



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

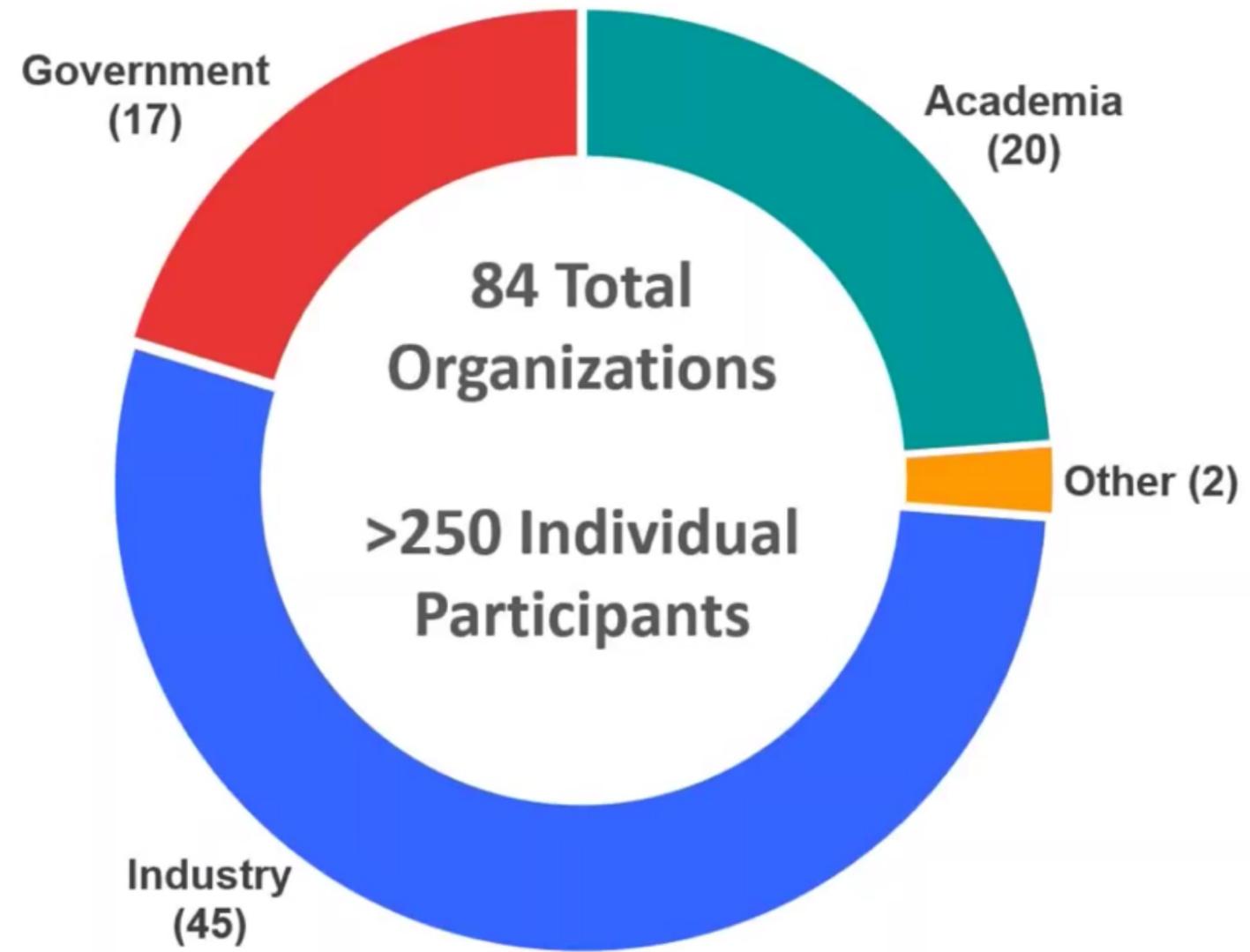
Content

- **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



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



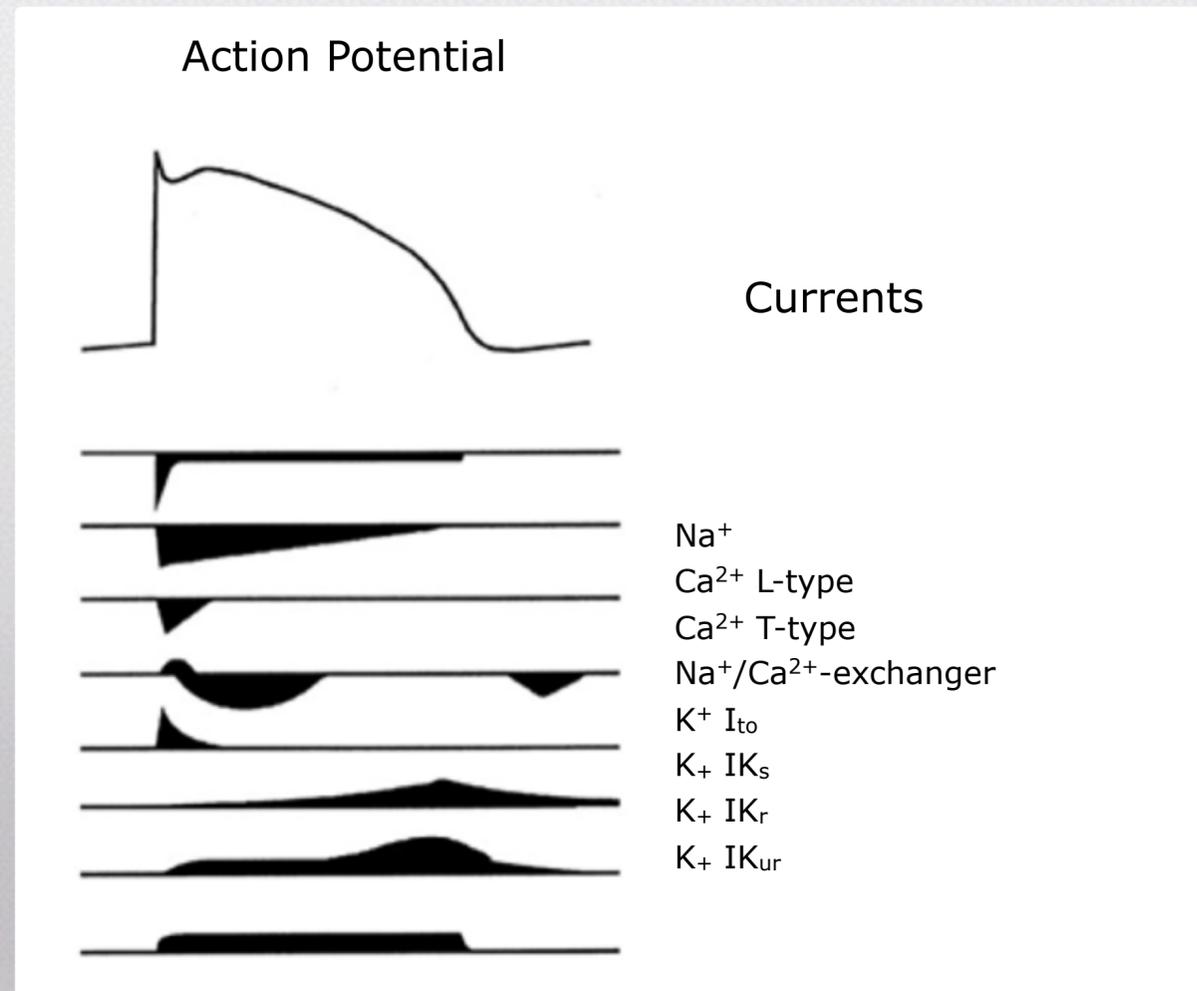
- ◆ Myocyte Phase 2 Study Protocol Development
- ◆ Educational Webinars kick-off

- ◆ Myocyte Phase 2 Study Data Collection
- ◆ Educational Webinars

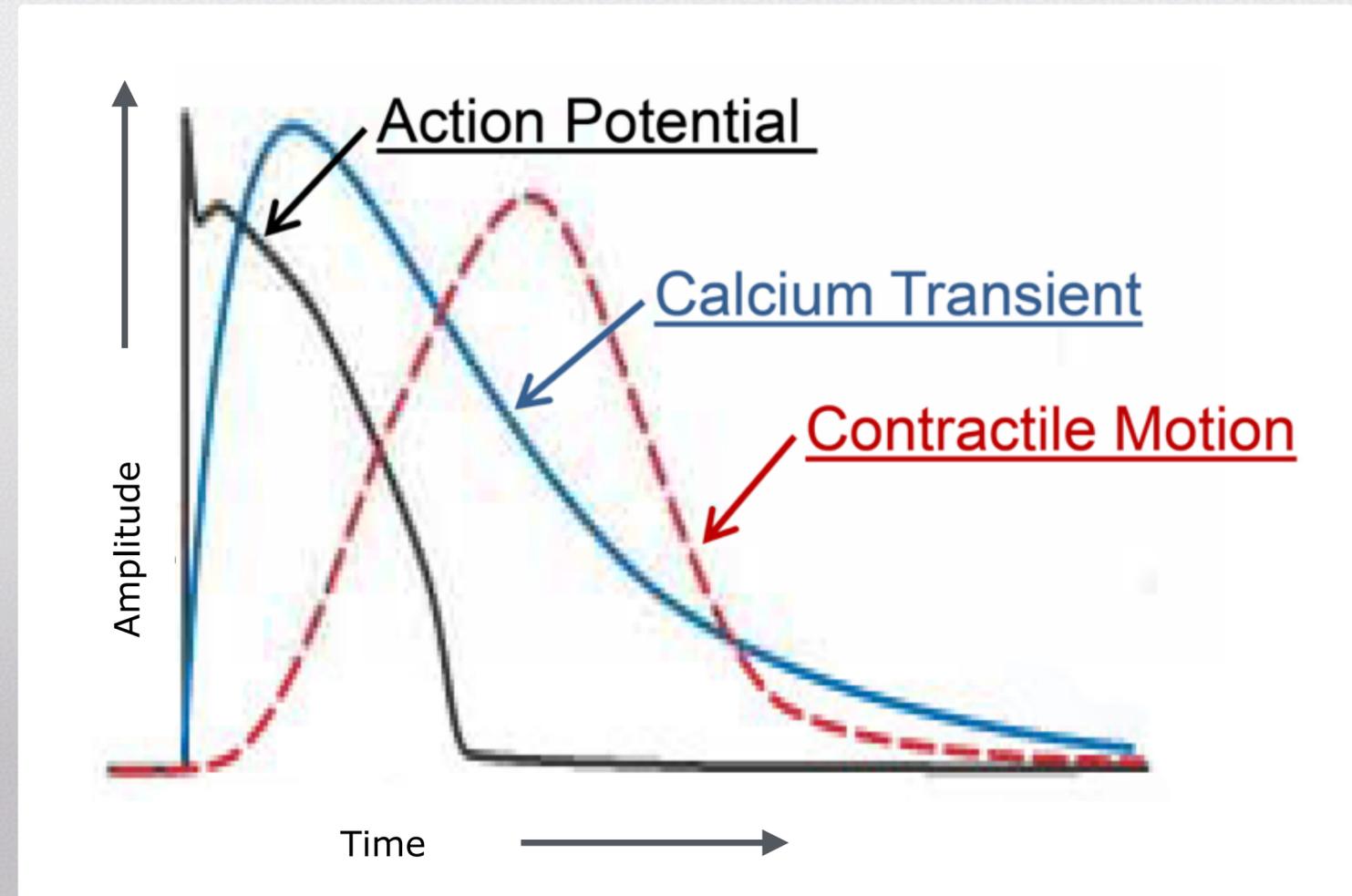
- ◆ Myocyte Phase 2 manuscript drafting
- ◆ Educational Webinars

Excitation-Contraction Coupling

A)



B)





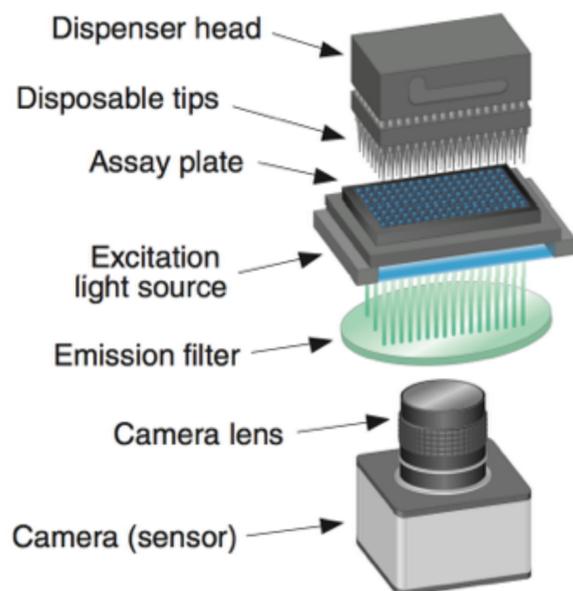
High Throughput Kinetic Plate Reader Assays

Plate Reader System - Hamamatsu

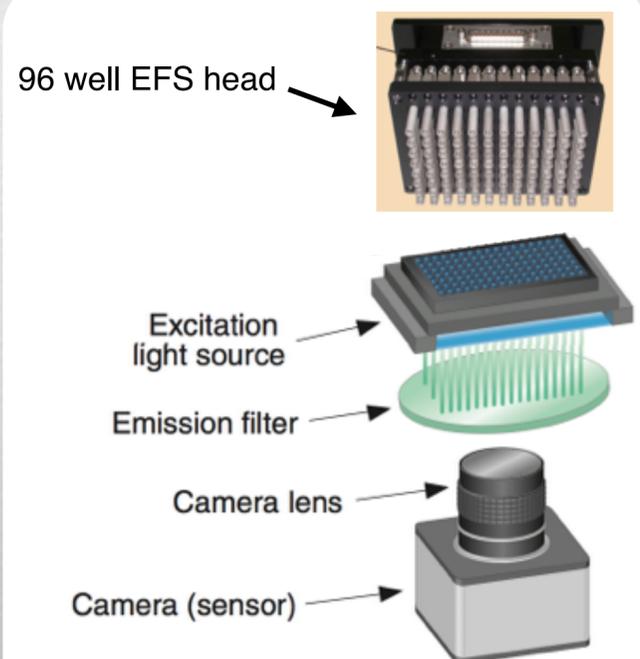
Hamamatsu FDSS μ Cell

Hamamatsu FDSS 7000EX

Setup A Pipettor Head



Setup B EFS Head

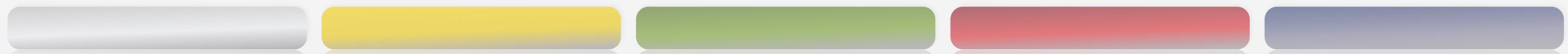


Both systems can be equipped with a temperature control

HAMAMATSU

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)

Company

Characterization

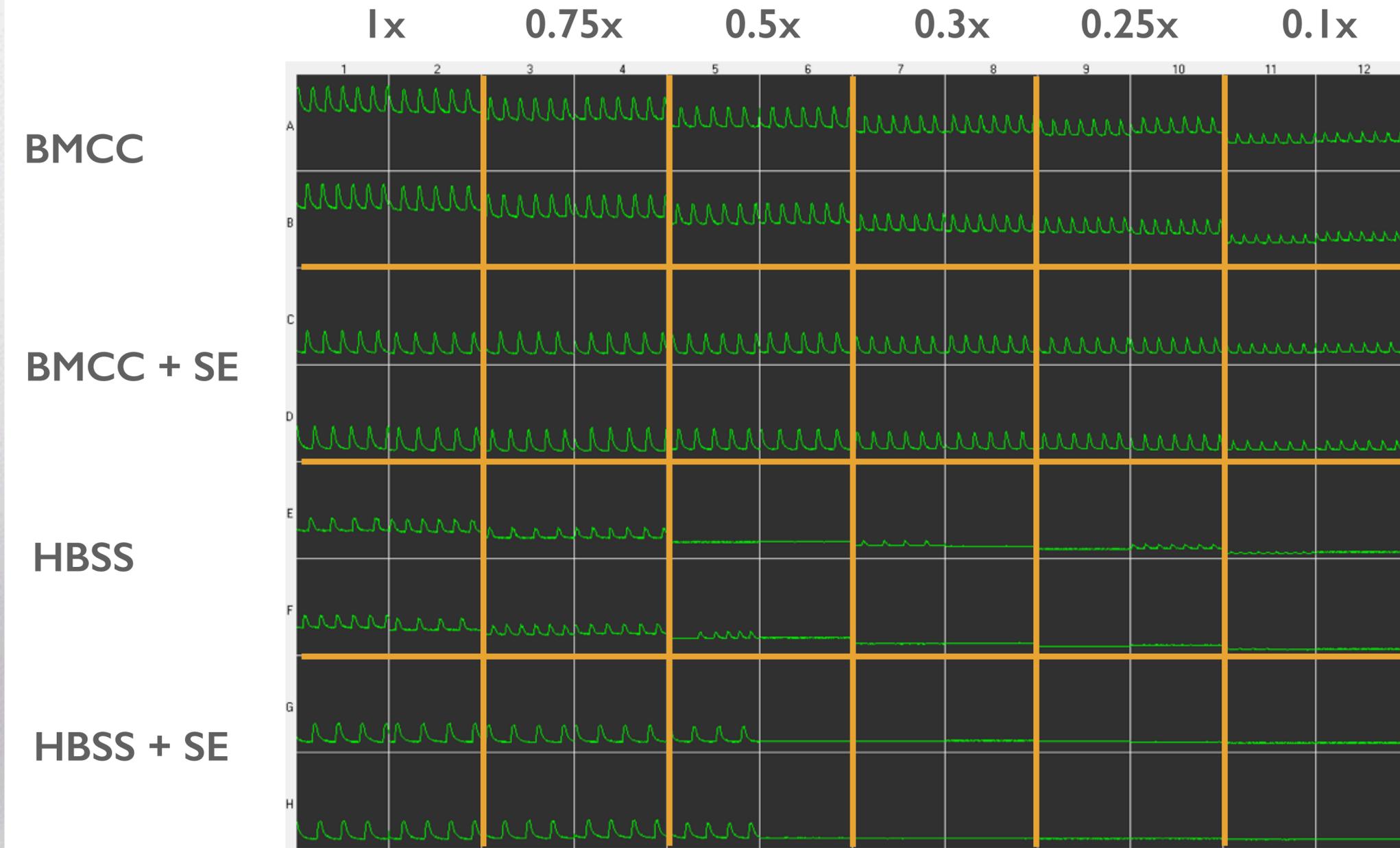
Product/Format

Service

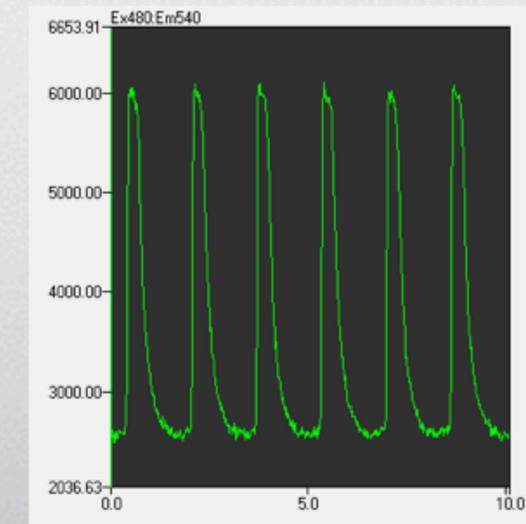
ACTOne™ Non-Wash Calcium Dye Kit Codex

Results

20 min

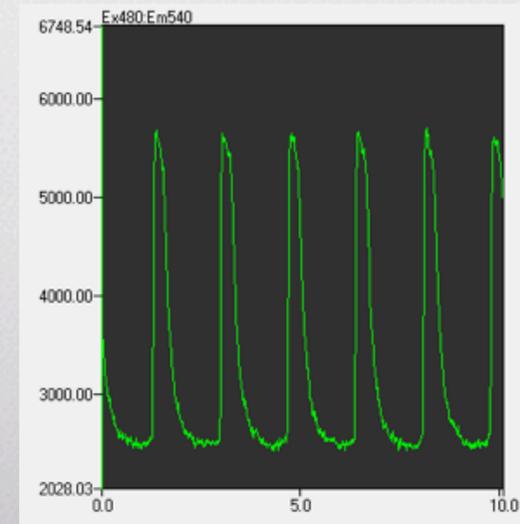


Well C8

0.3x in BMCC +
Signal Enhancer

3400 AFU

Well C9

0.25x in BMCC +
Signal Enhancer

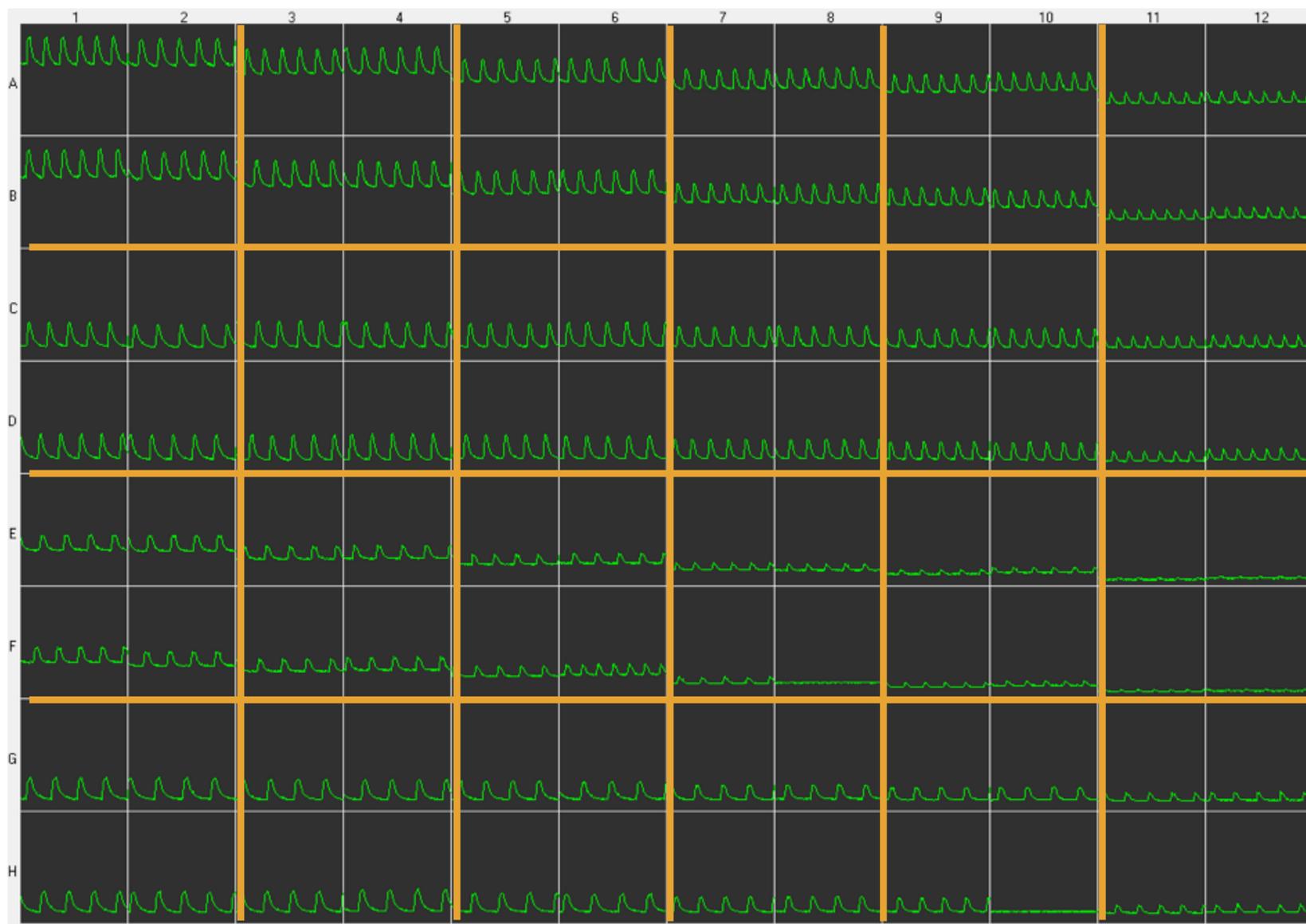
3000 AFU

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

Results

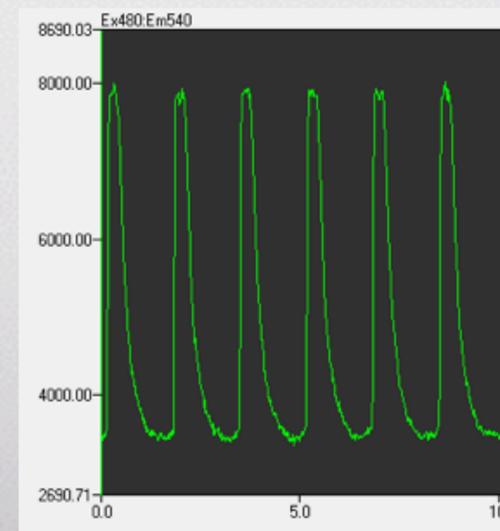
30 min

1x 0.75x 0.5x 0.3x 0.25x 0.1x



Well C8

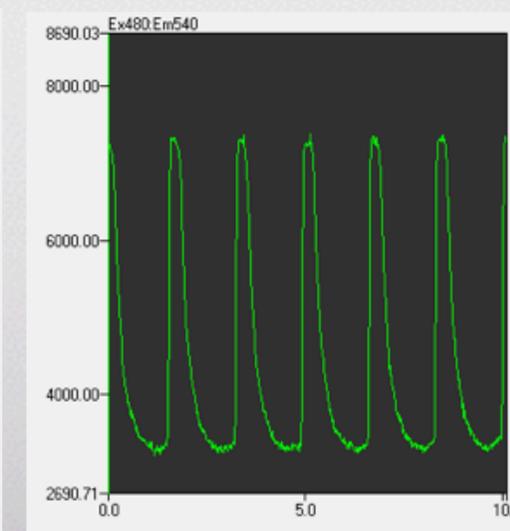
0.3x in BMCC +
Signal Enhancer



4200 AFU

Well C9

0.25x in BMCC +
Signal Enhancer



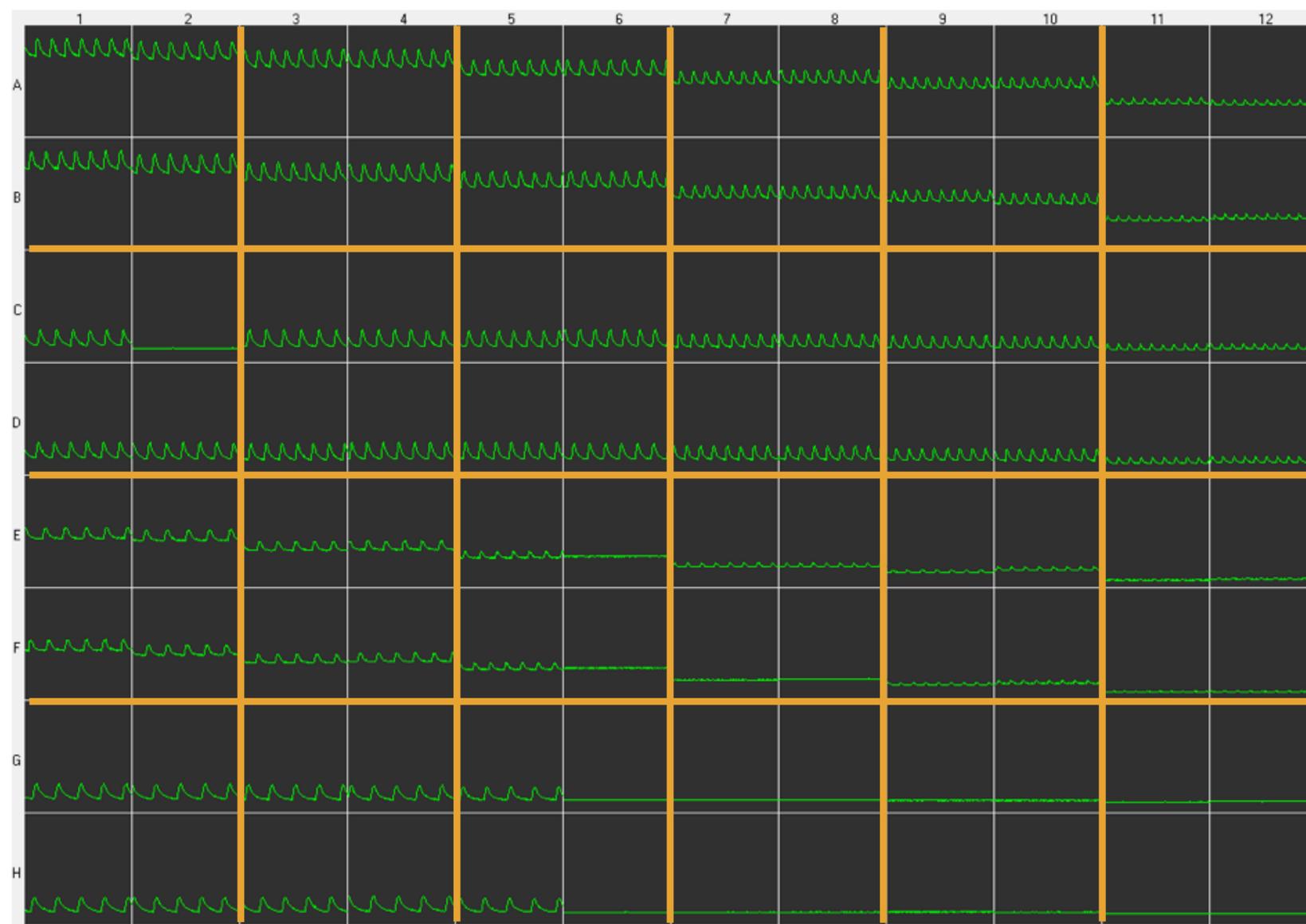
3300 AFU

Increase in background without Signal Enhancer.

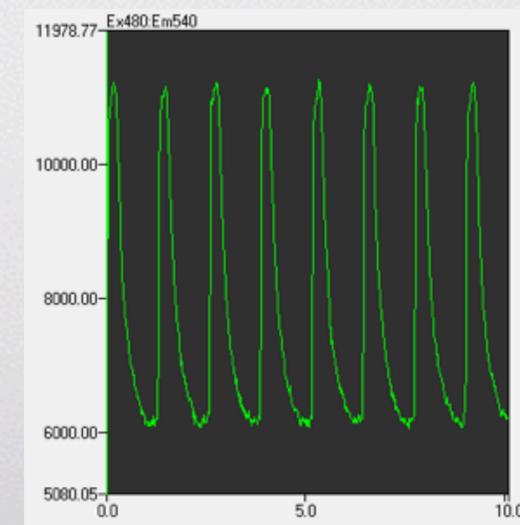
Results

60 min

1x 0.75x 0.5x 0.3x 0.25x 0.1x

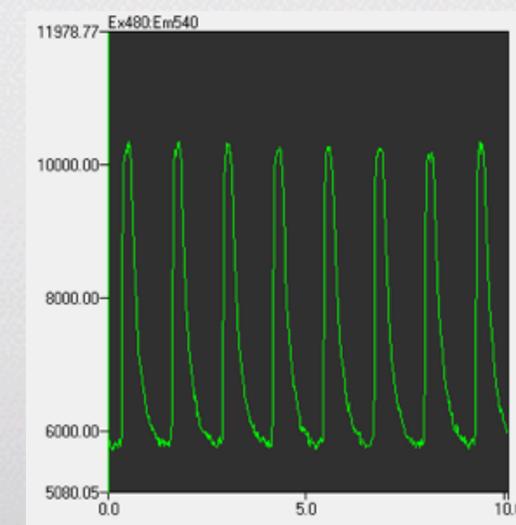


Well C8

0.3x in BMCC +
Signal Enhancer

5000 AFU

Well C9

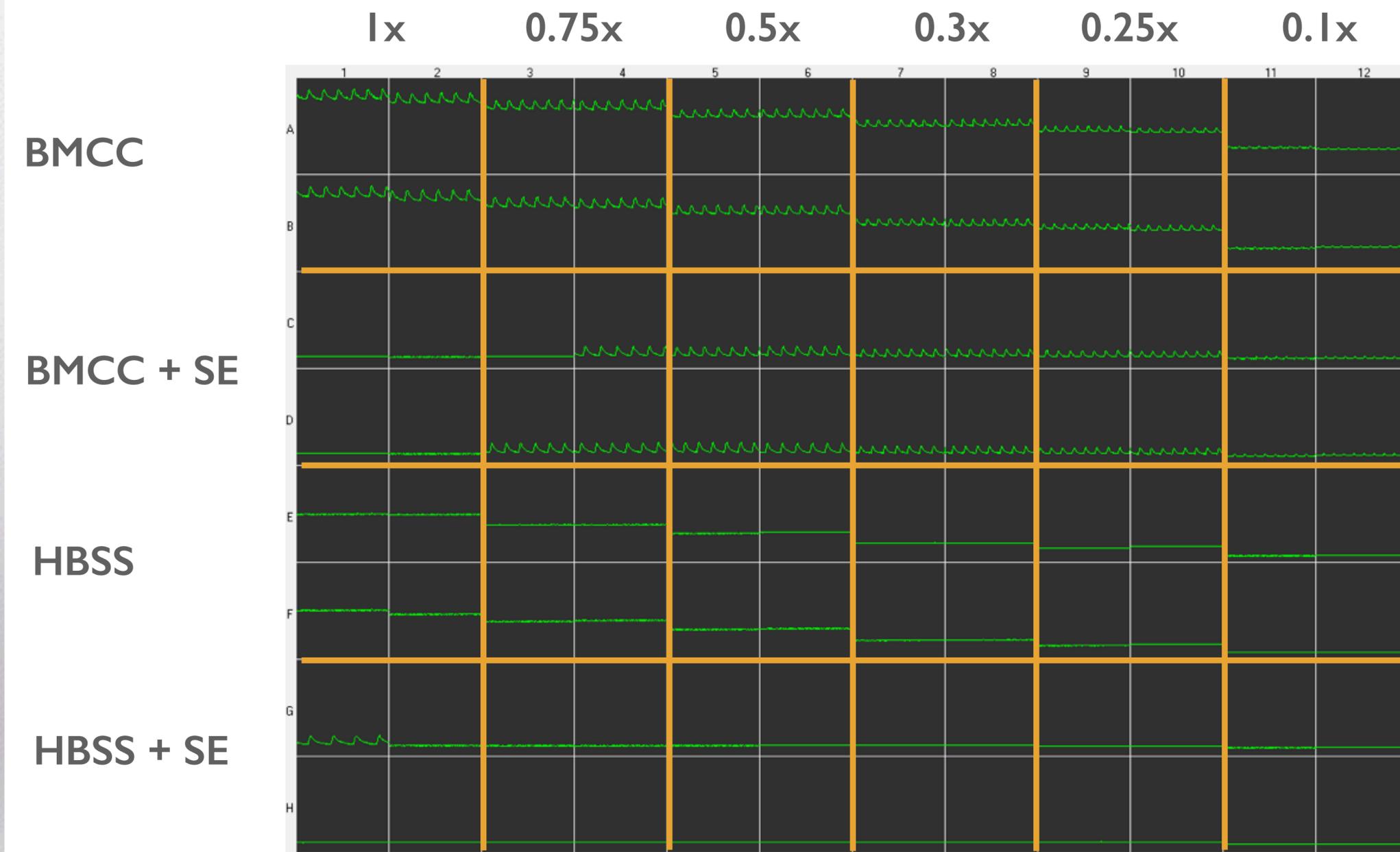
0.25x in BMCC +
Signal Enhancer

4000 AFU

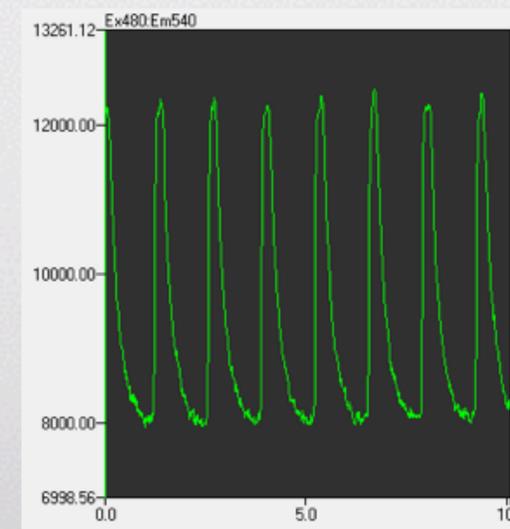
- Irregular Beating in HBSS buffer
- Further increase background

Results

90 min

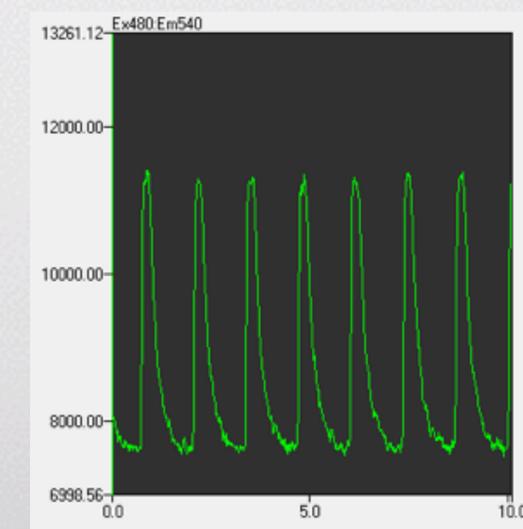


Well C8

0.3x in BMCC +
Signal Enhancer

4000 AFU

Well C9

0.25x in BMCC +
Signal Enhancer

3800 AFU

- 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

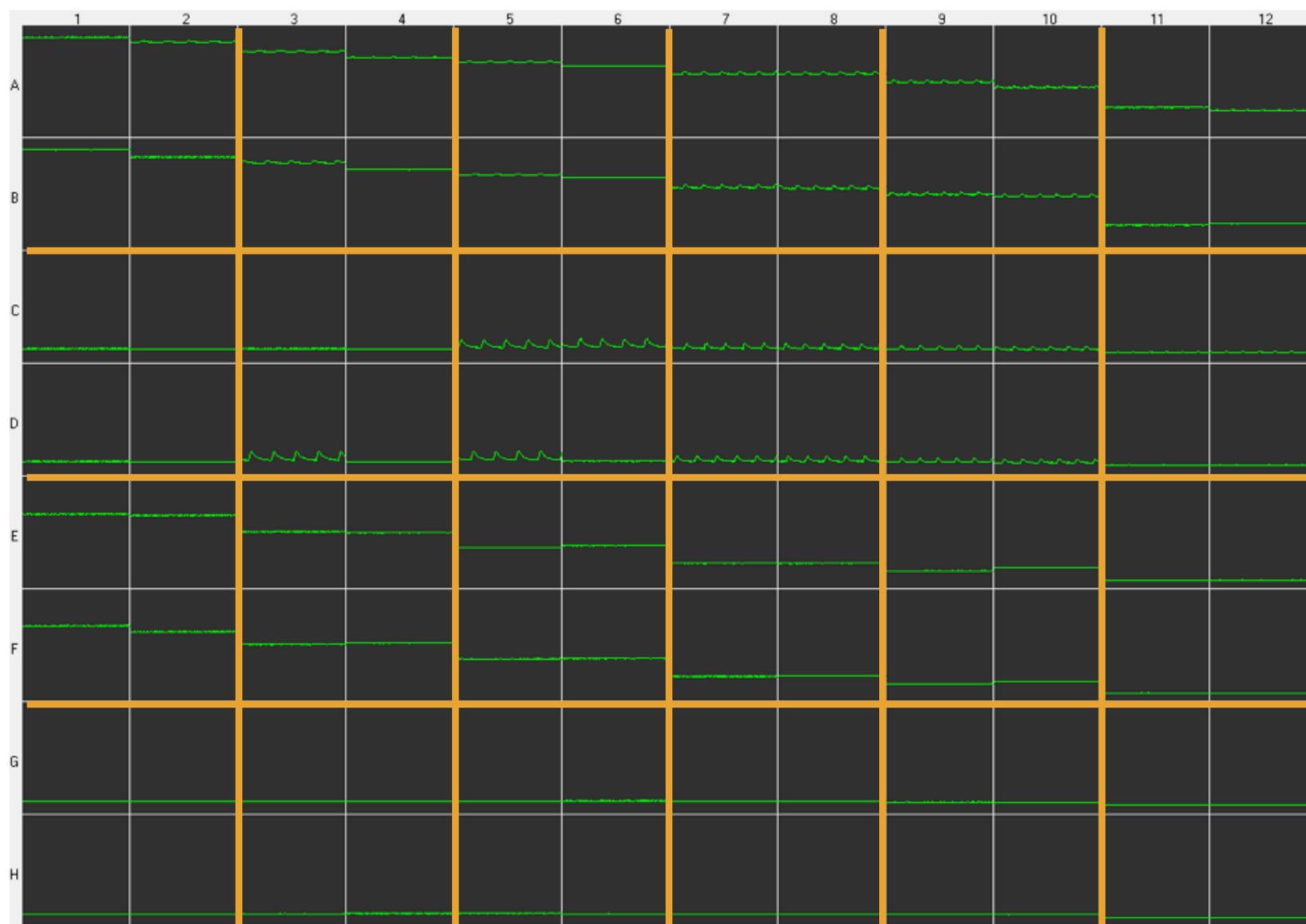
1x 0.75x 0.5x 0.3x 0.25x 0.1x

BMCC

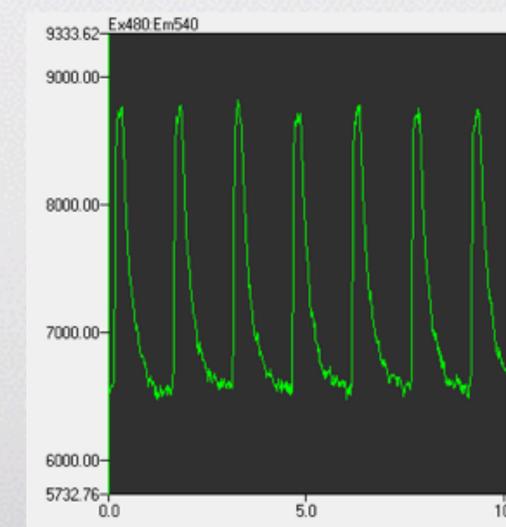
BMCC + SE

HBSS

HBSS + SE

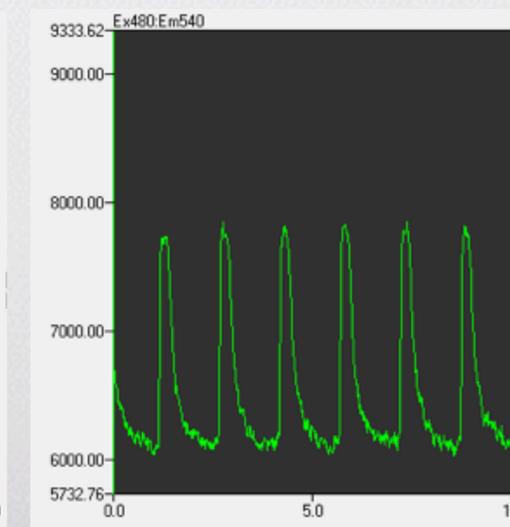


Well C8

0.3x in BMCC +
Signal Enhancer

2100 AFU

Well C9

0.25x in BMCC +
Signal Enhancer

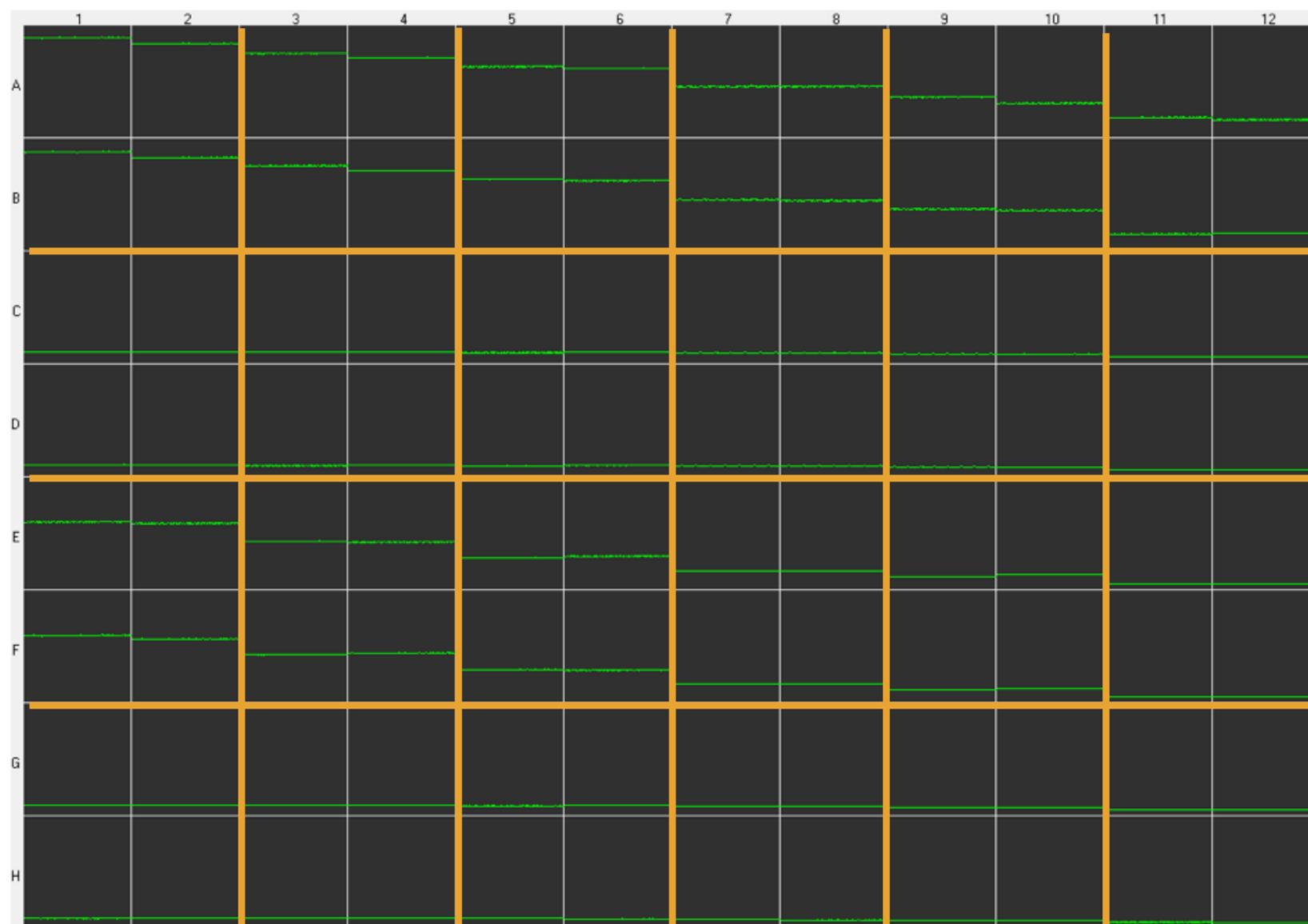
1900 AFU

- 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

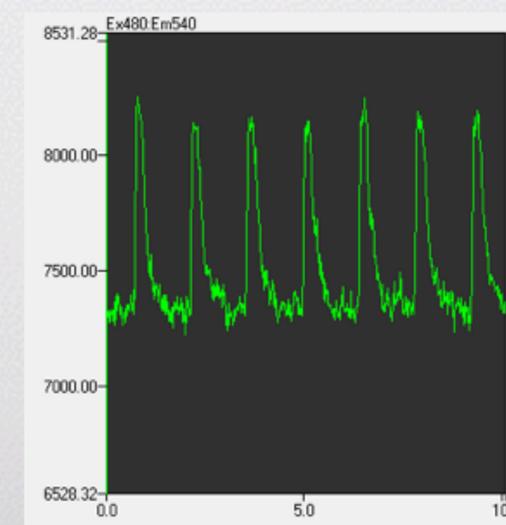
Results

240 min

1x 0.75x 0.5x 0.3x 0.25x 0.1x

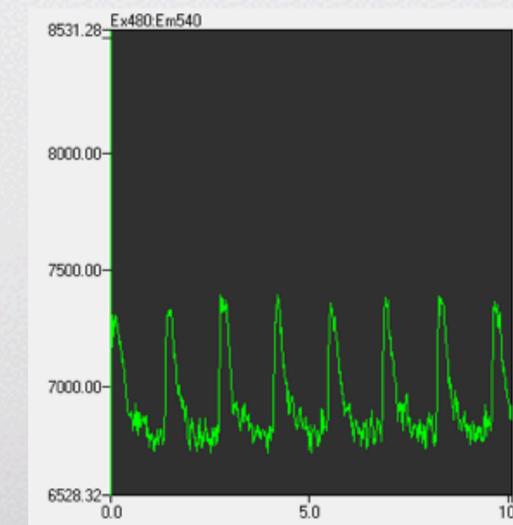


Well C8

0.3x in BMCC +
Signal Enhancer

ca. 1500 AFU

Well C9

0.25x in BMCC +
Signal Enhancer

ca. 500 AFU

- High 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.

Company

Characterization

Product/Format

Service

Cal-520™ , AM
AAT Bioquest

Protocol

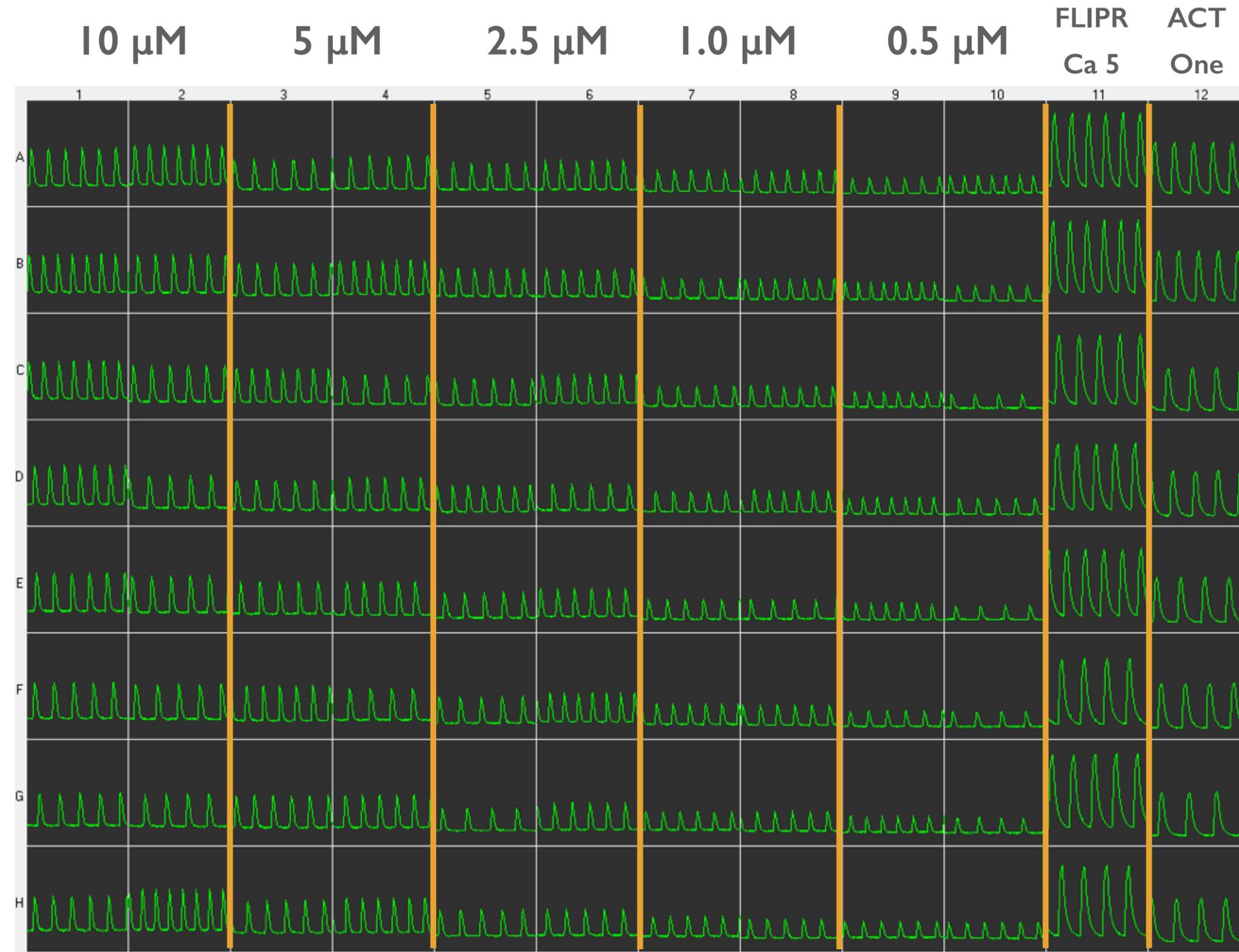
- Fresh Cor.4U Cardiomyocytes were seeded in Cor.4U Culture medium with 20k cells/well into a 96 well μ Clear plate from Greiner Bio One and cultured for 3 days.
- On the day of experiment, medium was exchanged for phenol red-free BMCC Medium at least 2 hours before the start of the experiment.
- The 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 μ M in BMCC Medium (without quencher or probenecide). The provider suggests to us concentrations between 10 μ M and 20 μ M.
- The following concentrations were tested with the Cor.4U Cardiomyocytes:
10 μ M, 5 μ M, 2.5 μ M, 1.0 μ M, 0.5 μ M
- 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.

Experimental Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												
	FLIPR Calcium 5 Assay Kit 1:6											
	Codex ACTOne Non-Wash Calcium Dye 0.3x											
	0.5 μ M Cal-520											
	1.0 μ M Cal-520											
	2.5 μ M Cal-520											
	5.0 μ M Cal-520											
	10 μ M Cal-520											

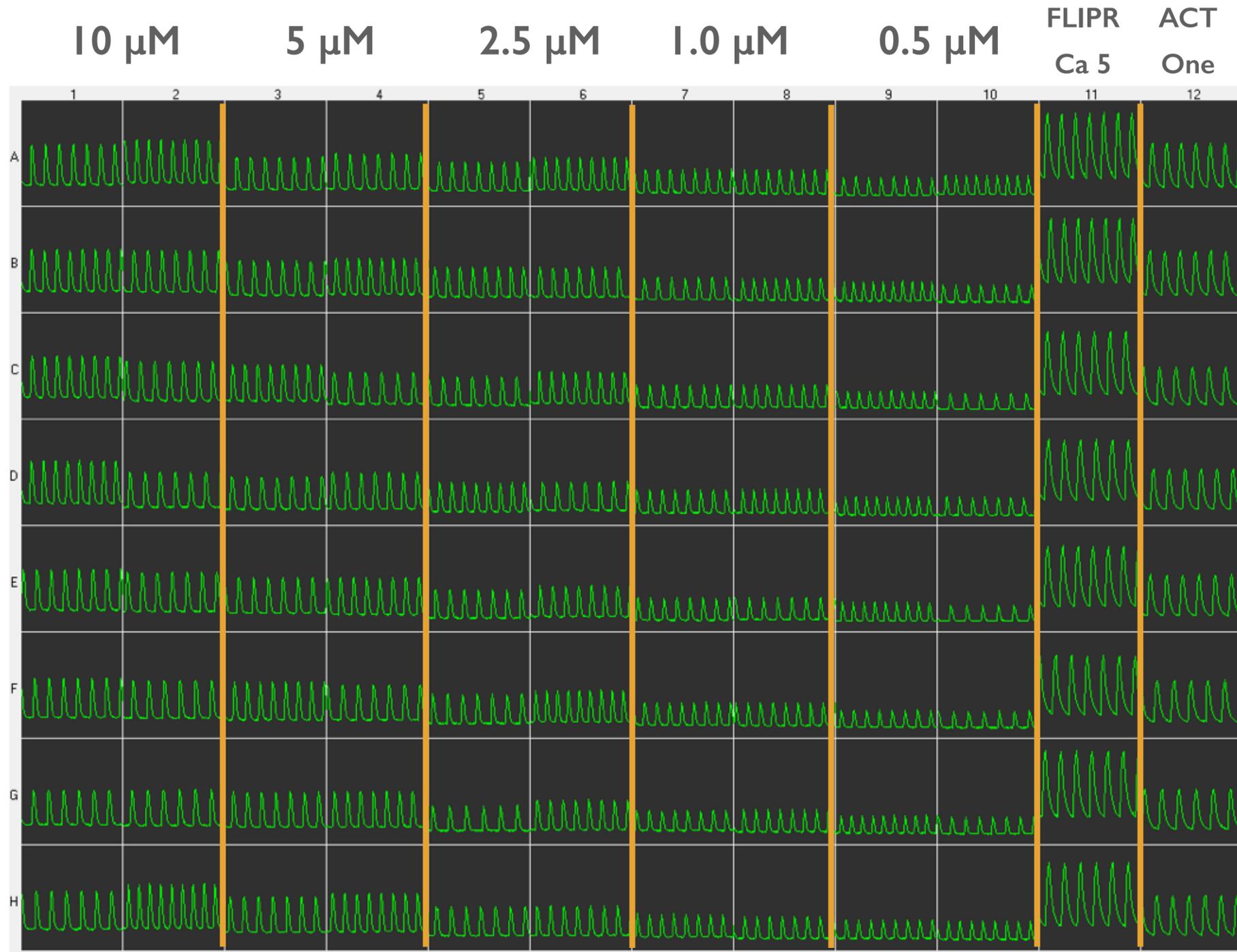
Results - Cal-520

30 min



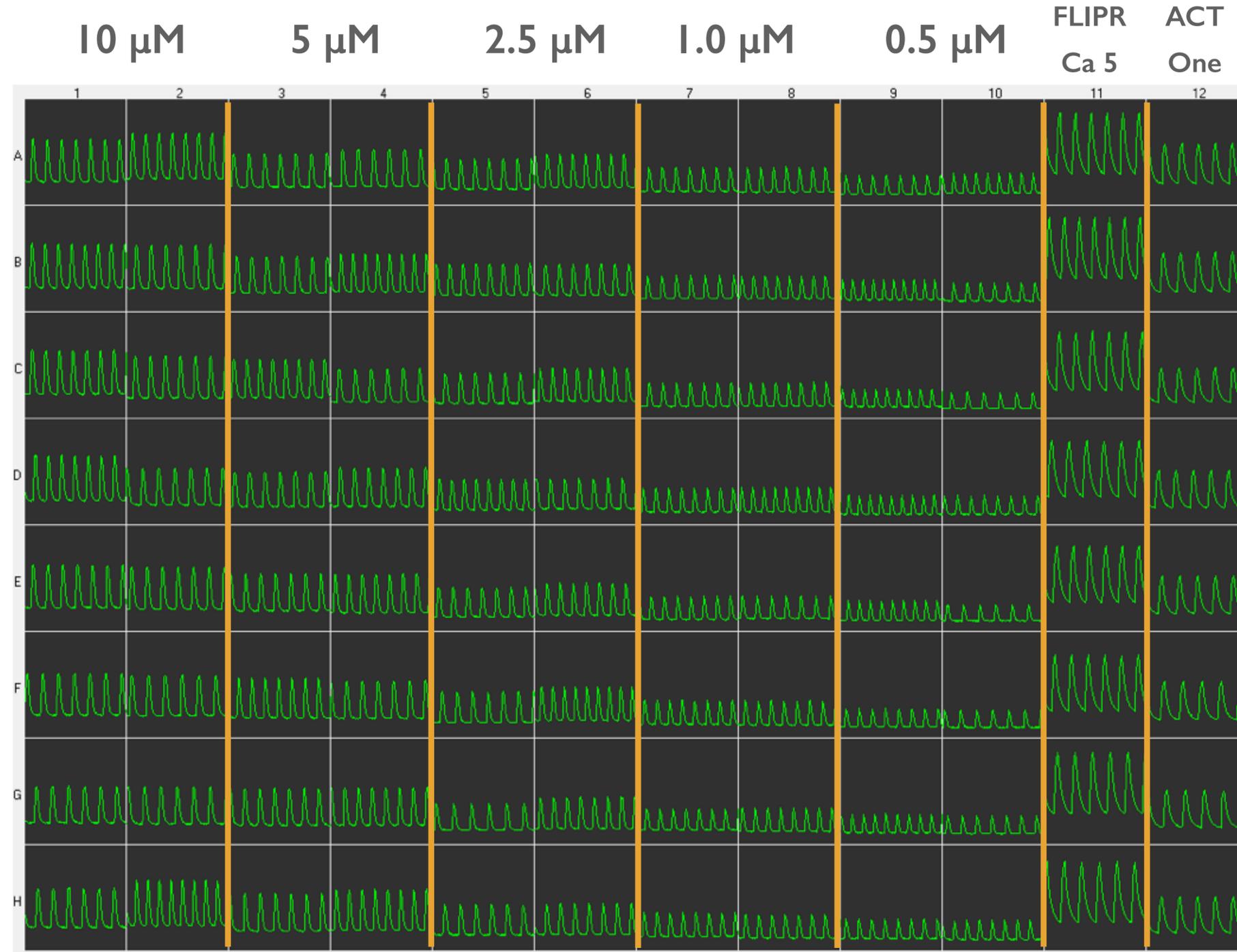
Results - Cal-520

45 min

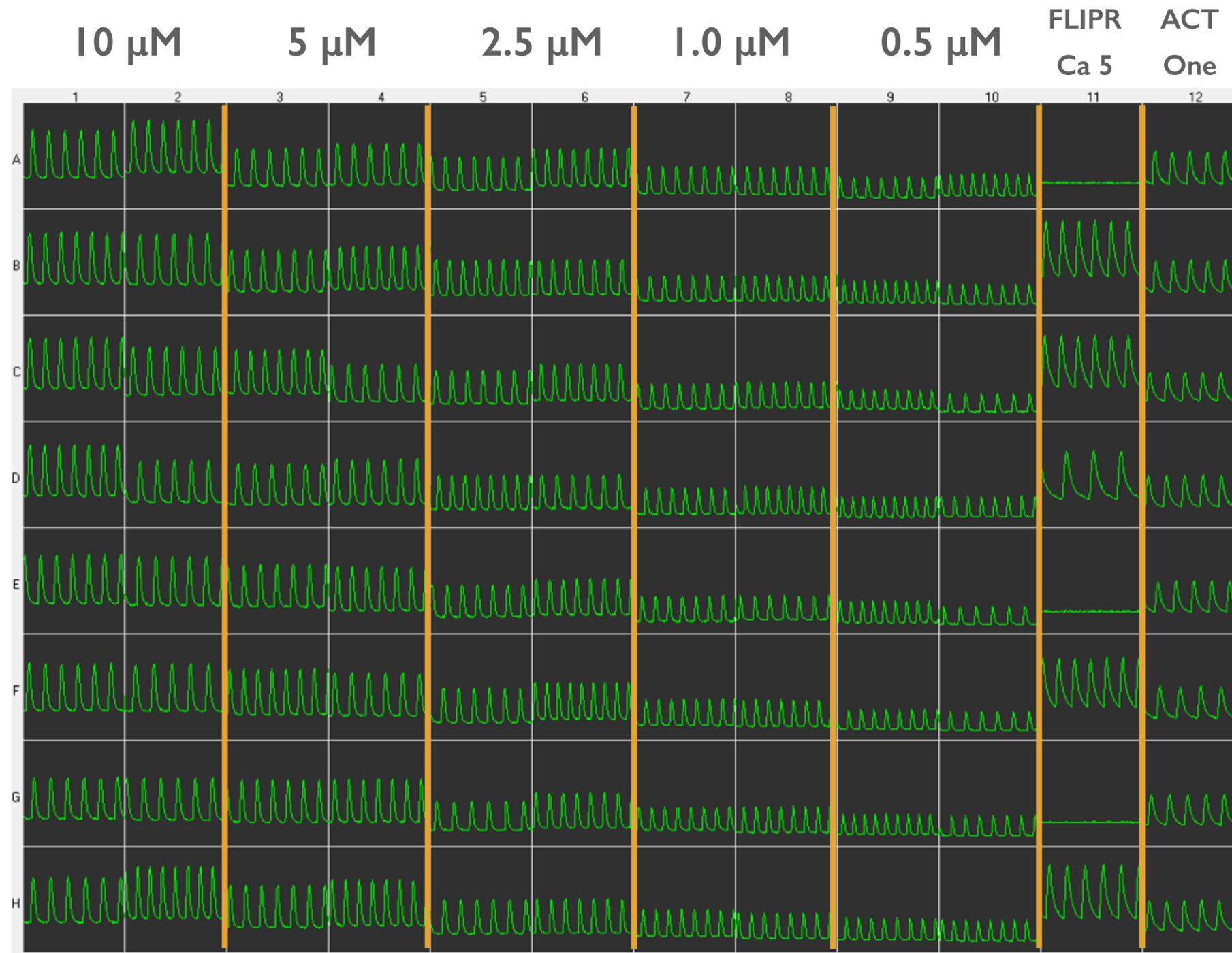


Results - Cal-520

60 min



Results - Cal-520

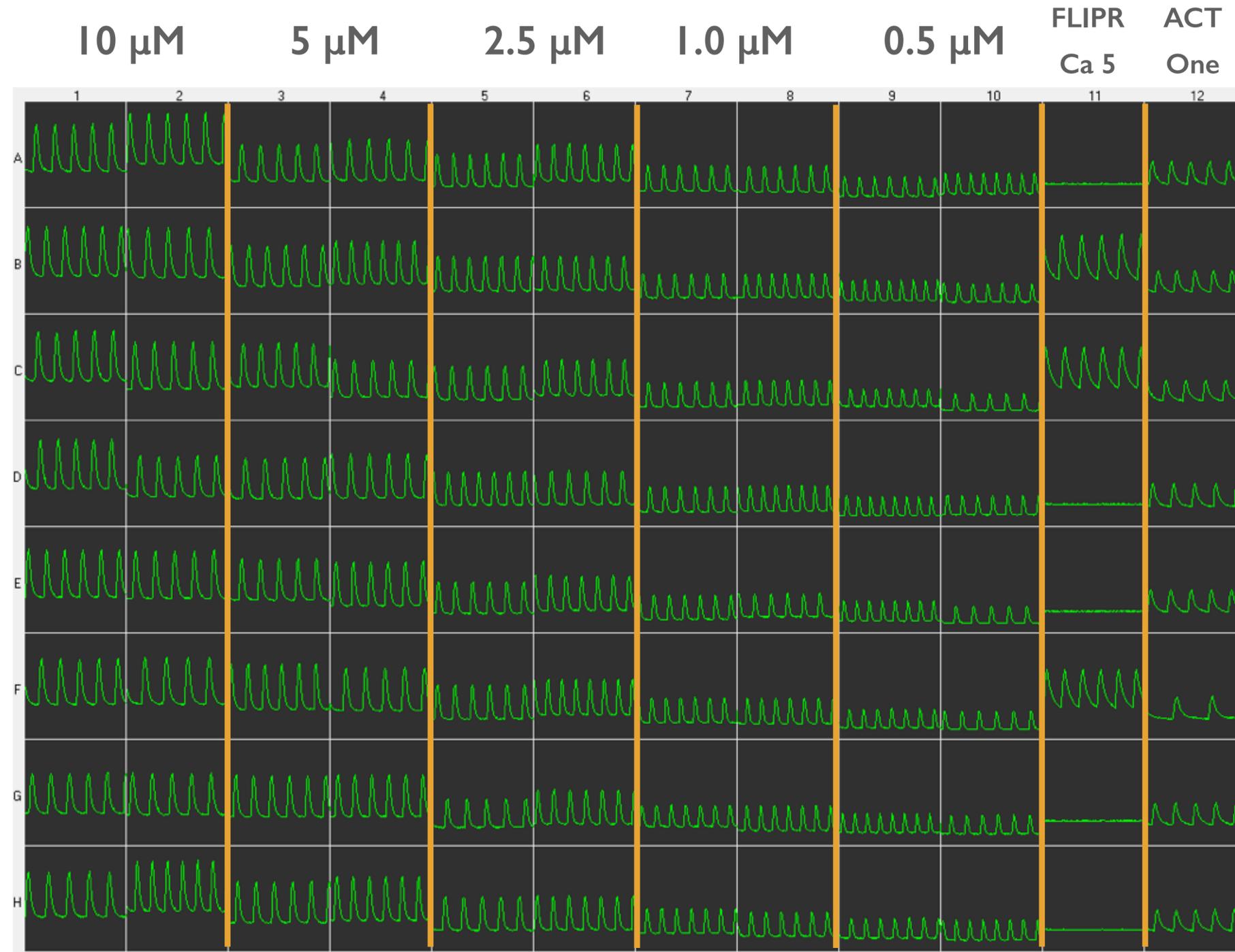


90 min

First arrest of the spontaneous calcium transients with the FLIPR Calcium 5 Assay Kit.

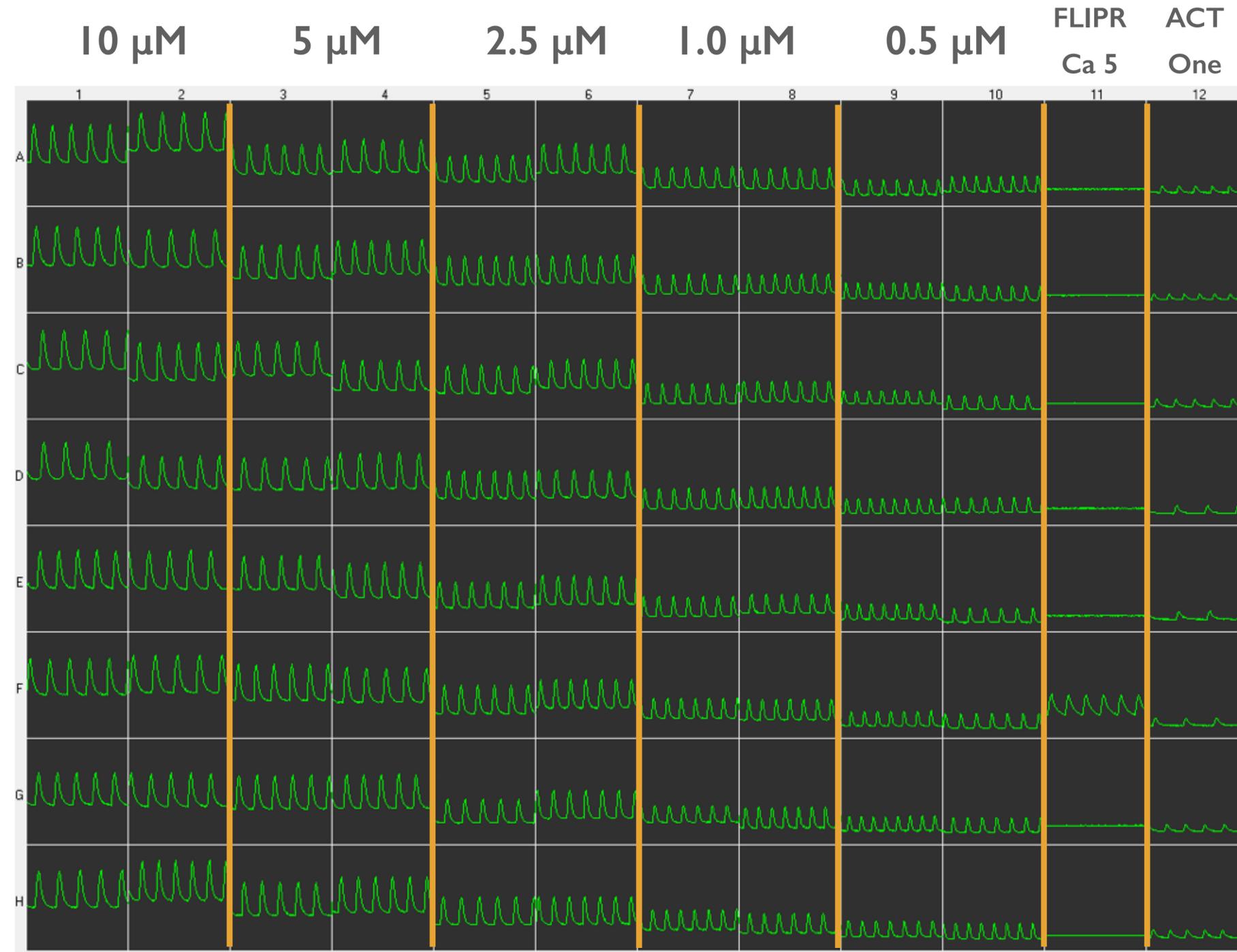
Results - Cal-520

120 min



XXX

Results - Cal-520

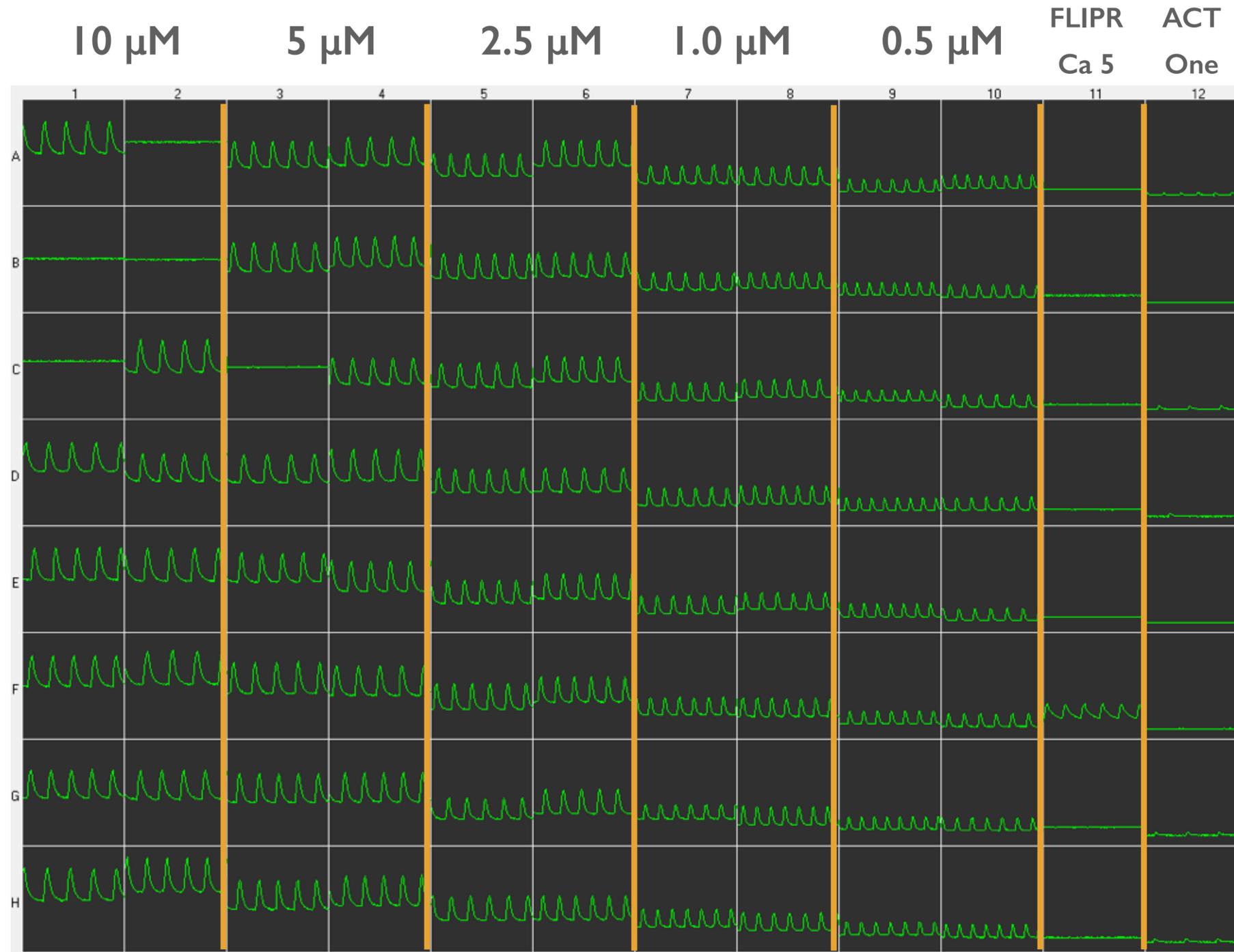


180 min

XXX

Results - Cal-520

240 min



First arrest at 10 μ M and 5 μ M Cal-520.

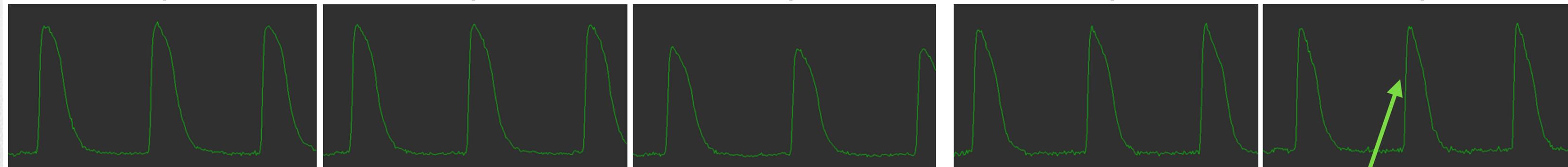
Decrease in fluorescent calcium transient amplitude with ACTOne.

Almost complete arrest with the FLIPRR Calcium 5 Assay Dye.

Morphological Differences and Changes Over Time of the Cal-520 Calcium Transients

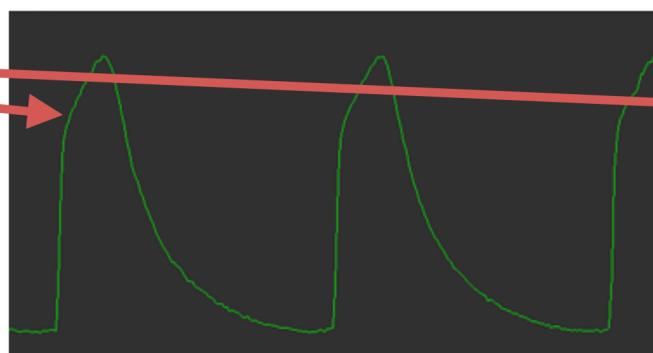
Results - Cal-520

30 min

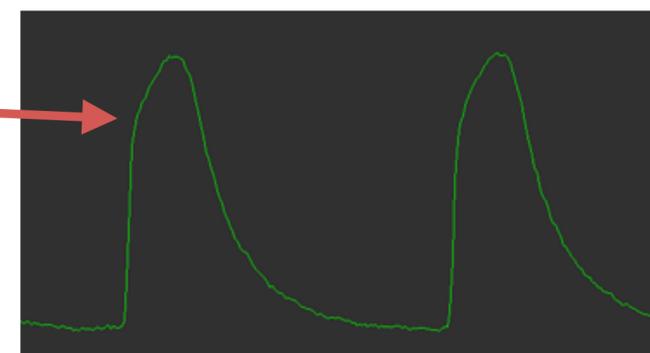
10 μM 5 μM 2.5 μM 1.0 μM 0.5 μM 

Bent fast rise of the calcium transient and prolonged peak width

FLIPR
Calcium 5



ACT
One



time windows: 4s

Physiological fast rise of the calcium transient and short peak width

- FLIPR Calcium 5 and Codex ACTOne reveal an slowed rise of the calcium transients from 80% to 100%.
- There is obviously a changes 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).

Results - Cal-520

90 min

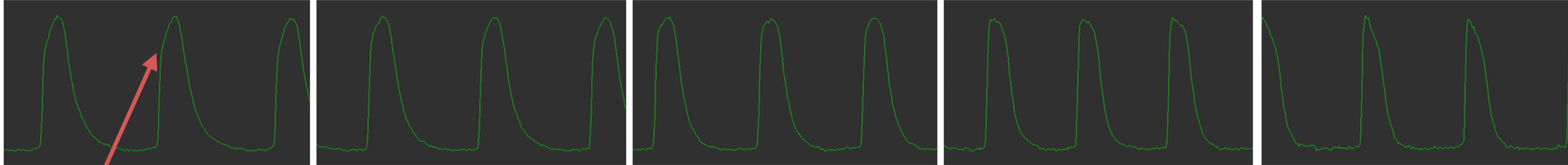
10 μ M

5 μ M

2.5 μ M

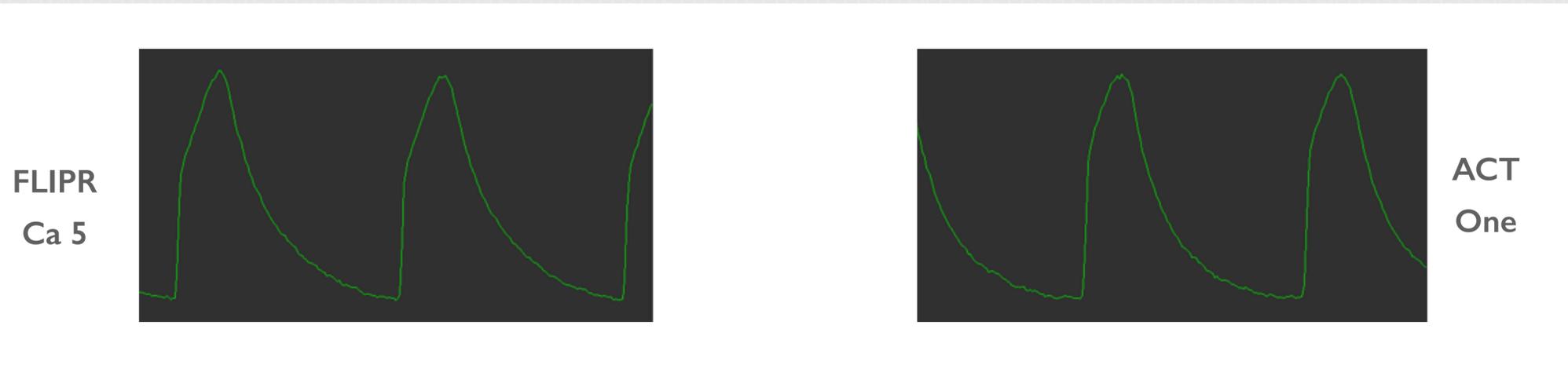
1.0 μ M

0.5 μ M



Bent fast rise of the calcium transient

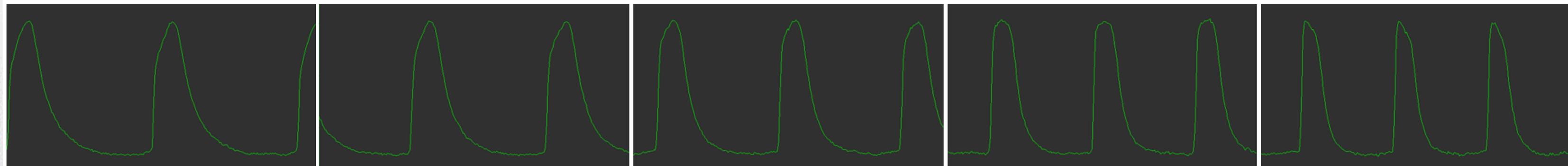
time windows: 4s



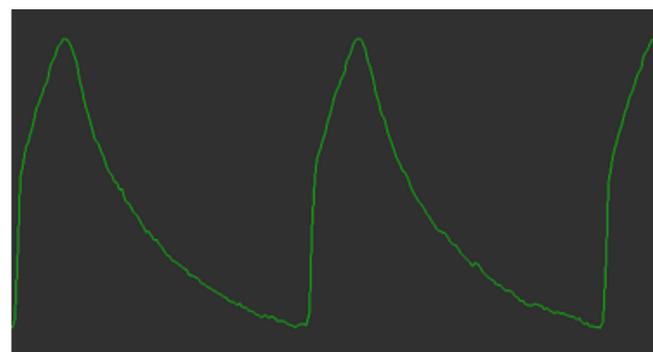
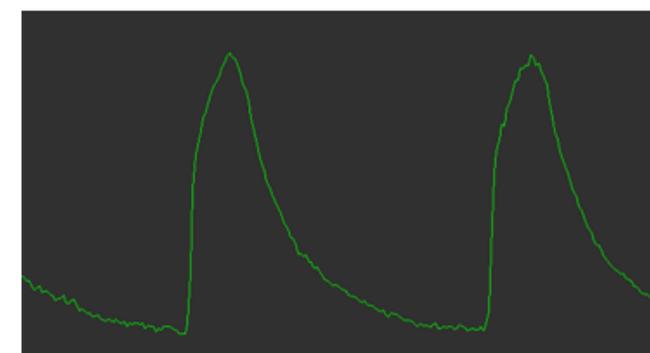
At higher concentrations of Cal-520 the slope at 80% to 100% starts to slow as well.

Results - Cal-520

120 min

10 μM 5 μM 2.5 μM 1.0 μM 0.5 μM 

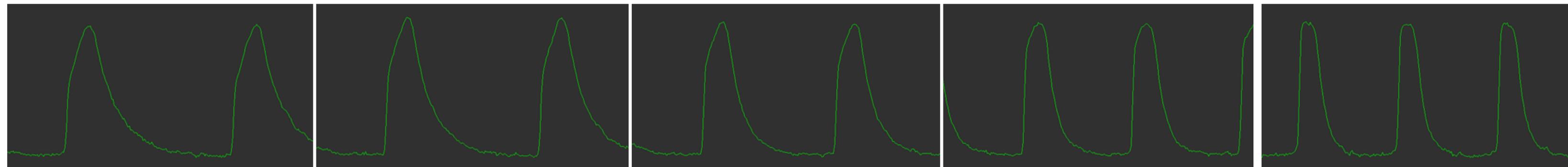
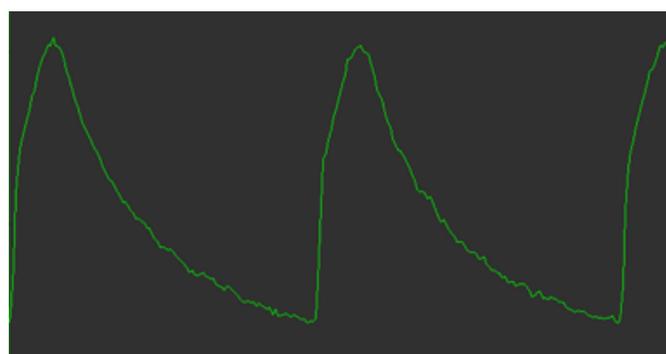
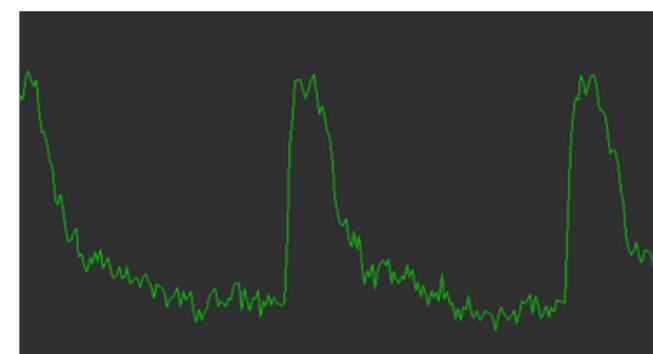
time windows: 4s

FLIPR
Ca 5ACT
One

At 10 μM Cal-520 the calcium transient peak with duration starts to prolong.

Results - Cal-520

240 min

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

time windows: 4s

- 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.

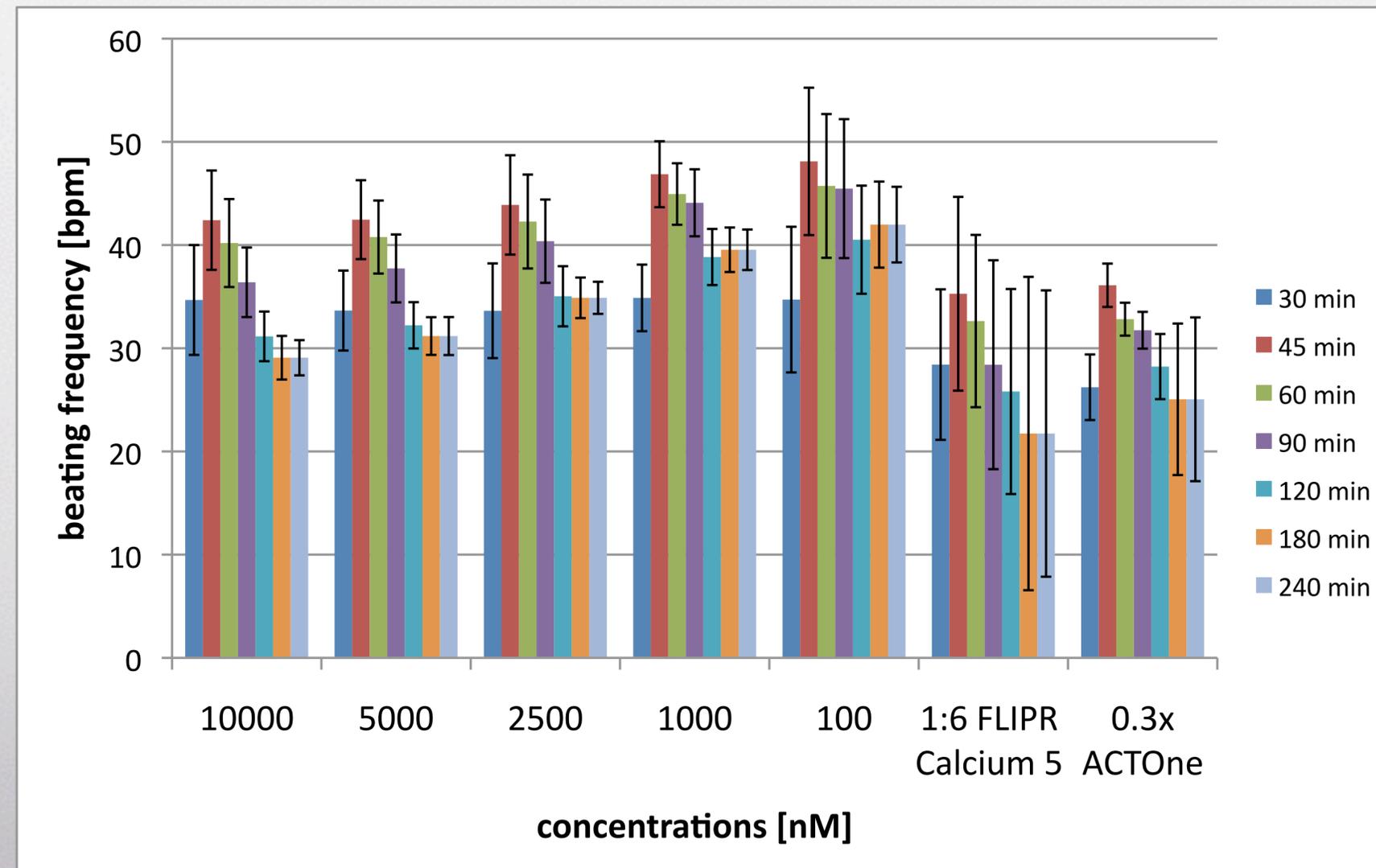
Quantitative Analysis of Non-Wash Cal-520 Calcium Transients Recorded from Cor.4U Cardiomyocytes

Overview - Arrest of Spontaneous Calcium Transient in Cor.4U Cardiomyocytes

Time [min]	10 μ M	5 μ M	2.5 μ M	1.0 μ M	0.5 μ M	1:6 FLIPR Calcium 5	0.3x Codex ACTOne
30	0/16	0/16	0/16	0/16	0/16	0/8	0/8
45	0/16	0/16	0/16	0/16	0/16	0/8	0/8
60	0/16	0/16	0/16	0/16	0/16	0/8	0/8
90	0/16	0/16	0/16	0/16	0/16	3/8	0/8
120	0/16	0/16	0/16	0/16	0/16	5/8	0/8
180	0/16	0/16	0/16	0/16	0/16	7/8	0/8
240	5/16	1/16	0/16	0/16	0/16	7/8	4/8

Results - Cal-520

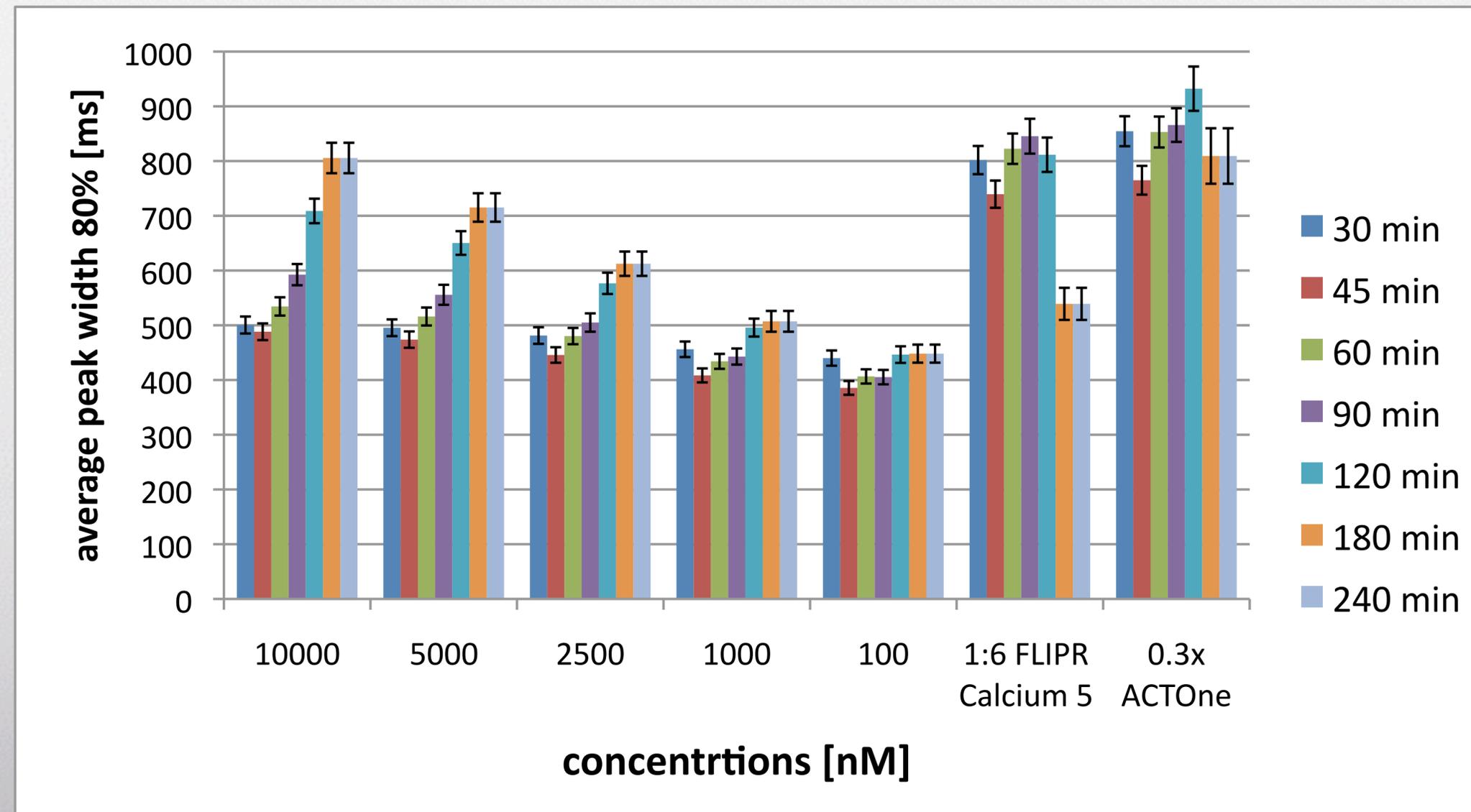
Beat Rate



- 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.

Results - Cal-520

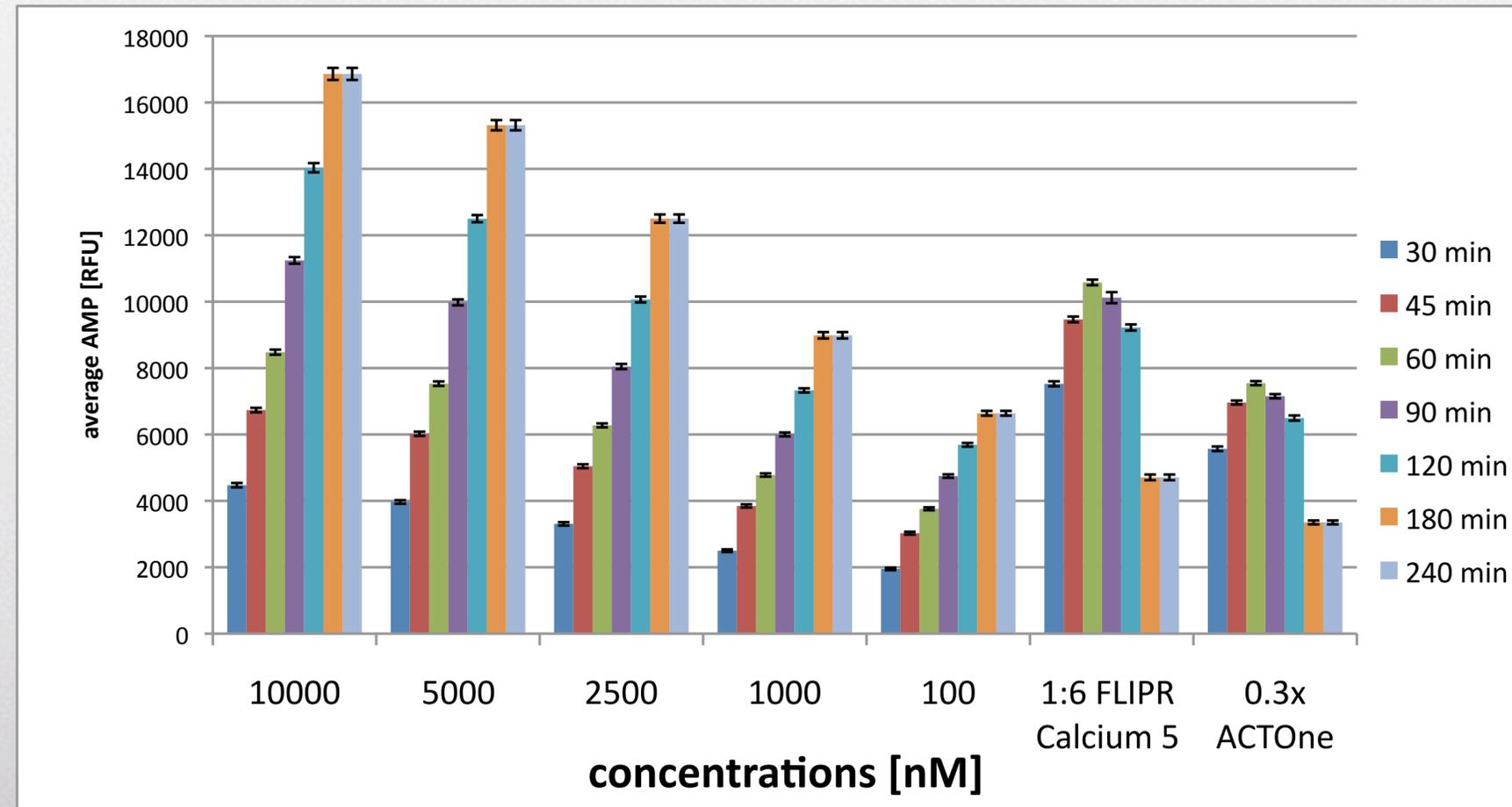
Peak Width (PW) 80%



- 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 Amplitude



- 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

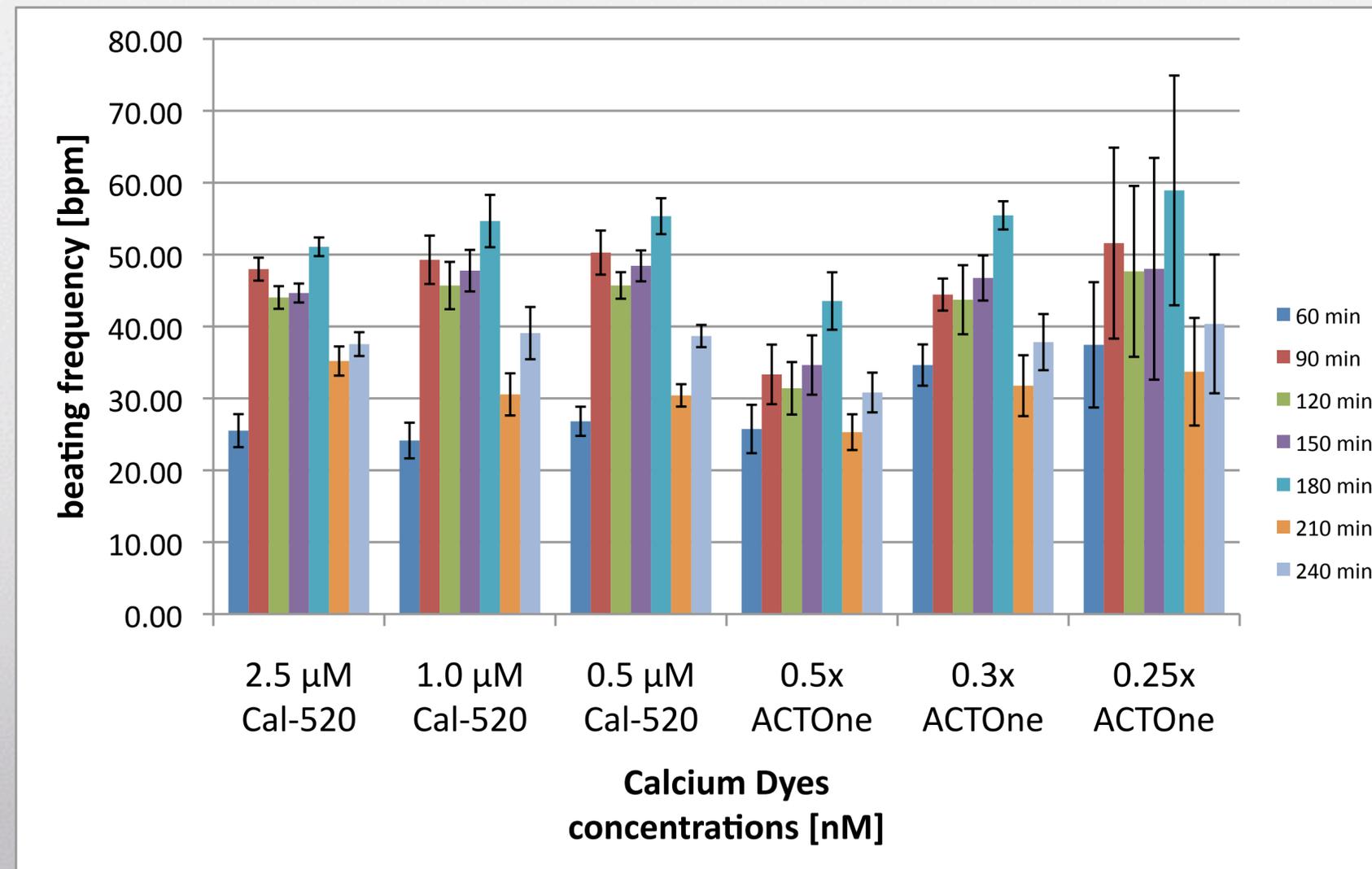
Cal-520™ , AM (AAT Bioquest)

and

ACTOne (Codex)

Results

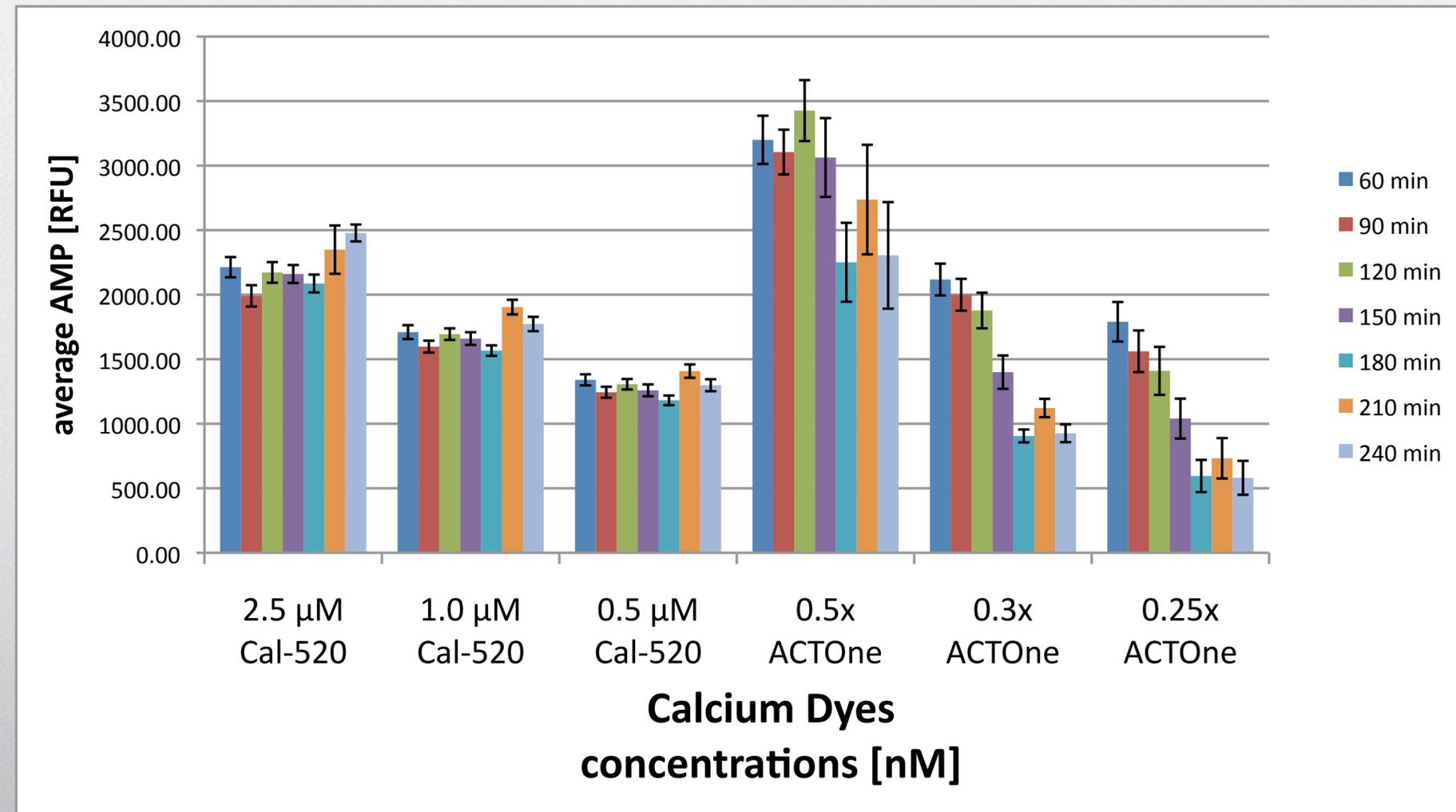
Beat Rate



- 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

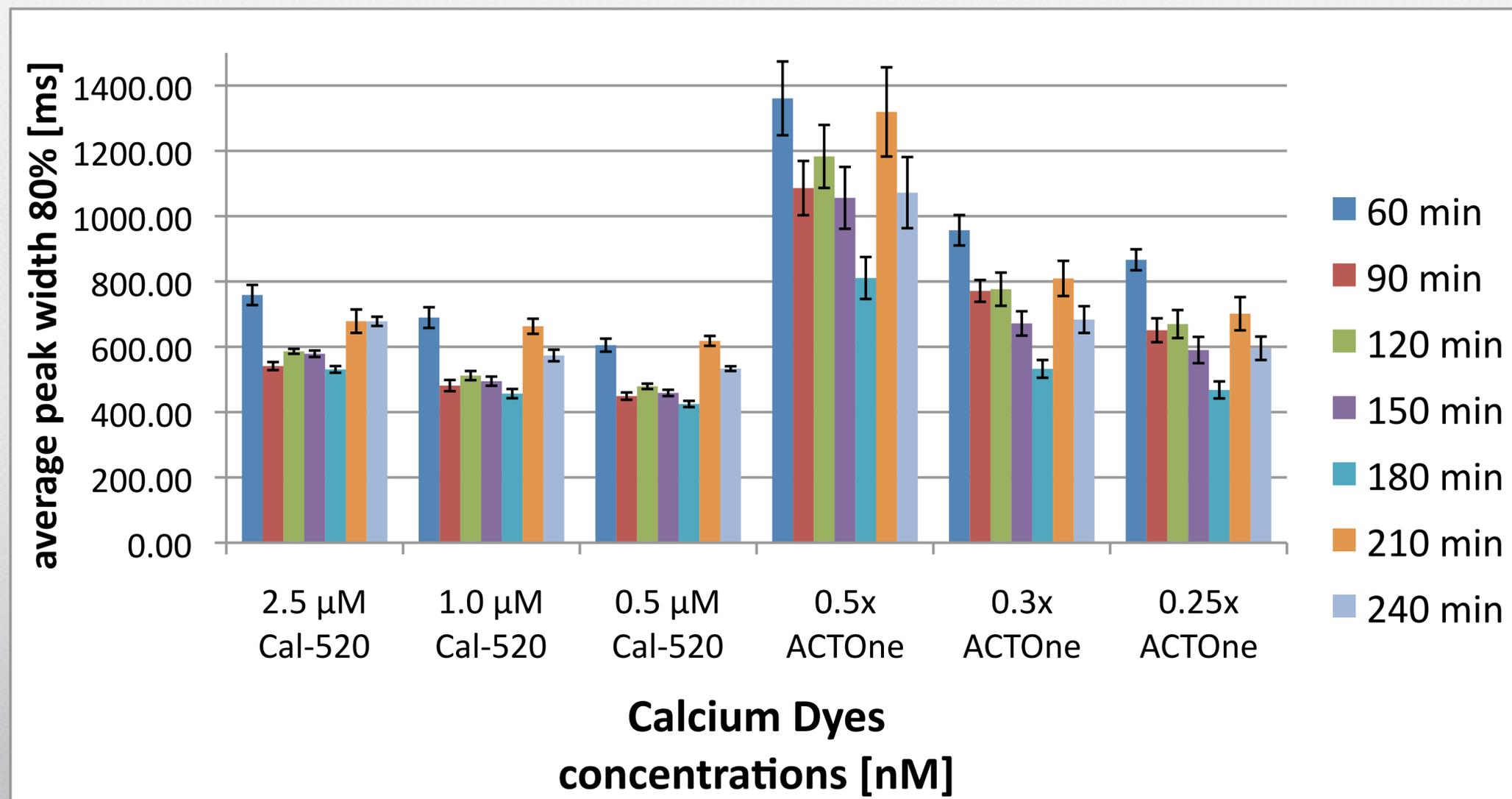
Calcium Transient Amplitude



- 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%



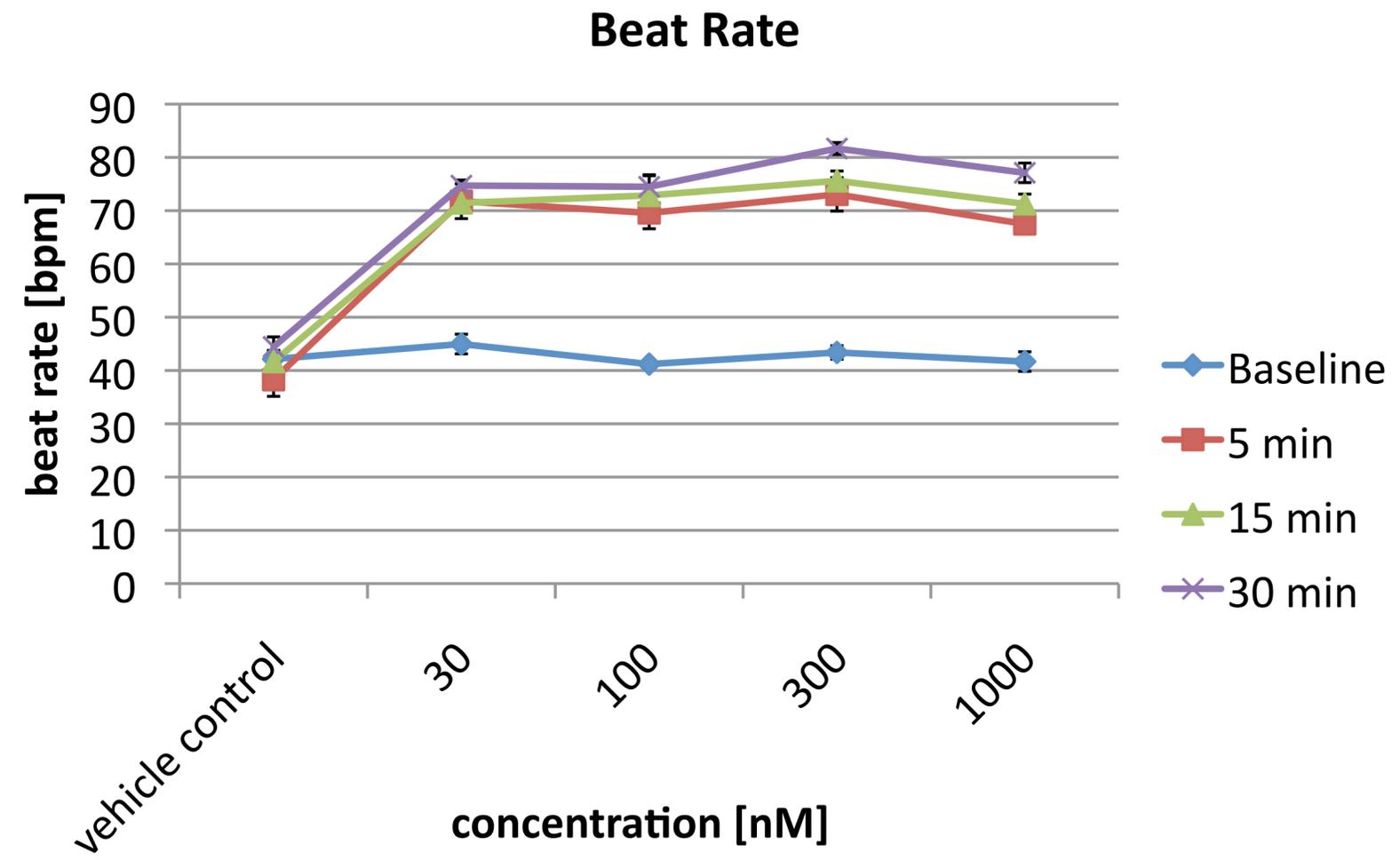
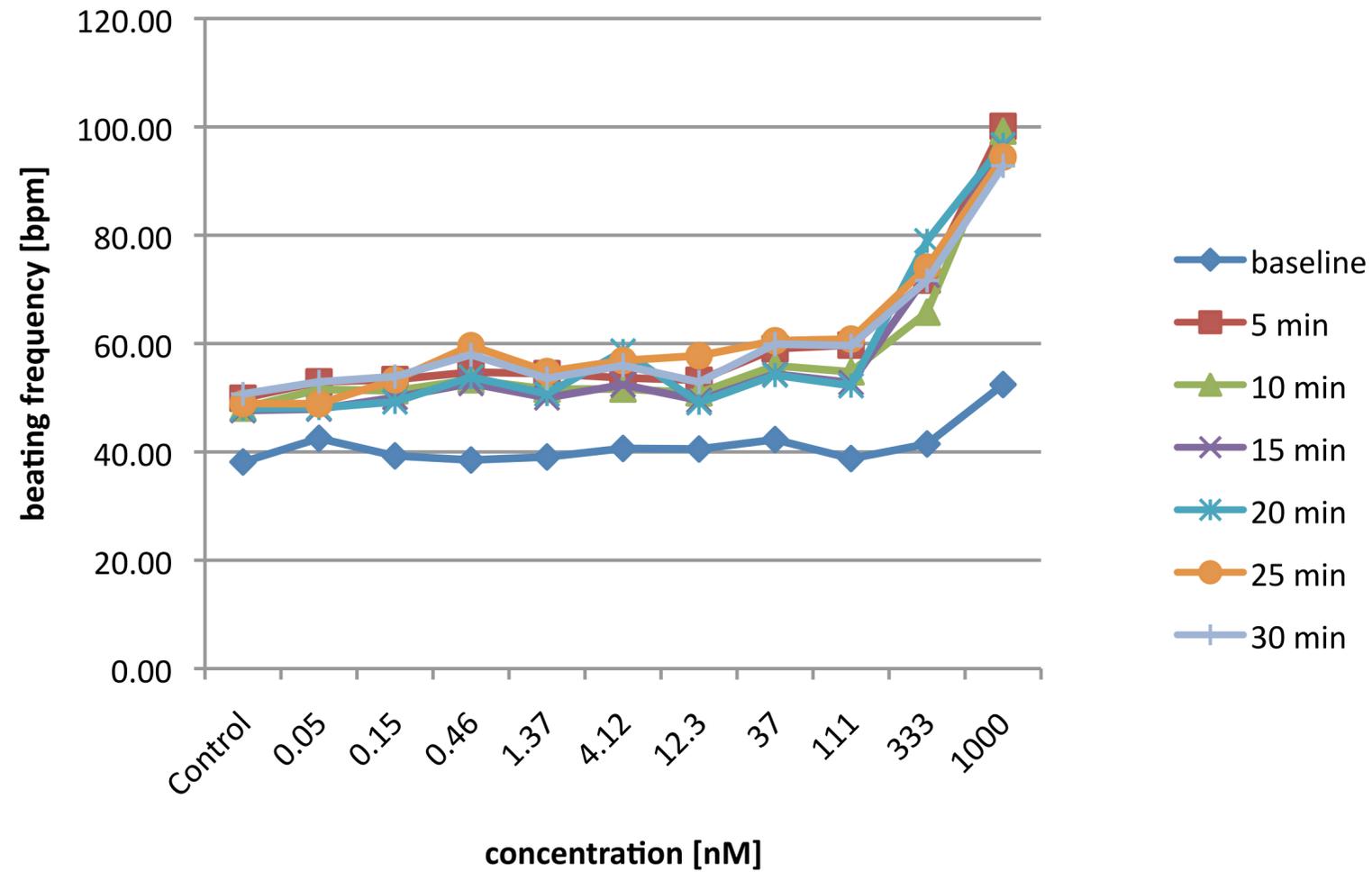
- 0.5x ACTOne peak width at 80% are doubled compared to 2.5 μM Cal-520 (and also 0.25x ACTOne) and almost 3x the values of 0.5 μM Cal-520.

Dye Effect on GPCR Agonist Pharmacology with Cor.4U Cardiomyocytes

Results

Calcium 5 Assay Kit Dye

Cal520



- 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