

Extended summary

Bioengineering shape, structure and function in primary and stem cell derived Cardiomyocytes: *in-vitro* and *in-silico* models of Excitation Contraction Coupling

Curriculum: Ingegneria dei Materiali delle Acque e dei Terreni

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Abstract. The pharmaceutical industry is facing a critical juncture: developing new compounds has become an extremely expensive process (~500M\$/compound that makes it to clinical trials) with such a low efficiency (per single disease target, ~5/5000 compounds make it to the later stages) that will not be sustainable much longer. Return on investment in fact is threatened by the toxicity that a compound may have on on/off-targets organs or in patients with particular genetics, a fact that might be overlooked during traditional clinical trials due to limited sample size. Recently a new paradigm has been proposed named "Fail Fast, Fail Cheap" (FFFC): that is, if better predictive models can be designed for the pre-clinical trial phases a compound's toxicity can be estimated when tests are cheaper, thus increasing the probability of the final ~5 compounds to be non-toxic. In this doctorate thesis the author investigates two strategies to achieve FFFC: i) a theoretical/computational approach to describe the biophysics of the biological systems of interest (*in-silico*) and male predictions regarding toxicity and efficacy and ii) an experimental approach with the use of primary and stem-cell derived cells (*in-vitro*). The work is focused on cardiac cells (cardiomyocytes) as cardiac toxicity has been introduced by regulatory agencies as one of the most important metric for drugs targeting any organ or disease.

Keywords. Computer Simulation, Mathematical Modelling, Heart, Excitation Contraction Coupling, Patch Clamp

1 Problem statement and objectives

The pipeline for new drugs can be divided in two stages: the pre-clinical trials stage, where thousands of compounds are screened for gross toxicity and efficacy and the clinical trials stage, where only an handful of compounds are used on patients and healthy subjects permitting a fine assessment of the overall drug performances. Each phase in each stage has its own metrics and indicators and regulatory agencies demand a very detailed report of the positive and negative results: only those compounds that undoubtedly have passed all the tests can be marketed. Owing to the sample size of clinical trials (a few thousands people at best) that cannot capture the genetic variability of the entire population, it is not uncommon for drugs to show side effects that were not observed in earlier stages. Given that the cost per stage tend to increase non-linearly as a compound is promoted to a later stage while withdrawing a drug from the market will almost certainly lead a company to bankruptcy - a new paradigm has been recently proposed, called "fail fast, fail cheap" [1, 2]. Within this paradigm, tools need to be developed that offer better predictive capabilities so that if a compound is doomed to fail - for example due to toxicity on cardiac cells, which are the focus of the doctorate thesis - the probability of observing this phenomenon would be higher in an earlier and cheaper stage. The patho-physiology of cardiac cells (or cardiomyocytes) is dominated by the process known as Excitation Contraction Coupling (ECC see fig 1); that is, cells receive electro-mechanical stimuli (Excitation) that are converted into mechanical actuation (Contraction) through fluxes of calcium and other ions (Coupling - see fig 2) from the extracellular space and intracellular organelles such as the Sarcoplasmic Reticulum (SR) [3]. Drugs specific for heart diseases tend to affect the excitation stage [4], while off-targets effects can be observed in all three stages [5].



Figure 2 - schematics of cardiac cell excitation contraction coupling (ECC) showing calcium ions, main ionic currents and the intracellular pathways responsible for calcium handling



Figure 1 – time courses of the membrane potential (A), calcium concentration in different compartments (B,C,D) and transmembrane currents of sodium (E), calcium (F), potassium (G, H,I, J) ions as well as of ions exchangers (K,L).



The doctorate thesis describes several strategies that can be employed to realize the "fail fast, fail cheap" paradigm and discusses them in relationship with methods commonly used in cardiac electrophysiology. In particular, the thesis tries to answer the following questions:

• Can mathematical modelling and computer simulation be reliably used to predict the effect of compounds and treatments on ECC [6,7]?

• Can this computational approach be converted into a Computer Aided Design tool for bioengineering? Can we use this tool to investigate the effect of different micro-environmental conditions on the cell shape, and its structure-function relationship [8]?

• Are there new technologies that can be reliably used to experimentally measure cell electrophysiology in a more automated way? Can these techniques be used on cardiac cells from animals and/or derived from stem cells [9].

Notably, the use of stem cell derived cardiomyocytes holds important promises for this paradigm shift. In fact, technologies are available to produce induced Pluripotent Stem Cell (iPSCs) a type of cell that can be derived directly from adult patients [10], offering the possibility to inexpensively test safety and toxicity of compounds on cells that show a vast range of genetic makeups.

2 Research planning and activities

The methods used can be divided in two categories: on one hand theoretical/computational tools and on the other hand experimental tools; the formers, comprising Ordinary Differential Equations (ODEs), Hidden Markov Models (HMM) and numerical integration are known as *in-silico* models while the latters, namely micro-contact printing and planar patch clamp, as *in-vitro* tools.

The author started from what is probably the cheapest way to determine whether or not a certain treatment has a probability of being toxic, namely to predict it on the base of what is know about the biological system and the compound. Owing to the complex and nonlinear nature of ECC though, this assessment can be done only computationally: first, biophysically sounding models need to be constructed and validated against the existing literature; secondly, the desired effect of the compound on its specific target is simulated in the model and predictions about on-/off-target effects can be obtained. Biophysically sound models are constructed starting from first principles such as conservation of mass and charge (see equations 1-5). As an ionic current flows from one side of the cell membrane to the other, it alters the membrane potential and produces a certain variation in the concentration of that ion on both sides: assuming all the compartments can be considered wellstirred these simple transport phenomenon can be described by means of source-sink ordinary differential equations (ODEs). These equations are non linear for a number of reasons: first, the driving force for a certain ionic specie is related to its electrochemical (Nernst) potential, which is a function of the concentration that is in turn affected by the flux itself. Secondly, in a liquid solution the semi-permeable cell membrane acts as a capacitance for the charged ions on both sides that cannot cross it unless specific transporters are available. Those transporters are known as ionic channels and their open probability (or equivalently the number of channels open at any given time) is a non-linear function of



other state variables, such as the membrane potential or the concentration of specific molecules. In particular, to mathematically characterize the ionic channels open probability Hidden Markov Models (see fig. 5C) are employed [11]. That is, the protein is assumed to exist in a finite number of distinct states, only a subset of which – the ones that conduct a measurable current - can be observed. If the structure of the protein is known the transition rates can be estimated of statistical mechanics ground [12], otherwise non-linear fitting and global optimization procedures can be employed to estimate them from data.

$$\frac{dV_m}{dt} = -\frac{1}{C_m} \sum_{l=j_k} I_l \tag{1}$$

$$\frac{d[j]_i}{dt} = \alpha \left(\sum_{k \text{ in } i} I_{j_k} - \sum_{k \text{ out } i} I_{j_k} \right)$$
(2)

$$I_{j_k} = G_{j_k} O_{j_k} \left(V_m - E_j \right)$$
 (3)

$$\mathbf{X} = \begin{bmatrix} X^1, X^2, \dots, O \end{bmatrix}$$
(4)

$$\frac{d\mathbf{X}}{dt} = \mathbf{R}(V_m, ...)\mathbf{X}$$
(5)

Model Equations - given a set of compartments (index i), of ionic species (index j) and of transporters (index k), conservation of charge (eq 1) and mass (eq. 2) describe the physics of the problem, with C_m being the membrane capacitance and α a factor converting current [A] in fluxes [M/sec] and the notation "k in/out i" means those transporters k that flow ions respectively in and out the comparment i. The currents involved in eq 1 and 2 can be expressed as the product of three terms: the maximum conductance (G), the difference between the actual membrane potential and the ion's Nernst potential (E) and the channel's open probability (O) that can be calculated using Hidden Markov Models (eq 4 and 5), where X is a vector containing the probability of the channel of being in state 1, 2, ...,N where N is the last state, by convention assumed to correspond to the open state and R is the connectivity matrix containing the non-linear transition rates, scuh that rmn is the rate of the

Moreover, more traditional *in-vitro* assays can be relied upon in developing "fail fast fail cheap" strategies: in the thesis two such methods are described namely micro-contact printing and planar patch clamp (PPC). Micro-contact printing [13] is a technique mutuated from materials science where soft-lithography is used to construct polydimethylsiloxane stamps that can be inked with an extracellular matrix protein and then use to control the cell shape and structure (see fig 3A). In this way the microenvironment of cells pertaining to different organs [14, 15] and/or pathophysiological conditions [16] can be reproduced on a laboratory culture dish offering a more realistic model that will potentially offer better predictive capabilities when used to screen compounds for safety and efficacy.

Additionally, as most of the drugs for heart diseases affect the cells electrical activity, advancements in patch clamp technologies (the experimental tool for electrophysiology) are pivotal in this area of research. Planar patch clamp (fig 3B) [**17,18**] has been recently introduced as a novel technique to obtain electrophysiology data, and in the thesis a commercially available system has been tested for the first time with primary and stem cell derived cardiomyocytes.



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Figure 4 – schematics of planar patch clamp arrangement, showing fluid lines for intra- and extracellular solutions, as well as the glass chip featuring the small aperture responsible for cell

Figure 3 - mouse Embrionic Stem Cell derived cardiomyocytes seeded on fibronecting islands shapes with different geometries (scalebar 10 µm).

3 Analysis and discussion of main results

Three sets of results are discussed in the doctorate thesis. First, traditional modelling frameworks have been applied to study *in-silico* ECC in human cardiomyocytes with particular reference to conditions, compounds and treatments whose effects can be predicted using mathematical modelling.

A detailed mathematical model for Ca handling and ionic currents in the human ventricular myocyte is proposed where ion channels and transporters have been modeled on the basis of the most recent experimental data from human ventricular myocytes. The model has then been validated against a wide set of experimental data including action potential duration (APD) adaptation and restitution, frequency-dependent increase in Ca transient peak and [Na]i. Among other things, the model has been used to investigate the effects of blocking K currents on APD and repolarization reserve: IKs block does not affect the former and slightly reduces the latter; IK1 blockade modestly increases APD and more strongly reduces repolarization reserve; IKr blockers significantly prolong APD, an effect exacerbated as pacing frequency is decreased, in good agreement with experimental results in human myocytes. We conclude that this model provides a useful framework to explore excitation–contraction coupling mechanisms and repolarization abnormalities as oserved in disease states treated with K-channel blockers available on the market such as HMR-1556 and dofetilide (see fig. 4).





Figure 4 - comparison between experimental (A) and simulation (B) results with known rapid (IKr) and slow (IKs) potassium channel blockers. The model reproduces the lengthening of the Action Potential Duration at 90% repolarization (APD90).

Second, a novel framework has been developed for ECC modelling that can automatically assembly the set of ODEs needed to describe the effects and interactions of ionic channels, in particular membrane-bound L-type calcium channels (LCC) and SR-bound Ryanodine Receptor (RyR) channels. This framework was used to speculate on the role of cell ultrastructure in affecting the cell structure-function relationship in neonatal rat ventricular myocyte. This new framework builds upon the local control theory of ECC. In the local control theory the open probability of LCCs is assumed to be modulated by the calcium concentration in the surrounding of the channels, due to the cell ultrastucture. In fact LCCs and RyRs face each other so closely in the so-called dyadic space that they behave as a Calcium Release Unit (CaRU). This being the case, we speculated that by uCPing cells on substrates we could affect this structural organization with effects on functional readouts that can explain the experimental results. In particular we uCPed neonatal rat ventricular myocytes (fig 5A) and observed the calcium transient (fig 5B) by virtue of fluorescent dyes and confocal microscopy. We then tested in-silico many different models for many different values of relevant parameters and show that, by letting the dynamics of LCC and RyR gating more strongly influence each-other, for example allowing synchronous transitions to happen (see red arrow in fig 5C), traces similar to those observed in our experiments can be obtained (fig 5 B,D)

The thesis describes also a new method to automatically assembly the set of ODEs that describes the dynamics of the CaRUs starting from the description of LCCs and RyRs. The algorithm is based on certain tensor operators (see equations in fig 5C) used in graph theory that can be applied to a symbolic representation in the computer of the HMM description of the ionic channels. Using symbolic computer algebra the tensor operators knowns as knonecker sum and porduct can be applied to the symbolic descriptions of single channels yielding a symbolic description of the CaRU. This description can then be converted into a numerical representation usable in the numerical integration routine to simulate the whole cell response (AP, CaT, ...). This framework is novel in ECC modelling and promising as it allows that kind of automation needed in Computer Aided Design tools.



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Figure 5 - modeling of ECC in mCP neonatal rat ventricular myocytes. mCP cells (A) show calcium transients (B) that considerably lengthen with increasing aspect ratio, exhibiting a characteristic bell shape. Using Hidden Markov Models (C) and introducing synchronous transitions (red arrow) similar time courses for the cytoplasmic calcium concentration can be obtained (D).

Finally, given the importance that the study of ionic channels has in characterizing the physiology of cardiomyocytes, planar patch clamp is discussed as a valid, versatile alternative to traditional patch clamp, when high throughput is required.

Planar patch clamp substitutes traditional cell impalement through a sharp glass pipette with a microfluidic design that flows cell through a small aperture (see fig. 3B). In this way the manual critical steps required to bring the pipette in close contact with the cell without killing it can be avoided, speeding up the execution and removing one of this technique's technical challenges. Planar patch clamp is a relatively new technique that have been mostly used with cell-lines, that is immortalized cells that can be kept in the laboratory for a long time and that are easy to genetically manipulate to over-express a single certain channel, thus making the collection of data easier and more robust. On the other hand, this has reduced the predictive power of this tool, by removing all those effects associated with the interaction of currents. In the thesis a single protocol was only slightly tweaked to accommodate experiments with neonatal rat and mouse ventricular myocytes (NRVMs and NMVMs) as well as cardiomyocytes derived from mouse embryonic stem cells (mESCs) and induced pluripotent stem cell (miPSCs). All the major electrophysiology readouts could be obtained, suggesting this technique can be used to screen multiple compounds against multiple biological targets. Additionally, the stem cell populations perform relatively well with respect to these tests, showing the whole-cell electrophysiological response, the action potential (fig. 6 A,B) as well as a variety of ionic currents (fig 6 C,D). Moreover, pharmacological studies with the sodium channel blocker TTX could be performed with automatic (fig 7 A,B) and manual (fig 7 C,D) cell perfusion.





Figure 6 – Electrophysiology characterization of Neonatal Rat Ventricular Myocytes (NRVM) with planar patch clamp. Action Potential train at 1 Hz (A) and single AP shape (B) can be recorded, as well as calcium and sodium currents



Figure 7 – Pharmacological (TTX) characterization of mouse induced Pluripotent Stem Cell with planar patch clamp. Sodim current elicited at 0 V is reduced by increasing concentration of TTX (A, B) when the solutions are exchanged with an automated perfusion systems. Similarly, manual wash in wash out experiments can be performed and an almost complete current recover can

4 Conclusions

Two of the results presented in this work deserved further comments for their potential enabling capacity. From the theoretical point of view, the algorithm that automatically couples the sets of ODEs describing different ionic channels is a first important step towards the development of those CAD tools that have made possible to obtain incredible successes in other field of engineering. Additionally, by automating the process of building computational models, these will become accessible to more researchers in different fields, possibly reaching that momentum that seem to be necessary for a given tool to be accepted in for example, the pharmaceutical industry. From the experimental perspective, having demonstrated the versatility of a commercially available planar patch clamp system is important, as it will allow for more data to be collected with implication for both computational model development and compound screening.



Finally the use of stem cells holds incredible promises for the future of health care. In the doctorate only stem cell from non-human sources were used and compared against their murine counterpart but the possibility in a future that might not be far away, to extract skin cells from a patient and "transform" them into, for example, cardiomyocyte will certainly be key not only for drug development but also for directly treating diseases.

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