Scalable Multi-Parametric Imaging of Excitable Tissue

Peter Lee

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy at the University of Oxford

Balliol College
University Oxford

Department of Physics
and
Life Sciences Interface Doctoral Training Centre

Trinity Term 2012
Scalable Multi-Parametric Imaging of Excitable Tissue

Peter Lee, Balliol College

Thesis submitted for the degree of Doctor of Philosophy
at the University of Oxford, Trinity 2012

Abstract

The field of cardiac electrophysiological imaging has advanced tremendously in the past three decades with developments in fluorescent dyes, photodetectors, optical filters, illumination sources, computers and electronics. This thesis describes several scalable multi-parametric imaging systems and their application to cardiac tissue preparations at various levels of complexity. Using off-the-shelf components, single-camera multi-parametric optical mapping systems are described for various fluorescent dye combinations and single-element photodiode-based fibre-optic detection systems are described for drug-testing applications. The instruments described take advantage of modern voltage-sensitive dyes, multi-band optical filters and powerful light-emitting-diodes, from the ultraviolet to the red.

The two electrophysiological parameters focused on were transmembrane voltage and the intracellular calcium concentration. Several voltage and calcium dye combinations were established, which produce no signal cross-talk. Furthermore, second- and third-generation voltage dyes were characterized in cardiac tissue, in vitro and in vivo.

The developed systems were then applied to isolated Langendorff-perfused whole-hearts, in vivo whole-hearts, thin ventricular tissue-slices and human induced pluripotent stem cell-derived cardiac tissue. The interventions applied include accurately-timed electrical and mechanical local stimulation of the whole-heart to generate ectopic beats, cardiotoxic drugs and flash-photolysis of caged-compounds.

With the high-throughput demands of drug discovery and testing, further development of scalable optical electrophysiological systems may prove critical in reducing attrition and costs. And for in vivo optical mapping, development of minimally-invasive and clinically-relevant optical systems will be essential in validating existing theories based on in vitro experiments and exploring cardiac function and behaviour with the heart intact in the organism.
Acknowledgements

I would like to acknowledge the support and guidance of my supervisors, Professor Peter Kohl and Professor Paul Ewart. I would like to thank Professor Elspeth Garman and Professor David Gavaghan at the Life Sciences Interface Doctoral Training Centre for opening the door to my doctorate and providing the biological, mathematical and computational training needed to conduct research at the life sciences interface. I am grateful to the Clarendon Fund for funding my studies at Oxford.

I could not have undertaken my research projects without the support from the Kohl team, Ewart team and my US collaborators. In particular, I would like to thank Dr. Christian Bollensdorff, Dr. Alan Garny, Ms. Ken Wang, Dr. Ben Williams, Dr. Christopher Woods, Professor Euan Ashley, Dr. Todd Herron, Dr. Matthew Klos, Professor José Jalife, Dr. Ping Yang, Professor Leslie Loew and Professor Derek Terrar.

I would like to thank Mr. Rob Harris, who recently retired from the Physics Research Workshop for his support in machining and Dr. Piers Nye, my college advisor, for personal and professional support.

I would like to thank my sister, mother and father for encouraging and supporting my decision to pursue a doctorate at Oxford University.

Finally, I would like to thank Professor Denis Noble, whose book The Music of Life, inspired me to pursue a doctorate at Oxford in the field of cardiac electrophysiology.

Peter Lee
February 2012
Abbreviations

AP        Action Potential
APD       Action Potential Duration
\([\text{Ca}^{2+}]_i\)  Intracellular Calcium Concentration
CaT       Calcium Transient
CICR      Calcium Induced Calcium Release
CMOS      Complementary Metal-Oxide Semiconductor
DMSO      Dimethyl Sulfoxide
EAM       Electroanatomic Mapping
EMCCD     Electron-Multiplied Charge-Coupled Device
iPSC      Induced Pluripotent Stem Cell
iPSC-CM   Induced Pluripotent Stem Cell-Derived Cardiomyocyte
LAD       Left Anterior Descending Coronary Artery
LED       Light Emitting Diode
MEC       Mechano-Electric Coupling
MEF       Mechano-Electric Feedback
NADH      Nicotinamide Adenine Dinucleotide – Hydrogen (reduced)
NP-EGTA    O-Nitrophenyl Ethylene-Glycol-Tetraacetic-Acid
PIN photodiode  P-type, Intrinsic, N-type photodiode with a lightly-doped near-intrinsic semiconductor region between the p-type and n-type semiconductor regions
PDA       Photodiode Array
SAN       Sinoatrial Node
\(V_m\)    Membrane Voltage
“Authority in science exists to be questioned, since heresy is the spring from which new ideas flow.”

John C. Polanyi
Publications
* Tentative article title and author list

Peer-Reviewed Journals

* Characterization of Action-Potential and Calcium-Transient Heterogeneity Patterns in the Rabbit Left-Ventricular Free Wall using Ventricular Tissue-Slices and Multi-Parametric Imaging
Wang K, Lee P, Kohl P, Gavaghan DJ, Bollensdorff C.
In Preparation

* Quantitative Assessment of Cell-Type Distribution in the Rabbit Heart
Burton RA, Lee P, Garny AF, Mansoori T, Grau V, Kohl P.
In Preparation

* Post-cardiac arrest myocardial depression is supported by CAMKII and ryanodine receptor dependent calcium memory and mitigated by Alda-1
Woods CE et al.
In Preparation

* Heart Slices and Multi-Parametric Imaging: Powerful Tools for Pharmacology
Lee P et al.
In Preparation

* Scalable Whole-Heart Electrophysiological Imaging Platform for Drug-Testing, Modelling and Education Applications
In Preparation

Palette of Fluorinated Voltage-Sensitive Hemicyanine Dyes
Proceedings of the National Academy of Sciences USA, 2012 (accepted)

Cardiac Electrophysiological Imaging Systems Scalable for High-Throughput Drug-Testing
Pflügers Archiv, 2012 (accepted)

Simultaneous measurement and modulation of multiple physiological parameters in the isolated heart using optical techniques

In situ Optical Mapping of Voltage and Calcium in the Heart.
Simultaneous Voltage and Calcium Mapping of Genetically Purified Human iPS Cell-Derived Cardiac Myocyte Monolayers
Circulation Research 110(12):1556-63 (2012)

Single-sensor system for spatially-resolved, continuous and multi-parametric optical mapping of cardiac tissue
Lee P, Bollensdorff C, Quinn TA, Wuskell JP, Loew LM, Kohl P.
Heart Rhythm 8(9):1482-1491 (2011)

Interpreting Optical Mapping Recordings in the Ischemic Heart: A Combined Experimental and Computational Investigation

Temporal pixel multiplexing for simultaneous high-speed high-resolution imaging
Bub G, Tecza M, Helmes M, Lee P, Kohl P.

Invited Reviews

A System for In Vitro and In Vivo Electrophysiological Imaging of the Heart
Lee P, Woods CE.
Drug Discovery & Development Magazine, June 2012

Multi-Parametric Imaging in Cardiac Electrophysiology
Lee P, Bollensdorff C.
Microscopy and Analysis Magazine, July 2012

Optical Imaging of Voltage and Calcium in Cardiac Cells & Tissues
Herron TJ, Lee P, Jalife J.

Book Chapter

Teaching Evolution in a Historical Context: From the Wisdom of the Ancient Greeks to Genetic Algorithms
Ferrari M. Lee P, Taylor RS.
Contents

1 Introduction 1

2 Background 4
  2.1 The Heart 4
    2.1.1 Anatomy and Cardiac Cycle 4
    2.1.2 Conduction Pathway 6
    2.1.3 Arrhythmias 7
    2.1.4 Cardiomyocytes 8
    2.1.5 Cardiac Excitation-contraction Coupling 10
    2.1.6 Multicellular Electrical Impulse Propagation 11
  2.2 Photodiodes 12
  2.3 Light-Emitting-Diodes (LEDs) 13
  2.4 Mathematical Modelling and Simulations 14
    2.4.1 The FitzHugh-Nagumo Model 14
    2.4.2 Computer Simulations 15
  2.5 Optical Mapping 16
    2.5.1 Photodetectors for Optical Mapping 18
    2.5.2 Illumination 21
    2.5.3 Voltage-Sensitive Dyes 22
    2.5.4 Calcium-Sensitive Dyes 25
    2.5.5 Motion Effects on Optical Recordings 27
    2.5.6 Photon Scattering Effects 27
    2.5.7 Simultaneous Voltage and Calcium Optical Mapping 28
  2.6 Recent Insight from Applications of Optical Mapping 31
    2.6.1 Single Cell Optical Recording 31
    2.6.2 Monolayer Optical Mapping 33
    2.6.3 Optical Mapping of the Sinoatrial Node 34
    2.6.4 Whole Heart Optical Mapping 35
    2.6.5 Endoscopic Mapping of AF 35
    2.6.6 Scroll Waves in the Human Ventricles 36
    2.6.7 Transmural Optical Mapping Using Optrodes 37
    2.6.8 Human Heart Optical Mapping 38
    2.6.9 Panoramic Optical Mapping 38

3 Materials and Methods 40
  3.1 Experimental Models 40
    3.1.1 Langendorff-Perfused Isolated Whole-Hearts 40
    3.1.2 Ventricular Tissue-Slices 41
    3.1.3 Human iPS-CM Monolayers 42
    3.1.4 In Vivo Rat Whole-Heart 42
  3.2 Compound Loading in Whole-Hearts, Tissue-Slices and Monolayers 44
    3.2.1 Voltage and Calcium Dye Loading 44
    3.2.2 Caged-Compound (ex. NP-EGTA) Loading 45
    3.2.3 Pharmaceutical Compound (ex. Nifedipine) Loading 45
  3.3 Fluorescence Imaging and Detection Setups 45
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Ratiometric Voltage and Ratiometric Calcium Optical Mapping System using a Single-Sensor coupled with ECG-Based Local Electrical/Mechanical Stimulation</td>
<td>47</td>
</tr>
<tr>
<td>4.1</td>
<td>Introduction</td>
<td>47</td>
</tr>
<tr>
<td>4.2</td>
<td>Isolated perfused heart preparation</td>
<td>50</td>
</tr>
<tr>
<td>4.3</td>
<td>Instrumentation</td>
<td>50</td>
</tr>
<tr>
<td>4.4</td>
<td>Single-sensor multi-parametric optical mapping</td>
<td>53</td>
</tr>
<tr>
<td>4.5</td>
<td>Results</td>
<td>54</td>
</tr>
<tr>
<td>4.6</td>
<td>Discussion</td>
<td>63</td>
</tr>
<tr>
<td>4.7</td>
<td>Conclusion</td>
<td>65</td>
</tr>
<tr>
<td>4.8</td>
<td>Supplemental figures</td>
<td>66</td>
</tr>
<tr>
<td>5</td>
<td>Simultaneous Voltage and Calcium Mapping of Genetically Purified Human iPS Cell-Derived Cardiac Myocyte Monolayers</td>
<td>71</td>
</tr>
<tr>
<td>5.1</td>
<td>Introduction</td>
<td>71</td>
</tr>
<tr>
<td>5.2</td>
<td>Instrument design details</td>
<td>72</td>
</tr>
<tr>
<td>5.3</td>
<td>Neonatal rat ventricular myocyte monolayers</td>
<td>74</td>
</tr>
<tr>
<td>5.4</td>
<td>Rat ventricular tissue-slices</td>
<td>75</td>
</tr>
<tr>
<td>5.5</td>
<td>Isolated perfused rat whole-heart</td>
<td>75</td>
</tr>
<tr>
<td>5.6</td>
<td>Reference applications of the imaging platform for simultaneous voltage and ratiometric calcium mapping in cardiac tissue</td>
<td>75</td>
</tr>
<tr>
<td>5.7</td>
<td>Human iPSC-CM monolayers</td>
<td>78</td>
</tr>
<tr>
<td>5.8</td>
<td>Immunofluorescence and human iPSC-CM structure</td>
<td>78</td>
</tr>
<tr>
<td>5.9</td>
<td>Flow cytometry</td>
<td>80</td>
</tr>
<tr>
<td>5.10</td>
<td>Simultaneous voltage and calcium mapping in human iPSC-CM monolayers</td>
<td>83</td>
</tr>
<tr>
<td>5.11</td>
<td>Discussion</td>
<td>86</td>
</tr>
<tr>
<td>5.12</td>
<td>Supplemental figures</td>
<td>89</td>
</tr>
<tr>
<td>6</td>
<td>In Vivo Optical Mapping of Voltage and Calcium in the Heart</td>
<td>90</td>
</tr>
<tr>
<td>6.1</td>
<td>Introduction</td>
<td>90</td>
</tr>
<tr>
<td>6.2</td>
<td>Results</td>
<td>91</td>
</tr>
<tr>
<td>6.3</td>
<td>Discussion</td>
<td>98</td>
</tr>
<tr>
<td>6.4</td>
<td>Imaging system</td>
<td>99</td>
</tr>
<tr>
<td>6.5</td>
<td><em>In vivo</em> rat animal model</td>
<td>100</td>
</tr>
<tr>
<td>7</td>
<td>Inexpensive Cardiac Electrophysiological Imaging Systems Scalable for High-Throughput Drug-Testing</td>
<td>102</td>
</tr>
<tr>
<td>7.1</td>
<td>Introduction</td>
<td>102</td>
</tr>
<tr>
<td>7.2</td>
<td>Isolated Langendorff-perfused guinea-pig whole-heart</td>
<td>105</td>
</tr>
<tr>
<td>7.3</td>
<td>Guinea-pig left-ventricular tissue-slices</td>
<td>106</td>
</tr>
<tr>
<td>7.4</td>
<td>Camera-based multi-parametric imaging</td>
<td>106</td>
</tr>
<tr>
<td>7.5</td>
<td>Consumer camera-based whole-heart optical mapping</td>
<td>107</td>
</tr>
<tr>
<td>7.6</td>
<td>Optical-fibre-based multi-parametric detection</td>
<td>109</td>
</tr>
<tr>
<td>7.7</td>
<td>Image processing</td>
<td>110</td>
</tr>
<tr>
<td>7.8</td>
<td>Results &amp; Discussion</td>
<td>110</td>
</tr>
<tr>
<td>7.9</td>
<td>Conclusion</td>
<td>119</td>
</tr>
</tbody>
</table>
Simultaneous Measurement and Modulation of Multiple Physiological Parameters in the Isolated Heart using Optical Techniques

8.1 Introduction
8.2 Multi-parametric optical mapping instrumentation
8.3 Caged-compound (NP-EGTA) loading and local flash-photolysis
8.4 Results
8.5 Discussion

Conclusions and Outlook

9.1 Conclusions
9.2 Outlook on High-Throughput In Vitro and In Vivo Electrophysiological Imaging of the Heart

References

Appendices

A1 Software
A1.1 MATLAB Software for 2D Cardiac Tissue Simulations
A1.2 Microcontroller Firmware for Camera and LED Coordination
A1.3 MATLAB Software to Communicate with Microcontroller
A1.4 MATLAB Software for Optical Mapping Data Analysis

A2 Electronics
A2.1 Computer- and Camera-Controlled LED Output Modulation
1 Introduction

The goal of the research reported in this thesis was to advance the field of cardiac electrophysiological imaging using fluorescence methods. Various attempts are described to leverage recent advances in fluorescent dyes, photodetectors, optical filters, illumination sources, computers and electronics. First, the relevant background that was needed before embarking on these endeavours is covered. Key topics include cardiac tissue structure and electrophysiology, norm and patho-physiological properties, modern photodetectors and light-emitting-diodes (LEDs), basic mathematical characterizations of multi-cellular cardiac electrical activity, optical mapping of single-cells, monolayers and whole-hearts. Although the field of cardiac imaging has made tremendous strides over the past three decades with the introduction of voltage-sensitive dyes, there are still many areas where advances need to be made. In particular, applying developments in firstly, the optics and electronics field and secondly, the medical device industry, will enable high-throughput and in vivo measurements to be made.

For a majority of the experiments, the simple, but effective, Langendorff-perfused isolated whole-heart preparation was used. As the bulk of the described research focused on developing and validating various methodologies, rats were mainly used because of their low cost. Ventricular tissue slices were then extensively explored as a more physiologically-relevant 2D model compared to the more popular neonatal rat ventricular myocyte (NRVM) monolayers. In collaboration with the University of Michigan and the University of Wisconsin, monolayers formed from human induced-pluripotent-stem-cell-derived cardiomyocytes (iPS-CM) were explored as an exciting new 2D model that can be electrically and mechanically stimulated and imaged using similar approaches developed for the Langendorff-perfused heart. In collaboration with Stanford University, in vivo multi-parametric imaging through blood-perfused tissue was explored to make the transition to studying cardiac electrophysiology with the heart still intact in the animal. Various voltage- and calcium-sensitive dye combinations were explored to establish negligible crosstalk and novel dye-combinations for multi-parametric imaging. Local electrical and mechanical stimulation, drugs and caged-compounds were used to perturb electrophysiological parameters.
Individual projects (culminating in manuscripts) are then discussed:

**Project 1**
Ratiometric imaging of voltage and calcium transients is a method that was developed to eliminate/minimize the effects of uneven dye loading, photobleaching and motion artifacts. Ratiometric approaches also permit quantification of transient amplitudes. This project describes a system for ratiometric imaging of voltage and calcium, using a single camera system and multi-band emission filters. For locally perturbing the heart, ECG-coordinated local electrical and mechanical stimulators were developed and applied while imaging. It was found that downstream consequences of local electrical and mechanical stimulation are indistinguishable.

**Project 2**
With the recent commercial availability of human iPS-CMs, patient specific studies are now possible. In this study, the formation of large confluent monolayers was explored. Monolayers were formed on silicone elastomer membranes firstly, to enable future studies on stretching and secondly, because past studies have shown differences in cell development trajectories between rigid and “soft” environments. It was found that monolayers formed on silicone membranes have higher electrical conduction speeds than those formed on rigid plastic petri dishes. A multi-parametric imaging system using a popular voltage dye and low-affinity ratiometric calcium dye was developed and applied to imaging these monolayers. The same system was also applied to NRVM monolayers, rat ventricular tissue-slices and rat whole-hearts, demonstrating the system’s versatility.

**Project 3**
With the advent of near-infrared 2nd-generation voltage sensitive dyes, cardiac researchers have demonstrated high signal quality in blood-perfused isolated heart preparations. Imaging of red calcium dyes has also been done in blood-perfused isolated hearts. Taking advantage of the isosbestic point of one of these 2nd-generation voltage dyes, a simultaneous voltage and calcium imaging system for blood-perfused tissue was developed and applied to an in vivo rat heart model. Use of such voltage dyes for angiography and contrasting infarct versus non-infarct tissue was also established.
Project 4
The photodetectors used in cardiac imaging are typically very expensive, making high-throughput measurements impractical. The goal of this study was to develop systems based on low-cost scientific cameras, high-speed consumer digital cameras, optical-fibres and single-element photodiodes for multi-parametric imaging/detection in whole-hearts and tissue-slices. Drugs were then applied to demonstrate the sufficient signal quality yielded by such inexpensive systems for high-throughput drug testing applications.

Project 5
Taking advantage of the isosbestic point of two near-infrared 2nd-generation voltage sensitive dyes, two different voltage- and calcium-dye imaging systems were developed. A proof-of-principle three-parameter (voltage, calcium and NADH) imaging system was also developed using a single camera and triple-band emission filter. Using mirrors, a voltage and calcium panoramic imaging system for the whole heart was also developed. And as an optical perturbation tool, flash-photolysis of a caged-compound was demonstrated using ultraviolet LEDs.

Conclusions and a discussion of future work complete the thesis. References and appendices containing software and electronic circuit schematics can be found in appendices.