synthesis and muscle contraction. Transporters which bring sodium into the cell may create ‘hotspots’ (indicated by red colour) proximal to the transporter, whereas transporters which extrude sodium may create ‘coldspots’ (indicated by white colour). There could also be cross talk between transporters such as NKA and NCX (indicated by pink) in order to regulate these sodium microenvironments. These sodium microenvironments may also be present in the nucleoplasm of cells and may be established through the interaction of NCX and NKA in the inner nuclear envelope. NCX = Sodium-calcium exchanger, NKA = Sodium-potassium antiporter, NHE = Sodium-proton exchanger, RyRs = Ryanodine receptor, SR = Sarcoplasmic reticulum, N = Nucleoplasm, and NL = Nuclear lumen.
1.2 - Approaches to Analysing Sodium in Live Cells

1.2.1 - Sodium Probe – CoroNa Green

The advent of the sodium specific fluorescent molecule CoroNa has enabled the study of sodium both in vitro and in vivo (Meier et al., 2006). CoroNa with an excitation and emission wavelength of 492 nm and 516 nm respectively is comprised of a fluorescein molecule attached to a crown-ether (Figure 1.02) (Meier et al., 2006, ThermoFisher, 2005). Consequently, this enables selective binding of sodium ions in the presence of other monovalent cations (Meier et al., 2006, ThermoFisher, 2005). Upon binding to sodium and excitation, CoroNa displays a dose-dependent increase in fluorescence at physiological concentrations (Figure 1.02) (Meier et al., 2006). This fluorophore has previously been employed to investigate sodium distributions in regenerating amphibian limbs (Tseng et al., 2010) and in following sodium influxes of stimulated neurons (Tyler et al., 2008).

![Figure 1.02 – CoroNa Green. (A) CoroNa is comprised of a fluorescein molecule attached to a crown-ether. (B) At 516 nm, CoroNa displays a dose dependent increase in fluorescence upon binding to sodium ions. Figure adapted from ThermoFisher (2005).](image-url)
1.2.2 - Fluorescence Microscopy

Fluorescence microscopy has become an important tool for biological and biomedical research because of the attributes that are not readily accessible with traditional optical microscopes (Muller, 2006). The employment of various fluorescent probes has enabled the visualisation and identification of sub-microscopic cellular components amongst non-fluorescing structures (Mason, 1999). Fluorescence microscopy works by firstly, irradiating the target molecules with a laser at a specific wavelength, and then, collecting the emission at a different wavelength (Mason, 1999, Muller, 2006). The principles of fluorescence microscopy can be applied to CoroNa to study sodium in live cells. However, employing traditional fluorescence microscopy has limitations, as it allows secondary light from areas of non-interest such as different focal planes to interfere with the resolution of components that are in focus (Mason, 1999, Muller, 2006).

1.2.3 - Confocal Microscopy

Confocal microscopy can address these limitations (Claxton et al., 2006). In contrast to wide field microscopes which bathe the entire specimen with light, confocal microscopes employ focused laser beams that scan across the specimen in specific areas to produce optical slice images of the specimen (Claxton et al., 2006). The focused laser beam used to excite the fluorophore is first passed through a dichromatic mirror, and once excited, the fluorophore emits light that is then captured and passed back through the dichromatic filter and into the Photon Multiplier Tube (PMT) (see Figure 1.05) (Claxton et al., 2006).

Laser scanning of the specimen can occur in three dimensions ‘x’, ‘y’ and ‘z’, generating multiple slices that can then be further processed to produce three dimensional images (Claxton et al., 2006). In addition, the confocal microscope can
perform analysis in the fourth dimension to enable the investigation of samples across time (Claxton et al., 2006). The employment of an aperture pinhole situated in front of the PMT detector further reduces background fluorescence from areas of non-interest (Claxton et al., 2006). These improvements on the fundamentals of light microscopy, has made confocal microscopy an invaluable tool that has opened new avenues of exploring cellular processes (Claxton et al., 2006).

1.2.4 - Spectral Imaging

Exclusively quantifying the fluorescence intensity of CoroNa is not expected to provide information about the molecular environment of sodium in live cells. Spectral imaging is distinct from a purely imaging technique that only provides the intensity of the fluorescence at each pixel \((x, y)\) (Garini et al., 2006). In spectral imaging the intensity of each wavelength in each pixel is collected to produce a three-dimensional \((x, y, \lambda)\) image, as depicted visually in Figure 1.03. As a consequence, spectral imaging is time-consuming, which stands in contrast to the requirements of live cell analysis (Garini et al., 2006). Therefore, compromises must be made in the quality of the spectral image if temporal resolution is required. This approach has previously been utilised to unmix fluorescent dyes which have overlapping emission spectra (Garini et al., 2006). A major challenge of spectral imaging is that complex algorithms are necessary for analysing the spectral data. The emission spectra are typically broad and extend into multiple spectral channels (Garini et al., 2006). For unmixing, the contribution of each fluorescent molecule to the total signal needs to be extracted from the spectral data. Therefore, a simpler approach to analysis is required in order to increase the utility of this method (Garini et al., 2006).
1.2.5 - Spectral Phasor Approach

The phasor approach to spectral imaging provides a simplified, graphical representation of the spectral data (Cutrale et al., 2013). This approach works by firstly, collecting and then Fourier transforming the emission spectra of each pixel in the image, using the equations in Figure 1.04, to produce two numbers: the amplitude and phase (Fereidouni et al., 2012). These two numbers are then used as X- and Y- coordinates to construct a scatter plot, otherwise known as a Phasor plot (Figure 1.05) (Fereidouni et al., 2012). Within this Phasor plot, emission spectra are grouped together based on similarities in their phase and amplitude; emissions that are more similar are grouped closer together and those that are more dissimilar are grouped further apart (Figure 1.05) (Andrews et al., 2013, Cutrale et al., 2013, Fereidouni et al., 2012). Selecting regions of the phasor plot with coloured cursors enables mapping of the emission spectra back to the original fluorescence image. Therefore, this approach enables the detection of distinct emissions and spatially maps them back to a fluorescence image (Cutrale et al., 2013). The difference
observed in the spectral emissions of a fluorescent molecule is reportedly contingent on a number of factors including: pH, ion concentration, protein binding, and molecular interactions (Andrews et al., 2013, Cutrale et al., 2013, Fereidouni et al., 2012). In principle, each sodium microenvironment in the cell will have specific species of molecules, which may or may not be actively engaged with sodium ions. The shifts in the emissions (wavelength and width) of CoroNa are expected to reflect the content and activity of that microenvironment.

\[
g = \frac{\sum_\lambda I(\lambda) \cos \left(\frac{2\pi n \lambda}{L}\right)}{\sum_\lambda I(\lambda)} \quad s = \frac{\sum_\lambda I(\lambda) \sin \left(\frac{2\pi n \lambda}{L}\right)}{\sum_\lambda I(\lambda)}
\]

Figure 1.04 – Equations Used to Generate Phasor Plot (Cutrale et al., 2013).

Figure 1.05 - Reference Spectral Phasor Plot.
Emission maxima from 370 nm - 650nm is shown in the grid. The position on the semicircle is determined by the wavelength (indicated by the colour). The spectral width ranges from 500-4000 cm\(^{-1}\), the position in the phasor changes towards the centre with increasing spectral width. Image adopted from Fereidouni et al. (2012).
As can be seen in Figure 1.04, \( I \) represents the pixel intensity, \( L \) the complete range of wavelengths, and \( n \) the harmonic at which the transformation can be performed (Cutrale et al., 2013). Notably, the same data set can be calculated at both the first and second harmonics (Cutrale et al., 2013). The Phasor plot of the first harmonic encompasses \( 3/4\pi \) radians, while the second harmonic encompasses \( 4\pi \) radians (Figure 1.06) (Cutrale et al., 2013). Consequently, compared to the first harmonic, the data set in the second harmonic is distributed more broadly and linearly, thereby increasing its sensitivity in distinguishing the spectral peaks (Cutrale et al., 2013). Despite the fact that there is an aggregation of emissions, the identification of distinct wavelengths is still possible at the first harmonic.

Figure 1.06 – First and Second Harmonic Phasor Plot. (A) The first harmonic’s position in the plot starts in the first quadrant (Q1) encompassing the blue spectrum, and ends in third quadrant (Q3) which encompasses the orange spectrum. The spectra of the first harmonic are more aggregated in comparison to the second harmonic (B) which begins in the second quadrant (Q2) and ends in the first quadrant (Q1). Moving anti-clockwise around the origin increases the wavelength, whereas, moving radially away from the centre decreases the width. Image adapted from Braithwaite (2014).
1.2.6 - Previous Application of Spectral Phasor Approach

Previous studies have applied the Spectral Phasor approach to different fluorescent molecules both in vitro and in vivo (Andrews et al., 2013, Cutrale et al., 2013, Fereidouni et al., 2012). For example, Cutrale et al. (2013) employed this approach to characterise the spectral properties and conversion dynamics of three photo-activable proteins (Dronpa, Kaede, and KikGR). They were able to distinguish these photo-activable fluorescent proteins during photo-conversion based on their spectral fingerprints (Cutrale et al., 2013). Furthermore, by utilising this approach, they were able to determine the states (active and inactive) of these fluorescent proteins in a mixed solution containing all three proteins (Cutrale et al., 2013).

Andrews et al. (2013) employed this approach to distinguish different subspecies of RNA based on the spectral properties of Pyronin Y labelled RNA. By comparing the spectral properties of the Pyronin Y labelled RNA with reference to the structural components of the cell, they were able to infer the identities of the RNA species (Andrews et al., 2013). For instance, they found that RNA transcripts in the nucleolus exhibited similar spectral properties to those of RNA transcripts located in the cytoplasm (Andrews et al., 2013). As such, it was inferred that RNA species in the nucleolus represented ribosomal RNA and messenger ribonucleoprotein complexes in the cytoplasm (Figure 1.07).
Aims of Project

Although the application of the Spectral Phasor approach has been demonstrated for fluorescent molecules (Cutrale et al., 2013; Andrews et al., Fereidouni et al., 2012), it is still a relatively new approach. The application of this approach has not yet been reported for the sodium specific fluorophore CoroNa. Neither has this approach been developed to enable the analysis of dynamic changes occurring in sodium microenvironments. Therefore, the aims of this study were:

- To ascertain the applicability of the Spectral Phasor approach to CoroNa labelled sodium.
- To determine the potential of developing this approach by reducing the acquisition time.
- To establish the utility of the ‘developed parameters’ in analysing the early stages of differentiation (≤ 10 minutes) and performing three-dimensional spectral characterisation.
2.1 - Introduction

Applying the Spectral Phasor approach to the sodium specific fluorescent molecule, CoroNa, is expected to further elucidate the role of sodium microenvironments in cellular processes. The application of this approach to CoroNa has not yet been reported in the literature, and therefore necessitates investigation. To address this, an appropriate biological model, which enables modification of intracellular sodium activity, must first be developed. The myoblast progenitor stem cells, which is an adherent cell line, is expected to serve this purpose. These cells have the potential for infinite self-renewal when maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% Foetal Bovine Serum (FBS) (Yaffe, 1968). They were initially isolated from the rat thigh muscle, and when induced to differentiate by reducing FBS from 10% to 2%, they give rise to multinucleated myocytes (Yaffe and Saxel, 1977). These cells can be perturbed in different ways to illicit a biological response which can then be used to determine the applicability of the Spectral Phasor approach. For instance, since sodium has been implicated to be involved in the differentiation process (Rao et al., 1992, Wang et al., 1997), dynamic changes in sodium microenvironments is expected to occur in the cell during differentiation. These changes are expected to not only localise in the vicinity of the cytoplasmic membrane, where influxes of sodium have been reported to occur (Aronsen et al., 2013), but also in the nucleus, where it is ostensibly involved in gene regulation and expression (Galva et al., 2012, Garner, 2002). The fact that myoblast stem cells are adherent provides the opportunity to apply directional stimulation by utilising trypsin. Although the ability of certain sodium transporters to serve as cellular adhesion molecules is well known (Malhotra et al., 2000), it is still unknown whether inducing changes to the cytoskeleton will result in changes in sodium microenvironments. If the Spectral Phasor approach is applicable to CoroNa in myoblast stem cells, then the expected biological changes of differentiation must be...
detected by this approach. Furthermore, if sodium is involved with the cytoskeletal changes induced by trypsin, then the Spectral Phasor approach is expected to characterise these changes.

2.2 - Method

2.2.2 - Cell Culture

Rat (L6) myoblast stem cells were cultured at least 48 hours prior to imaging onto a 35 mm glass bottom dish (Fluorodish) containing cell growth media (500 µl of DMEM) supplemented with 10% Foetal Bovine Serum (FBS) and Penicillin/Streptomycin, and kept in a 37°C, 5% CO₂ humidified incubator.

2.2.3 - Determination of Optimum CoroNa Concentration

To determine the optimum concentration of CoroNa for Myoblast stem cells, cells were loaded with varying concentrations of CoroNa (0.1 µM, 0.25 µM, 0.5 µM, and 1 µM). Loading the cells with 0.5 µM of CoroNa appeared to be the optimum concentration in 500 µl of DMEM (Appendix 1). Consequently, this concentration was utilised for all the subsequent experiments.

2.2.4 - Biological Model

2.2.4-1 - Fixed Cells

One group of cells was first induced to differentiate by replacing the complete DMEM (10% FBS) with incomplete DMEM (2% FBS), and then fixed with
formaldehyde (4%) at 10, 20, 30, and 40 minutes post differentiation. Another group of cells were trypsinised and then fixed with 4% formaldehyde at 10, 20, 30, and 40 minutes post addition of trypsin. Cells were maintained in phosphate buffer saline (PBS) and kept in a -80 °C freezer for storage until confocal and spectral data acquisition.

2.2.4-2 - Live Cells

In addition to fixed cell analysis, Spectral Phasor analysis of live cells was also performed. Live Myoblast stem cells were either induced to differentiate or undergo trypsinisation (300µL of 0.005% Trypsin EDTA) immediately prior to confocal and spectral data acquisition. Spectral data was collected at 10, 20, 30, and 40 minutes post addition of treatment.

2.2.5 - Spectral Data Acquisition

All confocal and Spectral data acquisitions were performed using the Leica True Confocal Spectrometer - Spectro-Photometer 5 inverted confocal microscope with a HCX PL APO CS 63 x 1.2 water objective paired with an Argon 488 laser. Just prior to imaging, the cells were loaded with CoroNa, excited at 488 nm (2% of maximum laser power) and the emission was captured at 516 nm. For all spectral data acquisition, a detection range of 413 nm - 728 nm, scan speed of 100 Hz, band width of 9.7 nm, and 32 detection steps was employed to acquire spectral images with a resolution of 256 x 256.

For cells undergoing trypsinisation, the focal z-plane corresponding to the base of the cell was utilised for the characterisation of the sodium microenvironmental changes in the vicinity of cellular adhesion proteins. For differentiating cells, to ensure
repeatability the nucleolus was used as a frame of reference to find the appropriate focal slice (Appendix 2).

2.2.6 - Spectral Phasor Analysis

The spectral data was analysed using SimFCS 4.0 (Gratton); regions in the Phasor plot were selected using square coloured cursors, and the emissions were subsequently mapped to the original fluorescence image (Appendix 3). Wavelength and wave-width analysis of the emissions was performed by selecting the ‘angle sectors’ selection in the ‘views window’. Different parameters such as ‘height’, ‘length’, and ‘angle’ were modified to produce cone shaped cursors (wavelength analysis) and rectangle shaped cursors (wave-width analysis) (Appendix 4).

2.2.7 - Statistics

Twenty small square cursors (0.01 SimFCS units) were utilised to select regions of the phasor plot which corresponded to the respective groups (control, differentiation, etc.). The values of the cursors were recorded and loaded into Rstudios to produce boxplots depicting the distribution and ranges of emissions, and bar graphs displaying the average wavelength and width with standard error bars.

2.3 - Results

2.3.1 - Cells Undergoing Trypsinisation

2.3.1-1- Fixed

The phasor of fixed control cells and cells undergoing trypsinisation was located in the second quadrant of the first harmonic (Figure 2.01). The emissions of CoroNa at 10 minutes were most distinct in cells treated with trypsin compared to the control cells (Figure 2.01). Although this difference was greatest at 10 minutes, the difference in emissions at the other timeframes was also significant (Figure 2.02). The greatest difference in the width of the emission was observed to occur at 10 minutes (Figure 2.02C).
Fixed control cells and cells undergoing trypsinisation were analysed with cone shaped cursors in SimFCS 4.0; the respective values of those emissions are listed in the legend immediately underneath the spectral images. Modifying the parameters of the cursor in SimFCS to produce cone shape cursor enabled the analysis of the distribution of wavelengths irrespective of wavelength. The cursor angle was set to 1.5 degrees, and lent was changed to 16 to ensure that all the emissions were included. Utilising this approach made apparent that control cells exhibited emissions with wavelengths of 524.8 nm (aqua cursor), 529.5 nm (maroon cursor), 533.2 nm (light-green cursor), and 541.7 nm (yellow cursor). These wavelengths also appear dominant in cells at 20 and 40 minutes post additions of trypsin. The 516.4 nm (magenta cursor) and 508.8 nm (black cursor) wavelengths appeared to be distributed in the vicinity of the cytoplasmic membrane of control cells and in cells 20 minutes post addition of trypsin; however, in cells undergoing trypsinisation at 10 and 30 minutes, this wavelength appears more centrally in the cell. Besides the aforementioned wavelengths, cells exposed to trypsin for 10 minutes exhibited wavelengths of 504.5 nm (blue cursor) and 513.1 nm (red cursor).
2.3.1-2 - Live

The phasor location of live control cells and cells undergoing trypsinisation was located in the second quadrant of the first harmonic. In square cursor analysis, control cells appeared to exhibit emissions with a wavelength of 516.9 nm (blue cursor), 526.8 nm (yellow cursor), and 535.7 nm (light-green cursor) in the first 30 minutes (Figure 2.03A). At 40 minutes, the 516.9 nm wavelength (blue cursor) which was primarily found centrally in control cells at 10 – 30 minutes appeared prominent peripherally in the vicinity of the cytoplasmic membrane (Figure 2.03A).

Furthermore, the distribution of the 525.4 nm wavelength (aqua cursor) was more evident in control cells at 40 minutes than at any other timeframe (Figure 2.03A). Cells undergoing trypsinisation exhibited the 508.3 nm (red cursor) and 516.9 nm...
(blue cursor) wavelengths (Figure 2.03A). At 20 minutes post addition of trypsin, these emissions were evident peripherally in the cell while the 525.4 nm wavelength (aqua cursor) was apparent centrally in the cell (Figure 2.03). At 30 minutes post addition of trypsin the 525.4 nm (aqua cursor), 516.9 nm (blue cursor), and 508.3 nm (red cursor) wavelengths were apparent in the periphery of the cell while the 526.0 nm (maroon cursor) was evident centrally in the cell (Figure 2.03A).

In wavelength analysis, the 510.9 nm wavelength (aqua cursor) was apparent in the vicinity of the cytoplasmic membrane of control cells at 10 minutes, throughout the cell at 20 minutes, and back towards the periphery of the cells at 30 and 40 minutes (Figure 2.03B). The distribution of this wavelength was evident throughout the cell at 10 minutes post addition of trypsin, and in the vicinity of the cytoplasmic membrane at 20 – 40 minutes post addition of trypsin (Figure 2.03B). The 525.4 nm (maroon cursor) and 531.6 nm (magenta cursor) were more salient not only in control cells at 10, 30 and 40 minutes, but also in cells undergoing trypsinisation at 30 and 40 minutes (Figure 2.03B). The 517.8 nm (light-green cursor) and 521.6 nm (black cursor) wavelengths were evident centrally in cells undergoing trypsinisation at 20 minutes; however its distribution appeared more peripherally in cells at 30 and 40 minutes (Figure 2.03B).

In width analysis, control cells at 10 and 20 minutes primarily exhibited emissions with broad widths (69 nm - maroon cursor) (Figure 2.03C). The distribution of emissions with this width appeared to mainly towards the periphery of the cell at 20 and 30 minutes (Figure 2.03C). At 40 minutes, in cells undergoing trypsinisation, emissions with a width of 44.7 nm (yellow cursor) were primarily evident (Figure 2.03C). Of note is that through all these timeframes, emissions with 54.6 nm (blue cursor) were present. Cells undergoing trypsinisation at 10 and 20 minutes primarily exhibited emissions with a width of 44.7 nm (yellow cursor) throughout the cell (Figure 2.03C). The distribution of these emissions was more prominent towards the periphery of the cell at 30 and 40 minutes post addition of trypsin (Figure 2.03C).
Cells at 30 minutes post addition of trypsin exhibited emissions with widths of 28.5 nm - 37.9 nm (Figure 2.03C). At 40 minutes cells exhibited emissions with widths of 25.5 nm (aqua cursor) and 28.5 nm (black cursor) centrally.
Figure 2.03- Spectral Analysis of Live Control Cells and Cells Undergoing Trypsinisation. (A) In square cursor analysis specific wavelength and widths were selected and then remapped back to original fluorescence image to produce spectral images. (B) In wavelength analysis, the width was excluded and emissions from 504.6 nm to 534.0 nm were selected by cone shaped cursors. (C) In wave width analysis, rectangular cursors were utilised to encompass all wavelength except the background emissions. Values of cursors are placed immediately underneath the phasor plots.
The distribution of emissions in control cells and cells undergoing trypsinisation was quantitatively analysed, as seen in Figure 2.04. It appears that at 10 minutes, cells undergoing trypsinisation exhibit significantly shorter wavelengths compared to control cells (Figure 2.04A & B). However, at 20 minutes cells undergoing trypsinisation exhibited emissions that were larger than control groups, and also had shorter wave widths (Figure 2.04B & C). The difference between the wavelengths in control cells and cells undergoing trypsinisation only appeared significant at 10 minutes (Figure 2.04 C). Conversely, the width of emissions was different throughout the 40 minutes (Figure 2.04C).

**Figure 2.04 – Quantitative Analysis of Emissions in Live Control Cells and Cells Undergoing Trypsinisation.** (A) Boxplot of wavelength distribution of control cells (coral colour) and cells undergoing trypsinisation (cyan colour). Control cells exhibited emissions in the range of 508 nm – 540 nm; conversely, cells undergoing trypsinisation exhibited emissions in the range of 503 nm – 534 nm. On the x-axis of the boxplot C represents control cells, T represents cells undergoing trypsinisation; width of emission is indicated by the degree of darkness of the circles. (B) Depicts the average wavelength of control cells vs cells undergoing trypsinisation (with error bars). The average wavelength of control cells was 520 nm at 10 minutes, 525 nm at 30 minutes, and 520 nm at 40 minutes. In contrast, the average wavelength of cells undergoing trypsinisation was 512 nm at 10 minutes, 523 nm at 20 minutes, 525 nm at 30 minutes, and 522 nm at 40 minutes. The emissions of control cells appeared to oscillate: decrease in wavelength followed by an increase and then subsequent decrease once more. Cells undergoing trypsinisation exhibited wavelengths that appeared to increase up to 30 minutes, and then decrease at 40 minutes. (C) The width of emissions of both control cells and cells undergoing trypsinisation appeared to progressively decrease overtime; however, treated cells exhibited emissions with significantly smaller widths throughout the 40 minutes.
2.3.2 – Cells Undergoing Differentiation

Fixed Cells 2.3.2-1

The spectral images of fixed cells undergoing differentiation revealed that cells at 10 minutes post induction of differentiation exhibited the most dissimilar emissions to the control cells (Figure 2.05). The distribution pattern of emissions in differentiating cells most resembled the control cells at 20 – 40 minute timeframes (Figure 2.05). Emissions of the two groups appeared to overlap in all the timeframes (Figure 2.06A). Comparison of the average wavelengths revealed no significant difference between the two groups throughout the first 40 minutes (Figure 2.06B). The average width of emissions appeared to be significant 10 – 30 minutes but not at 40 minutes (Figure 2.06C).

Figure 2.05 - Wavelength Analysis of Fixed Undifferentiated and Differentiating Myoblast Cells. Control cells appeared to exhibit emissions with a wavelength of 538.7 nm in the nucleolus, 541.9 nm (light-green cursor) in the nuclear region; 533.6 nm (maroon cursor) and 536.3 nm (red cursor) more peripherally in the cell. Cells at 10, 20, 30, and 40 minutes primarily exhibited emissions with a wavelength of 533.6 nm (maroon cursor), 536.3 nm (red cursor) and 538.7 nm (black cursor). At 40 minutes the 544.7 nm (magenta cursor) appeared to surround the nucleus of cell which itself mainly exhibited emissions with a wavelength of 533.6 nm (maroon cursor), 536.3 nm (red cursor), 538.7 nm (black cursor).
The phasor position of live undifferentiated and differentiating cells was located in the second quadrant of the first harmonic (Figure 2.07). Square cursor analysis revealed that control cells at 10 minutes appeared to exhibit emissions with a wavelength of 532.8 nm (aqua cursor) in the cytoplasmic region, and 535.4 nm (red cursor) and 546.4 nm (blue cursor) in the nuclear region (Figure 2.07A). Conversely, cells 10 minutes post induction of differentiation appeared to exhibit emissions with wavelengths of 535.4 nm (red cursor) and 542.9 nm (black cursor) (Figure 2.07A). Notably, at 20 minutes the emissions distributed in control cells and differentiating cells appear to be similar – mainly exhibited wavelengths of 535.4 nm (red cursor) and 542.9 nm (black cursor) (Figure 2.07A). Control cells at 30 minutes appeared to exhibit the 546.1 nm (maroon cursor) wavelength in the vicinity of the nuclear membrane and nucleolus, while the 539.6 nm (dark-green cursor) wavelength was apparent in the nucleoplasm (Figure 2.07A). Cells induced to differentiate at 30
minutes appeared to exhibit a distribution of emissions that was similar to control cells at 10 minutes (Figure 2.07A). At 40 minutes both the control cells and cells induced to differentiate appeared to exhibit similar emissions to control cells at 30 minutes (Figure 2.07A).

Utilising wavelength analysis made apparent that control cells at 10 minutes and 20 minutes appeared to exhibit emissions with a wavelength of 536.4 nm (purple cursor), and 538.5 nm (blue cursor) throughout the cell (Figure 2.07B). At 30 and 40 minutes, in control cells the 543.1 nm (black cursor) and 545.1 nm (yellow cursor) wavelengths appeared to co-localise in the vicinity of the nuclear membrane (Figure 2.07B). Cells 10 minutes post induction of differentiation appeared to exhibit the 543.1 nm (black cursor) and 545.1 nm (yellow cursor) wavelengths more prominently in the nuclear membrane than control cells at 10 and 20 minutes (Figure 2.07B). At 20 minutes both the control cells and cells induced to differentiate exhibited similar spectral emissions, chiefly 534.1 nm (aqua cursor), 536.4 nm (purple cursor), 538.5 nm (blue cursor) and to a lesser degree the 540.8 nm (red cursor) (Figure 2.07B). At 30 minutes post induction of differentiation the 540.8 nm (red cursor) and 543.1 nm (black cursor) wavelengths began to appear near the nuclear membrane, whereas in control cells these emissions were more prominent (Figure 2.07B). At 40 minutes, control cells and cells induced to differentiate appeared to exhibit a similar distribution of emissions, with the exception of 545.1 nm (yellow cursor) wavelength which appeared more prominent in control cells compared to differentiating cells (Figure 2.07B).

Width analysis revealed that control cells at 10 minutes appeared to exhibit emissions with larger widths in the cytoplasmic region (37.9 nm (blue cursor) and 45.0 nm (yellow cursor)), and smaller widths in the nuclear region (29.1 nm (red cursor) and 25.9 nm (purple cursor)) (Figure 2.07C). Cells 10 minutes post induction of differentiation appeared to exhibit emissions with smaller widths in the
cytoplasmic region (23.8 nm (aqua cursor)), and larger widths in the nuclear region (25.9 nm (purple cursor)) (Figure 2.07C). Notably, at 20 minutes the emissions distributed in control cells and differentiating cells appear to be similar – mainly involving emissions with widths of 29.1 nm (red cursor), 25.9 (purple cursor), and 23.8 nm (aqua cursor) (Figure 2.07C). Control cells at 30 minutes appeared to exhibit emissions with widths of 18.3 nm (light-green cursor) in the nucleoplasm, and 19.7 nm (maroon cursor) proximal to the nuclear membrane (Figure 2.07C). Cells induced to differentiate at 30 minutes appeared to exhibit a distribution of emissions that was similar to control cells at 10 minutes (Figure 2.07C). At 40 minutes both the control cells and cells induced to differentiate appeared to exhibit similar emission widths to control cells at 30 minutes, primarily exhibiting emissions with a width of 18.3 nm (light-green cursor), 19.7 nm (maroon cursor) and 23.8 nm (aqua cursor).
Figure 2.07- Spectral Analysis of Live Undifferentiated and Differentiating Myoblast Stem Cells. (A) Square cursor analysis selecting specific wavelength and widths which are then remapped back to original fluorescence image to produce spectral images. (B) In wavelength analysis, the width was not considered and emissions from 527.8 nm to 547.8 nm were selected by cone shaped cursors. (C) In widths analysis rectangular cursors were utilised to select width in the range of 18.3 nm – 45.0 nm irrespective of the wavelength. Values of cursors are placed immediately underneath the phasor plots.
Statistical analysis revealed that the emission wavelengths were only significantly different at 10 minutes post induction of differentiation. After this timeframe, the emissions overlapped in their distribution (Figure 2.08A), and the error bars began to overlap (Figure 2.08B). Control cells appeared to exhibit a greater degree of variability, covering a larger range of emissions (525.5 nm – 541.5 nm) at 10 minutes compared to the subsequent timeframes (Figure 2.08A). In contrast to the wavelength, the width of the emission was observed to be significantly different throughout the 40 minutes (Figure 2.08C). Furthermore, compared to control cells, differentiating cells exhibited narrower widths in the first 20 minutes and broader width at 30 and 40 minutes (Figure 2.08C).

**Figure 2.08- Quantitative Analysis of Emission Emitted by Undifferentiated and Differentiating Cells.** (A) Boxplot generated depicting the distribution of emissions according to the wavelength. The emission of control cells spanned the range of 525.1 nm – 544.5 nm. Differentiating cells exhibited emissions in the range of 534.5 nm – 544.5 nm. Width is indicated by the darkness of the circles, darker circles indicate broader widths while lighter circles indicate more narrow widths, C = control, and D = differentiation. (B) At 10 minutes the average wavelength of control cells was 534 nm and differentiating cells was 540 nm, with non-overlapping error bars. In the subsequent time frames the error bars were overlapping which indicated no significant difference between. (B) Bar graph showing the average wavelength of emissions with error bars revealed significant difference only at 10 minutes. (C) The width of emissions in undifferentiated cells progressively decreased from 30 nm at 10 minutes to 20 nm at 40 minutes. Conversely, the width of differentiating cells was 25 nm at 10 minutes, which then decreased to 19 nm at 20 minutes, and increased again to 28 nm at 30 minutes, and decreased once more to 25 nm at 40 minutes. The difference in the width of the emission was significant throughout the first 40 minutes of differentiation.
2.4 - Discussion

2.4.1 – Fixation Does not Provide Accurate Snapshot of Sodium Microenvironment

Prior to research presented here, the applicability of the Spectral Phasor approach in distinguishing molecular environments had not been reported for the sodium dye, CoroNa. The present study sought to determine whether this approach could be applicable in distinguishing discrete sodium microenvironments in myoblast stem cells. To this end, Spectral Phasor analysis of fixed and live cells undergoing trypsinisation or differentiation was performed. It was determined that fixed cells trypsinised cells exhibited emissions that were distinct from those found in fixed control cells (Figure 2.01). The largest difference in the emission spectra was apparent at 20 minutes post addition of trypsin. This, however, was not the case when spectral analysis of live cells undergoing trypsinisation was performed. In live cells, the emissions appeared most distinct at 10 minutes post addition of trypsin. The difference in the emissions observed in fixed and live cells reflects the changes induced by the fixation process itself. This finding is in agreement with previous research which found that the fixation process led to the loss of numerous molecules such as carotenoids, lipids, and cholesterols (Hobro and Smith, 2016). Furthermore, they found that due to changes induced by fixation; the Raman spectral fingerprint was significantly different compared to live cells (Hobro and Smith, 2016).

Considering the fluxing dynamic activity of sodium in live cells, fixation was utilised to determine if this process provided a snapshot of sodium microenvironments without inducing significant alterations. Research presented here suggests that the fixation process itself induces changes in sodium microenvironments to such a degree that spectral shifts ordinarily observed in live cells were not apparent in fixed cells (Figures 2.01, 2.03, 2.05 & 2.07).
2.4.2 – Spatial and Temporal Sodium Microenvironmental Changes

Spectral Phasor analysis of sodium in live differentiating cells revealed that distinct microenvironments exist in the nucleus, nuclear membrane and cytoplasm of cells undergoing differentiation (Figures 2.05 & 2.07). Furthermore, it appears that the microenvironments in the vicinity of the nuclear membrane, found in differentiating cells at 10 minutes post induction of differentiation were also found in that region at 40 minutes post induction, and also in control cells at both 30 and 40 minutes (Figures 2.05 & 2.07). This similarity in emissions suggests that at these timeframes, these regions of the cell either have similar biochemical processes occurring, or distinct biochemical process that have overlapping emission. The overlapping of emissions in SPA is analogous to other forms of spectroscopy technique such as infrared spectroscopy where distinct functional groups exhibit the similar spectral fingerprint (Noda, 1990).

2.4.3 - Utility of Width vs Wavelength Analysis

The SimFCS 4.0 program enables the utilisation of four cursor shapes for the analysis of spectral data. These shapes include: circle (not used in this study), square, rectangle, and cone shaped. The square cursor appeared useful when the isolation of specific wavelengths and widths were required. For example, utilising the square cursors with a size of 0.01 units enabled the selection of specific emissions for quantitative analysis of emission (Figure 2.02, 2.04, 2.06, and 2.08).

The utility of the cone shaped cursors became apparent when selection of specific wavelengths irrespective of the width was desired. This type of analysis was most useful when the level of the fluorescence between cells fluctuated. The fluctuation of the fluorescence between cells resulted in emissions with broader widths for low
fluorescence, and more narrow width for higher fluorescence emissions. Utilising the square cursors in this situation would not enable the analysis of the same wavelength because of the width difference.

The rectangular shaped cursors were utilised for the analysis of emission width changes. This type of cursor shape enabled the analysis of wave width irrespective of the wavelength. Utilising these three analysis approaches in conjunction enabled the elucidation of distribution patterns which otherwise would not have been apparent. For instance, in live cells undergoing trypsinisation, there did not appear to be a discernible pattern in the distribution of emission when utilising the square cursors (Figure 2.03A). However, wavelength analysis through the use of cone shaped cursors made apparent changes that were occurring in the distribution pattern of emissions in control cells and in cells undergoing trypsinisation or differentiation (Figures 2.03B, 2.07B).

Width analysis also revealed that there were width changes that were contingent on both the time course and treatment and control cells (Figure 2.03C). An emission with a broad wave width is comprised of multiple wavelengths exhibiting intensities (Garini et al., 2006). These wavelengths themselves would be indicative of specific molecular environments or processes. Therefore, the broadness of the emission reflects the heterogeneity of the processes occurring in that molecular environment. Research presented here found emissions with broader widths in the first 20 minutes of trypsinisation and differentiation, and narrower widths in the 30 and 40 minute timeframes (Figures 2.03C, 2.04, 2.07C, and 2.08). This suggests that sodium is involved in more diverse cellular processes in the earlier timeframes, and less diverse cellular processes in the later timeframes. Therefore, for future experiments, width analysis of the spectral emissions of CoroNa could be used to indicate the level of sodium activity in the cell.
A case could be made that the apparent pattern of changes in the emission width is only an indicator of fluorescence intensity. Indeed, a correlation between the fluorescence intensity and the width of the emission was observed by Cutrale et al. (2013) and also in research presented here (Appendix 5). Cutrale et al. (2013) applied the Spectral Phasor approach to unmix and fingerprint the different states of photo-activable proteins Dronpa, Kaede and KikGR. Dronpa is a photo-activable protein that has photo-switching capabilities – the protein undergoes rapid photo-bleaching when excited with 488 nm light, leading to a ‘dark state’. This ‘dark state’ is reversed when the protein is irradiated with 400 nm light (Eisenstein, 2005). Cutrale et al. (2013) reported that because the spectrum of the ‘dark state’ did not have a particular phase and was not modulated, the phasor position was located in the centre of the plot. As the width increased, Cutrale et al. (2013) reported the phasor position also shifting towards the centre of the plot, making it indistinguishable from the background.

If the width of the emission was entirely contingent on the fluorescence intensity, then the broadest widths would be expected at 10 minutes. This is because the fluorescence intensity is partially dependent on the number of CoroNa molecules inside the cell (also contingent on the availability of sodium ions). At 10 minutes the number of CoroNa molecules inside the cell would be relatively fewer than at 20, 30 or 40 minutes. What is instead observed, in differentiating cells for example, is that the width of emissions is broadest not at 10 minutes but at 30 minutes (Figures 2.08C & 2.07C). Furthermore, the width of emissions oscillated independent of the fluorescence intensity. This lends support to the idea that the width of the emission is a reflection of the heterogeneity of wavelengths, and consequently biochemical microenvironments rather than the fluorescence intensity. These examples reveal that had only a single approach been taken for analysis (wavelength vs width), then it would not have been possible to elucidate the various pattern of changes in the molecular environment of sodium in the myoblast stem cell.
The current study has demonstrated the ability of the Spectral Phasor Approach in distinguishing distinct emissions spectra of CoroNa. These emissions represent different microenvironments differing in a number of ways including pH, ion concentration, protein binding, and molecular interactions (as reported by Fereidouni et al. (2012)). While the application of the Spectral Phasor analysis has been demonstrated, the challenge of optimising this approach for the study of sodium still remains. In its current form, spectral data acquisition requires at least 103 seconds for the generation of one optical slice. Considering that the largest spectral shifts occurred in the first 10 minutes post treatment, the utility of SPA in its current form appears limited for the analysis of nuance sodium microenvironmental changes by reducing the acquisition time. Therefore, there is a need for further development of this approach. Furthermore, only analysing a single optical slice of the cell is not representative of all the microenvironmental changes that occur in the cell. Therefore, efforts need to be made to develop the SPA to also enable three-dimensional acquisition of spectral data.
Optimising Spectral Phasor Analysis of Sodium Microenvironments in Live Cells

3.1 - Introduction

Optimising the Spectral Phasor Approach for CoroNa is expected to increase its utility for the analysis of sodium microenvironments in live cells. Since a spectral image contains significantly more information than a fluorescence image, the acquisition time required is also much longer (Garini et al., 2006). In the previous chapter the application of the Spectral Phasor approach was demonstrated for CoroNa; however, since this approach has not been optimised for the analysis of CoroNa labelled sodium, the acquisition of a single spectral image required 103 seconds. When performing analysis of live cells, long acquisition times are an issue for three reasons. Firstly, the light energy from the laser induces changes in the physiology of the cell, consequently introducing artefacts (Garini et al., 2006). Secondly, excessive exposure to light energy from the laser leads to photo bleaching which can also introduce artefacts in the spectral data acquired (Cutrale et al., 2013). Thirdly, the findings of experiments conducted in Chapter 2, suggests that the majority of changes in sodium microenvironments occurs within the first 10 minutes. Consequently, a low temporal resolution (103 seconds) would not enable the characterisation of nuance sodium microenvironmental changes occurring in this timeframe. Therefore, the experiments conducted in this chapter aimed to optimise the Spectral Phasor approach for CoroNa by identifying the parameters that would reduce the acquisition time without significantly reducing the quality of the spectral images.

3.2 - Method

Myoblast stem cells were cultured and maintained according to the protocol discussed in chapter 2.2.2 – Cell Culture. Moreover, the treatment regimen employed
in this chapter was also according to the protocol discussed in Chapter 2.2.4- Biological model – Live Cells. In this study, to determine the appropriate scan rate, spectral acquisition was performed at 100 Hz, 200 Hz, 400 Hz, and 700 Hz. Based on the findings, all the proceeding experiments were conducted at 100 Hz with a band width of 9.7 nm.

The acquisition range was initially narrowed by acquiring spectral data in three regions of the spectrum: 404 nm – 500 nm, 500 nm – 600 nm, and 600 nm – 700 nm using 32 steps, 17 steps, or 12 steps. The 500 nm – 600 nm region of the spectrum was identified as a region of interest and therefore spectral data was then collected in this range using 17 steps. This scan range was further narrowed by collecting emissions in the 500 nm – 550 nm and 550 nm – 600 nm sub region. Within this range, the 520 nm – 550 nm range was identified as a region of interest; consequently, spectral data was collected in this region utilising either 4 steps. The data was loaded into SimFCS 4.0 and the range of the phasor plot was calibrated based on the acquisition range (See Appendix 6 for method of calibration).

3.3 - Results

3.3.1 - Determining Appropriate Scan Rate

To determine the optimum scan speed of the laser, varying scan speeds were utilised to acquire spectral data of control cells. Increasing the scan speed resulted in a broader phasor distribution (Figure 3.01). Consequently, the resolution of the spatial distribution of the spectral information decreased with increasing scan speed (Figure 3.01).
3.3.2 - Ascertaining Most Pertinent Region of Spectrum

3.3.2-1 - 404 nm – 500 nm

In order to develop the Spectral Phasor approach for CoroNa labelled sodium, data was acquired in three different regions of the spectrum: 404 nm – 500 nm, 500 nm – 600 nm, and 600 nm – 700 nm. In addition, the number of steps was also modified to not only determine the appropriate step number for acquiring data in a 100 nm range, but also ascertain the effect of varying the step number. Figure 3.02 depicts the intensity of emissions in the three regions of the spectrum scanned, in relation to the step number. Performing a full range scan (413 nm – 728 nm) gave rise to a phasor that was located in the second quadrant of the first harmonic (Figure 3.02A). Scanning in the range of 404 nm – 500 nm with 17 steps resulted in a phasor that was
similarly positioned in the second quadrant of the first harmonic (Figure 3.02B). Capturing the spectral data with 12 steps caused a shift in the phasor location between the first and second quadrant (Figure 3.02B). Employing 32 steps caused the phasor to shift from the first/second quadrant to the fourth quadrant of the first harmonic (Figure 3.02B). Furthermore, compared to 12 and 17 steps which have phasors located further out from the centre of the plot, acquiring spectral data with 32 steps resulted in a phasor that extended radially out from the centre of the phasor plot (Figure 3.02B). The shift in the position of the phasor also shifted the wavelengths and width of the emissions. For example, utilising 12 steps resulted in emissions in the range of $\lambda$: 420.5 nm – 731.8 nm with widths of 3.3 nm – 3.8 nm (Figure 3.02B). Whereas collecting data with 17 steps resulted in emissions in the range of $\lambda$: 428.9 – 444.8 nm with width of 3.5 nm – 5.6 nm (Figure 3.02B). Furthermore, acquiring data with 32 steps gave rise to emissions in the range of $\lambda$: 485.3 – 492.5 nm with width of 6.1 nm – 100 nm (Figure 3.02B). The spectral image of cells in the 404 nm – 500 nm range did not appear to resemble the morphology of the cell and the intensity of spectral emissions seen in the full range image (Figure 3.02A & B).

3.3.2-2 - 500 – 600 nm

Scanning in the range of 500 nm – 600 nm gave rise to a phasor that closely resembled the phasor of the full range scan (Figure 3.02 A & C). Utilising 32 and 17 steps for scans in this range resulted in a phasor that was located in the second quadrant of the first harmonic, which was essentially in the same location of the full range scan (Figure 3.02 A & C). The emissions evident when utilising these two step numbers were almost identical ($\lambda$: 532.3 nm – 543.1 nm for 32 steps and 532.5 nm – 545.0 nm for 17 steps) (see Figure 3.02 C). Furthermore, acquiring spectral data in this range with 32 steps resulted in the emissions shifting radially outwards (narrower widths). Utilising 12 steps shifted the position of the phasor to the third quadrant of the first harmonic (see Figure 3.02C). This resulted in emission wavelengths in the
range of $\lambda$: 546.6 nm – 558.1 nm with width in the range of 8.3 nm – 100 nm.

Comparing the spectral images acquired with varying step number, it is apparent that utilising 12 steps resulted in emissions with the lowest intensity and 32 steps with the highest intensity. From these step numbers it appears that 17 steps most resembled the spectral emission of the full range scan.
Figure 3.02 – Phasor Position of Myoblast Cells at Different Emission Ranges Step Numbers. (A) Full range scan of control cell collected using 32 steps. (B) Scanning in the 404 nm – 500 nm region did not exhibit images that resembled the full range scan. (C) 500 nm – 600 nm and (D) 600 nm – 700 nm appeared to resemble the full range scan. The step appeared to shift the position of the phasor and consequently the emissions.
3.3.3 - Spectral Phasor Analysis of Live Cells Undergoing Trypsinisation

Control cells at 10 minutes exhibited the 531.6 nm (magenta cursor) and 534.1 nm (aqua cursor) wavelengths in the vicinity of the cytoplasmic membrane, while exhibiting the 537.8 nm (blue cursor) and 536.5 nm (black cursor) wavelengths more centrally in the cell (Figure 3.03). At 20 minutes the 534.1 nm wavelengths (aqua cursor) appeared more prominently centrally in the cell than the subsequent timeframes. Notably, the 531.6 nm wavelength (magenta cursor) was apparent in the periphery in control cells throughout the 40 minutes. Cells undergoing trypsinisation appeared to exhibit the 531.6 nm wavelength (magenta cursor) broadly throughout the cell at 10 minutes post addition of trypsin (Figure 3.03). By 20 minutes this emission was most apparent in the periphery of the cells (Figure 3.03). The control cells and cells undergoing trypsin appeared most similar from 20 – 40 minute timeframes. This was further confirmed by comparing the average wavelengths which demonstrated that the largest difference was indeed at 10 minutes (Figure 3.03), after which, the average emissions were not significantly different between the two groups (Figure 3.04).
Figure 3.03 – Spectral Analysis (500 nm – 600 nm) of Live Control Cells and Cells Undergoing Trypsinisation. The phasor of both control cells and cells undergoing trypsinisation was located in the second quadrant of the phasor plot. Cone shaped cursors were utilised to select distinct emissions and remap them to the fluorescence image to produce spectral images. The values for the cursors are given in the blue box below the phasor plot.
Spectral analysis of live cells made apparent distinct emissions found in undifferentiating and differentiating cells (Figure 3.05). Undifferentiated cells exhibited emissions in the range of 530.0 nm – 535.7 nm compared to differentiating cells which exhibited emissions in the range of 534.9 nm – 540.0 nm (Figure 3.05).

Comparison of average wavelengths revealed a significant difference in emissions exhibited between undifferentiated and differentiating cells throughout the first 40 minutes post induction of differentiation (Figure 3.06). In contrast, average width
was significantly different at only 20 minutes post induction of differentiation (Figure 3.06).

Figure 3.05 – Spectral Analysis (500 nm – 600 nm) of Live Undifferentiated and Differentiating Myoblast Stem Cells. The phasor of both undifferentiated cells and cells undergoing differentiation was located in the second quadrant of the phasor plot. Cone shaped cursors were utilised to select emissions in the range of 529.5 nm – 538.6 nm. Emissions found in undifferentiated cells did not appear to significantly overlap with emissions found in differentiating cells. The values for the cursors are given in the blue box below the phasor plot. Control cells exhibited the 530.7 nm (magenta cursor) in the cytosol in all the timeframes; however, at 30 minutes its distribution appeared more prominent in the nuclear membrane and nucleolus (Figure 3.05). Conversely, differentiating cells exhibited the 538.6 nm (maroon cursor) wavelength in the nucleolus and the nuclear membrane region (Figure 3.05). The 536.3 nm (aqua cursor) was found in both undifferentiated and differentiating cell - the distribution in undifferentiated cells was apparent sporadically in the cell, whereas in differentiating cells its distribution was evident in the nuclear region.
3.3.5 - Comparison of Wavelength Ranges

To develop the Spectral Phasor approach, the spectral data acquisition range needs to be narrowed further. Experiments were run to determine which sub region of the 500 nm – 600 nm range displayed emission shifts that resembled the distribution of emission found in 500 – 600 nm. Consequently, spectral data was collected in three regions: (a) 500 nm – 600 nm; (b) 500 nm – 550 nm, (c) 550 nm – 600 nm. Figure 3.07 depicts the comparison of these three ranges in differentiating cells. It appears that spectral data acquired in the range of 500 nm – 550 nm most resembled the spectral data collected in the range of 500 nm – 600 nm (Figure 3.07). The emissions selected in the 550 nm – 600 nm did not resemble the emissions evident in the 500 nm – 600
nm range. Altering the scanning range also altered the emissions (wavelength and width) without modifying its distribution (see Table 1.1)

Figure 3.07 – Comparison of Emission Distribution in Various Ranges. Emissions selected and mapped in the spectral images collected in the range of 500 nm – 600 nm were 535.1 nm (yellow cursor), 540.0 nm (blue cursor), and 538.0 nm (purple cursor). In spectral images derived from scans in the range of 500 nm – 550 nm, the distribution of the 532.7 nm (light blue cursor) wavelength resembled the distribution of 535.1 nm (yellow cursor) of the 500 nm – 600 nm scan. Furthermore, the 534.1 nm (maroon cursor) wavelength appeared to have the same distribution of 538.0 nm (purple cursor) wavelength of the 500 nm – 600 nm scan range. Lastly the distribution of 535.2 (magenta cursor) appeared to be the same as the 540.0nm (blue cursor) acquired in the 500 nm – 600 nm scan. Emissions collected in the 550 nm – 600 nm scan range gave rise to spectral images that did not resemble the 500 nm – 600 nm scan. However, the 543.3 nm (light-green cursor) did appear to localise primarily towards the centre of the cell. The 556.6 nm wavelength appeared to be distributed throughout the cell, while the distribution of the 560.1 nm wavelength appeared sporadic.
Table 1.1 Emission Comparisons Between 500 nm – 600 nm and 500 nm – 550 nm. The wavelength of emissions shifted at least 2.4 nm when scans were performed in the 500 nm – 550 nm region of the spectrum. Colours represent cursor colour

<table>
<thead>
<tr>
<th>500 nm – 600 nm range</th>
<th>500 – 550 nm</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>538.0 nm</td>
<td>534.1 nm</td>
<td>3.9 nm</td>
</tr>
<tr>
<td>535.1 nm</td>
<td>532.7 nm</td>
<td>2.4 nm</td>
</tr>
<tr>
<td>540.0 nm</td>
<td>535.2 nm</td>
<td>4.8 nm</td>
</tr>
</tbody>
</table>

Spectral Phasor Analysis (500 nm – 550 nm) of Live Cells Undergoing Trypsinisation

Analysis of live control cells and cells undergoing trypsinisation revealed that the largest shifts in the emissions occurred 10 minutes post addition of trypsin (Figures 3.08 & 3.09). Moreover, emissions evident throughout the cell at 10 minutes post addition of trypsin were only apparent in the periphery in trypsinised cells in the subsequent timeframes, and in control cells throughout the 40 minutes. The wavelength of control cells and cells undergoing trypsinisation appeared not to overlap at 10 minutes to the same degree as in the subsequent timeframes (Figure 3.09A). Although, the emissions were most distinct at 10 minutes post addition of trypsin, this difference was not significant (Figure 3.09B). However, a significant difference in the emission width was observed only at 10 minutes and not in the subsequent timeframes (Figure 3.09C).
Figure 3.08 – Spectral Analysis (500 nm – 550 nm) of Live Control Cells and Cells Undergoing Trypsinisation.

Control cells throughout the 40 minutes predominantly exhibited emissions with wavelengths of 534.2 nm, 535.2 nm, and 531.7 nm. Cells 10 minutes post addition of trypsin exhibited emissions with wavelengths of 528.4 nm (aqua cursor), 530.8 nm (red cursor), and 532.6 nm. At 20 – 40 minutes post addition of trypsin the spectral emissions were found to be similar to those found in control cells. Values of cursors are given below phasor plot.
3.3.6 - Spectral Phasor Analysis (500 nm – 550 nm) of Live Differentiating Cell

The emissions found in undifferentiated and differentiating cells were significantly more similar throughout the first 40 minutes than those found in the 500 nm – 600 nm scan (Figures 3.05 & 3.10). Although it was not significant, the largest difference between undifferentiated cells and cells undergoing differentiation appeared to be in the first 10 minutes (Figure 3.11). Compared to control cells at 10 minutes, cells 10 minutes post induction of differentiation exhibited shorter wavelengths (Figure 3.10 & 3.11). Control cells predominantly exhibited emission with wavelengths of 533.9 nm (aqua cursor), 532.0 nm (blue cursor), 531.1 nm (maroon cursor) and 533.4 nm (dark green), whereas differentiating cells exhibited wavelengths of 531.1 nm...
(maroon cursor), 530.6 nm (yellow), 529.4 nm (black cursor) and 528.7 nm (magenta cursor) (Figure 3.10). The pattern of distribution of emissions between control cells and cells 20 minutes post induction of differentiation appeared to be virtually identically at 20 minutes (Figure 3.10). The 531.1 nm (maroon cursor) and 531.7 nm (purple cursor) wavelengths appeared to localise in the nucleoplasm while the 532.2 nm (light green) and 532.0 nm (blue cursor) wavelengths appeared to localise in the nuclear membrane region. These wavelengths became even more prominent in control cells at 30 and 40 minutes, and in differentiating cells at 40 minutes. At 30 minutes post induction of differentiation, the 530.6 nm (yellow cursor), 531.1 nm (maroon cursor), and 531.7 nm (purple cursor) wavelengths were evident in differentiating cells (Figure 3.10). Comparison of the average widths revealed a significant difference between undifferentiated and differentiating cells from 20 – 40 minutes (Figure 3.11).

Figure 3.10 – Spectral Analysis (500 nm – 550 nm) of Live Undifferentiated and Differentiating Myoblast Stem Cells. The phasor of undifferentiated and differentiating cells were located in the third quadrant of the first harmonic. Cone shaped cursors selected emissions in the range of 528.7 nm – 533.9 nm. The values for the cursors are given in the blue box below the phasor plot.
3.3.7 - Comparison of Scan Ranges

To better understand the effect of varying the range over which spectral data is collected, the average wavelength of CoroNa in each experimental condition was plotted over time. Performing a full range scan (413 nm – 728 nm) resulted in emissions that were distributed in a larger range, in both trypsinisation and differentiation experimental conditions (Figure 3.12). Full range (413 nm -728 nm) scans gave rise to emissions which oscillated over the 40 minutes of differentiation (Figure 3.12A). Narrowing the scan range to 50 nm (red lines) still gave rise to an oscillatory pattern; however, the range and degree of oscillation was reduced (Figure 3.12A). Moreover, the average wavelengths of the narrower scan ranges were significantly lower than the full range scan in differentiating cells.
Compared to differentiating cells, cells undergoing trypsinisation exhibited smaller wavelengths in the full range scans (Figure 3.12B). In the full range scan the average wavelength of both control cells and cells undergoing trypsinisation appeared to exhibit a concave pattern of emissions (Figure 3.12B). This, however, was not the case for the narrower scan ranges; the average wavelength appeared to oscillate in both the 100 nm and 50 nm scan range. Moreover, in the narrower scan ranges the average wavelengths were significantly larger than the full range scan. Figure 3.12 also makes apparent that the most significant spectral shifts in live cells undergoing differentiation and trypsinisation occurred within the first 10 minutes (with the exception of differentiating cells in 500 nm – 600 nm scan range).

Wavelength Changes in Live Myoblast Stem Cells Undergoing Differentiation or Trypsinisation

A. Cells Undergoing Differentiation

B. Cells Undergoing Trypsinisation

Figure 3.12 – Comparison of Average Wavelength of Control and Treatment Groups in Varying Scan Ranges. (A) Full range scan of differentiating cells resulted in emissions over a larger range (534 nm – 542 nm) compared to the 100 nm scan range (532 nm – 538 nm) and 50 nm scan range (531.7 nm – 532.2 nm). (B) Full range scans of cells undergoing trypsinisation gave rise to emissions in the range of 496 nm – 526 nm. Scanning in the narrower ranges also reduced the emission range to 531 nm – 536 nm.
3.3.8 - 520 nm – 550 nm Scan Ranges

After narrowing the spectral acquisition range to 500 nm – 550 nm, experiments were conducted to further optimise this approach. Consequently, the spatial distribution of emissions of control cells at 10, 20, and 30 minutes was acquired at both the 500 nm – 550 nm, and 520 nm – 550 nm ranges. Although the pattern of distribution of emissions collected in the 30 nm range appeared to match the distribution pattern of emissions collected at the 50 nm range (Figure 3.13). The specific emissions between the two scan ranges were not the same. However, it was apparent that the distribution of emissions with smaller wavelengths (yellow cursors), in both scan ranges, were similar, just as the distribution of larger wavelengths were similar (blue cursors) (Figure 3.13).

Figure 3.13 – Comparison of Average Wavelength Distribution in the 500 nm – 550 nm and 520 nm – 550 nm Scan Range. In the 50 nm range, the 518.5 nm was apparent peripherally in the cell at 10 minutes, both centrally and peripherally at 20 minutes, and peripherally at 30 minutes. In the 500 nm – 550 nm scan range, the 519.6 nm wavelength appeared most prominently centrally throughout the first 30 minutes. In the 520 nm – 550 nm spectral acquisition range, the 528.0 nm appeared to match the distribution of 519.6 nm in the 50 nm range scan. Furthermore, the 524.5 nm appeared to exhibit a similar distribution pattern to the 518.5 nm in the 50 nm scan range.
Reducing the acquisition time of spectral data was contingent not only on narrowing the range in which data was collected, but also reducing the number of steps utilised (Table 1.2). Narrowing the acquisition range to 30 nm enabled to utilisation of 4 steps for scans, which lead to an acquisition time of 13.5 seconds (Table 1.2).

<table>
<thead>
<tr>
<th>Number of Steps</th>
<th>Range (nm)</th>
<th>Time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>32</td>
<td>413 nm – 728 nm</td>
<td>102</td>
</tr>
<tr>
<td>17</td>
<td>500 nm – 600 nm</td>
<td>51</td>
</tr>
<tr>
<td>12</td>
<td>500 nm – 550 nm</td>
<td>37</td>
</tr>
<tr>
<td>4</td>
<td>520 nm – 550 nm</td>
<td>13.5</td>
</tr>
</tbody>
</table>

Table 1.2 – Comparison of Acquisition Time Based on Range and Step Number. As the number of steps and acquisition range was reduced, the acquisition time also reduced.
3.4 - Discussion

Research presented here aimed to optimise the Spectral Phasor approach for CoroNa by reducing the acquisition time. To this end, the most suitable laser scan rate was firstly determined by acquiring full range spectral data (413 nm – 728 nm) with varying scan speeds (from 100 Hz to 700 Hz). It was found that with increasing scan speed, the quality of the spectral images progressively decreased (Figure 3.01). This is because, reducing the scan speed also decreases the time spent at each pixel collecting photons (Garini et al., 2006). Due to the lower numbers of photons collected, distinguishing the photons from the background becomes increasingly difficult (Garini et al., 2006), consequently shifting the emissions towards the centre of the plot, as seen in Figure 3.01. Of these scan speeds, 100 Hz appeared to be most suitable based on the broadness of the phasor distribution, and the resolution of the spatial distribution of spectral emissions.

The acquisition time of the Spectral Phasor approach for the sodium specific fluorescent molecule was reduced by aiming to: (a) narrow the acquisition range; (b) reduce the step number utilised. This approach was undertaken to identify the most pertinent elements of the spectrum for the analysis of sodium in the trypsinisation and differentiation process. It was determined that the 500 nm – 550 nm region of spectrum enabled the analysis of cells undergoing differentiation or trypsinisation. However, the spectral shifts of the full range scan compared to the narrower scan range scans (100 nm and 50 nm) were significantly larger. This observed reduction in the variability of emissions in the narrower ranges suggests that critical spectral elements in other parts of the spectrum are missed during acquisition. By reducing the acquisition range, the contribution of spectral elements in other regions (e.g. perhaps 600 nm – 700 nm) is omitted. This suggests that a specific wavelength selected in the phasor plot does not only represent that specific wavelength, but rather, it also represents the contribution of other wavelengths collected in the acquisition range. As a consequence of this observation, emissions that have been
collected utilising different collection ranges during the same biological conditions are not comparable. Instead, what is comparable is the pattern of emission changes across the different acquisition ranges. This is further illustrated in Figures 3.07 and 3.12 which demonstrates the shifts in wavelength as a consequence of changing the scan range. Furthermore, in Figures 3.07 and 3.13 specifically, it becomes apparent that distinct wavelengths exhibit identical distribution patterns in the differentiating cell across the different scan ranges.

Notable is the difference in the emissions of CoroNa between the control cells of differentiating experiments and the control cells of the trypsinisation experiments (Figure 3.12). Control cells of the differentiation experiments on average exhibited larger wavelengths than control cells of the trypsinisation experiments (Figure 3.12). This can be explained by the focal plane utilised to acquire spectral data in the two experiments (Appendix 3). In the differentiation experiments, a focal plane which encompassed the nuclear membrane and nucleolus was utilised: (a) to ensure microenvironmental changes in the nucleus and nucleolus were included in the analysis; (b) to establish the repeatability of experiments by using the nucleolus and nuclear membrane as a landmark to identify the appropriate focal plane. In the trypsinisation experiments the focal plane corresponding to the base of the cell which housed the adhesion proteins was selected. Consequently, the difference in the emission spectra observed reflects the different sodium microenvironments in these two locations of the cell.

Experiments conducted here, further confirmed the findings of the previous chapter. In the full range experiments (chapter 2), the largest spectral shifts observed between the control and treated groups was at 10 minutes. After this timeframe, the emissions appeared to progressively become similar. Even though the spectral shifts of the narrower scan ranges (100 nm and 50 nm) were significantly smaller than the full range scans, the same pattern of spectral shifts was observed (Figure 3.12). This
finding confirms that the most significant changes in sodium microenvironments occur within 10 minutes post treatment. Furthermore, it highlights the utility of the narrower scan ranges (particularly the 50 nm range) in distinguishing these sodium microenvironmental changes.

Spectrally analysing differentiating cells in the 500 nm – 600 nm range appeared to contradict the pattern described. The emissions apparent in this collection range were distinct and did not overlap throughout the 40 minutes, a pattern not observed in the full range scan. The inconsistency observed may be a consequence of an unknown confounding variables. This idea is supported by data obtained in the 500 nm – 550 nm range which was in agreement with the results obtained in the full range scan (see Chapter 2). If the 500 nm – 600 nm data collected for live differentiating cells is excluded, the aforementioned pattern still holds true.

This study has demonstrated the reduction of the spectral acquisition time necessary for the analysis sodium microenvironments. The acquisition time was reduced from 103 seconds down to 13.5 seconds (Table 1.2) by acquiring spectral data exclusively in the 520 nm – 550 nm range for live cell analysis. What remains to be determined is whether acquiring spectral data in this range enables the distinction of sodium microenvironmental changes in live differentiating myoblast stem cells. Furthermore, the utility of this narrowed range in performing short-time frame analysis, and generating 3D spectral characterisation also requires confirmation.
4.1 - Introduction

A minute-by-minute spectral analysis of sodium microenvironments is expected to provide a nuance perspective of the fluxing activity of sodium in stem cell differentiation. From previous experiments it became apparent that the largest spectral shifts were occurring at 10 minutes post induction of differentiation and trypsinisation. In order to enable the potential analysis of sodium microenvironments at minute intervals, efforts were made to develop the Spectral Phasor Approach. Through these efforts, the acquisition time of spectral data was reduced down to 13.5 seconds by: (a) narrowing the spectral acquisition range to 520 nm – 550 nm region of the spectrum; (b) reducing the step number to 4 steps for the 30 nm range. The current study aimed to determine whether analysing spectral elements in this range would enable the characterisation of sodium microenvironment in live differentiating myoblast stem cells at a minute by minute basis. Furthermore, this study aimed to determine whether this reduction in acquisition time would enable three-dimensional spectral characterisation, through the acquisition of multiple z focal slices (x,y,λ,z).

4.2 - Method

The protocol for culturing myoblast stem cells was the same as discussed in Chapter 2.2.2 – Cell Culture. Just prior to analysis, cells were induced to differentiate and immediately loaded with 0.5 µM of CoroNa.
4.2.1 - Minute by Minute Spectral Analysis

Spectral data acquisition was initiated at 3 min post induction of differentiation, and scans at minute intervals were performed up to 30 minutes. In these scans, spectral data was only collected in the 520 nm – 550 nm range of the spectrum at 100 Hz using 4 steps with a band width of 9.7 nm. The data was loaded into SimFCS 4.0, and then the phasor plots generated were calibrated for 520 nm – 550 nm range by adjusting the minimum and maximum wavelengths in the lifeform window (Appendix 6).

4.2.2 - Three-dimensional Analysis

Whole cell spectral data was collected at 3, 10, and 20 minutes post induction of differentiation (Appendices 22 & 23). This was achieved by determining the highest and lowest z focal plane of the cell, and then acquiring multiple z focal slices at 1 micrometre steps. Despite the fact that the Leica SP5 system has the capability to automatically acquire three-dimensional spectral data (x,y,λ,z), SimFCS in its current form does not support the analysis of a single z stack file acquired through the Leica system. Therefore, z stack spectral data was collected by scanning each slice and then manually adjusting the z plane.
4.3 - Results

4.3.1- Minute Interval Spectral Analysis

The fluorescence intensity in control cells fluctuated throughout the first 20 minutes, whereas differentiating cells exhibited a progressive increase in fluorescence. The nuclear membrane integrity of control cells appeared compromised at 18 minutes, coinciding with an increase in fluorescence intensity (Figure 4.01A). Furthermore, control cells were observed to bleb at 28 minutes post addition of CoroNa (Appendix 18 & 19). Conversely, the nuclear integrity of differentiating cells did not appear compromised throughout the 30 minutes (Figure 4.01B). The microenvironmental changes observed via the utilisation of minute interval analysis, were distinct in both undifferentiated and differentiating cells (Figure 4.01). Moreover, the majority of microenvironmental changes occurred in the nuclear region, whereas microenvironments in the vicinity of the cytoplasmic membrane did not appear to change over the 20 minutes (Figure 4.01).

Quantitative analysis of spectral emission apparent in minute interval analysis revealed that the greatest difference occurred at 3 minutes (Figure 2). After which the emissions became increasingly similar up to 9 minutes, and increasingly dissimilar from 9 minutes up to 18 minutes (Figure 4.02).
Figure 4.01 – Minute Interval Spectral Analysis of Undifferentiated and Differentiating Myoblast Stem Cells.

The intensity of fluorescence is indicated by the colour gradient: blue represents low fluorescence, whereas red/orange represents high fluorescence. (A) In control cells the intensity of fluorescence was lowest at 3 minutes, after which the fluorescence was observed to progressively increase up to 9 minutes, particularly in the nucleus. From 9 minutes to 12 minutes, the fluorescence intensity was observed to decrease, and then increase after 12 minutes reaching the highest fluorescence at 20 minutes. (B) In stark contrast to control cells, the fluorescence intensity of CoroNa in cells induced to differentiate appeared lowest at 3 minutes but progressively increased up to 20 minutes. (A) In undifferentiated myoblast cells, the 527.1 nm emission (aqua cursor) was apparent near the nuclear membrane, cytoplasm and cytoplasmic membrane. While the distribution of this emission did not appear to change near the cytoplasmic membrane throughout the 20 minutes of observation. Close to the nuclear membrane and cytoplasm, the microenvironments which exhibited this emission began to shift to emissions with wavelengths of 527.4 nm (blue cursor) and 527.9 nm (purple cursor) at 4 minutes. The blue (527.4 nm) and purple (527.9 nm) coloured coded microenvironments were apparent up to 12 minutes, after which the emissions in the vicinity of the nuclear membrane shifted to 526.5 nm (red cursor). The distribution of the 526.1 nm emission (magenta cursor) did not shift significantly until the 526.5 nm emission (red cursor) was prominently apparent in the cell at 18 minutes. (B) In differentiating cells the 527.1 nm (aqua cursor) and 528.3 nm (green cursor) emissions were heterogeneously distributed. Similar to the undifferentiated cells, after the 3 minutes the distribution of 527.1 nm (aqua cursor) wavelength was seen to undergo a reduction in the nucleus and cytoplasm, but did not significantly alter near the cytoplasmic membrane. The same microenvironments which exhibited the 527.1 nm (aqua cursor) or 528.3 nm (green cursor) wavelengths began to instead exhibit the 527.9 nm (purple cursor) and 527.4 nm (blue cursor) emissions after 6 minutes post induction of differentiation. The distribution of the 526.1 nm (magenta cursor) wavelength was mainly apparent near the nuclear membrane and the nucleolus, from 6 minutes to 18 minutes post induction of differentiation. However, the prevalence of this emission in the nuclear membrane and nucleolus fluctuated – it became more prevalent near the nuclear membrane up 9 minutes, and then less prevalent after 9 minutes.
The largest difference in the average emissions exhibited by undifferentiated and differentiating cells was observed at 3 minutes (526.3 nm for undifferentiated cells and 527.1 nm for differentiating cells). From 3 minutes to 9 minutes the difference between the average emissions began to reduce, and was most similar at 9 minutes (526.6 nm for undifferentiated cells and 526.7 nm for differentiating cells). After this point of time, differentiating myoblast cells exhibited an increase and then a decrease, followed by a plateauing of emissions (526.8 nm at 18 minutes). Whereas control cells after 9 minutes, progressively exhibited smaller and smaller wavelengths, eventually reaching 526.2 nm.

Figure 4.02 – Average Wavelengths Exhibited in the Initial 18 minutes of Differentiation.
4.3.2 - Three-dimensional Analysis

Three-dimensional intensity and spectral images of the whole cell (refer to Appendix 23), nuclear region and cytoplasmic region was generated.

4.3.2-1 - Nuclear Region

Three-dimensional models of the nuclear region at 3 minutes revealed that control cells exhibited emissions in the range of 522.3 nm – 525.7 nm, whereas differentiating cells exhibited emissions in the range of 526.4 nm – 528.0 nm (Figure 4.03 A & B). The emissions of control cells appeared to localise in specific nuclear regions (Figure 4.03A). Conversely, the emissions of differentiating cells appeared to exhibit a greater degree of dispersion in its distribution (Figure 4.03B).

The nuclear region of control cells at 10 minutes exhibited distinct emissions from those found in differentiating cells (Figure 4.04). As seen in the nuclear region of differentiating cells at 3 minutes, the distribution of emissions at 10 minutes also appeared to be more heterogeneous than undifferentiated cells (Figure 4.04). At 20 minutes the emissions found in differentiating cells resembled those found in undifferentiated cells (Figure 4.05).
Figure 4.03 – Three-Dimensional Characterisation of the Nuclear Region of Undifferentiated and Differentiating Myoblast Stem Cells at 3 Minutes. (A) Three emissions appeared most prominently in the nuclear region of undifferentiated cells: 523.7 nm (green), 525.1 nm (red), and 525.3 nm (blue). The 523.7 nm emission appeared most dominant towards the top of the nuclear region, the 525.1 nm emission towards the middle, and the 525.3 nm towards the lower section of the nuclear region. (B) In the nuclear region of differentiating cells, the emissions most prominent were 526.4 nm (yellow cursor), 526.8 nm (blue cursor), 528.0 nm (red cursor), and 528.2 nm (green cursor). The emissions in the nuclear region of differentiating appeared more dispersed than those found in undifferentiated cells.
Figure 4.04 – Three-Dimensional Characterisation of the Nuclear Region of Undifferentiated and Differentiating Myoblast Stem Cells at 10 Minutes. (A) Undifferentiated myoblast stem cells primarily exhibited three emissions: 525.2 nm (yellow cursor), 525.4 nm (aqua cursor), and 526.7 nm (green cursor). The 526.2 nm (red cursor) and 527.6 nm (blue cursor) emissions were apparent only in the top section of nuclear region. (B) Differentiating myoblast stem cells mainly exhibited the 527.6 nm emission (blue cursor) throughout the nuclear region. The distribution of the 526.7 nm (green cursor), 526.2 nm (red cursor), and 527.6 nm (aqua) emissions appeared sporadically in the nuclear region of differentiating cells at 10 minutes. The values for the cursors are given in the blue box immediately below the phasor plot. Cursor values are same for both undifferentiated and differentiating cell.
Figure 4.05 – Three-Dimensional Characterisation of the Nuclear Region of Undifferentiated and Differentiating Myoblast Stem Cells at 20 Minutes. Both the control cells (A) and (B) differentiating cells at 20 minutes primarily exhibited 527.8 nm (green cursor) and 526.0 nm (aqua cursor) emissions. Differentiating cell also appeared to exhibit the 529.0 nm in the upper periphery of the nuclear region. The values for the cursors are given in the blue box immediately below the phasor plot. Cursor values are same for both undifferentiated and differentiating cell.
4.3.2-2 - Cytoplasmic Membrane Region

Emissions found in the vicinity of the cytoplasmic membrane of undifferentiated cells were distinct to those found in differentiating cells throughout the first 20 minutes (Figure 4.06). At 3 minutes, the emissions localised at the base of undifferentiated cells were distinct to those found in the vicinity of the cytoplasmic membrane corresponding to the sides of the cell (Figure 4.06A). Conversely, the base of differentiating cells did not appear to exhibit emissions that were distinct from the cytoplasmic regions corresponding to the sides of the cell (Figure 4.06).

At 10 minutes, both the undifferentiated and differentiating myoblast stem cells appeared to exhibit emissions in what appeared to be two layers (Figure 4.07). For both groups, the outer layer was predominantly composed of the 525.7 nm wavelength (magenta cursor) (Figure 4.07). The inner layer of emission appeared distinct between the two groups; undifferentiated cells mainly exhibited the 525.4 nm (aqua cursor) emission (Figure 4.07A), whereas differentiating cells primarily exhibited the 527.6 nm (blue cursor) emission (Figure 4.07B).

At 20 minutes, the cytoplasmic membrane region of undifferentiated cells chiefly exhibited the 526.8 nm (blue cursor) emission in conjunction with the 527.8 nm (light-green) emission which displayed a sporadic distribution (Figure 4.08B). These two emissions were apparent in differentiating cells; however, their distribution appeared sporadic (Figure 4.08B). Differentiating cells at 20 minutes primarily exhibited the 529.0 nm (magenta cursor) emission in the vicinity of the cytoplasmic membrane region.
Figure 4.06 – Three-Dimensional Characterisation of the Cytoplasmic Membrane Region of Undifferentiated and Differentiating Myoblast Stem Cells at 3 Minutes.

(A) The base of undifferentiated myoblast stem cells mainly exhibited the 523.7 nm (green cursor), 525.1 nm (red cursor), and 525.3 nm (blue cursor) emissions. The 525.7 nm (magenta cursor) and 522.3 nm (aqua cursor) emissions were apparent in towards the cytoplasmic membrane corresponding to the sides of the cell. In contrast, differentiating cells exhibited the 528.2 nm (green cursor) emission primarily in the base of the cell. Towards the cytoplasmic membrane corresponding to the sides of the cell, the 528.2 nm (light-green cursor) emission along with the 526.8 nm (blue cursor) and 528.0 nm (red cursor) emissions exhibited a sporadic distribution. The values for the cursors are given in the blue box immediately below the phasor plots.
Figure 4.07 – Three-Dimensional Characterisation of the Cytoplasmic Membrane Region of Undifferentiated and Differentiating Myoblast Stem Cells at 10 Minutes. There appeared to be two layers corresponding to the cytoplasmic region of both undifferentiated (A) and differentiating myoblast (B). Both undifferentiated and differentiating cells exhibited the 525.7 nm (magenta cursor) emission in the outermost layer. (A) However, in the inner layer, control cells exhibited primarily 525.4 nm (aqua cursor) emission, and more sporadically the 525.2 nm (yellow cursor) and 526.2 nm (red cursor) emissions. (B) Differentiating cells primarily exhibited the 527.6 nm emission in the inner layer, while also sporadically exhibiting the 526.2 nm (red cursor), 525.4 nm (aqua cursor), and 526.7 nm (green cursor). The values for the cursors are given in the blue box immediately below the phasor plot.
Figure 4.08 – Three-Dimensional Characterisation of the Cytoplasmic Membrane Region of Undifferentiated and Differentiating Myoblast Stem Cells at 20 Minutes. (A) The cytoplasmic region of undifferentiated cells chiefly exhibited the 526.8 nm (blue cursor) and 527.8 nm (green cursor). A sporadic distribution of 526.2 nm (red cursor), 527.1 nm (yellow cursor) was also observed in the vicinity of the cytoplasmic membrane. (B) Differentiating cells predominantly exhibited the 529.0 nm (magenta cursor), while also exhibited the 526.8 nm (blue cursor and 527.8 nm (green cursor) in the cytoplasmic membrane region. The values for the cursors are given in the blue box immediately below the phasor plot.
4.3.3 – Comparing Emissions of Different Cellular Regions

4.3.3-1 - Whole Cell

The emissions in undifferentiated and differentiating cells were the most distinct at 3-minute, but became progressively similar with increasing time (Figure 4.09A). The average emission in differentiating cell increased at 10 minutes post induction of differentiation and then decreased at 20 minutes (Figure 4.09A); whereas the emissions of control cells progressively increased in the first 20 minutes. Undifferentiated and differentiating were spectrally the most similar at 20 minutes post induction of differentiation, with a difference of 0.1 nm in the emission (Figure 4.09A).

4.3.3-2 - Nuclear Region

When only the nuclear region of undifferentiated and differentiating cells were analysed, a different pattern of emission changes became apparent. The nuclear region of differentiating cells exhibited a decrease in the average wavelength at 10 minutes. This was in contrast to the emission change of the whole cell which exhibited an increase in the average emission 10 minutes. Similar to the whole cell, the emissions between the two groups progressively became similar, reaching most similarity at 20 minutes.

4.3.3-3 - Cytoplasmic Membrane Region

The largest difference in emissions between the undifferentiated and differentiating myoblast stems cells was apparent in the cytoplasmic membrane region (Figure 4.09C). Undifferentiated cells exhibited an average emission of 524.7 nm at 3-minute, which then increased to 526.0 nm at 10 minutes. After this time frame, the average emission appeared to plateau around 526.0 nm. Differentiating cells exhibited an
average emission of 526.9 nm at 3-minute, which then increased to 527.1 nm at 10 minutes. Similar to undifferentiated cells, the average emission of differentiating cells at 20-minute appeared to plateau; however, this plateau occurred at 527.0 nm. The difference in emissions exhibited in the cytoplasmic membrane region was greater than that observed in the emissions of the whole cell and the nuclear region (Figure 4.09).

Figure 4.09 – Average Wavelengths of Different Cellular Regions During Early Stages of Differentiation. (A) The average emission between undifferentiated and differentiating cells was most distinct at 3 minutes (525.7 nm and 526.4 nm respectively). The difference between the two groups decreased overtime, reaching most similar at 20 minutes (control: 526.6 nm, differentiating: 526.7 nm). (B) Analysis of emissions in the nuclear region revealed a similar pattern to the whole cell; however, the nuclear region experience a decrease in average wavelength at 10 minutes, while the whole cell experienced an increase. (C) The cytoplasmic region of the cells was also most distinct at 3 minutes and became increasingly similar over the 20 minutes. However, the cytoplasmic membrane region remained more distinct than the nuclear region.
4.4 - Discussion

4.4.1 – Minute- interval Analysis

The aim of research presented was to determine whether acquiring spectral data in the range of 520 nm – 550 nm would enable the identification of distinct emission changes in undifferentiated and differentiating myoblast stem cells. Furthermore, the current study sought to determine whether acquiring data in this range would enable three-dimensional spectral characterisation. Analysis in this region of the spectrum made apparent that cells undergoing differentiation exhibited a different pattern of emissions compared to undifferentiated cells. Minute-interval analysis revealed that the majority of changes occurred in the nuclear region, while the cytoplasmic region exhibited no significant change. This suggests that throughout the early stages of differentiation the microenvironments in the nuclear region experiences the most change. These observed changes may be a consequence of different proteins, enzymes and transcription factors being recruited to initiate the differentiation process. The largest difference was observed at 3 minutes; however, it is likely that significant changes occur before this timeframe. The apparatus setup necessitated at least 2.5 minutes, consequently hindering the ability to characterise spectral changes earlier than 3 minutes. Furthermore, 3 minutes was needed for the CoroNa molecules to translocate into the cell for sodium ion detection. The difference observed at 3 minutes progressively decreased up until 9 minutes post induction of differentiation, where the similarity between the two groups was the highest (Figure 4.02). This appears to contradict the results obtained in previous experiments which demonstrated that the largest spectral shifts occurred at 10 minute post induction of differentiation. This difference may be accounted by the fact that since the acquisition of spectral data in previous experiments necessitated longer timeframes, it is possible that spectral data collected also encompassed emission beyond the 10 minute timeframe (11-12 minutes). The fact that after 10 minutes the cells began to exhibit emissions which were becoming increasingly different lends support to this idea. Therefore, it is possible that the spectral differences observed in previous
experiments reflect the divergence observed at 11-12 minutes post induction of differentiation.

CoroNa in undifferentiated cells initially exhibited relatively low fluorescence levels (3 minute); however the intensity of fluorescence fluctuated over the first 18 minutes of observation (Figure 4.01). The initial increase of sodium (observed at 4 minutes) in the cell may be the cell's attempt to achieve equilibrium in intracellular sodium concentration. This may be the case because of the way CoroNa detects sodium ions – the crown ether section of the molecule traps a sodium ion thereby disrupting its availability for biochemical processes (Meier et al., 2006). As a consequence of this disruption, the cell may be attempting to increase intracellular concentrations of sodium.

Compared to undifferentiated myoblasts stem cells, differentiating cells exhibited a lower level of fluorescence (Figure 4.01). Furthermore, differentiating cells did not exhibit an oscillatory pattern of intensity, but rather, the intensity progressively increased, reaching the highest at 18 minutes post induction of differentiation (Figure 4.01). This reduced level of fluorescence may be a function of a few factors. Firstly, since differentiating cells undergo significant changes in their biochemical environments, it is possible that sodium becomes heavily involved in these processes (Page and Di Cera, 2006, Wang et al., 1997). As a consequence, the fluorescence intensity of CoroNa is limited because of its inability to trap sodium that is already engaged in the biochemical processes of differentiation (Meier et al., 2006). Another possibility is that when myoblast stem cells undergo differentiation, the permeability of the cytoplasmic membrane to CoroNa becomes reduced; a finding that has been reported for other fluorescent dyes (Aeschbacher et al., 1986). In this scenario fewer molecules enter the cell, consequently causing a reduction in fluorescence intensity.
4.4.2 - Three-dimensional modelling

Research presented here demonstrated that acquiring data in the 520 nm – 550 nm region of the spectrum enabled not only the analysis of cells at minute intervals. But due to the relatively short acquisition time, utilising this scan range also enabled the acquisition of spectral data at multiple focal planes, enabling the generation of 3D spectral models. Utilising these models provided a new way of characterising the biochemical changes occurring in the early stages of differentiation.

The 3D fluorescence intensity models made apparent the existence of a fluorescence intensity gradient from the nucleus to the cytoplasmic membrane. The highest fluorescence intensity of CoroNa bound sodium was observed in the nuclear region, which has been reported previously to have high concentrations of sodium relative to the cytoplasm (Garner, 2002; Galva 2012). An unexpected observation was that fluorescence intensity of CoroNa decreased proportionally to the distance from the nucleus in rat myoblast stem cells. The decrease in fluorescence intensity may be a function of two factors. Firstly, it may exclusively be the result of low sodium concentrations in that specific region of the cell. Secondly, it may be the result of unavailable sodium ions which are already involved with proteins and other macromolecules. Based on this, it is possible to interpret the results presented here in the following way, sodium engagement with macromolecules increases proportionally to the distance from the nucleus, and consequently causes the reduction in observable fluorescence (Page and Di Cera, 2006). This interpretation would be in agreement with findings from the previous chapters (Chapter 2 and 3) that demonstrated the width of emissions increases proportionally to the distance from the nucleus. Since broader widths are composed of multiple wavelengths, and each specific wavelength is an indicator of a biochemical environment, the broadness of an emission can indicate the degree of heterogeneity of sodium concentra
microenvironments. This suggests that the diversity in biochemical processes which utilises sodium, increases with increasing distance from the nucleus.

Three-dimensional spectral models revealed that undifferentiated cells exhibited emission that appeared to have locality in distinct section of the nuclear region throughout the first 20 minutes (Figures 4.03A – 4.05A). In contrast, the cells in the early stages of differentiation (≤ 10 minutes) exhibited greater heterogeneity in the distribution of emissions in the nuclear region (Figures 4.03B – 4.05B). However, at 20 minutes the nuclear region of cells induced to differentiate resembled control cells – emissions appeared to localise in distinct nuclear regions. These findings suggest that a wave of changes involving sodium occurs in the nucleus in the first 10 minutes, and by 20 minutes the activity of sodium returns to normal (i.e. it resembles the control cells). Previous research conducted by Delgado et al. (2003), found that during the differentiation process there were multiple temporal waves of gene expression beginning immediately and lasting up to 24 hours post induction of differentiation in mouse C2C12 myoblast stem cells. In each wave, the expression of a specific group of genes was observed to increase by a factor of at least three. In this cell line, the majority of genes expressed immediately post induction of differentiation (0 hour) were involved in cell signalling and transcriptional control. Although the C2C12 mouse myoblast cell line is a different species to the L6 rat myoblast cell line utilised in the present study, it is conceivable that the temporal waves of gene expression are analogous. If this assumption holds true, then there appears to be a correlation between the activity of sodium in the nucleus and the subsequent gene expression that is observed to occur (Delgado et al., 2003). It is even possible that the temporal wave of sodium changes serves as an initial signal to trigger the expression specific genes. This hypothesis, however, requires further experimental work aiming to firstly, determine the genes expressed in the first 20 minutes of differentiation in rat L6 Myoblast cells, and secondly, to disrupt the activity of sodium to determine if any substantial changes occur in the expression of those genes. While a cause and effect relationship has not been established, the
present study does provide support for the involvement of sodium in these nuclear events. This would be in agreement with previous *in vitro* studies which demonstrated the involvement of sodium in regulating DNA-protein interactions, and gene expression.

Three-dimensional spectral analysis also revealed that compared to the nuclear region and the whole cell, the cytoplasmic membrane region exhibited the largest difference in average emissions in the first 20 minutes (Figures 4.06 – 4.09). This suggests that while the microenvironments of sodium in the nuclear region become increasingly similar over time, the microenvironments in the vicinity of the cytoplasmic membrane remain distinct for at least 20 minutes post induction of differentiation. This observed difference is a reflection of the changes that occur in the vicinity of the cytoplasmic membrane of differentiating cells otherwise not present in undifferentiated cells. These changes include but are not limited to: (a) the expression of genes related to cell signalling (Delgado et al., 2003); (b) reported greater expression levels of proteins such as ezrin, radixin, and moeisin which are linker proteins that are involved in connecting the cytoplasmic membrane to the cytoskeleton (Sliogeryte et al., 2014).

4.4.3 - Analysis of Sodium Induces Biological Changes

Performing minute interval spectral acquisition led to morphological changes in cells. Notably, from 18 minutes the morphology of the undifferentiated cell appeared to alter, and at approximately 28 minutes the cells began to bleb (Figure 1, and Appendices 18 & 19). Coinciding with the blebbing of the cell was an increase in intracellular sodium concentrations indicated by fluorescence intensity. The blebbing observed may be an indication that the cell is undergoing apoptosis. It has been previously reported that cells undergoing apoptosis exhibit a significant increase in intracellular sodium concentrations (Arrebola et al., 2005, Panayiotidis et al., 2006).
the current study, this blebbing and apoptosis was not observed in cells where spectral data was collected intermittently (7 – 10 minute intervals). This suggests that the blebbing of the cell is caused by the combination of both the addition of CoroNa and the introduction of light energy from the laser. It is also possible that the laser alone is the cause of the blebbing. Further experiments in which cells are exposed to the argon 488 laser both at minute intervals and 10 minute intervals without the addition of CoroNa, will elucidate the contribution of the laser to this phenomena.

Worthy of noting is the resistance that differentiating cells appeared to exhibit against blebbing. While Undifferentiated cells underwent blebbing at 28 minutes, differentiating cells did not appear to bleb throughout the 30 minute observation. This finding agrees with previous research (Lombet et al., 2001, Sliogeryte et al., 2014, Xiao et al., 2011) which also reported resistance to blebbing in differentiating stem cells of a number of cell lines including: human mesenchymal stem cells, human neuroblastoma stem cells, and mouse myoblast stem cells. In majority of these cell lines, undifferentiated and differentiating cells both exhibited increased expression of pro-apoptotic proteins (caspases etc.); however, differentiating cells exhibited increased expression of anti-apoptotic proteins (Lombet et al., 2001, Xiao et al., 2011). In addition, differentiating cells also increased membrane – actin cortex adhesion, which consequently reduced the susceptibility to both osmotic and mechanical bleb formation (Sliogeryte et al., 2014).

The findings of the current study suggest that minute interval spectral analysis induces significant changes to live cells. This alteration of the cell occurred to such a degree that cells began to bleb as a consequence. As a result, any spectral changes observed through this method cannot be attributed to the natural biochemical changes involved in the differentiation process. Rather, these spectral changes also reflect the modifications induced by the observation. In future experiments, rather than collecting spectral data at every minute, a longer interval between each
acquisition needs to be utilised (for example, 3 min or 5 min intervals). Comparison to the 10 minute interval analysis will indicate whether these shorter time intervals induce any discernible changes.
Conclusion

The aim of this research project was to firstly determine the applicability of the Spectral Phasor Approach to the sodium specific fluorescent probe CoroNa. This aim was addressed in chapter 2 where full range scans were performed on fixed and live myoblast stem cells either undergoing trypsinisation or differentiation. Through these experiments the applicability of the Spectral Phasor approach was demonstrated; however, these series of experiments also highlighted the need to reduce the acquisition time. This was a necessity because in both differentiating cells and cells undergoing trypsinisation, the largest spectral shifts were observed to occur at approximately 10 minutes post treatment. The aim of chapter 3 was therefore to determine whether the Spectral Phasor approach could be better adapted for the analysis of sodium microenvironments in myoblast stem cells. To achieve this aim, spectral data was collected in three regions of the spectrum 404 nm – 500 nm, 500 nm – 600 nm, and 600 nm – 700 nm. Data across these three ranges were collected in order to further narrow regions of the spectrum that were pertinent to the analysis of sodium. From the three regions of the spectrum, the 500 nm – 600 nm spectral intensity images most resembled the image of the full range scan. This range was hypothesised to contain the majority of spectral information pertinent for the analysis of CoroNa labelled sodium. Therefore, focus was shifted towards this region of the spectrum. Although the efforts of this research project focused on the 500 nm – 600 nm range, the spectral intensity images of the 600 nm – 700 nm range indicated that significant spectral shifts occurred in this region of the spectrum (Chapter 3, Figure 2). The absence of these emissions (600 nm – 700 nm range) was reflected by a reduction in the size of the shifts during the analysis of fixed and live differentiating cells and cells undergoing trypsinisation. In full range scans the shifts occurred over a larger range (encompassing 55 nm); however, as the spectral acquisition range was narrowed, the shifts became progressively smaller (1 nm in 520 nm – 550 nm scan range). This reduction in the shift again suggests that contributing elements in the 600 nm – 700 nm region of the spectrum are neglected by utilising the more
narrowed range analysis approach. Nonetheless, in chapter 3 the spectral acquisition range was reduced from the full range (413 nm – 728 nm) down to 520 nm – 550 nm. At each point of reduction (500 nm – 600 nm to 500 nm – 550 nm, etc.) the spectral image in the narrower range was compared to the larger range to ensure that the two spectral images exhibited a similar distribution emissions.

The largest contributing factor to reducing the acquisition time was determined to be the number of steps utilised in the collection of the spectral data. Reducing the step number led to a reduction in the acquisition time; however, it also led to a reduction in the quality of the spectral image. To mitigate this side effect, various step numbers were tested for the different ranges utilised. It was determined that having too many steps in a specified range led to an overlapping of the steps. This consequently caused the phasor to shift towards the centre of the phasor plot, making it indistinguishable from the background (chapter 3, Figure 2). Conversely, having too few steps led to sections of the specified region being omitted, consequently limiting the number of wavelengths scanned. Therefore, with all these ranges, a steps number which covered the range without overlapping (hair line distance between the steps) was utilised to mitigate the aforementioned issue.

However, comparing the spectral images generated in the range of 520 nm – 550 nm with 4 steps, to the 500 nm – 550 nm range acquired with 12 steps, it was evident that the spatial resolution of the spectral information had greatly reduced. As a consequence, chapter 4 aimed to determine whether this 30 nm range would still enable the minute interval analysis of sodium microenvironment during differentiation. Furthermore, since the acquisition time had been reduced from 103 seconds down to 13.5 seconds, the potential for performing whole cell spectral analysis also needed to be determined.
Experiments in chapter 4 demonstrated that spectral data acquisition in this narrowed range enabled analysis of sodium microenvironments at minute intervals. From these experiments it was determined that the largest difference in spectral emissions occurred at 3 minutes post induction of differentiation. Because 3 minutes was the minimum time required for the CoroNa to enter the cells and detect sodium, and for the apparatus to be set up for data acquisition, it is likely that greater changes occur within the first 3 minutes that are not characterised. This was not a novel finding, and was in agreement with previous research that also reported sodium influxes as an antecedent to downstream event such as DNA synthesis, proliferation, and differentiation (Koch and Leffert, 1979, Rozengurt et al., 1981, Wang et al., 1997). The novel finding of research presented here was: firstly, that sodium microenvironments in the nucleus experienced an initial change (≤ 3 minutes), and then over the first 20 minutes of differentiation began to increasingly become similar to the control cells. This wave of change early in the differentiation process may be the signal that initiates the differentiation process in live myoblast stem cells. This would be in agreement with the experimental results of Delgado et al. (2003) who observed temporal waves of gene expression throughout the differentiation process. However, further experimental work is required to show a cause and effect relationship.

**Future Work**

There are a number of questions that remain to be answered and should be the focus of future investigations. The first is, which specific elements in the 600 nm – 700 nm range are relevant for the analysis of sodium microenvironments? This can be determined by the same approach utilised in chapter 3, which was to compare the distribution of emission in the spectral images when attempting to narrow the scan ranges. Once other spectral elements in the 600 nm – 700 nm range have been specified, then experiments where these spectral elements are included in the data acquisition need to be conducted. A comparison of the results to the findings of
chapter 4 will determine whether including these spectral elements provides an improved approach to analysing sodium microenvironments.

The second is, can the ‘new developed’ approach of Spectral Phasor analysis, characterize the spectral changes occurring in the first 10 minutes of trypsinisation? Employing trypsin as a treatment enabled cytoskeletal perturbation, which was used to identify any changes in sodium microenvironments. Generating 3D spectral models of cells in the first 10 minutes post addition of trypsin is expected to elucidate: (a) if there are directional sodium microenvironmental changes; (b) whether these changes propagate from the base of the cell towards the nucleus. Investigating this question is expected to elucidate sodium changes involved in cellular deadhesion, a process involved in cancer metastasis (Hanahan and Weinberg, 2000). The third question that needs answering is, is there a cause and effect relationship between the sodium microenvironmental change and gene expression? Future experiments need to firstly determine whether the same pattern of gene expression as described for C2C12 also exist in rat myoblast L6 cells. Then, compounds which inhibit sodium influxes (for example, amiloride) need to be utilised to determine if the inhibition of sodium influxes will lead to the suppression of the expression of genes found in the first wave. Furthermore, compounds which facilitate sodium influxes (amphotericin B) can be utilised to determine if sodium microenvironments enhance the expression of the genes in the first wave. These experiments will help determine whether there is a cause and effect relationship between the sodium influxes and the expression of genes involved in initiating differentiation. What is also possible is that rather than sodium being involved in only the first wave of gene expression, it may also be involved in subsequent waves of gene expression. This question can be answered by spectrally analysing cells at the expected time of the wave of gene expression. If sodium is involved in that specific wave, then a significant difference should be observed either during or before the wave. Furthermore, inhibition of sodium influxes at that specific time is expected to suppress the expression of those genes if sodium indeed plays a role.
Reference


Braithwaite, J. 2014. Spectral Phasor characterisation of Cytoskeletal Protein and Live Cell RNA Dynamics. Bachelor of Science (Honours), Western Sydney University.


Appendices

Appendix 1 - Determining Appropriate CoroNa Concentration for Rat Myoblast Stem Cells

Appendix Figure 1.0 – Fluorescence intensity of CoroNa. (A) CoroNa exhibited dose dependent increase in fluorescence in media containing varying concentrations of sodium. (B) Pseudo-bright field image of myoblast cell displays no fluorescence in the absence of CoroNa. (C – E) Loading myoblast cells with CoroNa exhibited a concentration dependent increase in fluorescence (0.1 – 1 µM) (C= cytoplasm, N= nucleus, arrow indicating nucleolus).
Appendix 2 - Optical Focal Planes Utilised for the Current Study

Appendix Figure 2.0 – Focal Slice Position Utilised for Differentiation and Trypsinisation Experiments. (A) Differentiation experiments utilised a focal slice that traversed the nucleus and nucleolus. (B) Generic Cell model depicting the position of focal slices utilised in this project. (C) Focal slice corresponding to the base of the cell was employed for the trypsinisation experiments.
Appendix 3 - Method for Loading Spectral Files in SimFCS 4.0

Appendix Figure 3.0 – Method for Loading Spectral files into SimFCS for Analysis. Spectral files were loaded into SimFCS by selecting ‘FLIM’ option which subsequently opened two other windows. In the ‘Lifetime Imaging Calculation’ window, the files were referenced by clicking on the ‘file’ tab and then selecting ‘Reference spectra files’. The referenced files were then loaded into SimFCS by click the ‘file’ tab in the ‘1’ window, and then clicking on the ‘Read and referenced and add’ option. Loaded referenced files are shown in (c).
Appendix 4- Modifying Cursor Shape

Appendix Figure 4.0 – Method for Adjusting Cursor Shape and Size. The cursor shape was adjusted by selecting the ‘view’ tab in the ‘I’ window. The rectangular shaped cursor was produced by selecting the ‘Angular sector’ option, and then employing a larger angle value than the length. Conversely, the cone shaped cursor was generated by utilising a larger length than angle. Square cursors were generated by selecting the ‘square’ option; the size of the square cursor was modified in the ‘cursor’ tab (green box).
Appendix 5 - Relationship Between Fluorescence and Emission Width

A. Varying Sodium Concentration

B. Varying Laser Power

Appendix Figure 5.0 - Spectral Phasor Analysis of CoroNa™ in Media. Phasor plot derived from SimFCS makes apparent the phasor location for both varying sodium concentration (a) and laser power (b) which are located in the first quadrant of the first harmonic. Increasing the concentration of sodium (NaCl) and increasing laser power appeared to shift the phasor position more radially, indicating a narrowing of spectral width. The increase in sodium concentration and laser power caused an increase in fluorescence intensity.
Appendix 6 - Method of Calibrating Phasor Based on Acquisition Range

Appendix Figure 6.0 – Method for Calibrating Phasor Plot based on the Spectral Acquisition Range. In the ‘Lifetime Imaging Calculation’ window, the calibration tab was clicked and the start and end wavelengths were adjusted according to the range in which the spectral emissions were collected.
Appendix Figure 7.0 – Spectral Analysis (413 nm – 728 nm) of Fixed Control Cells and Cells undergoing Trypsinisation. (A) Square cursor analysis; (B) Wavelength analysis; (C) Width analysis. Values for the cursor are placed immediately below the phasor plot.
Appendix 8 – Fixed Differentiated Cells (Full Range) Full Dataset

A. Square Cursor Analysis

<table>
<thead>
<tr>
<th>Control</th>
<th>10 min</th>
<th>20 min</th>
<th>30 min</th>
<th>40 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>λ: 526.8 nm Width: 36.8 nm</td>
<td>λ: 534.0 nm Width: 18.8 nm</td>
<td>λ: 532.5 nm Width: 23.1 nm</td>
<td>λ: 534.6 nm Width: 22.3 nm</td>
<td>λ: 534.6 nm Width: 26.4 nm</td>
</tr>
<tr>
<td>λ: 535.9 nm Width: 43.3 nm</td>
<td>λ: 539.7 nm Width: 20.9 nm</td>
<td>λ: 541.9 nm Width: 20.0 nm</td>
<td>λ: 541.9 nm Width: 25.4 nm</td>
<td>λ: 542.7 nm Width: 18.6 nm</td>
</tr>
</tbody>
</table>

B. Wavelength Analysis

<table>
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<th>20 min</th>
<th>30 min</th>
<th>40 min</th>
</tr>
</thead>
<tbody>
<tr>
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<td>λ: 530.8 nm</td>
<td>λ: 533.6 nm</td>
<td>λ: 536.3 nm</td>
<td>λ: 538.7 nm</td>
</tr>
<tr>
<td>λ: 544.7 nm</td>
<td>λ: 547.2 nm</td>
<td>λ: 550.3 nm</td>
<td>λ: 553.4 nm</td>
<td></td>
</tr>
</tbody>
</table>

C. Width Analysis

<table>
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<tr>
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<th>20 min</th>
<th>30 min</th>
<th>40 min</th>
</tr>
</thead>
<tbody>
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<td>Width: 15.9 nm</td>
<td>Width: 17 nm</td>
<td>Width: 19.6 nm</td>
<td>Width: 21.2 nm</td>
<td>Width: 23.1 nm</td>
</tr>
<tr>
<td>Width: 25.8 nm</td>
<td>Width: 29.2 nm</td>
<td>Width: 32.8 nm</td>
<td>Width: 38.1 nm</td>
<td>Width: 38.3 nm</td>
</tr>
</tbody>
</table>

Appendix Figure 8.0 – Spectral Analysis (413 nm – 728 nm) of Fixed Control cells and Cells Induced to Differentiate. (A) Square cursor analysis; (B) Wavelength analysis; (C) Width analysis. Values for the cursor are placed immediately below the phasor plot.
Appendix 9 – Spectral Analysis (Full range) of Live Differentiating Cells Replicate

A. Square Cursor Analysis

B. Wavelength Analysis

C. Width Analysis

Appendix Figure 9.0 – Spectral Analysis (413 nm – 728 nm) of Live Control cells and Cells Induced to Differentiate. (A) Square cursor analysis; (B) Wavelength analysis; (C) Width analysis. Values for the cursor are placed immediately below the phasor plot.
Appendix 10 - Spectral Analysis (500 nm - 600) of Fixed Cells Trypsinised

Appendix Figure 10.0 – Spectral Analysis (500 nm – 600 nm) of Fixed Control cells and Cells Undergoing Trypsinisation. (A) Square cursor analysis; (B) Wavelength analysis; (C) Width analysis. Values for the cursor are placed immediately below the phasor plot.
Appendix 11 - Spectral Analysis (500 nm - 600) of Live Cells Undergoing Trypsinisation Replicate

Appendix Figure 11.0 – Spectral Analysis (500 nm – 600 nm) of Live Control cells and Cells Undergoing Trypsinisation. (A) Square cursor analysis; (B) Wavelength analysis; (C) Width analysis. Values for the cursor are placed immediately below the phasor plot.
Appendix – 12 Spectral Analysis (500 nm - 600 nm) of Fixed Differentiated Cells

A. Square Cursor Analysis

B. Wavelength Analysis

C. Width Analysis

Appendix Figure 12.0 – Spectral Analysis (500 nm – 600 nm) of Fixed Control cells and Cells Undergoing Differentiation. (A) Square cursor analysis; (B) Wavelength analysis; (C) Width analysis. Values for the cursor are placed immediately below the phasor plot.
Appendix – 13 Spectral Analysis (500 nm - 600) of Live Cells Undergoing Differentiation Replicate

Appendix Figure 13.0 – Spectral Analysis (500 nm – 600 nm) of Live Control cells and Cells Undergoing Differentiation. (A) Square cursor analysis; (B) Wavelength analysis; (C) Width analysis. Values for the cursor are placed immediately below the phasor plot.
Appendix 14 - Spectral Analysis (500 nm - 550) of Fixed Trypsinised Cells

Appendix Figure 14.0 – Spectral Analysis (500 nm – 550 nm) of Fixed Control cells and Cells Undergoing Trypsinisation. (A) Square cursor analysis; (B) Wavelength analysis; (C) Width analysis. Values for the cursor are placed immediately below the phasor plot.
Appendix 15 - Spectral Analysis (500 nm - 550) of Live Cells Undergoing Trypsinisation Replicate

Appendix Figure 15.0 – Spectral Analysis (500 nm – 550 nm) of Live Control cells and Cells Undergoing Trypsinisation. (A) Square cursor analysis; (B) Wavelength analysis; (C) Width analysis. Values for the cursor are placed immediately below the phasor plot.
Appendix 16 - Spectral Analysis (500 nm - 550) of Fixed Cells Differentiated Cells

Figure 16.0 – Spectral Analysis (500 nm – 550 nm) of Fixed Control cells and Cells Undergoing Differentiation. (A) Square cursor analysis; (B) Wavelength analysis; (C) Width analysis. Values for the cursor are placed immediately below the phasor plot.
Appendix 17 - Spectral Analysis (500 nm - 550) of Fixed Differentiated Cells Replicate

A. Square Cursor Analysis

B. Wavelength Analysis

C. Width Analysis

Appendix Figure 17.0 – Spectral Analysis (500 nm – 550 nm) of Live Control cells and Cells Undergoing Differentiation. (A) Square cursor analysis; (B) Wavelength analysis; (C) Width analysis. Values for the cursor are placed immediately below the phasor plot.
Appendix 18 – Minute Interval Spectral Analysis (520 nm - 550) of Live Control Cells Full Dataset

Appendix Figure 18.0 – Minute Interval Full Spectral Data of Undifferentiated Myoblast Stem Cells. Fluorescence images are represented by ‘FI’ and Spectral images are indicated by ‘SP’. Control cells began to bleb at approximately 28 minutes. Values for the cursor are placed immediately below the phasor plot. ‘FI’ represents fluorescence images, and ‘SP’ represents spectral images.
Appendix 19 – Minute Interval Spectral Analysis (520 nm - 550) of Live Control Cells Replicate

Appendix Figure 19 – Minute Interval Full Spectral Data of Undifferentiated Myoblast Stem Cells replicate.
Fluorescence images are represented by ‘F’ and Spectral images are indicated by ‘SP’. Control cells began to bleb at approximately 29 minutes. Values for the cursor are placed immediately below the phasor plot. ‘FI’ represents fluorescence images, and ‘SP’ represents spectral images.
Appendix 20 – Minute Interval Spectral Analysis (520 nm - 550) of Live Differentiating Cells Full Dataset

Appendix Figure 20 – Minute Interval Full Spectral Data of Differentiating Myoblast Stem Cells. Fluorescence images are represented by ‘FI’ and Spectral images are indicated by ‘SP’. Differentiating Cells did not appear to bleb within the first 30 minutes. Values for the cursor are placed immediately below the phasor plot. *FI* represents fluorescence images, and *SP* represents spectral images.
Appendix 20 – Minute Interval Spectral Analysis (520 nm - 550) of Live Differentiating Cells Replicate

Appendix Figure 20 – Minute Interval Spectral Analysis of Differentiating Replicate. Fluorescence images are represented by ‘FI’ and Spectral images are indicated by ‘SP’. Differentiating Cells did not appear to bleb within the first 30 minutes. Values for the cursor are placed immediately below the phasor plot. ‘FI’ represents fluorescence images, and ‘SP’ represents spectral images.
Appendix Figure 21 – Method for Generating 3D spectral Models. The distribution and intensity of emissions selected by coloured cursors were saved as separate files the 3D program in SimFCS could recognise. This was accomplished by clicking on the ‘file’ tab and then selecting the ‘Save color masks and intensity’ (A). The 3D program was opened by clicking on the ‘file’ tab and then selecting the ‘Go to 3D program without saving’ option (A). In the 3D program, the two files mask files previously generated were loaded by clicking the ‘file’ tab, selecting ‘open mask file’, and then ‘New style’ (B). The size of the cell was adjusted by clicking the Fix/tiles tab and adjusting the z-plane distance (blue box). The cross sectional 3D spectral images were generated by utilising the Dissection tool (green box).
Appendix Figure 22 - Method to Generating 3D intensity images. To generate 3D intensity the referenced spectral files were loaded. This was accomplished by clicking the ‘file’ tab, and selecting the ‘Open file for 3D (bin, int, ref, BHZ, BH)’ option (A). The size of the cell was adjusted by modifying the distance of the z focal slice (see appendix 21 -blue box). Specific regions of the cell was isolated based on the intensity histogram (red box) (b). Through the use of the intensity histogram, the cytoplasmic membrane region and nuclear region can be isolated.
Appendix 23 – Whole-Cell 3D Intensity and Spectral Images

A23.1 - Undifferentiated and Differentiating Cells at 3 minutes

A. Undifferentiated

3D Intensity 3D Spectral Cross section 3D Cross section 3D
image spectral image intensity image

B. Differentiating

3D Intensity 3D Spectral Cross section 3D Cross section 3D
image spectral image intensity image

Figure 23.01 – Three-Dimensional Intensity and Spectral Models of Undifferentiated and Differentiating Myoblast Stem Cells at 3 Minutes. (A) Undifferentiated cells; (B) Differentiating cells at 3 minutes. Values for cursors are placed immediately below phasor plot.
Figure 23.02 – Three-Dimensional Intensity and Spectral Models of the Nuclear Region of Undifferentiated and Differentiating Myoblast Stem Cells at 10 Minutes. (A) Undifferentiated cells; (B) Differentiating cells at 10 minutes. Values for cursors are placed immediately below phasor plot.
Figure 23.03 – Three-Dimensional Intensity and Spectral Models of the Nuclear Region of Undifferentiated and Differentiating Myoblast Stem Cells at 10 Minutes. (A) Undifferentiated cells; (B) Differentiating cells at 20 minutes. Values for cursors are placed immediately below phasor plot.