High throughput screening of kinase inhibitor drugs on cardiac function using engineered heart tissue constructs

By

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Abstract

Protein kinase inhibitors (KIs) are used as highly specific cancer targeting agents for their ability to prevent kinase molecules from activating signalling pathways that regulate cellular behaviour, however they have recently been linked to toxicities on the heart that were not detected during clinical trials. We hypothesize that a high-throughput, high-fidelity screening platform for evaluating the physiological effects of KIs on human myocardium could be established using high-content screening assays on hiPSC-derived cardiomyocytes (hiPSC-CM), and engineered heart tissue constructs termed Biowires™. 80 KIs were screened using high-content screening assays and artificial neural network modelling, and the least detrimental inhibitor on cardiac function was examined on the Biowire. 3D Biowire tissues were less sensitive to functional improvements and detrimental effects on viability of KIs compared to 2D monolayer cultures. In conclusion, we propose a system linking high throughput screening with empirical modelling to observe the effects of KIs on cardiac tissue.
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Table of Contents
1. Introduction .................................................................................................................. 1
2. Literature Review ........................................................................................................ 3
   2.1 Kinases as cancer treatment targets .......................................................................... 3
   2.2 Kinases in the heart .................................................................................................... 5
   2.3 Clinical cardiotoxicity of kinase inhibitors ................................................................. 8
   2.4 High throughput screening for cardiotoxicity ............................................................ 9
      2.4.1 Assessment of cardiac function using monolayer CM cultures ......................... 11
      2.4.2 3D Tissue testing ............................................................................................... 13
      2.4.3 Imagining techniques ........................................................................................ 14
3. Objectives and Hypothesis ........................................................................................... 16
   3.1 Hypothesis ................................................................................................................. 16
   3.2 Objectives .................................................................................................................. 16
4. Materials and Methods ............................................................................................... 17
   4.1 Monolayer Kinase Inhibitor Screening ..................................................................... 17
      4.1.1 CM cell culture .................................................................................................... 17
      4.1.2 Calcium flux measurement .................................................................................. 17
      4.1.3 Cell viability assessment .................................................................................... 18
      4.1.4 Preparation of kinase inhibitor solutions ......................................................... 19
   4.2 Artificial Neural Network (ANN) Modelling ............................................................. 21
      4.2.1 ANN design ........................................................................................................ 21
      4.2.2 Validation using control network ....................................................................... 21
   4.3 3D Biwire Kinase Inhibitor Screening ...................................................................... 22
      4.3.1 Biwire culture ..................................................................................................... 22
      4.3.2 Testing on Biwire platform ................................................................................ 22
      4.3.2 Tissue viability analysis ..................................................................................... 23
   4.4 Tissue Imaging and Force of Contraction Analysis ................................................ 24
      4.4.1 Wire tracking ImageJ algorithm ......................................................................... 24
      4.4.2 Force of contraction analysis MATLAB algorithm ............................................. 25
   4.5 Statistical Analysis ................................................................................................... 27
5. Development of Measurement and Analysis Techniques ............................................ 28
   5.1 Introduction ............................................................................................................... 28
   5.2 Results ....................................................................................................................... 28
      5.2.1 Measuring wire contraction using automated SpotTracker algorithm............. 28
Table of Figures

Figure 1 Inoculation chamber for Biowire Testing ............................................................. 24
Figure 2 Passive tension measurement in blue fluorescence videos ..................................... 26
Figure 3 Contraction parameters evaluated for each tissue during drug testing using MATLAB program .......................................................................................................................... 27
Figure 4 Relationship between manual and SpotTracker tracking of polymer wire movement with large wire displacement .................................................................................................. 34
Figure 5 Relationship between manual and SpotTracker tracking of polymer wire movement with small wire displacement .................................................................................................. 34
Figure 6 Example of force of contraction profile from MATLAB ............................................ 35
Figure 7 Effects of ROI size and time averaging (TA) length of calcium transient measurement ...... 35
Figure 8 Confirmation of simultaneous wire movement and calcium transient measurement .......... 36
Figure 9 Comparison between samples with visible polymer wires vs samples with obstructed polymer wires ............................................................................................................................ 36
Figure 10 Kinase inhibitor screening workflow ......................................................................... 51
Figure 11 Validation of monolayer testing assays using control compounds ............................ 52
Figure 12 Comparison of the effects of negative chronotrophic drugs on CMs ........................ 53
Figure 13 Effect of kinase inhibitors on iPSC-derived CM monolayers .................................... 54
Figure 14 Designing the architecture of control neural networks ............................................. 55
Figure 15 Performance of control neural networks ................................................................... 57
Figure 16 Prediction capabilities of control neural networks ...................................................... 58
Figure 17 Neural network approach to model and predict kinase inhibitors with significant effects on CMs ............................................................................................................................ 59
Figure 18 Tissue characterization ............................................................................................. 61
Figure 19 Viability of Biowire tissues after 24hr exposure to kinase inhibitor molecules ............... 62
Figure 20 Confirmation of Biowire performance and representative contraction traces ............... 63
Figure 21 Effect of kinase inhibitor drugs on tissue contraction ................................................ 64
Figure 22 Representative traces of tissue contraction after acute and prolonged (24hr) exposure to inhibitor GW703087X (#44), GW829055X (#24), and Sunitinib (stimulated at 1Hz) ...................... 65
Figure 23 Monolayer results for kinase inhibitor GW703087X (#44) and GW829055X (#24) ........... 66

Table of Tables

Table 1 Select clinically available KI-based cancer therapies, their kinase target, and observed cardiotoxicities .......................................................................................................................... 7
Table 2 Reference compounds for calcium transient testing ................................................... 17
Table 3 Experimental labels, kinase targets, and labels per the PKIs .......................................... 20
Table 4 Comparison of various control ANN designed around 0.1 µM results. Network chosen is highlighted in red .................................................................................................................. 55
Table 5 Comparison of various control ANN designed around 1 µM results. Network chosen is highlighted in red .................................................................................................................. 56
Table 6 Comparison of various control ANN designed around 10 µM results. Network chosen is highlighted in red .................................................................................................................. 56
1. Introduction

Heart disease is a pandemic that accounted for over 32% of deaths in 2010 alone. It is one of the largest drains on our health care system, with an associated cost of $315.4 billion in 2014\(^1\). One major issue in health care is the long-term cardiovascular effects of various pharmaceutical drugs not detected during clinical trials\(^2\). If, during or after clinical trials, it is determined a drug has adverse effects on non-target organs, including the heart, the drug will be recalled and millions of dollars will have been wasted. The lack of early identification of these cardiovascular complications can lead to a severe strain on the health care system, and the loss of millions of dollars in drug discovery.

In 2003, the cost associated with the development of a new drug ranged between $92 million to $883 million per drug\(^3\). Today, these costs have risen to $1395 million, an increase of 8.5% above the general price inflation\(^4\). Along with these staggering costs, there appears to be a significant decrease in the number of new chemical compounds that are being approved by the FDA; the FDA approved an all time low of 17 new molecular entities in 2007 and that number has only slightly risen in the last few years. The primary causes of rejection are efficacy and off-target toxicity once in clinical trials\(^5\). An emerging fear in the pharmaceutical industry are the challenges to the current business model, primarily from growing concerns regarding safety and efficacy\(^6\), leading to a decrease in profitability\(^7\). To avoid significant losses in profits and revenue, pharmaceutical companies need to increase R&D productivity and effectiveness to maintain innovation and stay competitive with the generic pharmaceutical industry. Some have suggested a shift from the traditional drug discovery process to a “fast fail” model; by increasing the amount of research conducted in the inexpensive Phase I of clinical trials, technical
uncertainty would be resolved early in the pipeline, before expensive later development stages (Phase II and Phase III)\textsuperscript{7}. Preclinical screening and “proof of concept” platforms are crucial elements in this new model of drug discovery and analysis.

One of the key technical factors that contribute to technical failure are the strength and quality of target validation. Overall, when researchers have a high level of confidence in the biological role of the target in human disease there were more successful projects\textsuperscript{8}. Kinases are a specific area of biology that is grossly underreported, although they have been shown to be incredibly important as targets for disease treatment\textsuperscript{9}. When the human genome was plotted in the 2000s, over 500 protein kinases were identified\textsuperscript{10}. Although many of these protein kinases have been linked to human disease, in the last 20 years around 65\% of kinase papers published focussed on the same 50 kinases heavily investigated in the 90s\textsuperscript{11}. Out of those 500 kinases, over 100 have completely unknown function, and 50\% are largely uncharacterized\textsuperscript{12}. For example, even with the knowledge that 11 kinases are key nodes in breast cancer signalling pathways, CDC2 has received more attention than the others combined\textsuperscript{11}. There is evidence that academic research focuses primarily on a kinase target space that largely mirrors the activity in commercial drug development\textsuperscript{12}. The lack of identification and characterization probes are highly detrimental to the future of kinase research. Tools for understanding and studying kinase activity in the body, specifically in a pre-clinical setting, are urgently needed to stimulate new drug discovery efforts.
2. Literature Review

The text in this literature review contributed significantly to the following publication:


2.1 Kinases as cancer treatment targets

Cancer treatment has progressed tremendously in the past several years due to targeted therapeutics, where anti-cancer drugs are designed to target specific tumor cells and not the rest of the body. A significant emerging trend in targeted cancer therapeutics are the use of compounds that inhibit kinase activity in cells. By catalyzing the transfer of the γ-phosphate group from adenosine triphosphate (ATP) to amino acid residues on other proteins, kinases are essential in activating signaling pathways that regulate cell growth, differentiation, metabolism, migration, and programmed cell death. Certain kinase molecules are required for cell growth and viability on a general level throughout the body, while others are tissue specific. Their importance in proliferation and survival are typically due to regulation of the cell cycle, and provision of anti-apoptotic factors to the cell. For example, AKT1 has been implicated in cell proliferation and survival in a variety of cell types by preventing progression through the M-G1 phase of the cell cycle, and has been shown to promote cell survival by inactivating apoptotic machinery. Similarly, CDK2 has been implicated in the survival and proliferation of eukaryotic cells by allowing the cell to progress through the G1 phase of the cell cycle. In this work, inhibitors for over 15 different kinase proteins were investigated. A thorough
overview of these kinase targets and their implication in cell proliferation, survival, and oncogenesis can be found in Appendix A.

Cancer is typically characterized by aberrations in cellular growth, proliferation, and survival pathways; unregulated cell proliferation, resistance to apoptotic signals, unabated cell cycle progression, sustained angiogenesis, and unlimited replication numbers are all indicative of a cancerous cell\textsuperscript{22}. Tumor growth is heavily governed by cancer cells’ abilities to proliferate and migrate rapidly, as well as their high vascular demand. Protein kinases are heavily implicated in the regulation of these processes. Mutation of the gene of a certain regulation pathway can lead to overexpression and cancer formation\textsuperscript{23}. For example, epidermal growth factor receptor (EGFR), a member of the ErbB family, is a cell-surface receptor whose activation can lead to cell growth and proliferation\textsuperscript{24}. Overexpression of EGFR is implicated in several solid tumor cancers, such as non-small cell lung cancer, and cancers of the breast, prostate, and colon\textsuperscript{25}. Other examples of kinase overexpression in solid tumor cancers is the increased presence of vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptor (VEGFR) in renal cell carcinoma\textsuperscript{26}, and the overexpression of b-RAF in malignant melanomas due to a point mutation in the b-RAF-coding gene\textsuperscript{27}.

In addition to their implication in tumor growth, kinases have been linked to non-tumorigenic cancers, such as the kinase complex Bcr-Abl’s antiapoptotic properties in chronic myeloid leukemia (CML)\textsuperscript{28}, and the role of anaplastic lymphoma kinase (ALK) in anaplastic large cell lymphoma\textsuperscript{29}. This is merely a glimpse at the impact of kinase molecules in cancer. Researchers have implicated a significant number of kinases in both solid and hematologic cancers\textsuperscript{23}. Both
monoclonal antibody-based kinase inhibitors (KIs) and small molecule KIs have been approved for clinical treatment of a variety of cancers, and there are over 12 clinically approved KI-based cancer treatments on the market, from pharmaceutical companies such as Pfizer, AstraZeneca, Genentech, Bayer, Novartis and GlaxoSmithKline (a selection can be seen in Table 1)\(^\text{10}\).

### 2.2 Kinases in the heart

Due to their high metabolic rate, contractile cells, including cardiomyocytes (CMs), require a constant supply of ATP. The sliding filament theory dictates that during muscle contraction, actin and myosin filaments slide past each other to create movement. The sliding motion is facilitated by the formation of cross bridges, whereby the thin actin filament temporarily binds to a myosin head, causing a conformational change in the myosin that moves the thin filament, and then releases to repeat the cycle. Cross bridge formation is induced by a rise of intracellular calcium ions ([Ca\(^{2+}\)]), and the generation of force and repetition of the contraction cycle is governed by the presence of ATP. Actin and myosin form a very weak attraction, however when ATP is bound to the myosin head and hydrolyzes to ADP and a Pi domain, the binding affinity increases. The Pi domain is released during the power stroke, the conformational change leading to force generation, and subsequently ADP is slowly released. A new ATP molecule is then required to release the myosin head from the actin filament to repeat the cycle\(^\text{31}\). As such, any perturbation to the mitochondrial function resulting in the interruption of the ATP supply in CMs can have drastic effect on cardiac tissue\(^\text{32}\). Inhibition of various kinases could potentially have detrimental effects on CM health and function, such as reduction in left ventricular ejection fraction (LVEF), myocardial infarction and congestive heart failure\(^\text{33}\).
In addition to the metabolic demand of CMs, certain kinases molecules have been determined as crucial in cell survival and repair after cardiac injury or under stress. Although it is difficult to predict the role of various kinases in the human heart, we can make assumptions based off rodent models and KI-treated mice. There is substantial evidence to suggest the ErbB family of proteins are implicated in CM function. For example, Erbb2 has been found to induce proliferation in the neo-natal heart\textsuperscript{34}, as well as has some cardioprotective functions by preventing cardiomyopathy\textsuperscript{35,36}. In addition, chronic treatment of mice with EGFR inhibitors led to decrease left ventricle wall thickness and showed an increase in fibrosis in the heart tissue\textsuperscript{37}. Similarly, AKT1 has been implicated in physiological hypertrophy that is necessary for neo-natal growth and as a response to long-term exercise in knockout mice\textsuperscript{38}.

We have shown that kinases expression is responsible for positive regulation of cardiac development and maintenance, however there are examples of kinases as down-regulators of hypertrophy and cardiac remodelling. For example, GSK-3β has been show to be a key negative regulator of normal growth and pressure overload (PO) stress response in the heart\textsuperscript{39,40}, and is implicated in calcium handling by downregulating sarcoplasmic reticulum ATPase in transgenic mice and knock-in mice\textsuperscript{41}. In addition, researchers have shown that GSK-3β is responsible for restoring calcium sensitivity after cardiac injury in dog HF models\textsuperscript{42}, and deletion can lead to increased CM proliferation following PO and MI in knockout mice\textsuperscript{43}. 
Table 1 Selection of clinically available KI-based cancer therapies, their kinase target, and observed cardiotoxicities.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Molecule type</th>
<th>Kinase Target</th>
<th>Cancer Type</th>
<th>Cardiac Toxicity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trastuzumab (Genentech, 1998)</td>
<td>Monoclonal antibody</td>
<td>ERBB2</td>
<td>Breast cancer</td>
<td>Congestive heart failure and depression of LVEF</td>
<td>44,45</td>
</tr>
<tr>
<td>Sunitinib (Pfizer, 2006)</td>
<td>Small molecule</td>
<td>VEGFR</td>
<td>Renal cell carcinoma</td>
<td>Acute coronary symptoms (MI), heart failure, LV dysfunction</td>
<td>46, 47</td>
</tr>
<tr>
<td>Lapatinib (GlaxoSmithKline, 2007)</td>
<td>Small molecule</td>
<td>ERBB2</td>
<td>Breast cancer (HER2 Positive)</td>
<td>Rare occurrences of decreased LVEF (reversible)</td>
<td>48, 49</td>
</tr>
<tr>
<td>Imatinib (Novartis, 2001)</td>
<td>Small molecule</td>
<td>Bcr-Abl</td>
<td>Myeloid leukemia</td>
<td>Isolated instances of HF, mitochondrial corruption</td>
<td>50, 51</td>
</tr>
<tr>
<td>Sorafenib (Bayer, 2005)</td>
<td>Small molecule</td>
<td>VEGFR, KIT, RAF</td>
<td>Renal cell carcinoma, liver cancer</td>
<td>Acute coronary symptoms (MI), heart failure. Increased incidences after treatment with Sunitinib</td>
<td>46, 52</td>
</tr>
<tr>
<td>Gefitinib (Astrazeneca, 2003)</td>
<td>Small molecule</td>
<td>EGFR</td>
<td>Lung cancer and other solid malignancies</td>
<td>No evidence of toxicity (one case of a patient with recurring MI, no link to gefitinib)</td>
<td>53</td>
</tr>
<tr>
<td>Erlotinib (OSI Pharma, 2004)</td>
<td>Small molecule</td>
<td>EGFR</td>
<td>Non-small-cell lung cancer, pancreatic cancers</td>
<td>Evidence of cardiac damage when administered in rats</td>
<td>54</td>
</tr>
<tr>
<td>Nilotinib (Novartis, 2007)</td>
<td>Small molecule</td>
<td>Bcr-Abl</td>
<td>Myeloid leukemia</td>
<td>QT prolongation</td>
<td>55, 56</td>
</tr>
</tbody>
</table>
2.3 Clinical cardiotoxicity of kinase inhibitors

As stated previously, KI-based cancer therapies have been very successful at reducing tumor size and preventing metastasis. However, repeated and prolonged use of these drugs have been linked to increased incidences of cardiovascular complications\textsuperscript{13,33,57}. After extended use, it has been observed that several KIs approved by the Food and Drug Administration (FDA) in the United States, such as Sunitinib, have induced or exacerbated cardiovascular diseases (CVDs) in patients who underwent repeated treatment\textsuperscript{47,58}. Several others have been shown to induce a variety of cardiovascular complications, ranging from QT prolongation to LVEF depression, as identified in Table 1, and their symptoms can range from mild to severe\textsuperscript{13}. Although the causes of these toxicities are not well understood, it has been suggested that lack of target specificity and unintended mitochondrial toxicity is a cause of CM damage during KI treatment\textsuperscript{32,59}. The first reported incidence of cardiotoxicity in KI use was trastuzumab, the monoclonal antibody that targets Erbb2, where in 4 of 5 randomized trials there was an increase of 5-17\% in the frequency of asymptomatic LVEF decrease and an increase of 1-3\% in the incidence of chronic heart failure\textsuperscript{44,45,60,61}. KI-based cancer treatment methods can vary between single-kinase inhibitors, such as trastuzumab, or multi-kinase inhibitors, aiming to inhibit vascular growth and tumor cell proliferation and survival. Sunitinib is one example of a multi-kinase inhibitor that has also been linked to cardiotoxicity, targeting the VEGFR1-3, PDGFR, KIT, FLT3, CSF1R, and RET kinases. It has been proposed that due to the unspecified nature of the inhibitor, it carries a higher risk of off-target activity and cardiotoxicity. These fears were confirmed when researchers such as Telli et al. reported that 15\% of patients undergoing Sunitinib treatment developed symptomatic heart failure\textsuperscript{62}. There silver lining to these findings is that with careful monitoring and cardiac treatment, Sunitinib toxicity can be mediated and the effects can be reversible\textsuperscript{46}. 
Due to the limitations of clinical trials and the ambiguity regarding the causes of CVD, the effect of these drugs could not be determined before being approved for use with patients\textsuperscript{30,57}. Identifying these adverse effects prior to clinical trails is imperative to conserving cost and reducing the negative side effects of anti-cancer drugs on patients.

2.4 High throughput screening for cardiotoxicity

High-throughput screening is a widely-used approach that appeals to the pharmaceutical industry, due to it’s ability to perform expedited screening of large libraries of compounds while minimizing costs of experimentation. There are several approved drugs that began as part of high-throughput screening initiatives, many of which are the KI molecules we’ve discussed previously\textsuperscript{63}. Unfortunately, pre-clinical high-throughput screening in cardiovascular research is still difficult to preform on an industrially relevant level due to the difficulty of acquiring reliable cardiac cells and tissues in a cost-effective manner. CMs are inherently contractile cells, and few existing protocols exist to measure CM function (contractility and electrophysiology) reliably with minimal interference to the cells. As such, researchers need to maximize the amount of information generated from each test performed and minimize the amount of resources consumed.

When assessing the potential cardiotoxicities of a new chemical entity, there are certain parameters one must assess. Initial viability testing must be performed to eliminate compounds that are cytotoxic. Following confirmation that the compound is not cytotoxic, we must assess its effect on both CM electrophysiology and CM contractility. The FDA requires researchers to
evaluate a compound’s capability to inhibit the cardiac human ether-a-go-go (hERG) channel, the ion channel primarily responsible for the electrical activity of the heart, as a safety measurement for cardiotoxicity. Prolongation of the QT interval of the repolarization event in the heart’s electrical cycle is indicative of alterations to the hERG channel, and can be an indicator of early cardiotoxicity. Patch clamping is a current method for assessing CM electrophysiology, however it must be performed manually on individual cells, and completely ignores cell-to-cell conduction that occurs in tissues.

Currently, immortalized animal or tumor-based cell lines are used as screening tools for cardiotoxicity, however, these results do not translate to human toxicities. Chinese hamster ovary (CHO) and human embryonic kidney (HEK) cell lines have been engineered to express the hERG channel to assess cardiotoxicity and meet FDA requirements. Neo-natal rat CMs, and other animal models, can be cost-effective alternatives to engineered cells, however Lu et al. demonstrated that there is significant interspecies variation in the ion channel currents in that contribute to the repolarization of CMs, and are thus differently sensitive to various drugs. Primary CMs from human donors provide a more accurate means of anticipating the effect of cardiotoxic compounds, however complications arise with low donor availability, problematic isolation techniques, and poor viability after isolation. Human induced pluripotent stem cell-derived CMs (hiPSC-CM) and human embryonic stem cell-derived CMs (hESC-CM) are exciting alternatives to donor and animal-based CMs, as they display many of the characteristics of normal in vivo CMs, including functional properties such as ion channel expression, electrophysiology and biochemical responses. In addition to healthy hiPSC-CM, Liang et al. demonstrated the ability to derive a library of hiPSC-CM from patients suffering hereditary
cardiac disorders, such as long QT syndrome (LQT), familial hypertrophic cardiomyopathy (HCM), and familial dilated cardiomyopathy (DCM), and demonstrated their differences in susceptibilities to cardiotoxic compounds more accurately than standard healthy control hiPSC-CM screening assays.

2.4.1 Assessment of cardiac function using monolayer CM cultures

To meet the pharmaceutical industry’s requirement for high-throughput, cost effective screening on CMs, there has been significant research into means of measuring electrophysiology using monolayer CMs. As stated previously, it is difficult to assess changes to CM electrophysiology in an automated, high throughput fashion. There has been significant research conducted recently examining extracellular field potentials in hESC-CM and hiPSC-CM using multi-well microelectrode array (MEA) systems, whereby cardiomyocytes are seeded directly onto an integrated microelectrode substrate. This provides a simple and cost effective means of measuring action potential characteristics and propagation in CMs. Braam et al., Caspi et al., and Harris et al. have successfully demonstrated that dose-dependent QT-prolongation can be measured in CMs using hERG-channel blocking compounds. Natarajan et al. improved on MEA-based action potential (AP) platform by patterning CM monolayers, by changing surface topography, onto the MEA surface for guided AP propagation. The advantage of this system is its multi-well and automated capabilities, leading it to be a medium throughput alternative to the traditional patch-clamp method of measuring CM electrophysiology.

An aspect of cardiac function that has yet to be addressed is the measurement of contractility of CMs. Understanding electrophysiological responses to CMs is important in determining cardiotoxicity, however we must as well understand the effects on CM contractility, as this can
be an indicator for ventricular dilation and cardiomyopathy. However, a challenge arises when trying to measure the contractile force of CM monolayer, as typically monolayers are culture on hard in-elastic polystyrene dishes. Park et al. have demonstrated that CMs cultured on a flexible polymer microcantilever substrate can be used to measure contractile forces in CM monolayers\textsuperscript{74}. Similarly, Grosberg et al. developed a platform they termed “heart on a chip”, whereby CMs could be seeded onto polymeric thin films to measure contractility and stained with voltage sensitive dye to measure AP propagation\textsuperscript{75}.

Although these platforms have combined the ability to measure contractility and electrophysiology, we still encounter the issue of lack of high throughput capability due to the difficulty of manufacturing of the platforms, and the ability to screen multiple compounds simultaneously. An alternative to the real-time measurement of contractile force is the measurement of calcium flux across the CM membrane. Spurgeon et al. have demonstrated simultaneous measurement of contraction and calcium flux in single cell CMs, validating the use of calcium flux measurement as a surrogate measurement to contraction in CM monolayer\textsuperscript{76}. With this knowledge, various groups have developed high throughput screening techniques to classify a vast library of compounds quickly using CM monolayers in a multi-well setup and non-invasive measurement techniques and readily available equipment, such as the FLIPR Tetra plate reader. Sirenko et al. have demonstrated the use of fast kinetic fluorescent imaging of intracellular [Ca\textsuperscript{2+}] and classify compounds are cardiotoxic or cardiosafe\textsuperscript{77}. Pointon et al. demonstrated the relationship between fast kinetic fluorescent imaging of intracellular [Ca\textsuperscript{2+}] and CM contractility to observe both positive and negative chronotropic effects and determine IC\textsubscript{50} values of various compounds\textsuperscript{78}.

The advantage of calcium flux measurement to measure
contraction is that it is a non-invasive measurement technique, and allows researchers to subsequently measure cell viability by a variety of means, such as measurement of ATP content or cell imaging\textsuperscript{78,79}. A recurring limitation of monolayer studies is the immature nature of hiPSC-CM and hESC-CM compared to adult and even fetal primary CM\textsuperscript{80}, and the lack of similarities between native 3D tissues and monolayer cells. To better mimic native adult myocardial physiology, we turn to 3D engineered heart tissue constructs as pharmacological testing tools.

\textbf{2.4.2 3D Tissue testing}

It is widely accepted that 3D tissue cultures have greater physiological relevance when compared to 2D monolayer cultures, in such areas as gene and protein expression, cell-cell signalling pathways, and cell-ECM interactions\textsuperscript{81,82}. Engineered heart tissue constructs have been developed to address many facets of the treatment of CVD, such as cell-based cardiac repair and predictive toxicology on cardiac tissues. They have an advantage over monolayer 2D cultures as they are a better representation of physiological conditions. Several platforms already exist to test the effects of drugs on cardiac tissues in vitro. Many of these platforms involve the formation of cardiac tissues around polymer cantilevers to constrain contraction and allow for simple quantification. Using beam bending theory, force can be calculated from the deflection of the cantilever, the elastic modulus of the post material, and the dimensions of the post itself. Mechanical stiffness of these cantilevers can be varied to alter CM contraction\textsuperscript{83}. A significant variant between existing engineered cardiac tissue devices is cardiomyocyte source. Certain groups have used rat CMs as their cell source\textsuperscript{83,84,85}, although as stated previously, these cells can be easily acquired but they cannot accurately replicate the effects of drugs on human tissue. Other groups have used CMs derived from human embryonic stem cells (hESCs) cultured
around poly(dimethylsiloxane) (PDMS) posts to measure contraction. It was previously stated that certain cardiomyocyte sources may not mimic adult human physiology. As such, their use in engineered cardiac tissues may not lead to high-fidelity models of the adult human myocardium.

In contrast to the cantilever method, which did not incorporate cardiac vasculature, Xiao et al. developed a perfusable cardiac tissue to mimic the native cardiac bundle, by forming cardiac tissue around microtubing that can be perfused with compounds. Using this platform, they illustrated the negative inotropic effects of nitric oxide on cardiac tissue. A common limitation of these platforms, and of 2D monolayer platform is the immaturity of tissues, which can lead to incorrect results in investigating toxicological compounds.

It has been well documented that the application of electrical stimulation while culturing cells and tissues is essential for CM maturation. As such, the Radisic lab has developed the Biowire, a matured three-dimensional (3D) engineered heart tissue platform, that is formed by electrically stimulating hESC-derived CMs during culture to enhance tissue cell maturation. More recently, we developed approaches to analyze contractile force in Biowire tissues. This platform may be ideal for high-content pre-clinical screening because of the relative ease of analysis of contractile force, measured using fluorescent microscopy without the need for additional cell-monitoring assays.

2.4.3 Imagining techniques
As stated previously, when we investigate tissue contractility and electrophysiology, we aim to use non-invasive techniques in order preserve tissue response and eliminate any interferences
from imaging techniques. Kinetic fluorescent ion sensitive assays can be used to measure calcium flux across cell membranes; paired with a fluorescent plate reader (for example, Molecular Devices’ FLIPR Tetra system) multiple compounds can be investigated simultaneously. When observing cardiac tissue contraction, it is of utmost importance to measure force of contraction in a manner that does not hinder tissue function or damage tissue for long-term studies. An emerging method of analysis is using sophisticated optical mapping techniques to observe and quantify tissue contraction. Many of these methods are borrowed from analysis of migratory cells and cell movement. Certain groups have used edge detection software to monitor the movement of a contracting post. The limitations of this approach is the necessity of having very visible contrast between the tissue and contracting element. Tissue variability does not always permit this, and as such can be difficult to analyze. Instead, fluorescent optical mapping can be used to better predict the movement of construct at high resolution. Optical mapping involves tracking the position of a fluorescent point (or several fluorescent points) from frame to frame in a video. These softwares have already been employed to track cell migration, chromosome division, and fluorescent microbeads. However, there is no such software that can track the deflection of a wire under fluorescent light. In this work, we will be using the theory behind such technology to develop our own means of optical mapping to track force of contraction and calcium transients.
3. Objectives and Hypothesis

3.1 Hypothesis

I hypothesize that micro-scale cardiac tissues termed Biowires, matured under electrical stimulation, will provide a high-fidelity model to study and quantify the physiological effects of kinase inhibitors on the viability and function of human myocardium.

3.2 Objectives

1. Perform preliminary studies of multiple kinase inhibitors on human cardiomyocytes plated as a monolayer, using high-content screening assays from Molecular Devices to assess cell viability and basic cardiac function.

2. Examine the full functional effects of non-detrimental kinase inhibiting molecules on Biowire platform.

3. To validate the use of engineered heart tissues for pre-clinical screening.
4. Materials and Methods

Sections 4.1, 4.2, 4.3, and 4.5 are included in the following publication:

Conant, G., Ahadian, S., Zhao, Y., Radisic, M. “High-throughput screening of kinase inhibitor compounds”. Scientific Reports. In progress.

4.1 Monolayer Kinase Inhibitor Screening

4.1.1 CM cell culture

iCell CMs (hiPSC-CMs), plating medium, and maintenance medium were purchased from Cellular Dynamics International, USA. Cells were plated and maintained according to supplier’s instructions in a 384-well black clear-bottom microplates (Greiner Bio-One, Austria). Microplates were coated with 50 µL of 0.2 wt% gelatin per well and incubated for 2 hr at 37°C and 5% CO₂. The cells were suspended in plating medium at density of 2x10⁵ viable cells/mL. Gelatin was aspirated and cells were plated at 8000 viable cells/well. Cells were maintained at 37°C and 5% CO₂ during culture. Culture medium was changed every 48 hr by removing 25 µL of old cell medium and replacing with 25 µL fresh maintenance medium. The data were collected 8 days after the plating.

4.1.2 Calcium flux measurement

Prior to testing, maintenance medium was aspirated and 25 µL maintenance medium + 20 mM HEPES was added to the wells and incubated at 37°C and 5% CO₂ for 1 hr. EarlyTox Cardiotoxicity Kit (Molecular Devices, USA) was used for the measurements. Cells were equilibrated with 25 µL of calcium reagent dye for 100 min at 37°C and 5% CO₂. Compounds to be tested were added to the cells after 100 min and cells were incubated for a subsequent 20 min
at 37°C and 5% CO₂. Cells were then examined using a Spectramax I3 plate reader (Molecular Devices), maintained at 37°C during testing. Calcium flux measurements were taken at 0.1s intervals for 40 s, and the results were recorded in the SoftMax Pro software (Molecular Devices). Calcium flux traces were then analyzed using a custom MATLAB program. Peak magnitude was described as the average peak magnitude (defined as the difference between the peak maximum and the baseline for that peak) across the entire trace. Frequency was determined by counting the number of peaks present in the time frame (40 s). Prior to inhibitor testing, Nifedipine, a calcium channel blocker, and thapsigargin, a sarco/endoplasmic reticulum calcium ATPase (SERCA) channel blocker, were used to validate the calcium transient assay. Compounds were examined at physiologically-relevant concentrations (Nifedipine: 0.01 µM, 0.1 µM, 1 µM, 10 µM, 100 µM; Thapsigargin: 0.002 µM, 0.02 µM, 0.2 µM, 2 µM, 20 µM) in triplicate. Subsequently, each plate of inhibitors included 5 reference compounds (Table 2).

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### 4.1.3 Cell viability assessment

Following calcium flux measurements, calcium dye was removed, 40 µL maintenance medium was plated, and cells were returned to 37°C and 5% CO₂ incubator. After 24 hr of initial inhibitor application, cells were treated with EarlyTox Cell Integrity Kit (Molecular Devices, USA) to
assess cell viability. Medium and inhibitors were removed and cells were equilibrated with 25 µL of a 50:50 solution of maintenance medium-to-Dulbecco’s phosphate-buffered saline (DPBS) (+CaCl₂ +MgCl₂). Viability stain was prepared in a 1:1000 dye-to-DPBS (+CaCl₂ +MgCl₂) solution. Cells were then stained with 25 µL of dye solution and incubated for 30 min at 37°C and 5% CO₂. Well images were taken using MiniMax 300 Imaging Cytometer (Molecular Devices) at 713 nm and 541 nm wavelengths for total and dead cells, respectively. Cells were counted using the SoftMax Pro software. Cell viability was determined as the ratio of live cells compared to the total number of cells present in each well. The number of live cells was calculated by subtracting the number of dead cells from the total number of cells.

4.1.4 Preparation of kinase inhibitor solutions

Published kinase inhibitors in a DMSO solution was provided by GlaxoSmithKline Inc. The names of the inhibitors were coded to blind experimenters and prevent any inherent bias during testing (coded names can be found in Table 3). To ensure DMSO concentrations were less than 0.1% in the well, inhibitors were diluted in maintenance medium 100X at minimum. A serial dilution was performed to create a 1000X and 10000X stock dilution of each kinase inhibitor in maintenance medium. Each inhibitor concentration was examined in triplicate. The same volume of stock solution was added to each well, to achieve a final inhibitor concentration of either 10 µM, 1µM, and 0.1µM in the well. A total of 80 independent small kinase inhibitors were tested in a blinded fashion. Cells were incubated with compounds for 20 min before testing.
In order to blind ourselves to the kinase targets of each inhibitor, the compounds provided by GSK were coded with an experimental number. Researchers performing monolayer experiments were not made aware of the kinase targets until completion of monolayer studies.

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4.2 Artificial Neural Network (ANN) Modelling

4.2.1 ANN design

Before the neural network modeling, the data set was normalized to eliminate any variation between samples, using the following equation.

\[
\text{Normalized data} = \frac{(\text{Experimental data} - \text{Blank data})}{\text{Blank data}}
\]

All ANNs were built using the Neural Network Toolbox available in MATLAB. We selected for compounds that had a minimal effect on CM viability and function. As such, we decided that the input layer of the network would comprise of inhibitor concentration, kinase target, normalized beat frequency, normalized cell viability, and normalized number of live cells, while the output layer corresponded to normalized peak magnitude. Several networks were tested, and the final number of layers, number of neurons per layer, and the transfer functions in each hidden layer were optimized using trial and error as described previously\textsuperscript{98,99}. 90\% of the data was chosen as the training set, while 10\% was used as the testing set. Networks were trained using the Levenberg-Marquardt back propagation algorithm\textsuperscript{100}. The limits for our design parameters were chosen to identify the inhibitors that led to the maximum increase in peak magnitude, while minimizing detrimental effects to CM viability. The performance of the network was assessed by examining the root mean squared error (RMSE) and correlation coefficient (R\textsuperscript{2}).

4.2.2 Validation using control network

To ensure our ANN was providing results consistent with literature, a series of control networks were designed to illustrate the effects of the kinase inhibitors on cell viability. A network was
designed for each inhibitor concentration, for a total of 3 ANNs. The input parameters were designated as normalized number of live cells and the kinase target, with the output layer being normalized cell viability (Fig. S1A). The optimal architecture of the network was determined and validated using the methods described above.

4.3 3D Biowire Kinase Inhibitor Screening

4.3.1 Biowire culture

Biowire tissues were generated as previously described from iCell CMs (hiPSC-CMs). Briefly, tissues were formed in 3D microwells treated with 0.2µL 24U/mL thrombin (Sigma, USA). Each tissue was comprised of 150 000 viable CMs and 15 000 human ventricular cardiac fibroblasts (Lonza, Switzerland) suspended in 2 µL of collagen/matrigel/fibrinogen gel (75 vol% collagen/matrigel, 25vol% fibrinogen). Tissues compacted for 7 days in plating medium supplemented with 10µg/mL aprotinin (Sigma, USA). On day 7, the excitation threshold (ET) and maximum capture rate (MCR) were assessed. Tissues were then stimulated for 14 days using biphasic field pulses (6V/cm) at frequencies ranging from 1 to 6 Hz, increasing by 0.83 Hz every 2 days. ET and MCR were assessed every 7 days. On day 21, tissues were being stimulated at 3Hz, 6 V/cm, and were ready for testing.

4.3.2 Testing on Biowire platform

An inoculation chamber was designed to allow for both tissue stimulation and drug perfusion during testing (Fig. 1). Prior to the start of the experiment, the ET and MCR of the tissues were assessed. Experiments were carried out in an environmental chamber (37ºC and 5% CO₂) and tissues were stimulated at 1Hz and the measured ET. To validate Biowire performance,
Nifedipine was tested at 0.01 µM, 0.1 µM, 1 µM, 10 µM, 100 µM, and 1000 µM. Inhibitors #44 (PKIs label GW703087X) and #24 (PKIs label GW829055X), selected by the ANN, and Sunitinib malate (Sutent, Pfizer, USA) were each examined at three concentrations: 0.1 µM, 1 µM, and 10 µM. Sunitinib was dissolved in DMSO, and the concentration of DMSO in the testing chamber did not exceed 0.1%. For each tissue, a baseline video was taken without the presence of any compound addition. The drugs were then added incrementally from low to high concentration. Drugs were introduced via a perfusion system and then incubated in the stimulation chamber for 15 min. To observe wire deflection, an 80s video was taken under the blue fluorescence at 10x magnification. After acute testing, tissues were incubated with the highest compound concentration, and 24 hr later contraction was observed again at 10x under blue fluorescence.

4.3.2 Tissue viability analysis

24 hr after initial drug addition, tissue viability was assessed using propidium iodide (PI) and DAPI stains. Tissues were stained with PI at 24 hr and then fixed with 4% paraformaldehyde for 3 days. After fixation, tissues were stained with DAPI. Tissues viability was assessed by imaging using an Olympus IX81 Spinning Disk Confocal Microscope, and counting cells using a custom designed algorithm in ImageJ. Total number of cells was assessed by the blue (DAPI) nuclei count. The number of dead cells was assessed by the red (PI) nuclei count.
**Figure 1 Inoculation chamber for Biowire Testing.** A 3D printed inoculation chamber was installed in a 24-well plate for Biowire testing. To allow for electrical stimulation, the inoculation chamber was designed with slots for carbon electrodes to be placed and then connected to platinum wires which can be attached to a stimulator. Plastic tubing was installed into the chamber and connected to a syringe to allow for drug perfusion during testing.

### 4.4 Tissue Imaging and Force of Contraction Analysis

#### 4.4.1 Wire tracking ImageJ algorithm

By exploiting the auto-fluorescent properties of the polymer wire, we are able to measure the wire’s movement using simple fluorescent microscopy under the blue fluorescence, at 10x magnification. During video analysis, the movement of the wire was measured using an ImageJ plugin called SpotTracker. This imaging algorithm was designed to measure the movement of fluorescent particles while ignoring the significant amount of noise created by the imaging techniques, including the laser and signal loss due to photobleaching. First, an ROI 50 pixels high was drawn around the point of maximum curvature (25 pixels above and below the centre of curvature). Then, the SpotTracker enhancing filter of 30 pixels was applied to the image stack; 30 pixels was chosen as it is the width of the polymer wire. Following the first filter, we applied
a minimizing filter of 10 pixels to the data set to identify the point of maximum intensity in the wires. Then we perform a second application of the SpotTracker enhancing filter, this time around 1 pixel to ensure we are only measuring the displacement of the wire. Following the application of the filter, we applied the SpotTracker tracking algorithm to measure the centroid of our newly filtered image. We have chosen to measure the centroid of the image because it represents the point of maximum curvature in the wire.

4.4.2 Force of contraction analysis MATLAB algorithm

Once the trace of the wire was established, it was then analyzed using a peak analysis software developed for this project in MATLAB. The trace was first converted from pixel movement to micrometer and then normalized so the initial baseline in the tissue lay at 0 µm. Due to the inherent noise associated with our image acquisition system, we applied a moving average filter to our data set to smooth the baseline and eliminate noise, while maintaining the integrity of contraction peak. The passive tension in the tissue was calculated by manually measuring the relaxed deflection of the wire (Fig. 2). Using MATLAB’s built-in peak finding capabilities we performed multiple iterations so each peak in the trace was located. Since the tissues were stimulated during testing we could determine the expected number of peaks using the frequency of stimulation. For each peak, the baseline was calculated as the average of the lowest points between each peak location. This way we could calculate different baselines for each contraction and observe whether passive tension was changing throughout the course of testing. The algorithm will be described in detail in Section 5. To convert the movement of the wire from micrometers to force, a calibration curve was established using the CellScale Microsquisher (Canada). The polymer wire was displaced using probes of varying diameter, and the force was recorded as a function of the wire displacement. The width of the tissue of the wire was
measured, and the proper calibration curve was used accordingly. Active tension is defined as the resulting force when you subtract the passive tension (calculated from the baseline) from the total force measured in the tissue. The peak start time was determined as the last point below the baseline before the peak, and the peak end time was determined as the first point below the baseline of the peak. In addition, this software was also able to report contraction duration, contraction velocity, and relaxation velocity (Fig. 3).

![Passive tension measurement in blue fluorescence videos.](image)

Figure 2 Passive tension measurement in blue fluorescence videos. Passive tension was measured by determining the baseline of the wire with no deflection (green line), and the passive tension was measured from that line to the point of maximum curvature when the tissue was relaxed.
Figure 3 Contraction parameters evaluated for each tissue during drug testing using MATLAB program. The blue line represents the force calculated using the calibration curves. The red line indicates the baseline calculated for each peak. The black X indicates the peak location, the light green line indicates contraction velocity, the dark green line indicates relaxation velocity. The light green x indicates the start of the peak, the dark green x indicates the end of the peak.

4.5 Statistical Analysis

Results in figures are presented as means ± standard deviation (SD). All statistical tests were performed in SigmaPlot version 12.0. One-way repeated measures ANOVA and Fischer LSD or Tukey’s post-hoc test was used for dose-response tests. One-way ANOVA and Fischer LSD test were used for ET/MCR assessment. P-values <0.05 were considered statistically significant and indicated in the graphs (* or #).
5. Development of Measurement and Analysis Techniques

5.1 Introduction

The use of imaging techniques for the measurement of tissue contraction, while being non-invasive to the tissue, can be a large bottleneck to the efficiency of the research process. Automation of analysis techniques increases process efficiency and minimize the amount of time researchers must spend on analysis. In addition, the elimination of the manual analysis can greatly reduce variation due to human error, and will result in more consistent results. We have proposed several different methods to optimize the analysis of our Biowire tissues, in order to meet the criteria of high-throughput analysis. Using 2 readily available pieces of software, ImageJ and MATLAB, we propose an analysis protocol that eliminates human bias and provides consistent analysis of tissue contraction.

5.2 Results

5.2.1 Measuring wire contraction using automated SpotTracker algorithm

Videos of wire movement were taken under blue fluorescence, at 100 frames per second, for 80 seconds. 100 frames per second was found to be the ideal frame rate because it provided enough resolution to observe wire movement, while minimizing the file size of the videos. It became evident very quickly that manual analysis of wire movement would be time consuming and inaccurate, due to the length of the videos and the precise nature of calculating miniscule wire movements. We hoped to automate the analysis process to eliminate human bias when measuring the movement of the wire. SpotTracker was chosen as the final analysis tool because of its inherent ability to track the movement of a single fluorescent particle in a series of images. It provided us with the most consistent results and proved to be the most accurate representation of
wire movement amongst all the tissues. We compared the results from SpotTracker to our manual tracking results; when the beating was large and distinct, we saw a strong relationship between the manual and SpotTracker results for wire location (Fig. 4), as indicated by an \( R^2 \) value of 0.978, however when the beating was less pronounced, there was a less strong relationship between the manual and SpotTracker results for wire location (Fig. 5), as indicated by an \( R^2 \) value of 0.671. This method also greatly reduced the amount of time to analyze a single video, from several hours to approximately 5 mins per video.

### 5.2.2 Trace analysis using MATLAB programming

Analysis of these traces was initial performed manually, leading to a significant bottleneck in experiment efficiency. As stated previously, we were interested in measuring several parameters per peak, including peak magnitude, peak duration, baseline value, contractile slope and relaxation slope. A sophisticated MATLAB program was developed to accurately identify wire movement and evaluate contraction parameters. The program is able to perform measurements of multiple files in a batch manner. After several iterations of the program was developed, the final analysis algorithm is as follows (Appendix B):

1. Tissue width along wire and passive tension baseline are measured in ImageJ.
2. The trace is normalized to the baseline value of the first 5 peaks, then added to the passive tension baseline value. The trace is then convert from pixels to μm.
3. A moving average filter of 4 frames is applied to the data to eliminate noise in the baseline, while not degrading the signal.
4. The peaks are found using the findpeaks function in MATLAB.
5. A checkpoint was implemented to allow the user to ensure that all the peaks were identified, and not the baseline noise.

6. If all the peaks were not identified, the program iterates through the findpeaks function again, adjusting the minimum peak height and distance between peaks.

7. The user can once again check to ensure all peaks are found. This iterative process continues until all peaks are found.

8. Once the user is confident all the peaks are identified, the program identifies the baseline by performing 3 iterations of averaging the points between peaks.

9. With the baseline established, the program will then find the beginning contraction by identifying the last value lower than the baseline before the maximum, and the end of relaxation by identifying the first value lower than the baseline after the maximum.

10. The trace is then converted to total force using the pre-determined calibration curves. If the thickness of the tissue of the wire is between two of the predetermined curves, then the force is interpolated between those two curves.

11. Passive tension is calculated as the force at the baseline, and active tension is calculated as the force from the baseline to the contraction peak magnitude.

12. The results are then exported to an Excel spreadsheet.

**Fig. 6** illustrates the results of our program in identifying the peak magnitude, baseline, and beginning and end of contraction. With this MATLAB algorithm, we are able to analyze a complete data set in a matter of seconds, as opposed to manually where the researcher could only analyze a selection of peaks over several hours. In addition, this automated analysis tool
provided consistency between each data set to identify the baseline, and did no rely on the researcher’s judgement to identify the baseline value or the beginning and ending of the peaks.

5.2.3 Simultaneous quantification of contraction and calcium transient

In addition to quantifying contractile force, we also investigated the possibility of measuring calcium transients in our 3D tissues. Using calcium-sensitive Fluo4 fluorescent stains, we measured both the calcium flux in the tissues and tissue contraction simultaneously. To measure calcium flux in the tissue, we selected a small region of interest in the center of the tissue and the average fluorescence of that region is measured from frame to frame. The location of the region of interest was determined to be very important, as the edges of the tissues led to movement artifacts not related to calcium flux. Other factors that heavily influenced the results of the calcium transient analysis were the size of the region of interest, and the time averaging constant. Fig. 7 illustrates the degradation of the signal as you increase the size of the region of interest, and as you increase the time averaging constant. Fig. 8 demonstrates our ability to simultaneously measure contractile force and calcium transient. It is evident that the peaks of both the wire movement and calcium flux occur at the same instance of time, confirming our ability to simultaneously measure calcium transients and force of contraction.

5.3 Discussion

A key aspect of this project was to develop a high-throughput platform to analyze the effect of kinase inhibitors on cardiac tissue. Analysis of large quantities of data can be a bottle neck for the efficiency of data, and the effort put into ensuring one has developed a high-throughput platform might be in vain should the analysis be cumbersome and inefficient. We present an
analysis protocol that automates the analysis of tissue contraction videos, and provides consistent and unbiased results.

We have demonstrated that SpotTracker is an efficient and accurate tool that can be used to measure the movement of the fluorescent polymer wire during cardiac tissue contraction. Several different analysis methods were examined before deciding upon using SpotTracker as our method of analysis. Originally, we tried thresholding the image to create a mask highlighting the wire. However, due to the variation in the tissues, occasionally the wire was less visible behind the tissue and analysis of the tissues were inconsistent (Fig. 9). Edge detection methods were not successful either. We tried adjusting the contrast of the image and filtering the data set, however we observed degradation in the signal and the differences observed were due to artifacts. In contrast, SpotTracker consistently identified the point of maximum curvature, and could trace the wire movement regardless of tissue obstruction. It is evident that when the wire movement is distinct, the SpotTracker and manual results are very similar. However, when the wire movement is miniscule, there is a lack of correlation between the manual and SpotTracker results (Fig. 4,5). We can attribute this discrepancy to the fact that our manual analysis can only detect single pixel movement, and SpotTracker can interpolate differences in intensities between pixels, thus providing a more accurate result. Once we were confident in our ability to measure wire displacement, we began our investigation into different means of analyzing that trace of movement.

MATLAB was an ideal choice of data analysis software as it is a simple programming language with several built-in functions that can perform a myriad of tasks, from peak identification to
edge detection. We were also able to easily design a batch analysis form of the software to expedite the analysis process even further. After performing a student’s t-test comparing manual vs MATLAB analysis, we observed a significant difference between the two groups (p<0.001). Although there was a significant difference, we still felt comfortable using our MATLAB algorithm as our analysis method, for it provided consistent results and removed any human bias from analysis.

5.4 Conclusions and Future Work

In this work, we demonstrated the need for the automation of imaging and data analysis for the Biowire platform, to increase efficiency as well as ensure consistency in results. One limitation of this work so far is our ability to image a single tissue at a time. In an ideal world, we would be able to image several tissues simultaneously, using multiple fluorescence channels. Future work for this project would be to investigate means of multiple simultaneous tissue imaging at different fluorescent channels to increase the efficiency of imaging.
5.5 Measurement and Analysis Technique Figures

**Figure 4** Relationship between manual and SpotTracker tracking of polymer wire movement with large wire displacement. There was a significant relationship between manual and SpotTracker analysis of wire movement, represented by an $R^2$ value of 0.978.

**Figure 5** Relationship between manual and SpotTracker tracking of polymer wire movement with small wire displacement. When the wire movement was not pronounced, there was not a significant relationship between the manual and SpotTracker analysis, represented by an $R^2$ value of 0.671.
Figure 6 Example of force of contraction profile from MATLAB. Analysis of force of contraction profile using particle tracking software (MATLAB). The peak is identified with red x, baseline is pink line, start of peak is green circle, and end of peak is blue circle.

Figure 7 Effects of ROI size and time averaging (TA) length of calcium transient measurement.
Figure 8 Confirmation of simultaneous wire movement and calcium transient measurement. Validation of our platform's ability to simultaneously measure tissue contraction and calcium transients. The peaks of contraction and of calcium transients lie at the same time point, indicating that the same contraction was observed.

Figure 9 Comparison between samples with visible polymer wires vs samples with obstructed polymer wires. When the wire is obstructed, the MATLAB algorithm could not identify the wire, and thus not track wire movement.
6. KI Screening on Engineered Heart Tissue Constructs

Section 6 text and figures is taken from the cited paper below. As first author, I wrote the manuscript, and performed all the bench-top experiments. The artificial neural network was created in MATLAB by Dr. Samad Ahadian, and the results were analyzed by myself.

Conant, G., Ahadian, S., Zhao, Y., Radisic, M. “High-throughput screening of kinase inhibitor compounds”. Scientific Reports. In progress.

6.1 Introduction

Cancer treatment has progressed tremendously due to targeted therapeutics, wherein anti-cancer drugs are designed to specifically attack tumor cells and not the rest of the body\cite{13}. Many of these anti-cancer drugs inhibit kinase activity in the cells. Kinases regulate cell growth, differentiation, metabolism, migration, and programmed cell death signaling pathways by catalyzing the transfer of phosphate residues from adenosine triphosphate (ATP) to tyrosine residues on the target protein\cite{13}. However, non-tumorigenic but highly metabolic cells can also be affected by kinase inhibitors. Cardiomyocytes (CMs) require a constant supply of ATP due to their high metabolic rate and any perturbation in the mitochondrial function of CMs can have drastic effect on cardiac tissue. CM force generation and repetition of the contraction cycle is governed by the presence of ATP according to the sliding filament theory\cite{31}; hydrolysis of ATP bound to the myosin filament head increases the binding affinity of actin and myosin, release of the Pi domain is required for the conformational change leading to force generation, and a subsequent ATP molecule is required to release the myosin from the actin filament to repeat the cycle. Kinase inhibition could detrimentally effect CM health and function by impeding typical contraction, resulting in a
reduction in left ventricular ejection fraction (LVEF), a myocardial infarction and/or congestive heart failure\textsuperscript{33}. After extended use, it has been observed that several tyrosine kinase inhibitors approved by the Food and Drug Administration (FDA) in the United States, such as Sunitinib, have induced or exacerbated cardiovascular disease in patients who underwent repeated treatment\textsuperscript{47,58}. Identifying these adverse effects prior to clinical trials is imperative to conserving cost and reducing the negative impact of anti-cancer drugs on patients.

High-throughput screening is a widely-used approach that appeals to the pharmaceutical industry because it allows for expedited research while minimizing costs associated with drug discovery\textsuperscript{63}. In the cardiac sphere, these screens typically involve the exposure of two-dimensional (2D) CM monolayers to drugs at a given dose for a given time, after which an endpoint measurement is acquired. High-throughput cardiac assays are limited by the acquisition of reliable human cardiac cells and tissues at low cost. Human adult CMs are considered to be terminally differentiated, thus they cannot be expanded at appreciable rates from cardiac biopsies\textsuperscript{101}. Due to the difficulty of acquiring a viable, high-fidelity cell source, researchers need to maximize the amount of information generated from each test performed and minimize the amount of resources consumed.

High-throughput 2D monolayer screens of small molecules can generate a vast amount of data, however it remains to be determined how this data can be effectively analyzed. In many cases, a comprehensive understanding of the molecular pathways targeted by these, often new, molecules is lacking. One possible strategy is to employ an artificial neural network (ANN) to model the data. ANNs are inspired by the central nervous system and allow researchers to make complex
non-linear connections between dependent and independent variables without a deep understanding of the underlying mechanisms involved in the process under investigation\textsuperscript{102}. A typical ANN involves a set of given inputs (independent variables) that are related to outputs (dependent variables) via transfer functions. The weight and bias of each transfer function is adjusted to minimize the error in the network. ANNs have been used as a powerful modeling technique in different research fields to date\textsuperscript{102,103,98}.

While high-throughput screens provide a quick readout of a few parameters for a large number of compounds to efficiently cull the test population, they do not provide a detailed high-content functional analysis. Conversely, engineered cardiac tissues (ECTs) have been developed to generate high-fidelity tissues with improved myocardial maturity and more predictive toxicology, as well as more comprehensive and physiologically-relevant functional readouts. Several platforms already exist to test the effects of drugs on cardiac tissues \textit{in vitro}, mainly relying on rat cell sources\textsuperscript{84}. While these cells are easily acquired, they cannot accurately replicate the effects of drugs on human tissue due to the differences between rat and human cardiac physiology. Other groups have used CMs derived from human embryonic stem cells (hESC-CMs) or human induced pluripotent stem cells (hiPSC-CMs)\textsuperscript{104}, cultured around polydimethylsiloxane (PDMS) posts to measure contraction, however limitations in tissue maturity have been noted\textsuperscript{86}. We developed the Biowire\textsuperscript{TM}, a three-dimensional (3D) ECT platform, designed to align and electrically-stimulate hiPSC-CMs during culture, and thereby generate improved cell maturation levels\textsuperscript{89}. More recently, we developed the second-generation Biowire\textsuperscript{TM II} platform to both mechanically- and electrically-stimulate hiPSC-CMs during culture for even greater cell maturation, and to provide direct, non-destructive contractility...
measurements\textsuperscript{90}. Moreover, the Biowire II platform was designed as a comprehensive high-content cardiac functional assay.

In this study, we propose a high-throughput, high-content screening method for examining the effects of kinase inhibitors on human cardiac physiology and function. Here, we screened small molecules from the GalxoSmithKline published kinase inhibitor set (PKIs){\textsuperscript{97,105,106}. We first used a traditional high-throughput 2D assay to screen the effect of kinase inhibitors on hiPSC-CM monolayers (Fig. 10A). We then analyzed the acquired data using a custom ANN to select candidate kinase inhibitors (Fig. 10B). Finally, a high-content screen of the selected kinase inhibitors was performed using 3D ECT constructs (Biowires) (Fig. 10C).

6.2 Results

6.2.1 Validation of Assay Performance Using Compounds with Known Cardiac Effects

At higher concentrations, calcium flux was completely hindered by the application of the known compounds nifedipine and thapsigargin (Fig. 11A), as expected and thus no beating was observed in human cardiac monolayers. These results were used to determine the level of background noise recorded by the software, in order to determine the minimum amplitude required to identify a contraction. As expected, there were no appreciable qualitative differences in cell viability between the low and high concentrations of control compounds (Fig. 11B). There was no statistically significant effect of the drugs on cell viability and cell number (Fig. 12), which is consistent with literature\textsuperscript{107}. However, there was a statistically significant concentration-dependent effect of nifedipine and thapsigargin on calcium transients, consistent with previous literature\textsuperscript{95,96}. 
6.2.2 Results of Monolayer Testing

With the limits of the calcium transient assay defined, we were able to begin our screen of 80 different kinase inhibitor molecules, from the GlaxoSmithKline published kinase inhibitor set, targeting 23 different pathways. Reference compounds (Table 2) were included on each plate. Overall, the trends observed in each parameter were consistent amongst kinase targets, and any discrepancies were attributed to difference in specificity of the individual inhibitor molecules (Fig. 13). The majority of the compounds either had no effect or resulted in a decrease in cell viability, live cell number, and Ca\(^{2+}\) transient frequency, whereas application of some compounds resulted in a profound increase in Ca\(^{2+}\) transient magnitude (Fig. 13). From the monolayer screen, one could identify many compounds that enhanced Ca\(^{2+}\) transient magnitude without negatively affecting cell viability, live cell number, or transient frequency (e.g. in EGFR/ErbB2 family, inhibitors GW703087X(#44) and GSK980961A (#12), Fig. 13) It should be noted that these immature CMs are not perfect predictors of inhibitor effect on adult tissue. As such, further testing is required on a more mature tissue to validate monolayer results.

6.2.3 ANN Modeling

The monolayer experiments provided us with a significant amount of data that needed to be processed to select compounds for 3D testing. We used an ANN approach to analyze the experimental data acquired and provide an empirical tool for identifying inhibitors with the least detrimental effects on CMs. The control ANNs allowed us to validate the possibility of using ANNs to accurately analyze the effect of kinase inhibitors on CM viability (Fig. 14, 15, Tables 3-5). Some of the tested kinase inhibitors targeted the known pathways, whose effects on cell
viability were investigated before. We chose the control ANN output parameter to be normalized cell viability, because we would be able to confirm our results with the known effects of pathways on cell viability. **Fig. 16** illustrates the results of the control ANN predictions. Our findings were in line with what we expected from the literature\textsuperscript{13,33,57,30,108,109}. For example, c-RAF is a kinase required for cell growth and proliferation, as well promoting survival by antagonizing apoptosis\textsuperscript{110,111}. Our results indicate that inhibition of the c-RAF pathway significantly decreases the viability of CMs (**Fig.16**).

With our results validated, we proceeded to predict the effects of the inhibitors on calcium transients, while restraining the effects on cell viability, live cell number, and contraction frequency (**Fig. 17**). Specifically, we designed an ANN to identify compounds that are likely to enhance Ca\textsuperscript{2+} transients without changing transient frequency and without decreasing cell viability and live cell number. This approach allowed us to analyze the data for each kinase inhibitor at various concentrations faster and with more accuracy than a manual approach. The chosen ANN architecture resulted in a correlation coefficient close to 1 (0.9379), and a minimal RMSE (0.1011) (**Fig. 17B,C**).

With our network performing adequately, we were then able to use it to predict compounds that would have a positive effect on calcium peak magnitude, while minimizing the detrimental effects on beat frequency, cell viability, and live cell number. ANN design parameters are listed in **Fig. 17D**. From these results, it was determined that inhibitor GW703087X (#44 according to our blinded screen), an EGFR/ErbB2 inhibitor, was the least detrimental to cardiac function and cell viability, while kinase GW829055X (#24 according to our blinded screen), an GSK3β
inhibitor, was the most detrimental to cardiac function and cell viability out of the 80 kinase inhibitors tested from the PKIs (Fig. 17F).

6.2.4 Kinase Inhibitor Testing on Human Engineered Cardiac Tissues- Biowires

The least and the most detrimental kinase inhibitors on cardiac function, live cell number and cell viability as a whole, as determined by the ANN were subsequently examined in a dose-response fashion on the Biowire. Tissue compaction of Biowires was monitored over 21 days of culture (Fig. 18A, 18Bi). After 7 days of compaction, there was no significant decrease in tissue diameter. In addition, ET decreased significantly after 7 and 14 days of electrical field stimulation (Fig. 18Bii) and MCR increased significantly after 7 and 14 days of stimulation (Fig. 18Biii) indicating improvements in tissue function. Therefore kinase inhibitors were screened after 21 days of Biowire cultivation, at which point both structural and functional properties of the tissues were stabilized.

Fig.19 illustrates the effect of each inhibitor on tissue viability after 24 hr of exposure to the compounds. There was no qualitative difference in cell size or shape for each group compared to control tissues (Fig.19A). In addition, no significant difference on viability was observed in any of the test groups compared to the control groups (Fig. 19B).

Four parameters were evaluated during tissue contraction: active tension in the tissue, beat duration, contraction slope, and relaxation slope (Fig. 20, 21). A dose response curve using nifedipine was performed, to validate the physiological relevance of the tissues (Fig. 20A, 20B). As expected, tissue contraction was significantly decreased at concentrations higher than 10 μM,
as assessed by the decrease in active tension, peak duration, rising slope and falling slope, indicating that the Biowires were sensitive to blockages of the L-type Ca\(^{2+}\) channels.

To confirm the capabilities of our tissues as a high-fidelity model for kinase inhibitor toxicity screening in cardiac tissue, we examined Sunitinib, a VEGFR inhibitor that has been documented to elicit acute coronary symptoms including myocardial infarction (MI), heart failure, and left ventricular dysfunction after prolonged and repeated exposure\(^{47,46}\). Significant decreases were observed in active tension, contraction slope, and relaxation slope at the acute time point upon Sunitinib application when compared to the untreated baseline. Additionally, a significant decrease was observed in contraction slope after 24 hr exposure of Biowires to Sunitinib (Fig 21, 22). This parallels the effects of Sunitinib reported in the literature.

Inhibitor GW703087X (#44), identified as the least detrimental by the ANN, did not elicit any significant differences to any of the parameters of interest at both acute and prolonged time points when compared to the untreated baseline measurements. In contrast, after 24 hr of exposure there was a significant decrease in contraction and relaxation slopes with the kinase GW829055X (#24), identified as detrimental by the ANN analysis.

We compared our Biowire results to our initial monolayer results, to determine the differences in the sensitivity of the monolayer vs 3D platform. Calcium transient magnitude after application of inhibitor GW703087X (#44) to CM monolayers, increased 3-fold compared to controls (Fig. 23); however these results did not translate to the 3D Biowire system (Fig. 21, 22). Relying on the
monolayer screen alone, one would conclude that GW703087X (kinase inhibitor #44) can enhance contractility without affecting cell viability.

Inhibitor GW829055X (#24) significantly decreased cell viability in the monolayer screen, while increasing Ca\(^{2+}\) transient magnitude at 1\(\mu\)M demonstrating oversensitivity and internally inconsistent results in the monolayer system. In the 3D Biowire system, there was no significant decrease in cell viability with the application of GW829055X (kinase inhibitor #24) and Ca\(^{2+}\) transient magnitude was only decreased at 24hr, demonstrating internally consistent results (Fig. 23). Although qualitatively, monolayer and 3D Biowire results exhibited similar trends, the magnitude of effects was different in the two systems.

6.3 Discussion

It has been well documented that overexpression of kinase molecules is typical of several aggressive forms of cancer\(^{112}\). The EGF kinase family, including EGFR (Erbb1) and Erbb2 (HER2), are overexpressed in 25\% of breast cancers\(^{113}\), and 10\% of lung cancers\(^{114}\). There are several clinically available EGFR or Erbb2 inhibiting cancer therapies, with low or minimal known cardiotoxicity. For example, trastuzumab, a monoclonal antibody Erbb2 inhibitor used to treat Erbb2\(^{+}\) breast cancer, has been shown to lead to congestive heart failure in 1.7-4.1\% of patients and a 10\% decrease in LVEF when used alone as treatment\(^{60,45,44}\). These detrimental effects tend to increase significantly when used simultaneously with anthracycline. Conversely, treatment with Lapatinib, a small molecule EGFR/Erbb2 inhibitor used to treat breast cancer, has not been linked to cardiotoxicity\(^{115,48}\). It is evident that even molecules that inhibit the same kinase pathway have varied associated toxicities\(^{13}\). In addition, there may be inherent differences
between the mode of efficacy of monoclonal antibody based kinase inhibitors, and small molecule kinase inhibitors. This indicates that the specificity of the molecule, and the point at which it inhibits in the pathway, are critical to its potential toxicities. In contrast to EGFR/Erbb2, GSK3β is implicated in mediating hypertrophy in the heart during pressure overload stress\textsuperscript{116}, and has been controversially implicated in cancer progression and tumorigenesis; it has been shown to function as a tumor suppressor in certain types of tumors, while promoting growth in others\textsuperscript{117}. For example, inhibition of GSK3β has prevented cell proliferation in colorectal cancer cells, whereas a decrease in GSK3β expression has been observed in non-melanoma skin cancer cells \textsuperscript{118}. As of 2011, there were no clinically available GSK3β-targeting cancer therapies\textsuperscript{30}.

The results from the experimental data and ANN indicate that use of an EGFR/Erbb2 inhibitor would lead to the least detrimental effects on cardiac function, cell viability and live cell number as a whole. Although the cardiac kinome is not fully understood, there have been recent advances in understanding the role of EGFR and Erbb2 in the heart. Erbb2 has been linked to CM proliferation through activation of ERK-MAPK and PI3K-AKT pathways that promote CM survival\textsuperscript{119}, while its inhibition can lead to CM death\textsuperscript{120}. In postnatal murine hearts, treatment with activated Erbb2 promoters has been shown to stimulate CM proliferation post MI\textsuperscript{34}. The role of EGFR is less understood in the heart. Recent findings suggest EGFR mediates pro-survival signalling during catecholamine stimulation\textsuperscript{121}, although its role in normal cardiac function is still unclear. The role of GSK3β has been well documented as a negative regulator of cardiac hypertrophy\textsuperscript{39} and has recently been implicated as regulator of ventricular remodelling in ischemic hearts\textsuperscript{122}. In addition, GSK3β has been identified as a regulator of calcium homeostasis in the heart during normal function and in diseased tissue\textsuperscript{41,42}. 
In this work, the inhibitors are all small molecules, not monoclonal antibodies. Our results align with what is expected from previous clinical studies in terms of the lack of toxicity of small molecules EGFR/ErbB2 inhibitors\textsuperscript{13,33}. These results indicate that although ErbB2 inhibiting molecules have led to some instances of cardiotoxicity, they should not be discounted as a cancer therapeutic agents due to the minimal detrimental effects.

To satisfy the condition of high-throughput screening, we had to face the challenge of efficient and unbiased analysis of large quantities of data. After considering various manual statistical approaches, we chose to use an automated technique. ANN modelling has become popular in health care as a diagnostic tool\textsuperscript{123,124}, but has also found significant use in the pharmaceutical sector for its ability to build predictive models of complex nonlinear relationships between molecular function and properties of biomolecules\textsuperscript{125,126}. ANN modelling has the ability to handle noisy data sets with no required knowledge of data source\textsuperscript{127}. ANNs have also been implicated as effective tools for high throughput screening analysis of pharmaceuticals and other active compounds\textsuperscript{125,128}.

Using this modeling technique, we were able to extract the most relevant information on the effects of kinase inhibitor molecules on CMs using two simple high content screening assays, and reduce the experimental effort and cost. One of the advantages of ANN modelling is the ability of the network to produce large number of data points from a significantly smaller set of experimental results. In this study, we tested 80 different kinase inhibitors at 3 concentrations and recorded 4 parameters for each condition, for a total of 240 experimental data points. Using
our predetermined limits for this experiment (Fig. 2C), the network obtained 125840 data points for this set of experiments. We can also use this network to predict the efficacy of other kinase inhibitor molecules on cardiac tissue in future experiments.

Sunitinib was chosen as a reference inhibitor because it has been documented to cause cardiac dysfunction after repeated and prolonged clinical use\textsuperscript{47,62,52,46}. Researchers have been able to mimic these results in ECTs, adult myocardium samples and zebra fish models\textsuperscript{129,130,131}. Rainer et al. examined the effect of Sunitinib exposure to adult human myocardium strips excised from patients undergoing heart surgery, and reported a decrease in force of over 40\% at 10μg/mL after 30 min of exposure\textsuperscript{130}. Similarly, Jacob et al. examined the effect of Sunitinib on rat ECTs and observed a significant decrease in force of contraction at concentrations higher than 1μM\textsuperscript{131}. Our findings agree with those of Jacob and Rainer, indicating that we can predict significant changes in tissue contractile force. In addition to the effect on contractile force, Sunitinib was implicated as having a significant effect on contraction slope and relaxation slope. Sunitinib has been implicated in affecting QT interval and action potential in the myocardium after both short and long term exposure\textsuperscript{132,133}, which is consistent with our findings in the Biowire system. Although Sunitinib had a significant effect on active tension, contraction and relaxation slopes in the tissues, there was no significant effect on cell viability. This is expected at the concentrations we were investigating\textsuperscript{131,132}.

With the assurance that our platform could detect cardiotoxicity caused by kinase inhibitors in a two-dimensional system, we began testing in the Biowires. Although we did not detect any
improvement in contraction or cell viability using the EGFR/ERBB2 inhibitor in the Biowire system (Fig. 21), as would be suggested by our monolayer studies (Fig. 23), there were no detrimental effects to cardiac function in the 3D system. This aligns with our expected results from the ANN, as well as the lack of evidence of cardiotoxicity using clinically available EGFR/ERBB2 inhibitors. The detrimental effects of the GSK3β inhibitor observed in the 3D Biowire system, were not as dramatic as anticipated from the monolayer results and as detected by the ANN.

These discrepancies could be the result of an increased sensitivity of the monolayers compared to 3D tissues, such that monolayers amplify both the positive and negative effects compared to the 3D tissues. The cell-to-cell interactions in the tissues may provide some cardioprotective function to the cells, preventing the significant acute detrimental effects on cardiac function or viability. It is possible that these detrimental effects would be replicated in the 3D culture at either higher concentrations or after a longer exposure time. Although inhibitor GW829055X (#24) had a significant detrimental effect on cell viability (Fig. 23), and inhibitor GW703087X (#44) significantly increased calcium transients in beating CM monolayers, these results were not observed in the human cardiac Biowire platform. Thus, 3D platforms eliminate oversensitivity, which is critical to decrease the incidence of both false positives and false negatives, in comparison to using monolayer assays alone.

Although no differences were observed in cell viability after 24 hr of incubation in the Biowire system, further testing is needed to determine whether a longer-term exposure of several months
would lead to cell death. In future studies, we will also examine the effect of the inhibitors after a repeated exposure over a period of time longer than 24hr, to better mimic the clinical treatment regiment. A limitation of this work is the lack of physiological information regarding these new kinase inhibitor molecules. Because we do not know therapeutic doses of the kinases, we are operating under the assumption that their activity would lie in the same range of current clinically available compounds.

6.4 Conclusion and Future Work

In conclusion, this study demonstrated the potential for high-fidelity analysis of kinase inhibitors on cardiac tissue—combining experimental results with empirical screening for high-throughput and unbiased analysis—that can be used in the pre-clinical screening phase of drug discovery. By first measuring contractile cell-specific parameters such as calcium transients and contraction frequency, in addition to viability, then analyzing the results using ANN modelling, we can efficiently quantify inhibitor effects on monolayers, and understand complex relationships that are not easily tested. This allows researchers to be more accurate when selecting compounds for a more costly and time-consuming 3D tissue testing. Though we have shown that this platform is effective at predicting and measuring the effect of kinase inhibitors, we hope in the future to examine other classes of pharmaceuticals that have been linked to cardiotoxicity.
Figure 10 Kinase inhibitor screening workflow. A) Cell number, viability, and Ca2+ transients were first assessed in a monolayer format using high-throughput screening assays. B) Monolayer results were used in an ANN to model and predict the effects of the kinase inhibitors. C) Inhibitors of interest from the ANN were then tested in 3D Biowire system.
Figure 11 Validation of monolayer testing assays using control compounds. A) Representative traces of calcium transient experiments on iPSC-derived CMs after 20-minute exposure to known compounds with negative chronotropic and inotropic properties, nifedipine and thapsigargin. B) Representative images taken from the cell viability experiments on iPSC-derived cardiomyocytes after 24-hour exposure to the compounds.
Figure 12 Comparison of the effects of negative chronotropic drugs on CMs. The solid line in each graph represents the results from the blank wells for each parameter, with one standard deviation above and below the average in dotted lines. Results were similar for both nifedipine and thapsigargin; Significant differences from blank controls are indicated by #.
Figure 13 Effect of kinase inhibitors on iPSC-derived CM monolayers. Each inhibitor was applied to the cells at 0.1 \( \mu \text{M} \), 1 \( \mu \text{M} \) and 10 \( \mu \text{M} \) in triplicate. The average of each set of triplicates was compared to the control average, and coded as being within 1, 2 or 3 standard deviations of the control compound effect. Heat maps were drawn to visualize changes in normalized cell viability, live cell number, Ca2+ transient magnitude, and Ca2+ transient frequency under spontaneous beating. Inhibitors are listed by experimental number and grouped according to kinase target for clarity. To decode experimental labels, PKIs names are provided in Table 3.
Figure 14 Designing the architecture of control neural network. A control network was designed to validate the capabilities of the neural network to predict compounds affecting cell viability. Normalized number of live cells and kinase target were used as inputs, and normalized cell viability was the output.

Table 4 Comparison of various control ANN designed around 0.1 µM results. Network chosen is highlighted in red.

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Table 5 Comparison of various control ANN designed around 1 µM results. Network chosen is highlighted in red.

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Table 6 Comparison of various control ANN designed around 10 µM results. Network chosen is highlighted in red.

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Figure 15 Performance of control neural networks. Performance of neural network designed to model and predict changes in normalized viability for cells exposed to kinase inhibitors at (A) 0.1 µM, (B) 1 µM and (C) 10 µM. As the number of epochs increased, RMSE decreased and converged at a minimum value, illustrated by the graph on the left. In addition, there was a significant relationship between the predicted change in normalized cell viability and the actual change in normalized cell viability, as illustrated by the graphs on the right. The red dotted line indicates the linear trend line observed, with the respective R2 highlighted. Predicted values were acquired from the neural network architecture illustrated in Figure S.1B. Monolayer results were normalized as follows: Normalized data = (Experimental data – Blank data)/ Blank data.
Figure 16 Prediction capabilities of control neural networks. A) Limits for normalized cell number were chosen as largest and smallest values in the experimental data. Total number of data points available for each network was 6970. B-D) Prediction capabilities of neural network designed to model and predict normalized cell viability for cells exposed to kinase inhibitors at 0.1 µM, 1 µM, and 10 µM respectively. Each inhibitor has 87 data points predicted by the neural network. The figure on the left indicates the predicted results for each inhibitor provided by the network. The figure on the right illustrates the relationship between the experimental results (blue) and the mean predicted results (orange) of the network in terms of normalized cell viability.
Figure 17 Neural network approach to model and predict kinase inhibitors with significant effects on CMs. A) Several network architectures were examined by altering number of hidden layer(s) and the number of neurons within them, as well as changing the transfer function of each neuron, with the goal of maximizing R² while minimizing RMSE. The network chosen is highlighted in red. B) Validation of network performance. After 1000 epochs (iterations), the RMSE converges on a single minimum value, 0.1011. C) Comparison between actual and predicted normalized Ca²⁺ peak magnitude. There is a correlation between the actual normalized peak magnitude values and the predicted normalized peak values, with an R² value of 0.9379. The expected trend line is illustrated as the red dotted line. D) Design parameters chosen for prediction network. These parameters were chosen in order to identify compounds that had the greatest effect on Ca²⁺ transient magnitude, while minimizing undesired effects on normalized cell viability, live cell number, and
frequency. From these limitations, we designed our network with 125,840 data points. The data points used in training the network were normalized as follows: Normalized data = (Experimental data – Blank data)/ Blank data. E) Predicted normalized value of Ca²⁺ peak magnitude for each inhibitor. There are 1573 data points for each compound F) The mean predicted value for each compound is illustrated by the blue line. The compound with the least detrimental effect was found to be inhibitor GW703087X (#44), an EGFR/ErbB2 inhibitor; the compound with the most significant negative effect was found to be inhibitor GW829055X (#24), an GSK3β inhibitor.
Figure 18 Tissue characterization. A) Representative images of tissue compaction taken at 4x magnification. After 2 days of compaction there was evidence of tissues beating spontaneously. B) Changes in tissue structure and function. i) Dimensions of tissue centre throughout maturation. There is a clear decrease in tissue diameter throughout the first 7 days of culture. After 7 days, tissue diameter did not change significantly (confirmed using ANOVA analysis). ii) ET of tissues throughout course of electrical stimulation. ET was measured 7 days after seeding before any stimulation occurred. After 7 and 14 days of stimulation, ET decreased significantly, indicated by * and confirmed using ANOVA analysis. iii) MCR
of tissues throughout course of electrical stimulation. After 7 days of stimulation there was a significant increase in maximum capture rate of the tissues, indicated by * and confirmed using ANOVA analysis. C) Biowire stimulation protocol. On day 7, tissue ET and MCR was assessed. Tissue stimulation began at 1.5x average ET and 1Hz. Every two days, frequency of stimulation was increased by 0.83 Hz, to a maximum of 6 Hz. After 14 days of stimulation, tissues were maintained at 3Hz. Orange bars indicate date of tissue assessment, blue bars indicate date of stimulation modification.

Figure 19 Viability of Biowire tissues after 24hr exposure to kinase inhibitor molecules. A) Representative images of Biowire tissues taken using confocal microscopy after 24hr exposure to kinase inhibitors. All cell nuclei were stained with DAPI (blue channel) and dead cells were stained with propidium iodide (red channel). B) Comparison of the effect of inhibitor GW703087X (#44), GW829055X (#24), and Sunitinib on tissue viability. ANOVA analysis indicated no statistically significant differences in viability between the different drug samples and the control.
Figure 20 Confirmation of Biowire performance and representative contraction traces. A) Effect of Nifedipine on tissue contraction, expressed as a difference from the baseline measurement. There was a statistically significant difference in active tension, peak duration, contraction slope, and relaxation slope from the untreated baseline at concentrations 10 μM and higher, indicated by #. B) Representative traces of tissue contraction after exposure to nifedipine (stimulated at 1Hz). Traces were acquired using ImageJ particle tracking software and converted to force using unique MATLAB software. Spontaneous secondary contractions were observed starting at 0.1 μM, indicated by the blue arrow. At 1000 μM no beating was observed.
Figure 21 Effect of kinase inhibitor drugs on tissue contraction. Active tension, peak duration, contraction slope, and relaxation slope were characterized for each tissue after acute and prolonged exposure to inhibitors GW703087X (#44), GW829055X (#24), and Sunitinib. No statistically significant differences were observed for inhibitor GW703087X (#44) at either acute or 24hr time points. There was a statistically significant decrease in contraction slope and relaxation slope after 24 hr exposure to inhibitor GW829055X (#24), indicated by #, and a significant decrease in relaxation slope between 10 μM acute and long term exposure, indicated by *. An acute effect on active tension, contraction slope, and relaxation slope was observed on tissues treated with the control compound Sunitinib when compared to baseline values, indicated by #. Differences between doses are indicated by *
Figure 22 Representative traces of tissue contraction after acute and prolonged (24hr) exposure to inhibitor GW703087X (#44), GW829055X (#24), and Sunitinib (stimulated at 1Hz). Traces were acquired using ImageJ particle tracking software and converted to force using specialized MATLAB software.
Figure 23 Monolayer results for kinase inhibitor GW703087X (#44) and GW829055X (#24). Kinase inhibitor GW703087X (#44) significantly increased calcium transient magnitude (indicated by #) at 0.1µM and 1µM (no beating was observed at 10µM). Kinase inhibitor GW829055X (#24) significantly decreased CM viability at all concentrations (indicated by #).
7. Contributions to the Literature

As stated previously, there has been a significant lack of research in the area of kinases and their role in cellular function. While some are thoroughly documented, many remain incredibly under-researched. In this work, we have directly demonstrated the effects of various kinase inhibitors on hiPSC-CM and matured engineered human cardiac tissues. A substantial amount of research has investigated the effects of kinases in rodent models, however very few studies have screened multiple kinases inhibitors on human tissues. Our platform provides a method for high-throughput screening of kinase inhibitors on human cardiac tissue, and the ANN modelling allows for an unbiased empirical modelling of the complex relationships between kinase inhibition and CM function.

Our results have also demonstrated that we can elicit physiologically relevant responses to kinase inhibition in our tissues, as is evident by the observed detrimental effect of Sunitinib. We have validated our results by examining the multitude of cardiotoxic reports of Sunitinib both clinically and *in vitro*. Our 3D platform mimics the human myocardium, and can be a surrogate testing tool for cardiotoxicity studies.

In addition to our work in investigating the effect of kinases in the body, we have also demonstrated the capabilities of 3D tissue-screening to eliminate oversensitivity from monolayer results. Oversensitivity can lead to both false positive and false negative results in terms of inhibitor effects, which can be highly detrimental later in the drug development process.
8. Future Work

There are several aspects of this work that should be further investigated. We have demonstrated the platform’s ability to study the effect of kinase inhibition in CM monolayers and cardiac tissue, and a next step of this project would be to investigate the remaining inhibitors from the PKIs. The acquisition of more data would help to train and test the network even further, providing a more accurate prediction model of KI effect. In addition, we would examine the effect of the inhibitor in our 3D Biowire tissues after a significantly longer exposure period, since the longer exposure better mimics the clinical treatment regimen. As well, we would investigate the effect of repeated exposure, as typical chemotherapy regiments require multiple exposures to the cancer therapeutic compounds.

It would also be worthwhile to investigate the specific mechanism of kinase inhibition of compounds of interest, by studying protein and gene expression in the tissues after KI exposure. As stated previously, each kinase inhibitor can block the signalling pathway at different instances, and thus can greatly affect cellular function. At certain points in the signalling pathway there might be other kinases that perform similar functions to the inhibited kinase, resulting in inhibition eliciting less of an effect on cardiac function. Gaining an understanding as to the mechanism of inhibition of these inhibitors might provide us with an understanding of the cause of cardiotoxicity due to kinase inhibition, and means of preventing such complications in the future could be made apparent.
9. Project Conclusions

In conclusion, this project has demonstrated that high-throughput screening of kinase inhibiting drugs on Biowires is a high-fidelity model for assessing the effect of KIs in the human body. Traditional monolayer screening can be effective means of rapid screening of multiple compounds on cardiac tissue and acquisition of large volumes of data, however it is plagued with the potential for oversensitivity. Examining the effects of KIs on 3D tissues provide a more accurate representation of their effect in the body, and can clarify any oversensitivities observed in the monolayer cultures.

ANN modelling has also been demonstrated to be a crucial tool to the success of this high-throughput platform. When screening multitude of compounds at different concentrations, and investigating several cellular functions, we generated thousands of data points. Identifying compounds of interest from this immense data set can be difficult, when comparing differences in multiple parameters. The benefit of ANN modelling is its ability to examine the differences between multiple parameters for each compound and make inferences without any required previous knowledge. In addition, sophisticated imaging and analysis techniques greatly increases the high-throughput capabilities of this platform by minimizing the time required for analysis and eliminating experimenter bias.

We hope that in the future, this platform can be adapted to other classes of compounds, and extended to other tissue platforms, with the goal of better understanding their effects on the body early in drug development, and reduce late stage drug attrition during clinical trials.
References


## Appendix A: Kinase Table

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<th>Effect on cell viability</th>
<th>Overexpression in Cancer</th>
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<td>AKT1</td>
<td>Essential for cell proliferation(^{134})</td>
<td>Promotes cell survival by inactivating apoptotic machinery(^{135})</td>
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<td>ALK5</td>
<td>Inhibitions prevents endothelial cell proliferation(^{137}). Inhibition prevents tumour angiogenesis(^{138}). Induces SMC differentiation in vessels(^{139}).</td>
<td>Activation of TGFβ through ALK5 promotes cell survival of mammary epithelial cells(^{140}).</td>
<td>Yes(^{141})</td>
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<td>b-RAF</td>
<td>Over-expression required for proliferation(^{142})</td>
<td>Supress apoptosis (^{142,143})</td>
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<td>CDK2/CDK4</td>
<td>Required for progression through G1 phase of cell cycle(^{144,145})</td>
<td>Protects podocytes from apoptosis(^{146})</td>
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<td>c-RAF</td>
<td>Required for growth and proliferation of NIH-3T3 cells(^{148,149})</td>
<td>Promotes cell survival by controlling ROS production(^{150}). Antagonizing apoptosis(^{151})</td>
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<td>EGFR/ErbB2</td>
<td>Inhibition of EGFR inhibited proliferation in cancer cells(^{152}). With ERBB3, drives breast cancer cell proliferation(^{153}). Knock out ErbB2 leads to reduced proliferation in tumor cells(^{154}).</td>
<td>Depletion of ERBB2 in myoblasts/myofibers led to apoptosis(^{155})</td>
<td>Yes(^{153})</td>
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<td>Inhibition prevented proliferation in colorectal cancer(^{156})</td>
<td>Implicated in cell survival during gestation(^{157}). Inhibition induced apoptosis in colorectal cancer(^{156}). Promotes apoptosis in neuronal cells and vascular smooth muscle cells(^{158,159})</td>
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<td>IGF-1R</td>
<td>Stimulates cell proliferation(^{160})</td>
<td>No correlation with cell survival in lung carcinoma (10). Promotes cell survival against apoptotic stimuli (11)</td>
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<td>IKKα/IKKβ</td>
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<td>Promotes cell survival by activating NF-kB pathway (12)</td>
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<td>JNK2/JNK3</td>
<td>Part of MAPK pathway, essential for progression to S phase of mitosis in endothelial cells(^{164,165}).</td>
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<td>Critical in progression through cell cycle(^{164}). Required for fibroblast cell proliferation, but not differentiation(^{168}).</td>
<td>Inhibition has no effect on cell survival(^{169})</td>
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<td>Normal cell mitosis not affected by PLK1 depletion, at high concentrations cancer cells severely affected(^{171}).</td>
<td>Normal cells can survive depletion, but not cancer cells(^{171})</td>
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<td>Inhibition leads to reduction in cell shrinkage and detachment(^{174}).</td>
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<td>With NGF signalling, required for airway smooth muscle cell proliferation(^{176}). Implicated with neuronal cell differentiation(^{177}).</td>
<td>Receptors mediate anti-apoptotic signals. Inhibition leads to apoptosis(^{178})</td>
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<td>VEGFR2</td>
<td>Promotes angiogenesis by inducing endothelial cell proliferation. Major mediator of mitogenic, angiogenic and permeability enhancing effects of VEGF(^{180}).</td>
<td>Activation is required for cell survival and angiogenesis in human umbilical vein endothelial cells(^{181}).</td>
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Appendix B: MATLAB Peak Finding Code

clear all;
Directory = uigetdir('Please Select the Experiment Directory');

FilesInDirectoryXLSX = dir(fullfile(Directory, '*.xlsx'));
numFiles = length(FilesInDirectoryXLSX);
TotFiles = numFiles;
for fileIDX = 1:TotFiles
    FileName{fileIDX} = FilesInDirectoryXLSX(fileIDX).name;
end

%% Importing Data Parameters
InputSheetInfo = inputdlg({'Max or Min', 'Frequency', 'Passive Tension Baseline (pixel)', 'Tissue diameter on wire (mm)', 'Tissue diameter in centre (mm)'},
MaxMin = char(InputSheetInfo(1,1));
Frequency = str2double(InputSheetInfo(2,1));
PTB = str2double(InputSheetInfo(3,1));
dOnWire = str2double(InputSheetInfo(4,1));
dInMiddle = str2double(InputSheetInfo(5,1));
if (7 ~= exist(fullfile(Directory, 'ResultsWITHFILTER'), 'dir'))
    mkdir(fullfile(Directory, 'ResultsWITHFILTER'));
end

SheetName = 'Sheet1';
Framerate = 100;
counter = 1;
c = 1;
pixtomm = 390;
pixtoum = 0.39;
PTBase = PTB/pixtoum;
tissuearea = pi*(dInMiddle/2)^2;
for fileIDX = 1:numFiles
    SheetName = 'Sheet1';
data = xlsread(char(fullfile(Directory, FileName(fileIDX))), 'B1:B30000');
    DS = data(1:501);
    if strcmp(MaxMin, 'Min') == 1 || strcmp(MaxMin,'min') == 1
        DS = (max(data) - DS);
    end
    D = DS(D < mean(D));
    BL = mean(D(D < mean(D)));
    if strcmp(MaxMin, 'Min') == 1 || strcmp(MaxMin,'min') == 1
        dataSet = (max(data) - data);
    else
        dataSet = data;
    end
%% Peak analysis

% Find baseline from relaxed, normalize dataSet, and convert to um
dataSet = (dataSet-BL)/pixtoum + PTBase;

%% Apply moving average filter (window size of 4, through trial and error)
b = [0.25 0.25 0.25 0.25];
dataSet = filter(b,1, dataSet);
dataSet(1:3) = [];
times = (1:1:length(dataSet))/Framerate);
%Beating check
DataMax = max(dataSet);
DataMin = min(dataSet);
Check = DataMax-DataMin;

if Check > 1

% Find Peaks
coeff = 0.7;
distcoeff = 5;
minpeakheight = coeff*(max(dataSet)-min(dataSet)) + min(dataSet);
minpeakdist = round((Framerate/Frequency)) - distcoeff;

[peaks locs] = findpeaks(dataSet, 'minpeakdistance', minpeakdist, 'minpeakheight', minpeakheight);

figure(counter);
hold on;
plot(times, dataSet, 'k');
plot(times(locs), dataSet(locs), 'xr');
xlabel('Time (s)')
ylabel('Movement (um)')
title({'Plot of ' num2str(Frequency) 'Hz'}))
pause
allPeaks = questdlg('Were all the peaks found (yes or no)', 'Peak Finding', 'Yes', 'No', 'No');
close(counter)

while strcmp(allPeaks,'No') == 1

    coeff = coeff - 0.1;
distcoeff = distcoeff + 5;
minpeakheight = coeff*(max(dataSet)-min(dataSet)) + min(dataSet);
minpeakdist = round((Framerate/Frequency)) - distcoeff;
if sign(minpeakdist) == -1
    minpeakdist = 1;
end
[peaks locs] = findpeaks(dataSet, 'minpeakdistance', minpeakdist, 'minpeakheight', minpeakheight);
figure(counter);
hold on;
plot(times, dataSet, 'k');
plot(times(locs), dataSet(locs), 'xr');
xlabel('Time (s)')
ylabel('Pixel Movement')
title({'Plot of ' num2str(Frequency) 'Hz'})
pause
allPeaks = questdlg('Were all the peaks found?', 'Peak Finding', 'Yes', 'No', 'Too Far', 'No');
close(counter)
if strcmp(allPeaks,'Too Far') == 1
    coeff = coeff + 0.1;
distcoeff = distcoeff -5;
minpeakheight = coeff*(max(dataSet)-min(dataSet)) + min(dataSet);
minpeakdist = round((Framerate/Frequency)) - distcoeff;
if sign(minpeakdist) == -1
    minpeakdist = 1;
end
[peaks locs] = findpeaks(dataSet, 'minpeakdistance', minpeakdist, 'minpeakheight', minpeakheight);
end

end

%% Find the baseline (if the frequency is below 3Hz. If it's over, it will not search for a baseline.
if Frequency <= 3
if locs(1) < 10
    locs(1) = [];
    peaks(1) = [];
end
if (length(times) - locs(end)) < 20
    locs(end) = [];
    peaks(end) = [];
end
Temp = zeros(length(locs), 2);
R90timeToPeak = zeros(length(locs), 1);
R90timeFromPeak = zeros(length(locs), 1);
baselineForThisPeak = zeros(length(peaks), 1);
amp10 = zeros(length(locs), 1);
R90 = zeros(length(locs), 2);
int1 = zeros(length(locs), 1);
int2 = zeros(length(locs), 1);
for peakIdx = 1 : (length(peaks))
    % Find baseline for each peak
    if peakIdx == 1
        temp = dataSet(1:locs(peakIdx));
    else
        temp = dataSet(locs(peakIdx) : locs((peakIdx+1)));
    end
    temp = temp(temp < mean(temp));
    baselineForThisPeak(peakIdx) = mean(temp(temp<mean(temp)));
    if isnan(baselineForThisPeak(peakIdx))
        baselineForThisPeak(peakIdx) = mean(temp);
    end
    % Set up baseline for whole frequency
    if peakIdx == 1
        baseline(1:(locs(peakIdx)-1)) = baselineForThisPeak(peakIdx);
    elseif peakIdx > 1 && peakIdx < (length(peaks))
        baseline(locs(peakIdx)-1:locs(peakIdx)) = baselineForThisPeak(peakIdx);
    else
        baseline(locs(peakIdx):length(times)) = baselineForThisPeak(peakIdx);
    end
end
for peakIdx = 1:length(peaks)
    differences = peaks(peakIdx) - baselineForThisPeak(peakIdx);
    amp10(peakIdx) = (0.1*differences) + baselineForThisPeak(peakIdx);
    Temp(peakIdx,:) = [find(dataSet(1 : locs(peakIdx)) < amp10(peakIdx), 1, 'last') ...
    find(dataSet(locs(peakIdx) + 1 : end) < amp10(peakIdx), 1, 'first') + locs(peakIdx)];
    L(peakIdx) = Temp(peakIdx,1) + 1;
    R(peakIdx) = Temp(peakIdx,2) - 1;
    % Rising R 90
    m1 = (dataSet(L(peakIdx)) - dataSet(Temp(peakIdx,1)))/((times(L(peakIdx)) - times(Temp(peakIdx,1))));
    b1 = dataSet(L(peakIdx)) - m1*times(L(peakIdx));
    int1 (peakIdx) = (amp10(peakIdx)-b1)/m1;
% Falling R90
m2 = (dataSet(Temp(peakIdx,2)) - dataSet(R(peakIdx)))/(times(Temp(peakIdx,2)) - times(R(peakIdx)));
b2 = dataSet(R(peakIdx)) - m2*times(R(peakIdx));
int2(peakIdx) = (amp10(peakIdx)-b2)/m2;
R90(peakIdx,:) = [int1(peakIdx), int2(peakIdx)];
end

figure(counter);
hold on;
plot(times, dataSet, 'k');
plot(times(locs), dataSet(locs), 'xr');
plot(times, baseline, 'm');
title({'Plot of ' num2str(Frequency) 'Hz'})

% Find the Max and Min values of the peaks
peakamp  = zeros(length(peaks),1);
PeakMinVal = zeros(length(peaks),1);
PeakMaxVal = zeros(length(peaks),1);
for peakIdx = 1:length(peaks)
    peakamp(peakIdx) = dataSet(locs(peakIdx)) - baselineForThisPeak(peakIdx);
    try
        PealkMinVal(peakIdx) = find(dataSet(1 : locs(peakIdx)) <= baselineForThisPeak(peakIdx), 1, 'last');
        s = locs(peakIdx) - PealkMinVal(peakIdx);
        if s >= minpeakdist
            temp = dataSet(locs(peakIdx-1):locs(peakIdx));
            [A I] = min(temp);
            PealkMinVal(peakIdx) = I+locs(peakIdx-1);
        end
    catch
        s = find(dataSet(1 : locs(peakIdx)) - baselineForThisPeak(peakIdx) < 0.001, 1, 'last');
        PealkMinVal(peakIdx) = s + locs(peakIdx);
    end
end
else
    if locs(1)<10
        locs(1) = [];
        peaks(1) = [];
    end
end

other
if (length(times) - locs(end)) < 20
    locs(end) = [ ];
    peaks(end) = [ ];
end
Temp = zeros(length(locs), 2);
R90timeToPeak = zeros(length(locs), 1);
R90timeFromPeak = zeros(length(locs), 1);
baselineForThisPeak = zeros(length(peaks), 1);
amp10 = zeros(length(locs), 1);
R90 = zeros(length(locs), 2);
int1 = zeros(length(locs), 1);
int2 = zeros(length(locs), 1);
baselineLoc = zeros(length(peaks), 1);
for peakIdx = 1 : (length(peaks))
    % find baseline
    if peakIdx == 1
        temp = dataSet(1:locs(peakIdx));
    else
        temp = dataSet(locs(peakIdx-1) : locs(peakIdx));
    end
    [baselineForThisPeak(peakIdx), loc] = min(temp);
    if peakIdx == 1
        baselineLoc(peakIdx) = loc;
    elseif peakIdx > 1 && peakIdx < (length(peaks))
        baseline(locs(peakIdx-1):(locs(peakIdx)-1)) = baselineForThisPeak(peakIdx);
    else
        baseline(locs(peakIdx-1):length(times)) = baselineForThisPeak(peakIdx);
    end
end
figure(counter);
hold on;
plot(times, dataSet, 'k');
xlabel('Time (s)');
ylabel('Movement (um)');
title({'Plot of ' num2str(Frequency) 'Hz'})
peakMinVal = zeros(length(peaks),1);
peakMaxVal = zeros(length(peaks),1);
for peakIdx = 1:length(peaks)
    peakMinVal(peakIdx) = baselineLoc(peakIdx);
    if peakIdx == length(peaks)
        [val, l] = min(dataSet(locs(peakIdx):end));
        peakMaxVal(peakIdx) = l + locs(peakIdx)-1;
    elseif peakIdx > 1 && peakIdx < (length(peaks))
        baseline(locs(peakIdx-1):(locs(peakIdx)-1)) = baselineForThisPeak(peakIdx);
    else
        baseline(locs(peakIdx-1):length(times)) = baselineForThisPeak(peakIdx);
    end
end
for peakIdx = 1:length(peaks)
    differences = peaks(peakIdx) - dataSet(peakMinVal(peakIdx));
    amp10(peakIdx) = (0.1*differences) + dataSet(peakMinVal(peakIdx));
    Temp(peakIdx,:) = [find(dataSet(1 : locs(peakIdx)) < amp10(peakIdx), 1, 'last') ... find(dataSet(locs(peakIdx)) + 1 : end) < amp10(peakIdx), 1, 'first') + locs(peakIdx)];
    L(peakIdx) = Temp(peakIdx,1) + 1;
    R(peakIdx) = Temp(peakIdx,2) - 1;
% Rising R 90
m1 = (dataSet(L(peakIdx)) - dataSet(Temp(peakIdx,1)))/(times(L(peakIdx)) - times(Temp(peakIdx,1)));
b1 = dataSet(L(peakIdx)) - m1*times(L(peakIdx));
int1(peakIdx) = (amp10(peakIdx) - b1)/m1;

% Falling R 90
m2 = (dataSet(Temp(peakIdx,2)) - dataSet(R(peakIdx)))/(times(Temp(peakIdx,2)) - times(R(peakIdx)));
b2 = dataSet(R(peakIdx)) - m2*times(R(peakIdx));
int2(peakIdx) = (amp10(peakIdx) - b2)/m2;

R90(peakIdx,:) = [int1(peakIdx), int2(peakIdx)];

end
figure(counter);
hold on;
plot(times, dataSet, 'k');
plot(times(locs), dataSet(locs), 'xr');
end

%% Plot data and peaks
plot(times(PeakMinVal), dataSet(PeakMinVal), 'og');
plot(times(PeakMaxVal), dataSet(PeakMaxVal), 'xb');
plot(R90(:,1), amp10, 'or');
plot(R90(:,2), amp10, 'ob');
ylabel ('Movement (um)');
xlabel ('Time (s)');
hold off
%close(counter)

%% Find distances and times to and from peaks
TimeToPeak = times(locs(1:(end))) - times(PeakMinVal);
TimeFromPeak = times(PeakMaxVal) - times(locs(1:(end)));
PeakDuration = times(PeakMaxVal) - times(PeakMinVal);

%% Beat Frequency
PeakToPeakTimes = diff(times(locs));
beatfrequencies = 1 ./ PeakToPeakTimes;

%% Peak Intensity
% To find peak intensity, subtract out the baseline for each peak
% PeakIntensity = peaks - baselineForThisPeak;
if Frequency <= 3
    PeakIntensity = peakamp;
else
    PeakIntensity = peaks - min(dataSet);
end

%% Calculate Force
x = dataSet;
if dOnWire >= 0.8
    Force = (0.000169*x.^3 - 0.00251*x.^2 + 0.697*x);
elseif dOnWire == 0.7
    Force = (0.0000621*x.^3 + 0.00295*x.^2 + 0.520*x);
elseif dOnWire <= 0.5
    Force = (0.0000459*x.^3 + 0.000789*x.^2 + 0.668*x);
elseif dOnWire > 0.5 && dOnWire < 0.7
    F1 = (0.0000459*x.^3 + 0.000789*x.^2 + 0.668*x);
    F2 = (0.0000621*x.^3 + 0.00295*x.^2 + 0.520*x);
    Fdiff = F2-F1;
    fracdiff = dOnWire - 0.5;
    x2 = fracdiff/0.2;
    Force = (x2*Fdiff)+F1;
elseif dOnWire > 0.7 && dOnWire < 0.8
    F2 = (0.000169*x.^3 - 0.00251*x.^2 + 0.697*x);
    F1 = (0.0000621*x.^3 + 0.00295*x.^2 + 0.520*x);
end
Fdiff = F2 - F1;
fracdiff = dOnWire - 0.7;
x2 = fracdiff/0.1;
Force = (x2*Fdiff)+F1;
end

PassiveTension = zeros(length(peaks),1);
ActiveTension = zeros(length(peaks),1);
for peakIdx = 1:length(peaks)
    if dOnWire >= 0.8
        PassiveTension(peakIdx) = (0.000169*baselineForThisPeak(peakIdx)^3 - 0.00251*baselineForThisPeak(peakIdx)^2 + 0.697)*baselineForThisPeak(peakIdx);
    elseif dOnWire == 0.7
        PassiveTension(peakIdx) = (0.0000621*baselineForThisPeak(peakIdx)^3 + 0.00295*baselineForThisPeak(peakIdx)^2 + 0.520)*baselineForThisPeak(peakIdx);
    elseif dOnWire <= 0.5
        PassiveTension(peakIdx) = (0.0000459*baselineForThisPeak(peakIdx)^3 + 0.00251*baselineForThisPeak(peakIdx)^2 + 0.697)*baselineForThisPeak(peakIdx);
    elseif dOnWire > 0.5 && dOnWire < 0.7
        A1 = (0.0000459*baselineForThisPeak(peakIdx)^3 + 0.00251*baselineForThisPeak(peakIdx)^2 + 0.697)*baselineForThisPeak(peakIdx);
        A2 = (0.0000621*baselineForThisPeak(peakIdx)^3 + 0.00295*baselineForThisPeak(peakIdx)^2 + 0.520)*baselineForThisPeak(peakIdx);
        Fdiff = A2 - A1;
        fracdiff = dOnWire - 0.7;
        mov2 = fracdiff/0.2;
        PassiveTension(peakIdx)= (mov2*Fdiff)+A1;
    elseif dOnWire > 0.7 && dOnWire < 0.8
        A1 = (0.0000459*baselineForThisPeak(peakIdx)^3 + 0.00295*baselineForThisPeak(peakIdx)^2 + 0.520)*baselineForThisPeak(peakIdx);
        A2 = (0.000169*baselineForThisPeak(peakIdx)^3 - 0.00251*baselineForThisPeak(peakIdx)^2 + 0.697)*baselineForThisPeak(peakIdx);
        Fdiff = A2 - A1;
        fracdiff = dOnWire - 0.7;
        mov2 = fracdiff/0.1;
        PassiveTension(peakIdx)= (mov2*Fdiff)+A1;
    end

ActiveTension(peakIdx) = Force(locs(peakIdx)) - PassiveTension(peakIdx);
end

for i = 1: (length(locs))
    contraction (i) = (Force(locs(i)) - Force(PeakMinVal(i)))/(times(locs(i)) - times(PeakMinVal(i)));
    relaxation (i) = (Force(PeakMaxVal(i)) - Force(locs(i)))/(times(PeakMaxVal(i)) - times(locs(i)));
end

R90(:,1) = times(locs)- R90(:,1);
R90(:,2) = R90(:,2) - times(locs);

outputVariable {c} = [times(locs(1:(end))), PassiveTension(1:end), ActiveTension(1:end), PeakDuration, TimeToPeak, TimeFromPeak, R90(:,1), R90(:,2), contraction, relaxation];

outputDirectory = fullfile(Directory, 'ResultsWITHFILTER');
scanName = FileName(fileIDX);
scanName = char(scanName);
scanName = scanName(1:end-5);
outputTitle = [{'Peak Location (s)'} {'Passive Tension (uN)'} {'Active Tension (uN)'} {'Peak Duration (s)'} {'Time to Peak (s)'} {'Time from Peak (s)'} {'R90 to Peak(s)'} {'R90 from Peak(s)'} {'Contraction slope (uN/s)'} {'Relaxation slope (uN/s)'}];
AvOutputTitle = [{'Variable:'} {'Average:'} {'Standard Deviation:'}];
AvOutputCat = [{'Passive Tension (uN)'} {'Active Tension (uN)'} {'Peak Duration (s)'} {'Time to Peak (s)'} {'Time from Peak (s)'} {'R90 to Peak'} {'R90 from Peak'} {'Contraction slope (uNs)'}... {'Relaxation slope (uNs)'}];

counter2 = 1;

temp2 = outputVariable{1,counter2};
SaveSheetName = [SheetName ' Contraction Parameters'];
excelOutputPath = [outputDirectory scanName ' Contraction Results2.xlsx'];
xlswrite(excelOutputPath, outputTitle, SaveSheetName, 'A1:J1');
xlswrite(excelOutputPath, temp2, SaveSheetName, ['A2:J' num2str(size(temp2,1)+1)]);
outputAverages = (mean(temp2(:, 2:10))).';
outputStDev = (std(temp2(:, 2:10))).';
xlswrite(excelOutputPath, AvOutputTitle, SaveSheetName, 'L1:N1');
xlswrite(excelOutputPath, AvOutputCat', SaveSheetName, 'L2:L10');
xlswrite(excelOutputPath, outputAverages, SaveSheetName, 'M2:M10');
xlswrite(excelOutputPath, outputStDev, SaveSheetName, 'N2:N10');
else
disp('No Beating Observed')
end
%% Save figures in folder
TotData = dataSet;
x = TotData;
TotTimes = times;
if dOnWire >= 0.8
TotForce = (0.000169*x.^3 - 0.00251*x.^2 + 0.697*x);
elseif dOnWire <= 0.8
TotForce = (0.0000621*x.^3 + 0.00295*x.^2 + 0.520*x);
elseif dOnWire <= 0.5
TotForce = (0.0000459*x.^3 + 0.000789*x.^2 + 0.668*x);
elseif dOnWire > 0.5 && dOnWire <= 0.7
F1 = (0.0000459*x.^3 + 0.000789*x.^2 + 0.668*x);
F2 = (0.0000621*x.^3 + 0.00295*x.^2 + 0.520*x);
Fdiff = F2-F1;
fracdiff = dOnWire - 0.5;
x2 = fracdiff/2;
TotForce = (x2*Fdiff)+F1;
elseif dOnWire > 0.7 && dOnWire <= 0.8
F2 = (0.000169*x.^3 - 0.00251*x.^2 + 0.697*x);
F1 = (0.0000621*x.^3 + 0.00295*x.^2 + 0.520*x);
Fdiff = F2-F1;
fracdiff = dOnWire - 0.7;
x2 = fracdiff/0.1;
TotForce = (x2*Fdiff)+F1;
end
outputDirectory = fullfile(Directory, 'ResultsWITHFILTER');
scanName = FileName(fileIDX);
scanName = char(scanName);
scanName = scanName(1:end-5);
SaveSheetName = [SheetName ' Force Trace'];
excelOutputPath = [outputDirectory scanName ' Contraction Results2.xlsx'];
outputTitle = [{'Time (s)'} {'Force (uN) above starting passive tension'}];
FinalTrace = [TotTimes, TotForce];
xlswrite(excelOutputPath, outputTitle, SaveSheetName, 'A1:B1');
xlswrite(excelOutputPath, FinalTrace, SaveSheetName, ['A2:B' num2str(size(FinalTrace,1)+1)]);

clearvars -except Directory FileName PTBase numFiles MaxMin Frequency dOnWire dInMiddle counter pixtomm pixtoum tissuearea Framerate c
counter = counter + 1;
end
Appendix C: Publications and Presentations

Publications:


Presentations:

Conant, G., Ahadian, S., Zhao, Y., Radisic, M. (2016, October) High-throughput analysis of kinase inhibitor drugs on cardiac function using engineered heart tissue constructs. Poster presented at the International Conference on Stem Cell Engineering, Toronto, ON.

Conant, G., Zhao, Y., Radisic, M. (2016, May) High throughput analysis of kinase inhibitor drugs on cardiac function using engineered heart tissue constructs. Oral presentation at the Ontario On a Chip Symposium, Toronto, ON.