Higher Throughput Calcium Transient Recording from hiPSC-derived Cor.4U Cardiomyocytes: Ready for CiPA Phase II Validation Study

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08.06.2016 Hamamatsu Application Workshop
Content

• The CiPA Initiative - Short Introduction

• Factors Influencing the Calcium Transient Assay

• Calcium Dyes Tested
  • FLIPR Calcium 5 Assay Kit (Molecular Devices)
  • ACTOne (Codex Biosolutions Inc.)
  • Cal-520 (AAT Bioquest)

• Conclusion
Comprehensive in vitro Proarrhythmia Assay
CiPA - Initiative
2014-2015 MEMBERS

- Government: 17
- Academia: 20
- Industry: 45

84 Total Organizations
>250 Individual Participants

Presented for: CiPA Members
CiPA - Overview of Working Groups

**COMMITTEE WORKING GROUPS OVERVIEW**

- **Proarrhythmia Working Group**
  - Main objective: Assess proarrhythmic risk

- **Cardiac Biomarkers Working Group**
  - Main objective: Development and application of biomarkers of CV toxicity

- **Cardiac Stem Cell Working Group**
  - Main objective: Understanding & characterizing stem cell-derived cardiomyocytes for use in CV safety assessments

- **Integrative Strategies Working Group**
  - Main objective: Assess predictability of preclinical CV models to human
CiPA Phase I - Pilot Study

- 3 Providers of pluripotent stem cell-derived cardiomyocytes
- 16 Volunteer sites
  - 12 sites; 3 microelectrode array platforms
  - 4 sites; 4 Voltage-sensing-optical (VSO) platforms
  - 8 blinded test compounds; 4 concentrations, 3 triplicates

- Study was accomplished End 2014
- Manuscript for publication is under discussion
CiPA Phase II - Validation Study

- 2 Providers of pluripotent stem cell-derived cardiomyocytes
- 5 core sites (funded by FDA grant)
  - 2 sites; 4 microelectrode array platforms
  - 3 sites; 3 Voltage-sensing-optical (VSO) platforms
  - Calcium Transient Assay (potential backup assay)
    - 3 sites: Janssen, Axiogenesis, Merck (USA)
- Compounds:
  - 28 blinded test compounds; 4 concentrations, 6 replicates
  - 4 calibration compounds
- Volunteer non-core test sites:
  - 12 blinded test compounds + 4 calibration compounds
CiPA Phase II - Validation Study

Next Steps

- Myocyte Phase 2 Study Initiated
- Educational Webinars
- Myocyte Phase 2 Study Data Analysis
- Educational Webinars
- Myocyte Phase 2 manuscript submission (close BAA)
- New project scoping

- Myocyte Phase 2 Study Protocol Development
- Educational Webinars kick-off
- Myocyte Phase 2 Study Data Collection
- Educational Webinars
- Myocyte Phase 2 manuscript drafting
- Educational Webinars
Excitation-Contraction Coupling

A) Action Potential

B) Calcium Transient

![Diagram showing currents and action potential over time](image)

- Na$^+$
- Ca$^{2+}$ L-type
- Ca$^{2+}$ T-type
- Na$^+$/Ca$^{2+}$-exchanger
- K$^+$ $I_{to}$
- K$^+$ $I_{Ks}$
- K$^+$ $I_{Kr}$
- K$^+$ $I_{Kr}$
High Throughput Kinetic Plate Reader Assays
Plate Reader System - Hamamatsu

Setup A
- Pipettor Head
  - Dispenser head
  - Disposable tips
  - Assay plate
  - Excitation light source
  - Emission filter
  - Camera lens
  - Camera (sensor)

Setup B
- EFS Head
  - 96 well EFS head
  - Excitation light source
  - Emission filter
  - Camera lens
  - Camera (sensor)

Hamamatsu FDSS µCell

Hamamatsu FDSS 7000EX

Both systems can be equipped with a temperature control.

Data generated in collaboration with Hamamatsu.
Important Factors Influencing the Calcium Transient Assay with hiPSC-derived Cardiomyocytes
Calcium Transient Assay - Important factors

- The calcium dyes
- Dye loading time
- Assay stability over time (assay window)
- Wash vs. non-wash
- Signal to noise ratio
  - Medium / buffer
  - Quencher
- Addition of organic anion transporter (e.g. probenecid)
ACTOne™ Non-Wash Calcium Dye Kit
Codex
### Experimental Layout

#### Dye Dilution

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- **BMCC**
- **HBSS + HEPES**
- **Signal Enhance (Quencher)**

**Codex Dye**: 0.1x, 0.25x, 0.3x, 0.5x, 0.75x, 1x
Results

20 min

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Well C8
0.3x in BMCC + Signal Enhancer

Well C9
0.25x in BMCC + Signal Enhancer

- Irregular beating of fresh Cor.4U Cardiomyocytes in HBSS Puffer

3400 AFU
3000 AFU
Results

30 min

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Well C8

0.3x in BMCC + Signal Enhancer

4200 AFU

Well C9

0.25x in BMCC + Signal Enhancer

3300 AFU

Increase in background without Signal Enhancer.
Results

60 min

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Well C8

0.3x in BMCC + Signal Enhancer

Well C9

0.25x in BMCC + Signal Enhancer

- Irregular Beating in HBSS buffer
- Further increase background

5000 AFU

4000 AFU
Results

90 min

- Amplitude at 0.3x and 0.25x is already decreasing in BMCC + SE
- Arrest with the highest dye concentration in BMCC + SE
- Complete arrest in HBSS buffer
Results

120 min

Well C8

0.3x in BMCC + Signal Enhancer

- 2 hours after start of dye loading the amplitude has decreased more than 50% compared to max
- Arrest occurs at higher dye concentration in BMCC

Well C9

0.25x in BMCC + Signal Enhancer

2100 AFU

1900 AFU
Results

240 min

Well C8

0.3x in BMCC + Signal Enhancer

ca. 1500 AFU

Well C9

0.25x in BMCC + Signal Enhancer

c. 500 AFU

- Hugh increase in background without Signal Enhancer.
- Remaining Amplitude was only 30% or 12.5% for the 0.3x or 0.25x diluted dye, respectively, compared to max amplitude after 60 min.
Cal-520™, AM
AAT Bioquest
Protocol

- Fresh Cor.4U Cardiomyocytes were seeded in Cor.4U Culture medium with 20k cells/well into a 96 well µClear plate from Greiner Bio One and cultured for 3 days.

- On the day of experiment, medium was exchanged for phenol red-free BMCC Medium at least 2 hours before the start of the experiment.

- The lyophilized dye was reconstituted in water-free DMSO as 5 mM stock concentrations and was cryopreserved in aliquots.

- The dye stock solution was dilute 1:500 to obtain the 1x working concentration of 10 µM in BMCC Medium (without quencher or probenecide). The provider suggests to us concentrations between 10 µM and 20 µM.

- The following concentrations were tested with the Cor.4U Cardiomyocytes:
  
  10 µM, 5 µM, 2.5 µM, 1.0 µM, 0.5 µM

- Recording in the FDSS7000EX were done from 30 min and up to 4 hours after loading with the calcium dye.

- As a reference FLIPR Calcium 5 Assay Kit dye (1:6 diluted of the recommended 1x solution) and the Codex ACTOne Non-Wash Calcium dye (at 0.3x concentration of the recommended 1x solution) were tested on the same plate.
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**FLIPR Calcium 5 Assay Kit 1:6**

- Codex ACTOne Non-Wash Calcium Dye 0.3x
  - 0.5 µM Cal-520
  - 1.0 µM Cal-520
  - 2.5 µM Cal-520
  - 5.0 µM Cal-520
  - 10 µM Cal-520
Results - Cal-520

XXX
Results - Cal-520

XXX

45 min

26

Presented for:

Characterization

Product/Format

Service

Company

2.5 µM

1.0 µM

0.5 µM

FLIPR Ca.5

ACT One

10 µM

5 µM

1.0 µM

0.5 µM
Results - Cal-520

<table>
<thead>
<tr>
<th></th>
<th>10 µM</th>
<th>5 µM</th>
<th>2.5 µM</th>
<th>1.0 µM</th>
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<th>FLIPR Ca.5</th>
<th>ACT One</th>
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XXX
Results - Cal-520

First arrest of the spontaneous calcium transients with the FLIPR Calcium 5 Assay Kit.
Results - Cal-520

10 µM  5 µM  2.5 µM  1.0 µM  0.5 µM  FLIPR  ACT  One

120 min
Results - Cal-520

<table>
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<th>Time</th>
<th>Concentration</th>
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<td>180 min</td>
<td>10 µM</td>
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- FLIPR
- ACT

XXX

Presented for:

Company

Characterization

Product/Format

Service
## Results - Cal-520

First arrest at 10 µM and 5 µM Cal-520.

Decrease in fluorescent calcium transient amplitude with ACTOne.

Almost complete arrest with the FLIPRR Calcium 5 Assay Dye.

<table>
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<tr>
<th>FLIPR Ca 5</th>
<th>10 µM</th>
<th>5 µM</th>
<th>2.5 µM</th>
<th>1.0 µM</th>
<th>0.5 µM</th>
<th>ACT One</th>
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240 min
Morphological Differences and Changes Over Time of the Cal-520 Calcium Transients
Results - Cal-520

- FLIPR Calcium 5 and Codex ACTOne reveal a slowed rise of the calcium transients from 80% to 100%.
- There is obviously a change in calcium transients which potentially indicates the start of toxic events at an early time point.
- Calcium transient durations are increased with the FLIPR Calcium 5 dye and the ACTOne dye at concentrations tested compared to Cal-520 dye (see also quantitative analysis).
At higher concentrations of Cal-520 the slope at 80% to 100% starts to slow as well.
At 10 µM Cal-520 the calcium transient peak with duration starts to prolong.
The amplitude of the ACTOne calcium dye decrease overtime if no probenecid is applied.

- Calcium transient duration with ActOne dye decreases with decreased amplitude

- Low concentration of Cal520 dye conserves physiological phenotype.
Quantitative Analysis of Non-Wash Cal-520 Calcium Transients Recorded from Cor.4U Cardiomyocytes
# Overview - Arrest of Spontaneous Calcium Transient in Cor.4U Cardiomyocytes

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<thead>
<tr>
<th>Time [min]</th>
<th>10µM</th>
<th>5 µM</th>
<th>2.5 µM</th>
<th>1.0 µM</th>
<th>0.5 µM</th>
<th>1:6 FLIPR Calcium 5</th>
<th>0.3x Codex ACTOne</th>
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- Beat rate is higher in Cal-520 Assay compared to the both other dyes, especially at the lowest dye concentration.

- Beat rate decreases with increasing dye concentrations.
- Cal-520 calcium transient PW30% and 80% increase over time in the highest concentrations (toxic effect?).
- FLIPR Calcium 5 and ACTOne dye PW80% values are almost twice as high compared to the lowest Cal-520 concentration (=> toxic or unphysiological?).
Results - Cal-520

- Calcium Transient amplitudes from Cal-520 increase over time (max after 3 hours) although no probenecid was added.
- FLIPR Calcium 5 and ACTOne dye amplitudes reach a maximum after 60 min.
Wash Assay Using

Cal-520™, AM (AAT Bioquest)

and

ACTOne (Codex)
Results

Beat Rate

- Beat rate at 1 hour is reduced due to wash step => requires longer incubation after wash.
- Beat rate with Cal-520 is 10 BPM higher compared to compared 0.5x ACTOne.
Results

Calcium Transient Amplitude

- Amplitude of Cal-520 calcium transients is absolutely stable during after 4 hours.
- ACTOne amplitudes are decreased after 3 hours.
Results

Peak Width (PW) 80%

- 0.5x ACTOne peak width at 80% are doubled compared to 2.5 µM Cal-520 (and also 0.25x ACTOne) and almost 3x the values of 0.5 µM Cal-520.
Dye Effect on GPCR Agonist Pharmacology
with Cor.4U Cardiomyocytes
Results

Calcium 5 Assay Kit Dye

- Right shift of isoproterenol increased beat rate with the Calcium 5 Assay Kit dye
- Cal520: More physiological isoproterenol effect
Conclusion

- Choice of the right calcium dye is important
- Cal520 at low concentrations revealed to be the most physiologic dye
  - Long-term stability (assay window)
  - Calcium transient and beating parameters
- No quencher is required for Cal520 when the right assay medium/buffer is chosen
- Washout is required for Cal520