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Higher throughput *in vitro* cardiac contractility recordings under physiological mechanical conditions

Whitepaper

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Significance

- Translatability of data obtained from hiPSC-CMs to human physiology is the subject of current scientific discussion
- Contractility data derived from hiPSC-CMs in an environment that reflects the mechanical properties of real human cardiac tissue in a higher throughput format (FLEXcyte 96) is physiologically relevant
- Example data on commercially available cell types with the FLEXcyte 96 system show a high degree of consistency with clinical data

1. Introduction

The aim of continued global efforts is to contribute towards the development of next generation assays reducing false positive and false negative results within drug development (e.g. Strauss *et al.*, 2019, Blinova *et al.*, 2018, Bot *et al.*, 2018). Improving patient safety and reducing animal tests while saving time, money and replacing unnecessary *in vivo* experiments with novel assays has a high priority (Breckwoldt *et al.*, 2019, Feric *et al.*, 2019, Tiburcy *et al.*, 2017, Keung *et al.*, 2019, Pfeiffer-Kaushik *et al.*, 2019).

By using human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) in a format beyond 2D monolayers, common issues with the translatability of pharmacological results from animal models to human physiology could be avoided. A lack of three dimensional (3D) physiological environments could explain why 2D hiPSC -CMs are considered immature (Wu and Hochedlinger, 2011, Denning *et al.*, 2016, Veerman *et al.*, 2015). Multiple studies have demonstrated that the phenotypic maturity of hiPSC-CMs can be improved by imitating physiological environments such as 3D matrices and structured scaffolds (Liau *et al.* 2011, Ribeiro *et al.*, 2017), co-culture with non-myocyte supporting cells (Zhang *et al.*, 2013, Nunes *et al.*, 2013, Eng *et al.*, 2016) or electrical pacing or mechanical conditioning upon elastomeric scaffolds (Hazeltine *et al.*, 2012, Hirt *et al.*, 2014, Eng *et al.*, 2016, Ruan *et al.*, 2016). Nonetheless, the discovery of key upstream drivers of cardiomyocyte maturation and combining these within one single assay remains a challenge (Mills *et al.*, 2017). 3D egineered heart tissues (Lemoine *et al.*, 2017, Breckwoldt *et al.*, 2019, Feric *et al.*, 2019, Tiburcy *et al.*, 2017, Keung *et al.*, 2019, Pfeiffer-Kaushik *et al.*, 2019), 3D hiPSC-CM co-cultures in a plate-based format (Burnham *et al.*, drafted manuscript) or hiPSC-CMs as cultured on flexible, hence increased physiological relevant substrates (Gossmann *et al.*, 2016), all represent innovative ways to achieve this.

Traditional systems for the quantification of cellular contraction rely on animal-based models, time-consuming experimental setups or indirect approaches. Integration into standard lab procedures remains a challenge for current *in vitro* systems. In this study, we augmented the well-established CardioExcyte 96 platform (Nanion Technologies) for cardiac contractility assessment in physiological cellular environment by developing the FLEXcyte 96 technology. The FLEXcyte 96 is available as an add-on for the CardioExcyte 96 system. Cells are cultured in a 96-well format on ultra-thin silicone membranes, forming a biohybrid material with physiologically relevant mechanical properties. While being deflected by the weight of the culture medium, rhythmic contraction of the cardiomyocytes lifts the membranes upwards.

In this manuscript we show that the flexible environment leverages maturation of hiPSC-CMs, thereby providing a reliable model for adult ventricular function. The pro-maturation environment enables observation of inotropic and chronotropic compound effects, which are typically hard to detect with 2D monolayers on overly stiff substrates. Furthermore, we show predictive recordings of contractile behavior in the presence of well known reference compounds.

The CardioExcyte 96 device supports highly resolved impedance-based measurements, MEA- like extracellular field potential (EFP) and viability recordings. The system is used for contractility and electrophysiology measurements of beating monolayers of cells and for impedance-based studies of a variety of other, non-contractile, cell types (McSweeney *et al*., 2019, Chavali *et al*., 2019, Cai *et al*., 2019, Wang *et al*., 2019, Meents *et al*., 2018, Kondrashov *et al*., 2018, Pugsley *et al*., 2018, Wang *et al.*, 2018, Bot *et al*., 2018, Obergrussberger *et al.*, 2016, Doerr *et al*., 2015). The FLEXcyte 96 add-on enlarges the application area of the setup by allowing the generation of physiologically relevant contractility data.

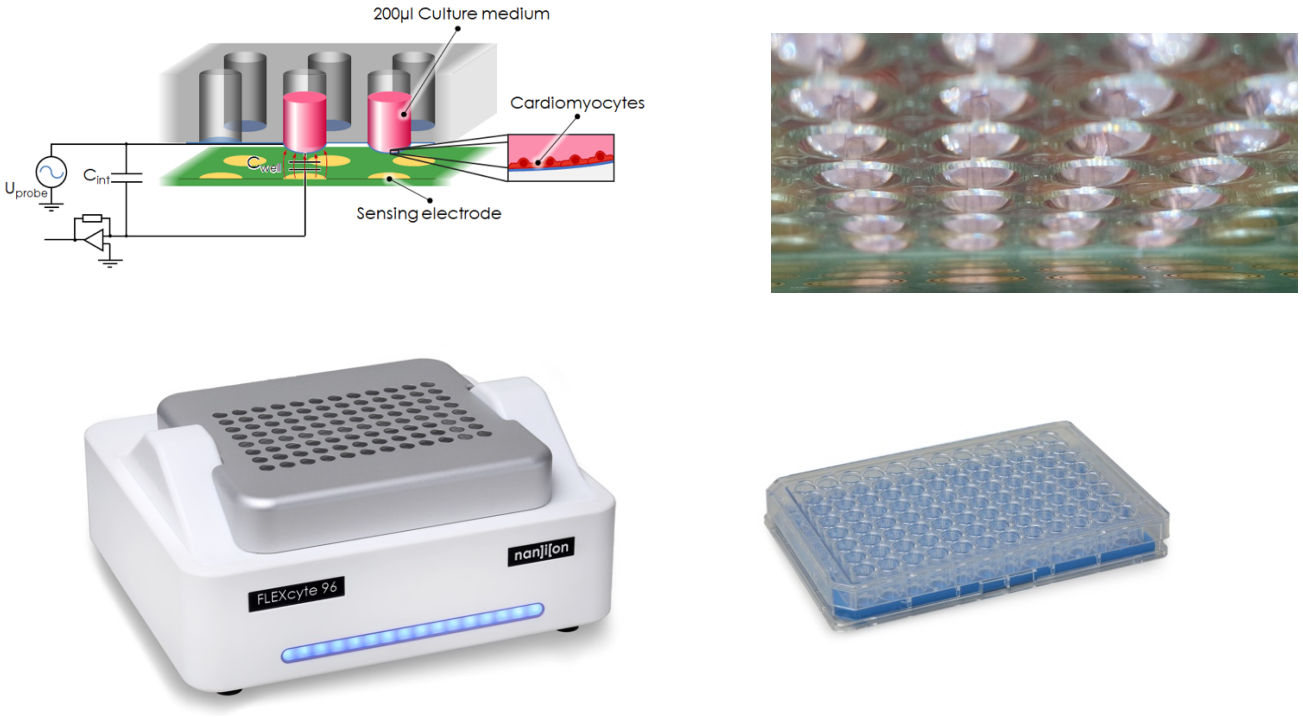


Fig. 1 Schematic graphical display of a portion of the FLEXcyte 96 consumable (top left), FLX-96. Flexible silicone membranes serve as substrates for the cardiomyocytes. The movement of the membranes due to synchronized cell beating is sensed by electrodes via capacitive signals. Photographic display of downward deflecting silicone membranes of the FLX-96 membrane plate (top right). The FLEXcyte 96 system (bottom left) is a benchtop device with integrated incubation system. Bottom right: Consumable, FLX-96 membrane plate.

2. Basic technological aspects: "Gain of confidence" and the potential of adopting FLEXcyte 96 technology in early drug discovery

The key to wider acceptance of new assay platforms is the production of validation data. The reliability and relevance of the FLEXcyte 96 needs to be established for a defined purpose with known predictivity by comparative testing of relevant well characterized drugs. Just like Haddrick & Simpson state for Organ-on-a-chip technology (Haddrick & Simpson, 2019), regulatory qualification is drug or model approval within a stated context of use. For example, the results from a specific model can be relied upon as decision-making data, but only within acknowledged boundaries or limitations of the data.

First of all, the following more "technical" considerations were used to create prerequisites for acceptance of the assay within a broad application area, aiming for an adoption of the technology in early drug discovery, putatively with a potential to replace a number of animal tests.

1. The use of electronic components in our system allows for a higher miniaturization of the sensors and thus allows for a simplified setup and handling, e.g. using the standard 96-well format.

2. The cost per sensor is reduced by eliminating optical elements for readout.

3. Through the purely electronic data readout process, parallelization can take place at a basic level in terms of data integration and processing.

The process takes into account advantages such as higher parallelization in comparison to optical methods as used in alternative approaches (Breckwoldt *et al.*, 2019, Feric *et al.*, 2019, Tiburcy *et al.*, 2017, Keung *et al.*, 2019, Pfeiffer-Kaushik *et al.*, 2019).

The goals of the FLEXcyte 96 assay are to be more predictive and biologically relevant compared with the technologies/ approaches that it putatively replaces in early drug discovery, coupled with providing a cost-efficient and reliable yet easy-to-operate system with a higher throughput using standard 96-well dimensions. The data generated on FLEXcyte 96 are readily quantifiable. For data handling, we used acquisition and analysis software packages which are already established in high throughput systems (HTS; SyncroPatch 384 /768i) and CardioExcyte 96 systems. These software programs have been designed for the readout and analysis of 96, 384 or 768 parallel recording channels. Supplemental figures 1 and 2 display an exemplary analysis routine. The reliability of data handling and optimized ease-of-use is well established (e.g. Kang *et al.*, 2019). Furthermore, in line with the *open data formatting* subteam of the CiPA initiative, the data output of the software packages is currently being adapted to the FDA (US Food and Drug Administration) -required formatting according with the ongoing proceedings of the initiative.

3. Methods

A key factor of the FLEXcyte 96 technology is a well-defined, approximately isotropic and homogeneous biomechanical cell tension environment (the basic, predecessor *CellDrum* method has been described in Gossmann *et al.*, 2016). The cells are cultured on flexible, 6 µm thin silicone membranes sealing the bottom of a 96-well plate. Due to the weight of the cell culture medium the membrane deflects downwards. The inner side of the membrane is covered with a monolayer of hiPSC-CMs. Mechanical rhythmic cell contraction and relaxation of the auto-contractile cardiomyocytes lifts the membrane up and down, respectively. The changes in deflection is measured via capacitive current, the mechanical stress is calculated by Laplace's Law.

The software package is assisting the user with the preparation of experiments, management of acquired data, and the evaluation and export of experimental results (in more detail described in Doerr *et al.*, 2015). The user can choose and configure several analysis parameters (see Fig. 2 and Doerr *et al.*, 2015), which are applied to the raw data right after recording and visualized in online analysis graphs progressing with the ongoing experiment. Parameters such as amplitude, beat rate, pulse width, etc., and, in addition, "primary" and "secondary" beats picturing arrhythmic events, can be distinguished.

As assistance in experiments including addition of compounds, the software allows virtual additions of pre-defined substances with specific concentrations (Doerr *et al.*, 2015). Markers are added to the measurement file, which can then be used for tracking the additions and for compound- and concentration-related data evaluation. This also includes elaborate IC_{50} analysis and calculation of dose-response curves.

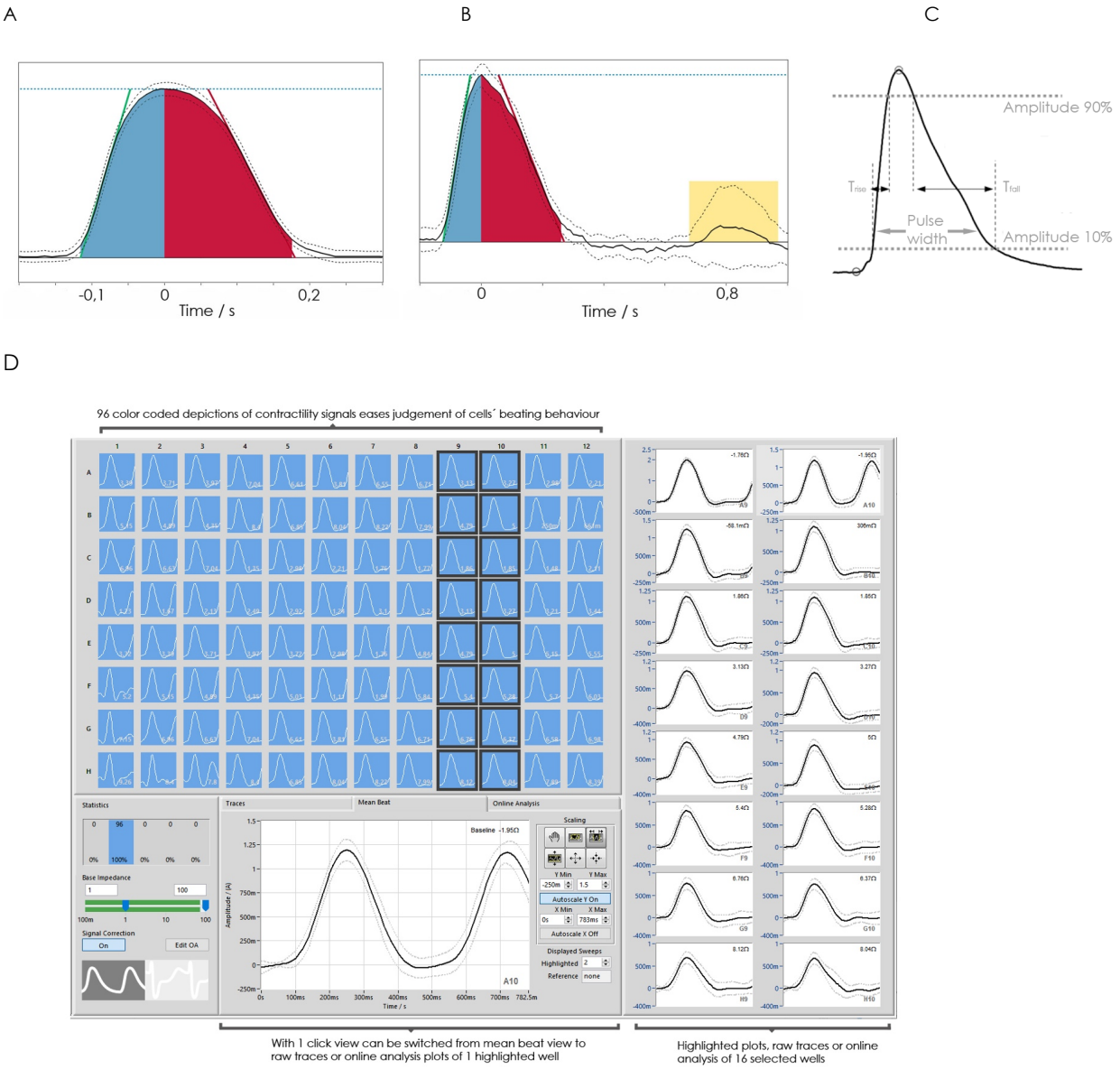


Fig 2. An adaptive signal detection algorithm extracts the positions and values of beating events. (A – C) Parameters such as beat intervals, amplitudes, rising and falling time are detected along with pulse widths and beat rate. Furthermore, integrals (blue and red, for contraction and relaxation) and (B) arrhythmia (yellow) are identified and quantified. Dotted lines represent standard deviation of mean signal shapes. (D) Graphical user interface during recordings. Contractile behavior of hiPSC-CMs (here Cardiosight-S®). Overview of mean beats including standard deviations from all 96 wells. Thus, arrhythmia or rhythmic beating can be judged at a glance.

4. Contractility data reproduce expected responses to reference compounds

The FLEXcyte 96 technology allows for precise quantification of drug actions on 96 samples at a time under defined auxotonic mechanical conditions. Mechanical contraction can be characterized with deep insight into the shape of the contraction-relaxation-cycle.

Acute compound effects: Positive inotropes such as isoproterenol, a beta-adrenoceptor agonist, typically evoke chronotropic rather than inotropic responses in 2D iPSC-CMs, which may reflect the relative underdevelopment of SR compared to sarcolemmal Ca^{2+} handling (Yang *et al.*, 2014). In Cardiosight-S® hiPS-CMs, isoproterenol showed an approx. 1.9 fold increase in contractile force at a concentration of 3 µM. (Fig. 3). Upon application of increasing isoproterenol concentrations (30 nM – 3 µM) an increase in beat rate is

observed, along with an increased beat amplitude. Fig. 4 displays the positive force-frequency relationship after 15 min incubation time. Next, we tested digoxin at concentrations of 1 nM, 10 nM, 100 nM, 1 µM and 10 µM, again using Cardiosight-S® hiPSC-CMs. Digoxin's primary mechanism of action is the ability to inhibit membrane-bound alpha subunits of sodium-potassium ATPase (sodium pump), mainly but not exclusively located in the human myocardium. This inhibition promotes sodium-calcium exchange, which increases the intracellular calcium concentration that is available to the contractile proteins, resulting in an increase in the force of myocardial contraction (Gheorghiade *et al.*, 2004). Digoxin toxicity is commonly associated with serum levels >2 ng/mL = 2.562 nmol/L (Dec, 2003), therapeutic levels such as 1.68 ng/ml (Reinbach *et al.*, 2003) are hence very close to toxic levels. We observed a beat rate (suppl. Fig. 1) and beat amplitude (Fig. 5) increase upon increasing concentrations up to 1 µM. At concentrations above 1 µM toxic effects did overrule the inotropic effect: at timepoints t = 30 and 60 min a cessation of beating was seen at 1 µM and 10 µM.

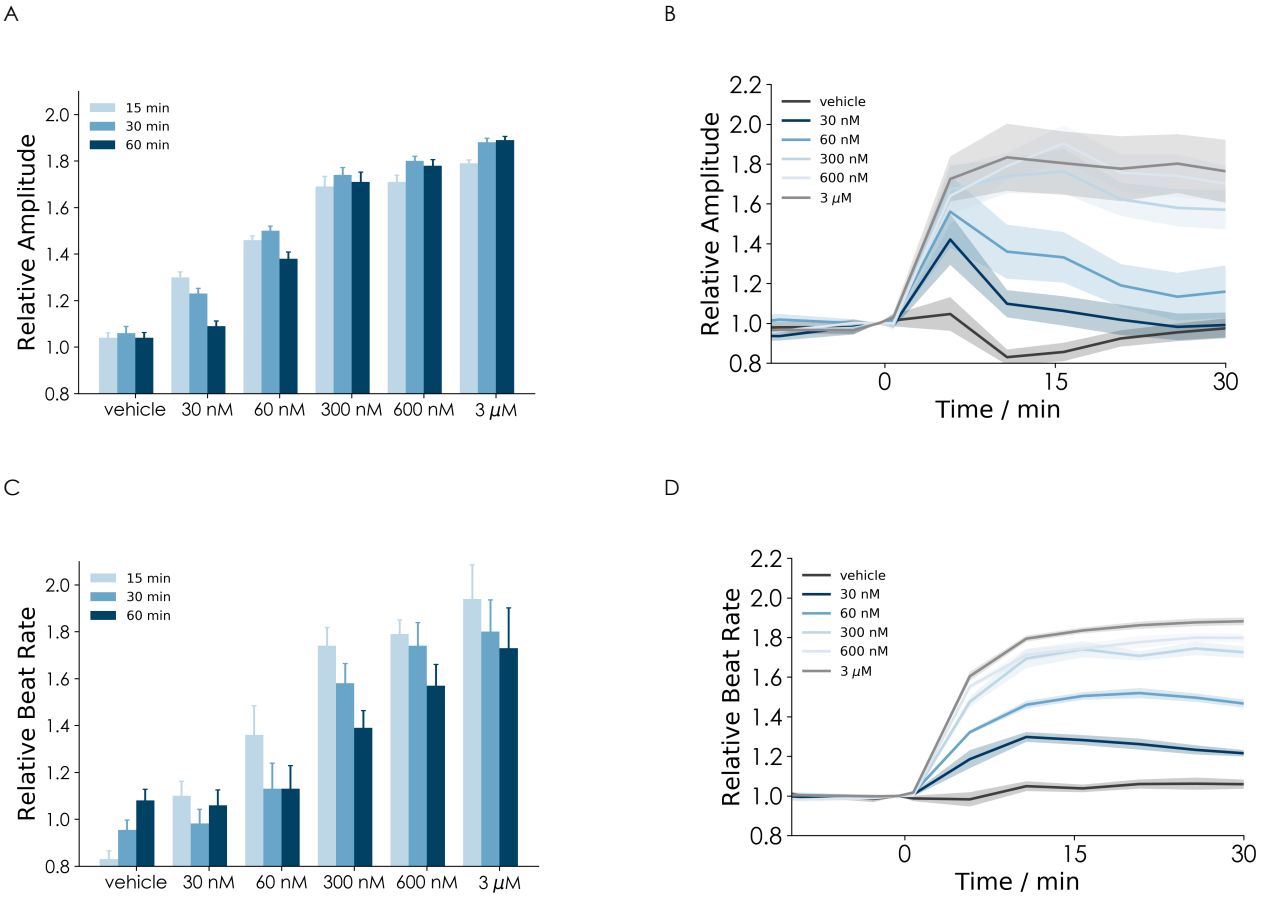


Fig. 3 Isoproterenol enhances contractile force in hiPSC-CMs (here Cardiosight-S®). (A) Relative beating amplitude at timepoints 15 min, 30 min and 60 min after vehicle or compound addition, plotted against the applied increasing concentrations of isoproterenol (30 nM – 3 µM). (B) Relative beating amplitude plotted against time. At timepoint "0", the isoproterenol concentrations (lines in shades of blue and grey represent different concentrations) were added to the wells. (C) Relative beat rate at timepoints 15 min, 30 min and 60 min after vehicle or compound addition, plotted against the applied increasing concentrations of isoproterenol (30 nM – 3 µM). (D) Relative beat rate plotted against time. At timepoint "0", the isoproterenol concentrations (lines in shades of blue and grey) were added to the wells. Envelopes of lines display standard error of the mean. Error bars: SEM, n = 6 – 8.

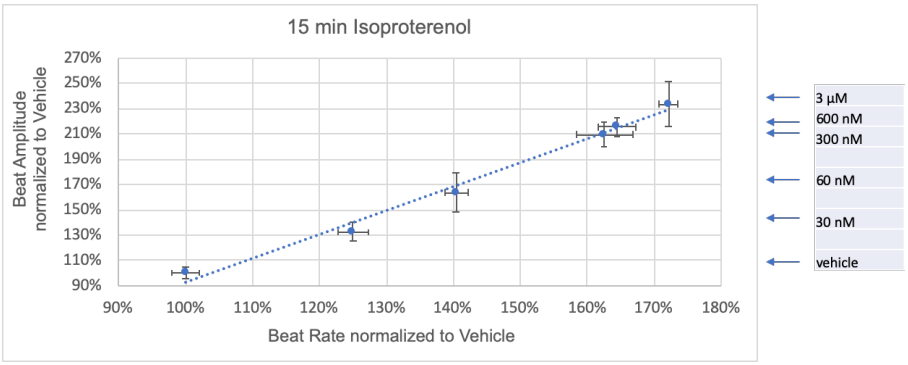
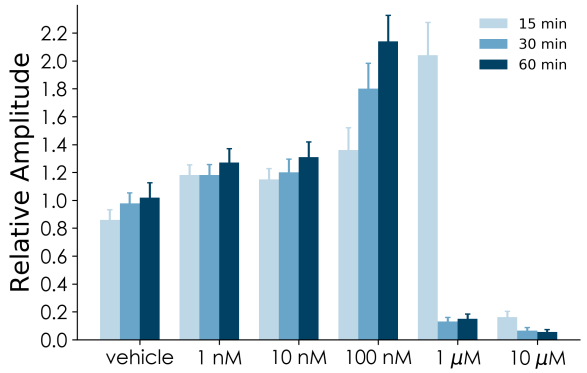


Fig. 4 iPSC-CMs show a positive force-frequency relationship. The graph is based on data from Cardiosight-S® hiPSC-CMs as seen in Fig. 3, at t = 15 min after isoproterenol application (30 nM, 60 nM, 300 nM, 600 nM and 3 µM).

Chronic compound effects: With the FLEXcyte 96 system, measurements for 5 days and longer are possible, revealing long-term effects on hiPSC-derived cardiomyocytes. Tyrosine kinase inhibitor gefitinib shows significant functional cardiotoxic effects during sub-chronic exposure in iCell CM² iPSC-CMs (Fig. 6). Both beat amplitude and rate decrease nearly linearly over the observed time frame of 120 hours at a gefitinib concentration of 10 µM. The beat amplitude decreases from 70% after 2 hours to 20% after 120 h, while the beat rate decreases from 70% to 40% in the same time frame.

A



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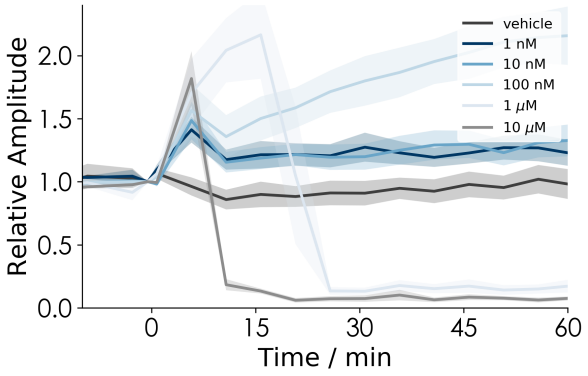

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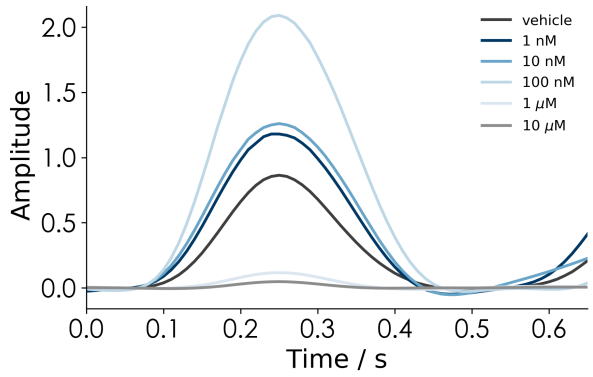

Fig. 5 (A, B) Digoxin at 1 nM – 10 µM, development of beating amplitude over 60 min, Cardiosight-S® hiPSC-CMs. (C) Overlay of mean beats at t = 60 min after digoxin application, shades of blue and grey represent vehicle or different concentrations. Error bars: SEM, n = 6 – 8.

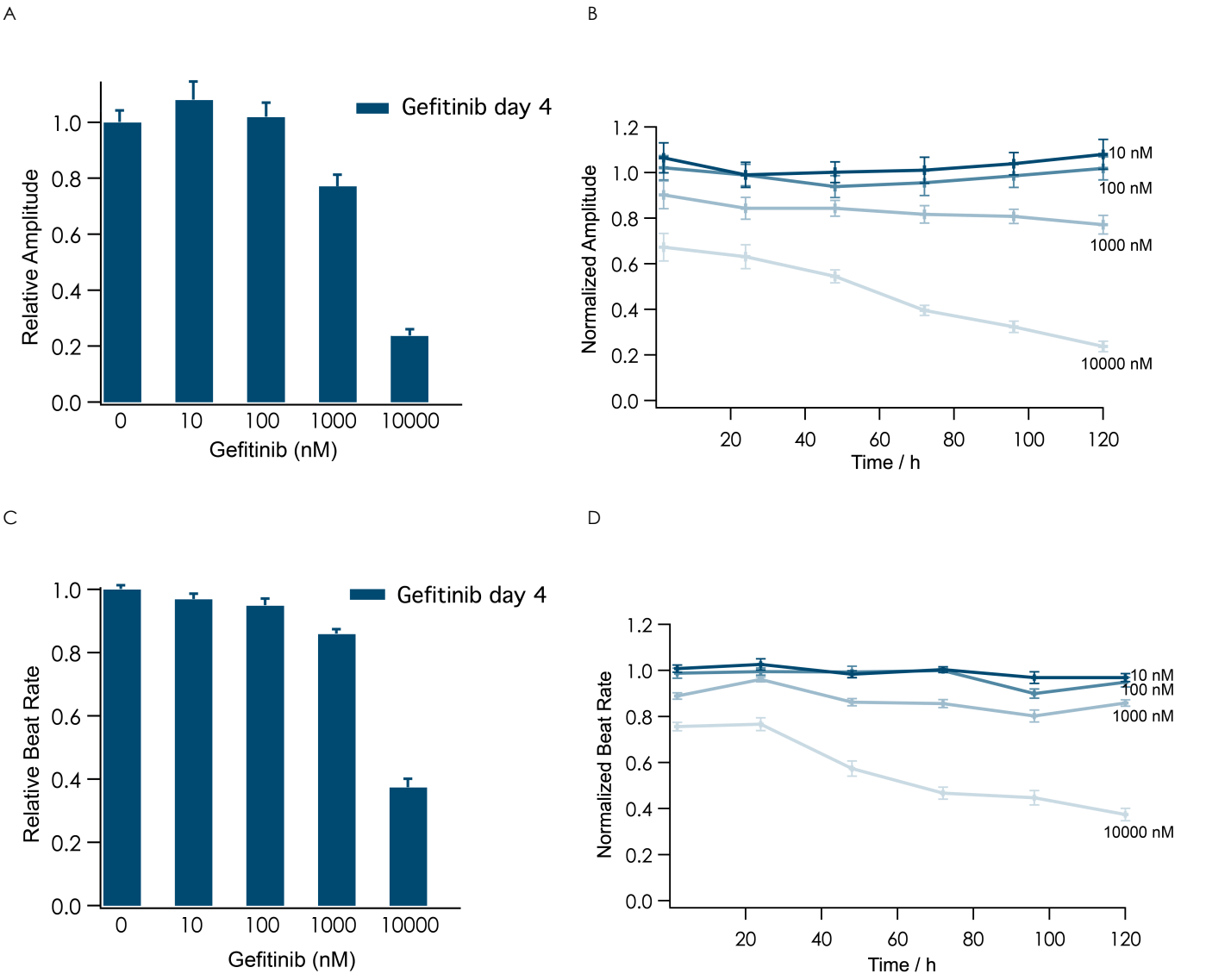

Fig. 6 (A) Gefitinib after 4 days of chronic incubation, increasing concentrations plotted against the beating amplitude as normalized to control. (B) Development of beating amplitude depending on the concentration (10 nM – 10000 nM). Shades of blue represent different concentrations. (C) Gefitinib after 4 days of chronic incubation, increasing concentrations plotted against the beating rate as normalized to control. (D) Development of beating rate depending on the concentration of gefitinib. Here, iCell CM² iPSC-CMs were used.

5. Pacing of hiPSC-CMs and combined investigation of contractility, extracellular field potentials and structural or morphological changes

Despite the well established predictive power of iPSC-CMs in drug safety assays (e.g. Obergrussberger *et al.*, 2016,) there are challenges to overcome due to relative immaturity of the cells before they can be utilized as an all-round predictive model for drug safety assays. Ruan *et al*. showed that pacing at physiological heart rates can improve functionality of hiPSC-CMs and responses to inotropic compounds (Ruan *et al.*, 2016).

The basic system CardioExcyte 96 offers for two independent pacing options. Electrical pacing via stimulus electrode or the stimulating optical lid, CardioExcyte 96 SOL, which uses LEDs for spatially uniform stimulation of cells transfected with light-gated ion channels such as Channelrhodopsin2 (ChR2) (Rehnelt *et al*., 2017).

The SOL can also be used with the FLEXcyte 96 setup. After transfection of hiPSC-CMs with ChR2 the cells can be stimulated by blue light. Every flash of light induces depolarization of the cells and thus an action potential is triggered. The advantages of optical stimulation over electrical stimulation include the highly precise timing: all cells of the beating network are stimulated exactly at the time of the light stimulus. In contrast, electrical stimulation propagates from an electrode across the well and thus cells are stimulated progressively. A mean beat calculation of precisely timed beats enables in-depth compound analysis and concentration response dependencies to be obtained.

6. Has FLEXcyte 96 the potential to replace animal tests?

Drug screening efforts with cardiac cells owning physiologically relevant mechanical properties and contractility as requested endpoint can be addressed with the FLEXcyte 96. The fact that readily available hiPSC-CMs are used, in addition to the 96-well throughput capability and the cost- and time-effective data generation could allow laboratories to refrain from using whole organs prepared from animals. Furthermore, organ specific, circulatory, metabolic or stress effects as present in whole organ studies are prevented. Whole organ studies are still required e.g. in infarct size studies or other approaches where the whole organ is investigated holistically.

The FLEXcyte 96 method is suitable for gaining positive force-frequency relationships, observing positive and negative inotropic effects and quantifiying contraction force. The system provides a tool to measure real contractility on 96 samples simultaneously. This enables the testing of drug candidates at early stages on human cardiac tissue. The multi-parameter analysis detects alterations in beat shape as well as frequency and arrhythmic events and provides a quantitative measure for these.

Computational models of drug actions have been implemented in the data analysis and successfully tested against inotropic and chronotropic effects (Gossmann *et al*., 2016). Data are relatable to *in vivo* data by translational modelling (Frotscher *et al.*, 2015 I, Frotscher *et al*., 2015 II, Frotscher et al., 2016, Tuan Duong *et al*., 2016).

7. Summary

During the approval process of new drugs, many lead candidates fail in the late stages of development mainly due to drug induced injury of organs. Impedance analysis of mammalian cells grown on planar film electrodes provides a label-free, non-invasive and unbiased observation of cellular properties addressing the biological response to drugs, toxins or stressors in general (Reiss & Wegener 2015). These approaches are tempered by the observation that hiPSC-CMs typically retain a fetal-like phenotype when the environment is not appropriate for physiologic development, i.e. regarding the mechanical environment. To address this, Nanion Technologies and innoVitro have developed a non-invasive, label-free, higher throughput device & assay for monitoring contractility of hiPSC-CMs in a more physiological environment.

We conclude that the FLEXcyte 96 system provides a versatile tool for *in vitro* cardiac contractility measurements addressing the demands for both physiological relevance and throughput.

Acknowledgements

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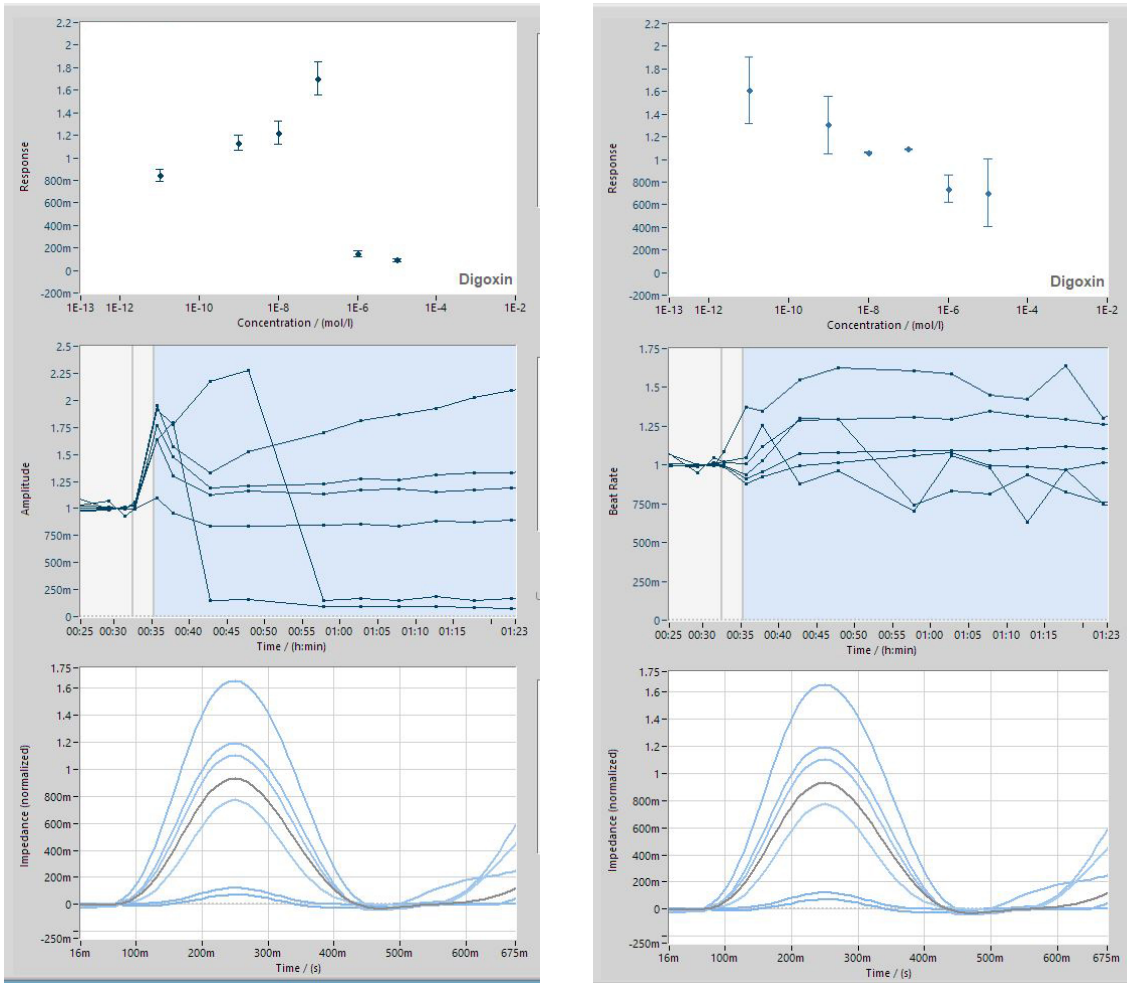
Supplement

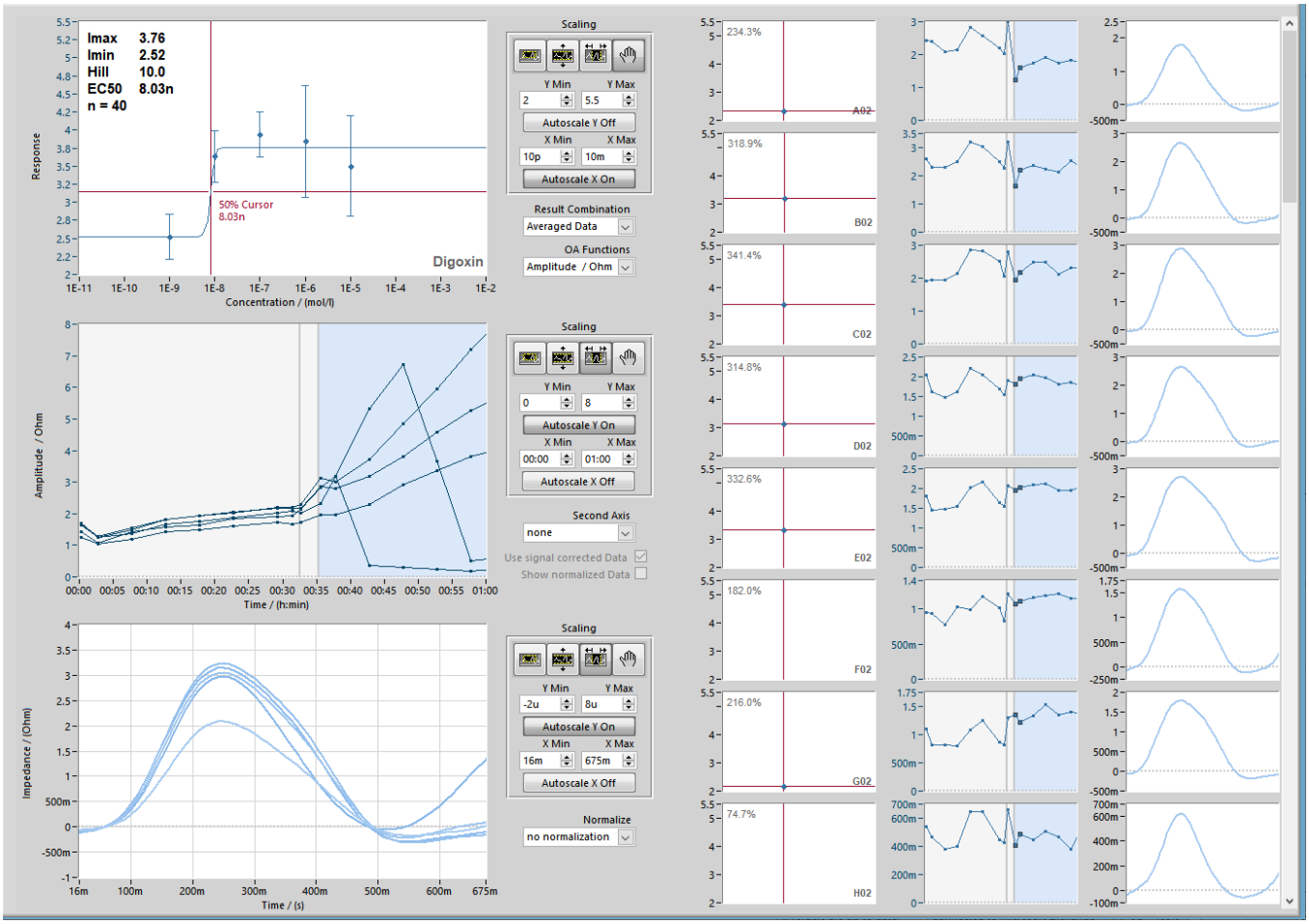
Supplemental figures 1 and 2 represent a typical layout of the analysis routine in *DataControl* 96. This software package comes along with every CardioExcyte 96 /FLEXcyte 96 system.

Getting the most out of data generated on higher throughput platforms and access to data are important considerations for companies and institutions. *DataControl*, the analysis software developed by Nanion and provided with the CardioExcyte 96, SyncroPatch 384/768PE and SURFE2R platforms, offers a multitude of analysis parameters to get the most out of safety and drug discovery data. The software is high-capacity yet easy to use and can be used for every stage of knowledge discovery. Key advantages are:

- The license provided with Nanion´s platforms is not user limited.
- *DataControl* can be installed on as many computers as needed by the user.
- Collaborative work in a department or beyond is made easy: define who can access the data, replay data and perform analysis.
- Analysis templates can be stored and shared to ensure consistency.



Supplemental Fig. 1 Compound effects as displayed in the *DataControl* software, an exemplary analysis routine for FLEXcyte 96 data. Concentration response relations (top windows; left: Amplitude response and right: Beat rate response, both plotted against compound (here digoxin) concentrations). Middle: Plots of parameters of choice, here beat amplitude (left, middle) or beat rate (right, middle) vs. time. In addition, raw data traces are displayed and overlayed (bottom).



Supplemental Fig. 2 Digoxin concentration response curves, mean IC_{50} calculation. An increase of beating amplitude in a concentration dependent manner can be observed up to a concentration of 1 µM. Above that, cytotoxic effects overrule positive inotropic effects.