Measuring the physiological response of single cardiomyocytes
An alternative career in science

Why I find it difficult to leave science:
Seeing what nobody has seen before
In this talk:

- Basic principles of calcium – contractility measurements

- Mechanical experiments on (intact myocytes) – mimicking the whole heart by measuring work-loops

- Case study: the RBM20 mutation and the importance of pre-activation
What is a cardiomyocyte

From Guyton & Hall, textbook of medical physiology

(pict. Camelliti et al. AJP 2010)
Why do we use cardiomyocytes?

- Ease of use, millions of cells
- Reductionist approach
- Can be cultured while retaining functionality
- Can easily be visualized
  - Allows mechanical manipulation
    - Electrophysiology (patch clamping)
    - Studying cell mechanics
  - Fluorescence
  - Motion detection
- Compound testing

Image courtesy of Ben Prosser
The intact myocyte is the smallest functional unit of the heart that can be used to study excitation–contraction (EC) coupling.

- Action potentials
- Calcium transients
- Force development (or cell shortening)

From D. Bers, 2002)
Using fluorescence to quantify intracellular ion concentrations

Principles of fluorescence: Stokes shift

\[ \lambda_{\text{ex}} < \lambda_{\text{em}} \]

- UV $\rightarrow$ Blue, green
- Blue $\rightarrow$ Green
- Blue $\rightarrow$ Yellow
- Etc.

\[ E = h\nu = \frac{hc}{\lambda} \]

Energy of photon = planck constant x frequency
(or planck x speed of light / wavelength)

(from Molecular Probes handbook)
Fluo-3 is an example of a single excitation-single emission dye

Excitation/emission spectra for Fluo-3
(graphs from Molecular Probes handbook)

Equilibrium reaction
How to load a cell with a salt?
AM-ester loading

Chemically link the dye to an ester group
Am-esters are non polar -> membrane permeable

Enzymes remove the estergroup, the salt remains -> dye accumulation

Sounds simple, but no cell is the same

Figure from molecular probes handbook
Epifluorescence measurements

- Di-chroic mirror (long pass)
- Cell, loaded with fluorescent dye
- Broad spectrum (white) excitation light (Xenon or mercury-arc lamp) or LED light
- Bandpass filter for excitation light
- Emission filter
- Fluorescence emission light
- Light detector (camera or photomultiplier tube)

Image from Chroma website
System Overview
Downside of single excitation-single emission dyes

-Dye bleaching or leakage changes the signal
-Cannot easily correct for loading or thickness of preparation
-Absolut concentration cannot be established (eg. a change in diastolic calcium)
The solution: Ratiometric dyes
Making use of a spectral shift upon Calcium binding

Excitation spectrum of Fura-2

(figure adapted from Molecular Probes Handbook)
Fura-2 ratiometric measurements, what does it look like

Ratiometric dyes exist for pH, Na⁺, Zn²⁺, Mg²⁺
The ratiometric principle is also used with FRET and Chameleons
Using Fura-2 in intact isolated cardiac myocytes
Standard assays using Ca++/contractility

pro-arrhythmic effects

The inotropic effects of compounds

Part Two: Force measurements on intact cells

Skinned vs. intact
The first intact myocyte force measurements

- Le Guennec et al, ‘90
- Force measurements on isolated intact myocytes
- Carbon rods, really low force levels
Introduction of MyoTak generated renewed interest in intact myocyte experiments

(images courtesy of B. Prosser)
It allows to study the effects of stretch on the cell.

Rat myocytes show very strong length dependent activation.
Can we do work-loops on single cells? And why?

- At Ionoptix we had just developed a new generation of force transducers; could we now do force control?
- measure mechanical work
- a great tool for diastolic dysfunction

Refresher: what is a PV/workloop
(LVP is ‘left ventricular pressure’, LVV is ‘left ventricular volume’)

(animations courtesy of G. Iribe)
LVP (or force)

LVV (or cell length)
100 ~ 10

End-systole

LVP (or force)

LVV (or cell length)
Pressure-Volume (PV) loop

Approximately 10

LVV (or cell length)

LVP (or force)
How to measure work loops in single cells

A major advantage of single cells over the working heart: it is really easy to change the pre-load
Recording @ 2 Hz
Recording @, 4 Hz
Recording @ 8 Hz
We can now control the force development during a contraction.

Varying pre- and afterload

End Diastolic and End Systolic force length relation

Force

Length

SL = 1.98 \mu m

2.02 \mu m

2.03 \mu m

Phase I  Phase II  Phase III  Phase IV

0.5 \mu N

50 ms

\mu m

50 ms
Case study

We had developed the method, we needed an experiment

- RBM20 mutation; causes DCM in humans
- Spontaneous mutation in an inbred rat strain (M. Greaser)
- RBM20 is involved in titin splicing
- The RBM20 mutation leads to an extremely elongated isoform of titin
- This is expected to lead to a strong reduction in passive stiffness
- Passive stiffness is thought to underly length dependent activation
Functional measurements in RBM20-mice

Passive stiffness is annihilated

Length dependent activation is severely affected

But the while the -/- were very sick, the +/- (HET) had normal hearts...
The HET even had increased running speed

(figures from Methawasin et al. Circulation 2014)
First, due diligence: ca++ contractility study

- Resting SarcLen
- % shortening
- Time to peak shortening
- Return to baseline 90%

- Diast Ca++
- Ca++ ampl
- Time to peak Ca++
- Ca++ re-uptake (tau)
Finding the after-load that delivers maximum external work…

\[ W = \Delta F \cdot \Delta l \]
Work generating capacity is impaired in HET
At high pacing frequencies not difference between WT and HET

- The difference in work-generating capacity disappears at high frequencies
- The WT have high diastolic stiffness compared to Het
Hypothesis 1: the difference at low frequencies is caused by a depressed rate of force development in HET compared to WT. This is due to a reduced LDA (null-hypothesis: there is no difference in the rate of force development between WT and Het)

Hypothesis 2: increased diastolic calcium levels at high pacing frequencies can partially compensate for a lack of LDA (but why don’t the WT benefit from increased diastolic calcium levels)
Force development in cardiac myocytes is time limited

**Het:** low length dependent activation compared to WT

But how does it catch up? Diastolic Ca^{++} based pre-activation
Method

Calcium Relax

Cantilever force probe

To Piezo

9,0 (Relax)

Pca 5,7

Pca 7,1

Pca 5,7

Pca 6,8

Pca 5,7

9,0 7,1 6,8 6,6 6,4

5,7 5,7 5,7 5,7 5,7

Calcium Relax
Dynamic contractions in skinned myocytes using rapid perfusion switching
Effect of diastolic calcium on rate of force development
In permeabilized myocytes, pre-activation does enhance force development

* $p_{\text{ca}} < 0.05$
The downside of pre-activation
Increased diastolic calcium impairs relaxation in permeabilized myocytes
So what is likely happening in the intact myocytes?

At high pacing frequencies, impaired relaxation is problematic

(intact, workloops)
Switching from 8Hz to 1 Hz
Conclusion:

- Diastolic calcium and length are to some extent interchangable
- Relaxation is as important as force development
- Work-loops can be a very sensitive tool to study diastolic (dys)function
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Current project:

Improving the throughput of Ca\(^{++}\)/Contractility experiments