Label-free analysis of cardiomyocyte beating using the Opera Phenix Plus system.

Key features

- Reliable quantification of cardiomyocyte beating frequency
- Fast frame rate imaging up to 100 fps to accurately capture fast cellular responses
- Tip-based on-board pipetting compatible with 96 and 384-well plates
- Stable environmental conditions with temperature, CO_2, and humidity control

Introduction

Drug-induced cardiotoxicity is a frequent cause of drug attrition during drug development and can lead to the withdrawal of drugs from the market.[1] *In-vitro* models using induced pluripotent stem-cell derived cardiomyocytes are commonly used to predict both safety and therapeutic efficacy of drugs, chemicals, environmental pollutants, cosmetic ingredients, or food additives.[2,3] The development of new, non-invasive approaches allowing the quick and reliable assessment of the cardiotoxic potential of a compound are necessary to improve drug safety.[4]

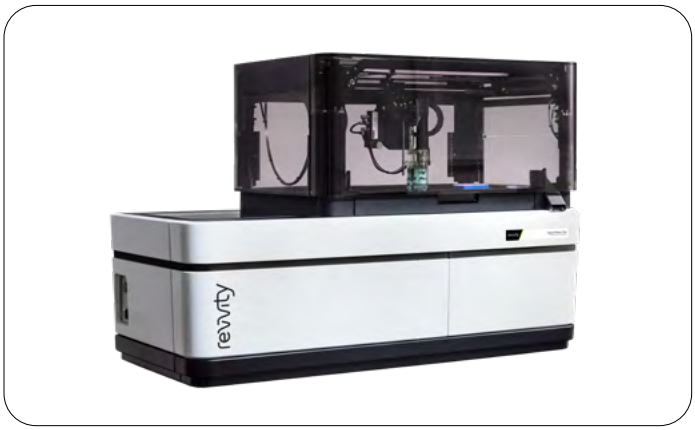
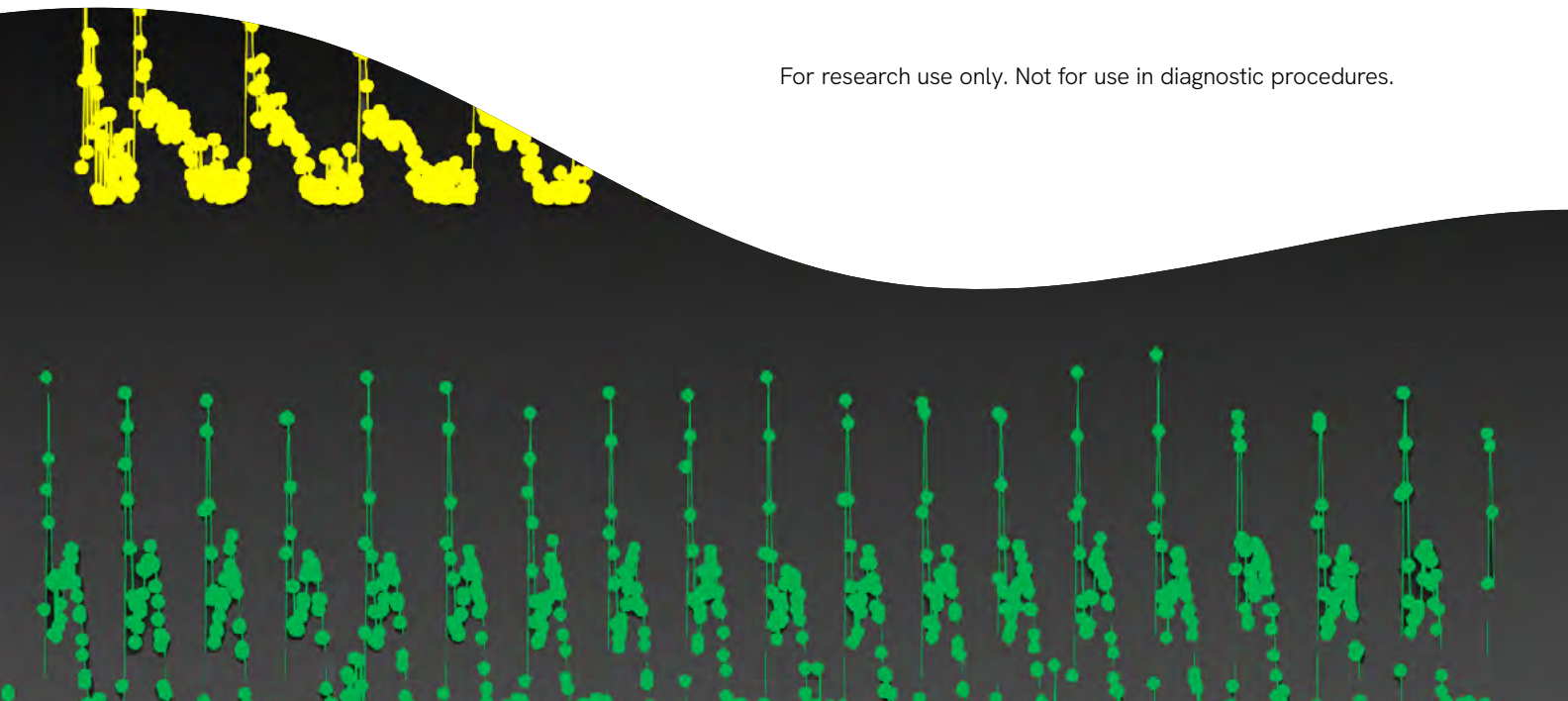


Figure 1: Opera Phenix Plus high content screening system.



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Cardiomyocyte beating assay

Materials and methods

Table 1: List of materials and devices used in cardiomyocyte assay.

Cells	CardioSight®-S hiPSC derived cardiomyocytes (Nexel, # C-002)
Growth medium	CardioSight media # CM-002, complemented with either 50x Maintenance-supplement # CS-001 or 50x Plating supplement # CS-020
Compounds	▪ Epinephrine hydrochloride (Cayman, # Cay21245-1) ▪ Ivabradine hydrochloride (Sigma, # SML0281) ▪ Nifedipine (Sigma, # N7634)
Solvent	DMSO (Sigma, # D2650)
Plate coating	20 µg/mL Human plasma fibronectin (Merck/Sigma, # FC010)
Microplates and sealing	▪ Assay Plate: PhenoPlate™ 96-well (Revvity, # 6055300) ▪ Compound Plate: StorPlate™ 96-well V-bottom (Revvity, # 6008290) ▪ Compound Plate Sealing: Pierce Heat Seal, 4titude, # 4ti-0531
Pipettor tips	200 µL Conductive tips sterile (Revvity, # 6001250)
Imaging instrument (Figure 1)	Opera Phenix® Plus high content screening system (Revvity, #HH14001000)

Cell culture

CardioSight®-S human induced pluripotent stem cell derived (hiPSC) cardiomyocytes were thawed, plated, and maintained according to the manufacturer's instructions with minor adaptations. 1.2 E5 cells in 200 µL plating medium per well were seeded into fibronectin-precoated PhenoPlate™ 96-well microplates. Medium was exchanged to maintenance medium one day post plating and every other day. On the experimental day, medium was renewed 3h before the experiment started. The hiPSC derived cardiomyocytes formed spontaneously and synchronously beating monolayers from day three onwards. Treatment experiments on the Opera Phenix® Plus system were performed between day three to six post plating.

Preparation of compound plates

Compounds were diluted in maintenance medium and provided in a StorPlate™-96V (100 µL per well). To minimize evaporation and for light protection, compound storage plates were heat-sealed with pierceable aluminium foils. Compound plates were preequilibrated inside the Opera Phenix Plus system for 30 minutes.

Monitoring of cardiomyocyte maturation

To find the optimal day to start compound experiments, plated cells were closely observed on a live-cell microscope in regular intervals for cell attachment, confluency, and contractility of the monolayer.

Automated on-board liquid handling and image acquisition

The compound plate and assay plate were transferred to a temperature, CO_2, and humidity equilibrated Opera Phenix Plus high-content screening system. Images were acquired in nonconfocal mode using a 20x long-working distance air objective. A time series measurement was set up in well-repeat mode using the brightfield channel with 5 ms exposure time, one field of view and one plane.

The experimental setup consisted of three sequences and one pipetting step between the first two sequences (Figure 2). The first sequence (baseline) included 1000 time points (tps) at 100 frames per second (fps), followed by an automated pipetting step transferring 20 µL 11x concentrated compound solution into the corresponding well of a 96-well assay plate. Then, a second imaging

sequence was used for incubation, containing two acquisitions with a 3-minute interval followed by the third and last sequence consisting of 3000 tps at 100 fps to capture the compound effect.

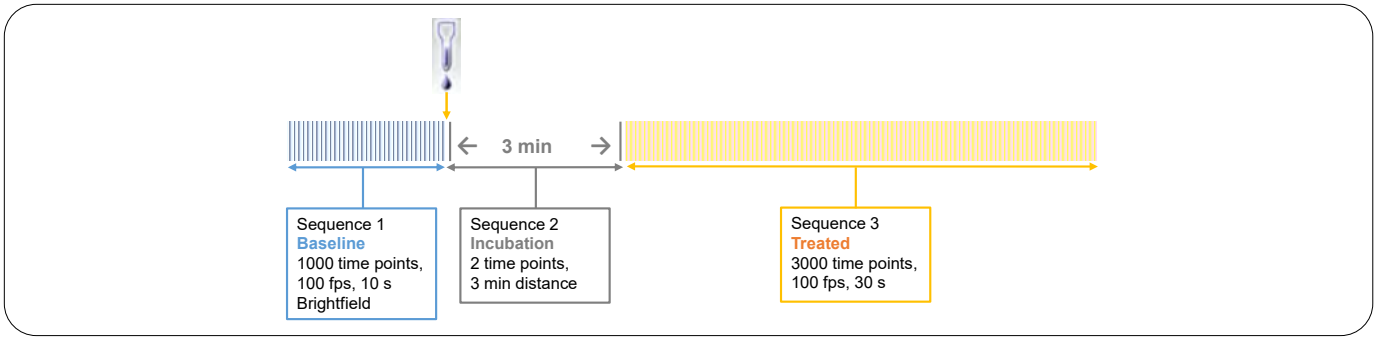


Figure 2: Image acquisition sequences: baseline beating frequency was recorded for 10s at 100 fps in the brightfield channel followed by an automated pipetting step transferring 20 µL into 200 µL (96-well). A second sequence of two measurements at a 3-minute distance was created to incubate the compound for 3 mins before measuring the beating frequency of treated cells for 30s at 100 fps in the brightfield channel.

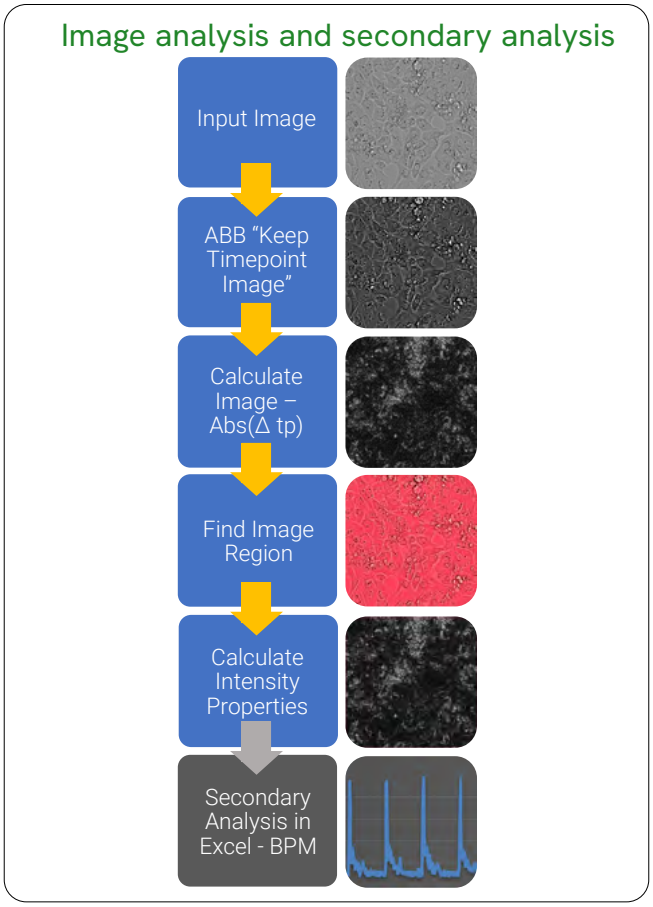


Figure 3: Image analysis sequence in the Harmony® high-content analysis software using the Keep Timepoint Image assay specific building block. Keeping the image of the previous timepoint (tp) in a time series, enables the per-pixel calculation of the absolute difference in the brightfield channel between two time points using Calculate Image. The whole image region is then subjected to a Calculate Intensity Properties building block, from which the mean result is plotted over time to analyze the beating frequency in beats per minute (BPM) in a secondary analysis using Excel.

Results and discussion

Assay development

A control microplate with human iPSC derived cardiomyocytes seeded into a fibronectin coated PhenoPlate 96-well plate was monitored visually over 6 days on a live-cell microscope. As shown in Figure 4, the hiPSC derived cardiomyocytes formed a monolayer within two to three days of culture. Cardiomyocyte contractility began around day three after thawing.

To analyze drug induced changes in the contraction frequency, the cardiomyocytes were imaged on an Opera Phenix Plus system equipped with a pipettor module. As fluorescence imaging at high frequencies might be phototoxic[4] and might affect contraction rates, we used brightfield imaging with a gentle far-red LED as the light source. To prove that brightfield imaging at high frequencies has no detectable effect on cardiomyocyte beating frequency, one well was imaged 12 times for 30s with 100 fps, each interspersed by a 5 min interval. As shown in Figure 5, this imaging setup did not affect the beating frequency.

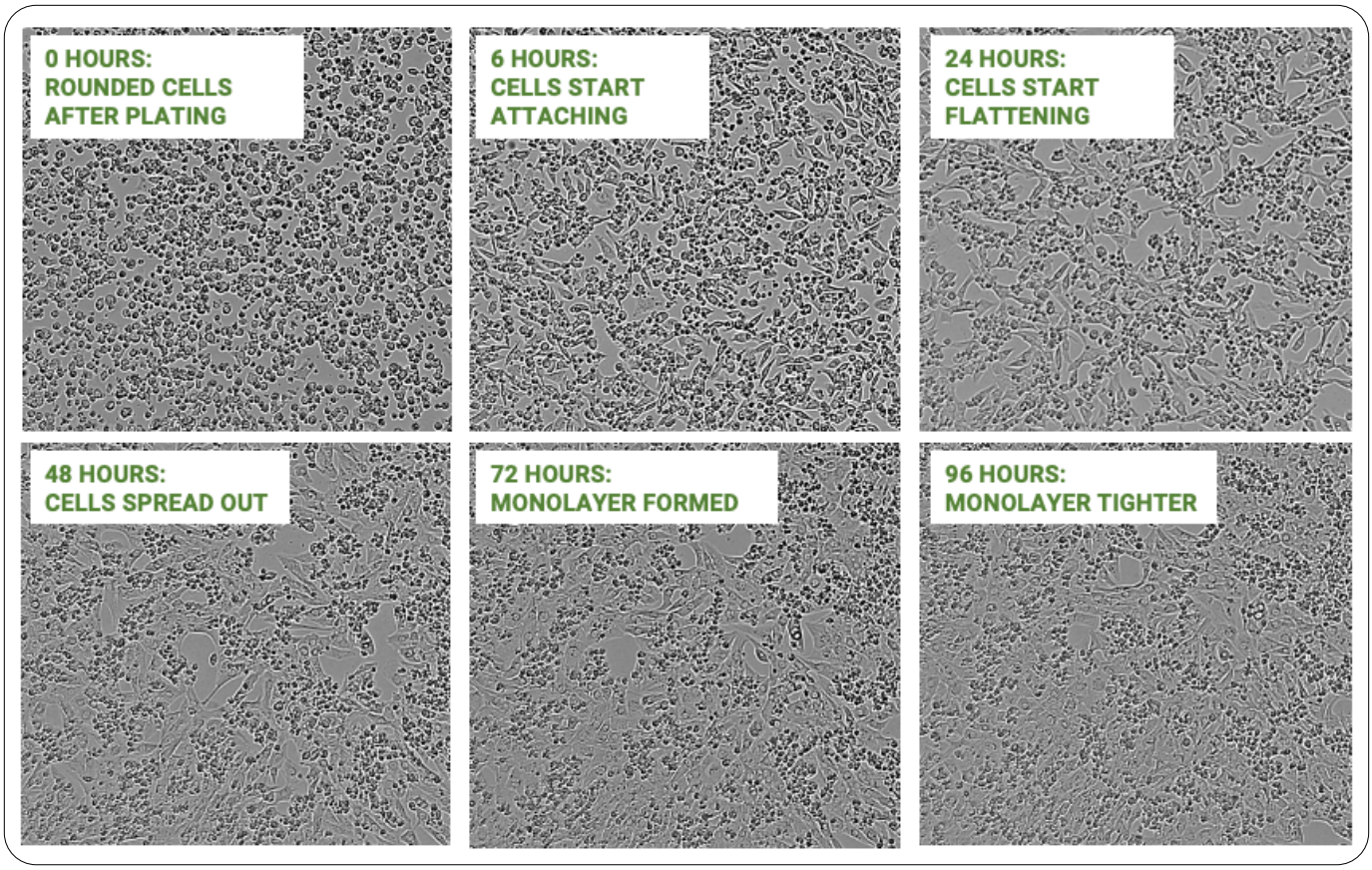


Figure 4: Monitoring of cardiomyocyte attachment and maturation on a live-cell culture microscope (4x objective, brightfield). As part of assay development, cells in a control microplate were closely observed after thawing to determine the optimal timing (day 3 to day 6) for compound experiments conducted on the Opera Phenix Plus system. Here, example images from days 0 to 4 are shown.

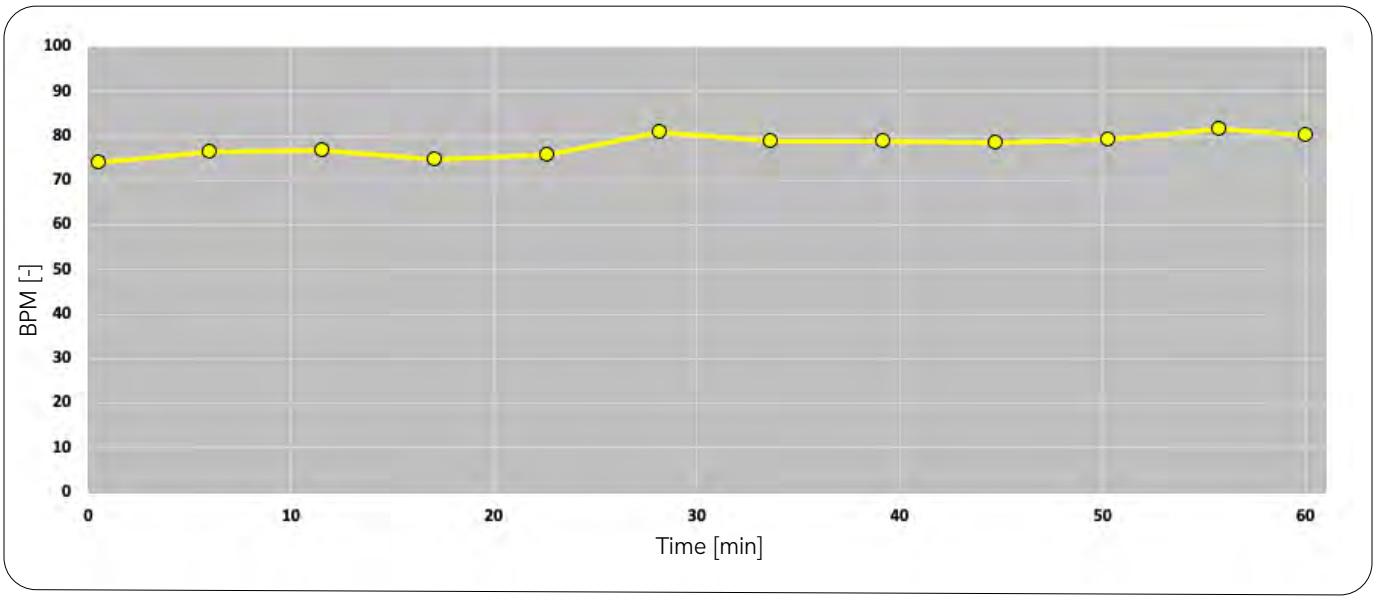


Figure 5: Human iPSC derived cardiomyocytes show a stable beating rate when imaged on the Opera Phenix Plus system. The environmental control option of the Opera Phenix Plus system stably controls temperature, CO_2, and humidity to ensure best possible imaging conditions. As the beating rate of cardiomyocytes can be sensitive to temperature shifts,[4,5] constant environmental conditions are an important prerequisite for cardiotoxicity assays. To analyze the effect of high frame rate brightfield imaging on cardiomyocyte beating, cells were repeatedly imaged for 30s with 100 fps followed by a 5-minute recovery interval.

Compound effects on cardiomyocyte beating rate

As a first compound, epinephrine (adrenaline) was added at different concentrations and its effect on cardiomyocyte beating frequency was analyzed. Epinephrine is supposed to increase cardiomyocyte beating frequency due to activation of adrenoceptors.[6] For each well a baseline sequence was acquired prior to treatment and the epinephrine induced changes were normalized to the respective baseline beating rate.

Figure 6 shows the resulting "heartbeat" curve derived by plotting the image analysis results (mean intensity) over time for baseline, and after addition of 1 µM epinephrine. As expected, epinephrine treatment led to an increase in cardiomyocyte beating rate. The epinephrine dependent increase of the beating rate can also be seen in this video created in the Harmony image analysis software (first 10s: baseline, last 30s: after treatment with 1 µm epinephrine).

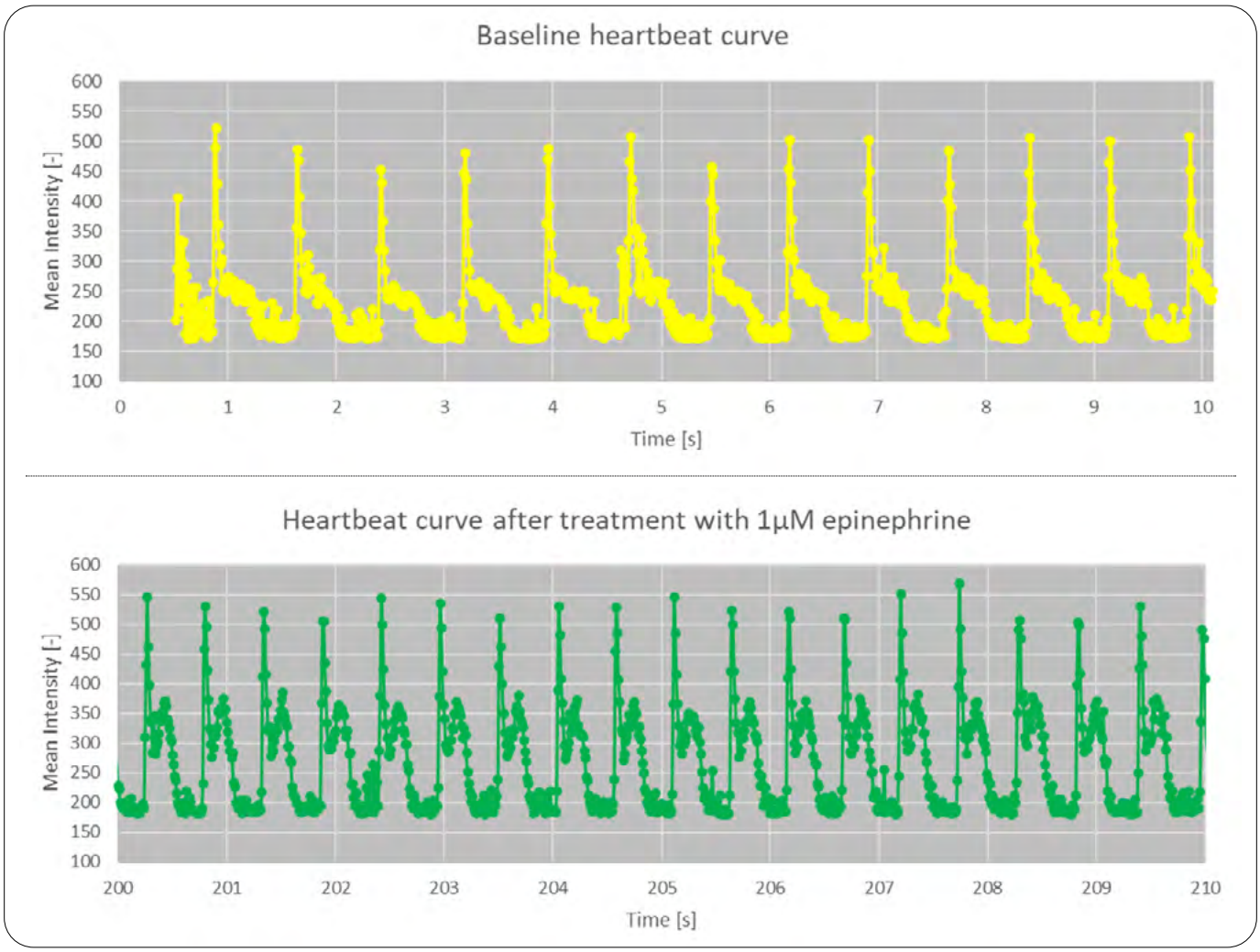
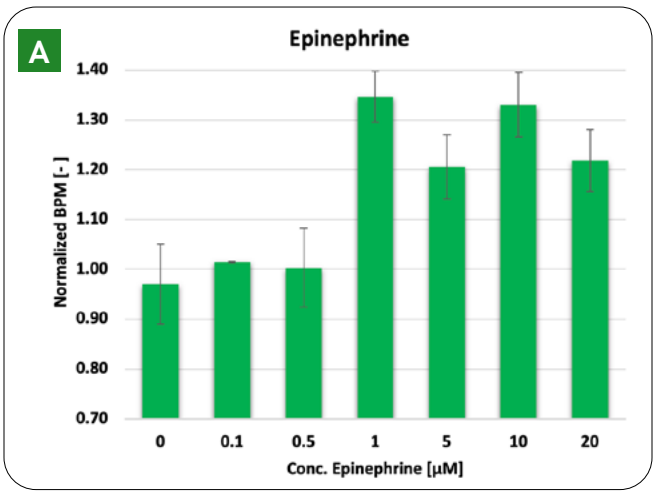


Figure 6: Analysis of cardiomyocyte beating in response to epinephrine using the Harmony analysis software. Epinephrine increases cardiomyocyte beating frequency. "Heartbeat" curves derived by plotting mean intensity shift between two successive images over 10s each are shown for baseline (yellow) and treatment (green).

Image analysis results were exported from the Harmony software and secondary analysis was performed in Microsoft Excel to calculate the beating rate in beats per minute (BPM) for each compound and concentration.

Figure 7A summarizes the effects of different epinephrine concentrations on cardiomyocyte beating rate. The beating frequency is unaltered up to a concentration of 0.5 µM epinephrine and then increases by 35% after addition of 1 µM epinephrine. The addition of ivabradine which blocks ion current and stabilizes the heart beating rate[7] led to a dose dependent decrease of cardiomyocyte beating rate as shown in Figure 7B. However, the contractions did not stop even at ivabradine concentrations of up to 20 µM. In addition, we applied different concentrations of nifedipine which acts on the calcium ion-flux and reduces the heart beating rate.[1] As can be seen in Figure 7C, a treatment with 0.005 µM nifedipine[1] completely eliminated the beating of cardiomyocytes without other detectable changes in overall cell morphology.



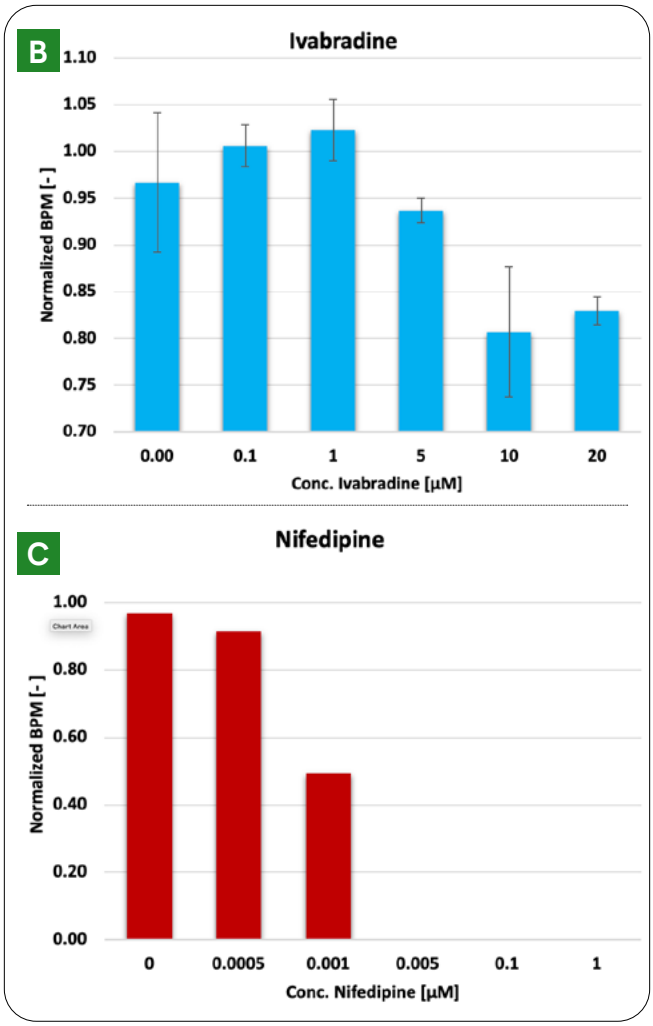


Figure 7: Opera Phenix Plus system equipped with an onboard liquid handling system allows for the quantification of compound dependent changes in cardiomyocyte beating rate. Shown are changes in normalized beats per minute (BPM) in response to three different compounds. Epinephrine (A) increases the beat rate while ivabradine (B) and nifedipine (C) decrease or completely eliminate the cardiomyocyte beating. Epinephrine and Ivabradine N = 3 wells, Nifedipine N = 2 wells, error bars = standard deviations of three replicates.

Conclusions

Reliable and scalable cardiotoxicity assays that keep cardiomyocytes at physiological conditions are very important for drug development.

The Opera Phenix Plus high-content screening system offers fast frame rate imaging of up to 100 fps synchronized with automated on-board pipetting enabling the analysis of compound induced changes in cardiomyocyte contractility. It also precisely controls and keeps temperature, CO_2, and humidity at optimal levels during the assay to prevent negative effects on your biological model systems. The ability to analyze changes in cardiomyocyte beating in brightfield images adds to this, as this imaging modality was shown to not affect cardiomyocyte beating over a time course much longer than the one used to detect compound effects. This is powered by image analysis tools in the Harmony software which make it easy to analyze fast frame rate time series data.

References

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