Chapter 20

Biomechanical Characterization of Human Pluripotent Stem Cell-Derived Cardiomyocytes by Use of Atomic Force Microscopy

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Abstract

Atomic force microscopy (AFM) is not only a high-resolution imaging technique but also a sensitive tool able to study biomechanical properties of bio-samples (biomolecules, cells) in native conditions—i.e., in buffered solutions (culturing media) and stable temperature (mostly 37 °C). Micromechanical transducers (cantilevers) are often used to map surface stiffness distribution, adhesion forces, and viscoelastic parameters of living cells; however, they can also be used to monitor time course of cardiomyocytes contraction dynamics (e.g. beating rate, relaxation time), together with other biomechanical properties. Here we describe the construction of an AFM-based biosensor setup designed to study the biomechanical properties of cardiomyocyte clusters, through the use of standard uncoated silicon nitride cantilevers. Force-time curves (mechanocardiograms, MCG) are recorded continuously in real time and in the presence of cardiomyocyte-contraction affecting drugs (e.g., isoproterenol, metoprolol) in the medium, under physiological conditions. The average value of contraction force and the beat rate, as basic biomechanical parameters, represent pharmacological indicators of different phenotype features. Robustness, low computational requirements, and optimal spatial sensitivity (detection limit 200 pN, respectively 20 nm displacement) are the main advantages of the presented method.

Key words Atomic force microscopy, Biomechanical characterization, Human stem cell, Cardiomyocyte contraction, Drug testing

1 Introduction

Cardiomyocyte contraction and relaxation are important cardiac functional parameters, undergoing alteration in most cardiac muscle pathologies. Often, these heart diseases have hidden causes at the cellular and genetic levels [1, 2] not clinically distinguishable and moreover inaccessible for diagnostics, as heart biopsy is an invasive procedure with significant risk, often giving only

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Nuno C. Santos and Filomena A. Carvalho (eds.), *Atomic Force Microscopy: Methods and Protocols*, Methods in Molecular Biology, vol. 1886, https://doi.org/10.1007/978-1-4939-8894-5_20,

morphological information [3, 4]. Recently, human cellular models became available, partly derived from human embryonic stem cells (hESC) [5] and disease-specific induced pluripotent stem cells (hiPSC), collectively called human pluripotent stem cells (hPSC) [6]. The essential stage is the differentiation into cardiomyocytes monolayers or clusters (three dimensional embryoid bodies, EBs) [7]: progenitor and maturing cells are important in drug and disease screening (reviewed in refs. 8, 9). The analysis of cardiomyocytes contractility phenotype represents an important approach in drug development and disease modeling [10]. Dynamic beating force and rate of the EBs contractions can be robustly monitored by micromechanical transducers, creating a platform for cell-based biosensors (CBB). These devices represent a novel powerful tool to understand and specifically treat various human diseases, including heart-related ones [9].

We constructed a sensitive and robust setup that employs uniformly sized EBs containing human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs), combined with an atomic force microscopy (AFM) force sensing platform, to measure biomechanical properties (absolute values of contractile forces together with the beat rate) in complex conditions and cellular models.

2 Materials

2.1 BioAFM Microscope

A standard BioAFM microscope JPK NanoWizard 3 (JPK, Berlin, Germany) with no custom modifications is used for all experiments, including proprietary software environment (Fig. 1). A motorized stage combined with a JPK Petri dish heater is mounted on the top of an inverted optical microscope Olympus IX-81S1F-3 (Olympus Corp., Tokyo, Japan). It is then possible to accurately and remotely move the sample, in a Petri dish, while the working temperature is kept at a constant level of 37.0 °C ± 0.1 °C. A plastic ring is used to hold down the Petri dish and provide needle access for media exchange, through polypropylene (PP) tubes. An Imaging Source DFK 31BF03 camera (Imaging Source Europe, Maisach, Germany) is mounted on the side interface of the Olympus microscope to follow the in situ processes and to calibrate the AFM system prior to measurements. To visualize objects at the cellular level, the top interface of the microscope equipped with an Olympus DP-74 camera is used. This specific optical path is also equipped with a dual IR filter (Perkin Elmer, Waltham, MA, USA) to block the emission of IR laser of the AFM, when, e.g., fluorescence is measured.

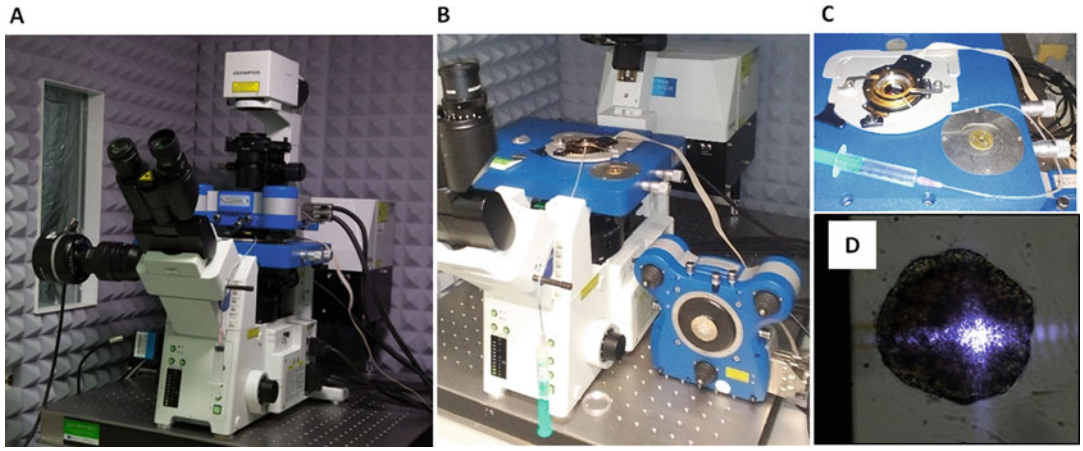

Fig. 1 JPK NanoWizard 3 BioAFM microscope placed on the stage of the Olympus IX-81S1F-3 optical microscope. (**a**) Complete system view in the acoustic enclosure chamber, (**b**) disassembled system with AFM head facing to the front, (**c**) a detailed view of the Petri dish heater placed on motorized stage, (**d**) microscope top view of the cantilever placed above the EB cluster (10×)

2.2 Buffers and Solutions for Biomechanical Studies

1. All the buffers, media, and solutions used in the study of hPSC-CMs biomechanics are prepared under UV sterilized clean box/flow box from sterile vials, preheated to physiological temperature and saturated with CO_2 in the inner environment of standard laboratory incubator Shellab (37 °C, 5% CO_2, Sheldon Manufacturing, Cornelius, OR, USA) for at least 20 min. The appropriate solution/buffer was removed from the incubator for only short period of the injection. Specific solutions are employed:

   (a) Tyrode's solution: 135 mM NaCl, 10 mM HEPES, 5.4 mM KCl, 0.9 mM $MgCl_2$; pH = 7.4. $CaCl_2$ has to be added immediately before experiments in the concentration of 1.8 mM, by diluting a 1 M stock solution 556-times. Similarly, crystalline glucose monohydrate (Sigma Aldrich, St. Louis, MO, USA) is added before starting the experiments, to reach the final concentration of 10 mM. Typically, 90 μL of 1 M $CaCl_2$ and 99.1 mg of solid glucose monohydrate are added to 50 mL of Tyrode's solution previously described (*see* **Note 1**). This solution serves as baseline for measurements and is the main medium to dissolve different drugs to perform drug dependency studies.

   (b) Mouse embryonic fibroblasts (MEF) medium: Knockout DMEM is supplemented with 10% fetal bovine serum, 1 mM β-mercaptoethanol, 1% penicillin-streptomycin 10,000 U/mL, 1% MEM non-essential amino acids 100× solution, and 2 mM L-glutamine. All reagents are

provided by ThermoFisher Scientific (Waltham, MA, USA).

(c) Stock solutions of selected drugs are either made by dissolving solid powder or by diluting medical solution in distilled water: 10 mM (2.5 mg/mL) isoproterenol hydrochloride (Sigma Aldrich), 1.5 mM (1 mg/mL) metoprolol tartrate (BETALOC, AstraZeneca, Prague, Czech Republic), and 0.1 M (19.4 mg/mL) caffeine (Sigma Aldrich).

(d) Working solutions for drug testing were prepared fresh on day of experiment from stock solution by adding 10 mL of complete Tyrode's solution (i.e., already containing 1.8 mM $CaCl_2$ and 10 mM glucose). The amount of stock solution of the appropriate drug was: isoproterenol 1 μL (1 μM final concentration), metoprolol 467 μL (70 μM), and caffeine 1 μL (10 μM).

3 Methods

3.1 Laboratory Environment

The bio-AFM experiments should be performed in the standard cell cultivation manner, in either clean or at last semi-clean room, equipped with UV-C germicide lamp (NBVE 110/55 PL, 150 $\mu W/cm^2$, capacity 199 m^3/h; Ultra-Viol, Zgierz, Poland) covering AFM work place and most of the other space of the room. UV shining of the work space for a 60 min period prior the experiment reduces sample contamination risk and allows repeated measurements. Use of adhesive doormat together with personal protective equipment is highly recommended. Portable UV lamp/AFM UV lamp shining in the inner space of sound-insulated AFM chamber allows equipment sterilization in about 15–30 min immediately before the sample is inserted into the AFM sample holder (*see* also **Note 2**).

3.2 BioAFM Preparation and Calibration

1. An uncoated silicon nitride rectangular cantilever (AppNano Hydra2R-100N; AppNano, Mountain View, CA, USA) with silicon pyramidal tip is properly fixed into the AFM glass block holder. This is then placed into the AFM measurement head, and a drop of distilled water is deposited (using standard pipette) on the top part of the chip, where the cantilever is located, to prevent entrapment of air bubbles during immersion.
2. A Petri dish (TPP, Trasadingen, Switzerland) internal diameter 34 mm (product number 93040) is filled with 2 mL of distilled water ($\sigma < 0.1$ μS/cm) and preheated inside the Petri dish heater to 37 °C, for 15 min. The dish plastic bottom serves as non-deformable sample surface for calibration purposes.

3. The AFM scanning head is placed in its housing on the motorized stage, the cantilever holder is dipped into the Petri dish, maintaining the position approximately 250 μm above the surface. The focal field of the optical microscope is then focused on the level of AFM cantilever, and the optimal laser position at the cantilever free extremity is achieved using the Nanowizard 3 head adjustment screws. The parameter "SUM," indicating the total laser reflection, is proceeding, and accepted, within the range 0.40–0.47 V. The position of the photodetector is then aligned to have zero coordinates (i.e. under 0.1 V), by rotating the appropriate screws of the AFM head. After this first alignment, a period of 15 min is necessary for temperature stabilization of the whole setup. Afterward, a minor readjustment of photodetector position takes place again.
4. The calibration of the setup starts with the automatic approach procedure, when the following parameters of the tip-surface interaction feedback loop were set: $I_{\text{Gain}} = 50$, $P_{\text{Gain}} = 0.001$, set Point = 1 V. A force-distance (FD) curve is recorded in a place randomly selected on the dish surface, when these parameters are used: Z-length 500 nm, closed loop compensation of the Z piezo position, time per curve 1 s, relative set point value 2.5 V. Cantilever deflection sensitivity is evaluated by linear fitting of the repulsive contact part of the FD curve. A value between 18.5 and 26.0 nm/mV is usually obtained for the Hydra2R-100N cantilevers. To measure real values of force (according to Hooke's law—*see* Eq. (1)), calibration of cantilever stiffness (spring constant) is necessary: the thermal noise calibration method is used for this purpose. The cantilever is lift back to 250 μm above the surface, as first step, to exclude surface damping and separate noise emissions coming either from the dish or from the cantilever. The thermal noise spectrum is recorded and averaged for 60 s, the resonance peak (15–20 kHz) is then fitted by the calibration module of the JPK software, thus giving values of the cantilever stiffness k—typically within the range 12–18 pN/nm.

$$F = -k\Delta h \tag{1}$$

where F is the measured force, k the spring constant, and Δh the cantilever vertical displacement (extension of the spring).
5. When cantilever stiffness and optical lever sensitivity are measured, and inserted as parameters into the software, the instrument is ready for measurements. However, if the laser beam is moved from the cantilever during the experiment, the calibration process needs to be repeated, to record precise and comparable values of force.

3.3 Culturing and Seeding of Cell Clusters

1. Either stem cell-derived cardiomyocytes or neonatal primary animal dissociated cells, organized in functional syncytium, are suitable for mechanical contraction analysis. Single cell form is feasible, despite low rate, irregular spontaneous contractions, and uneasy maintenance of viable cultures. Multicellular clusters or monolayers provide a better electrical stability with sustained contraction over time, as per our case.
2. Size-controlled cellular clusters can be achieved by forced aggregation of suspended hPSC into agarose microwells molded by a silicone stamp (1.5% agarose, VWR, Radnor, PA, US) [11]. Cardiac differentiation follows by stepwise supplementing of cytokines [12] on low-adhesive Petri dishes in initial hypoxic conditions (5% O_2, 5% CO_2, and 90% N_2), and later standard cell incubator atmosphere, at 37 °C. Contracting clusters appear within 2 weeks of differentiation. In order to account for maturity level, clusters are measured in similar differentiation state, e.g., 28 days post aggregation. Samples presenting round shape and adequate size allow repeatable evaluation, while seeding in advance (12–24 h) onto adherent 34 mm Petri dishes (TPP) allows stable sample adhesion and easier handling during measurements.

3.4 Recording of Mechanocardiograms

The mechanocardiogram (MCG) curve shows the interaction force between the AFM tip and the surface of the beating cell clusters, recorded in real time. An optimal position is verified by landing on multiple sites of the EB, and selecting the one with the highest positive (and, when possible, monophasic) contraction magnitude. There are two ways of MCG recording—active and passive mode (*see* also **Note 3** for more details).

1. Active mode (constant force mode): The force between tip and cell cluster surface is kept constant (given by the set point parameter, typically 6 nN) for the whole period of the experiment. JPK software contact imaging mode is used for this purpose; however, the X–Y movement of the AFM piezo is not used. The feedback loop of the AFM microscope (intensity set by I_{Gain} and P_{Gain} parameters equal to those used for calibration, i.e., 50 and 0.001, respectively) guarantees the constant force of interaction. The force-time curve shown in the oscilloscope window of the measurement software (Fig. 2) is in fact a force value uncompensated during contraction movements to keep the interaction constant. The force-time curve is recorded with a sampling frequency of 1 kHz (i.e., 1000 values per second), and the reference height channel is often recorded, too.
2. Passive mode (constant height mode): The MCG curve is recorded when the cantilever is kept in compensated constant

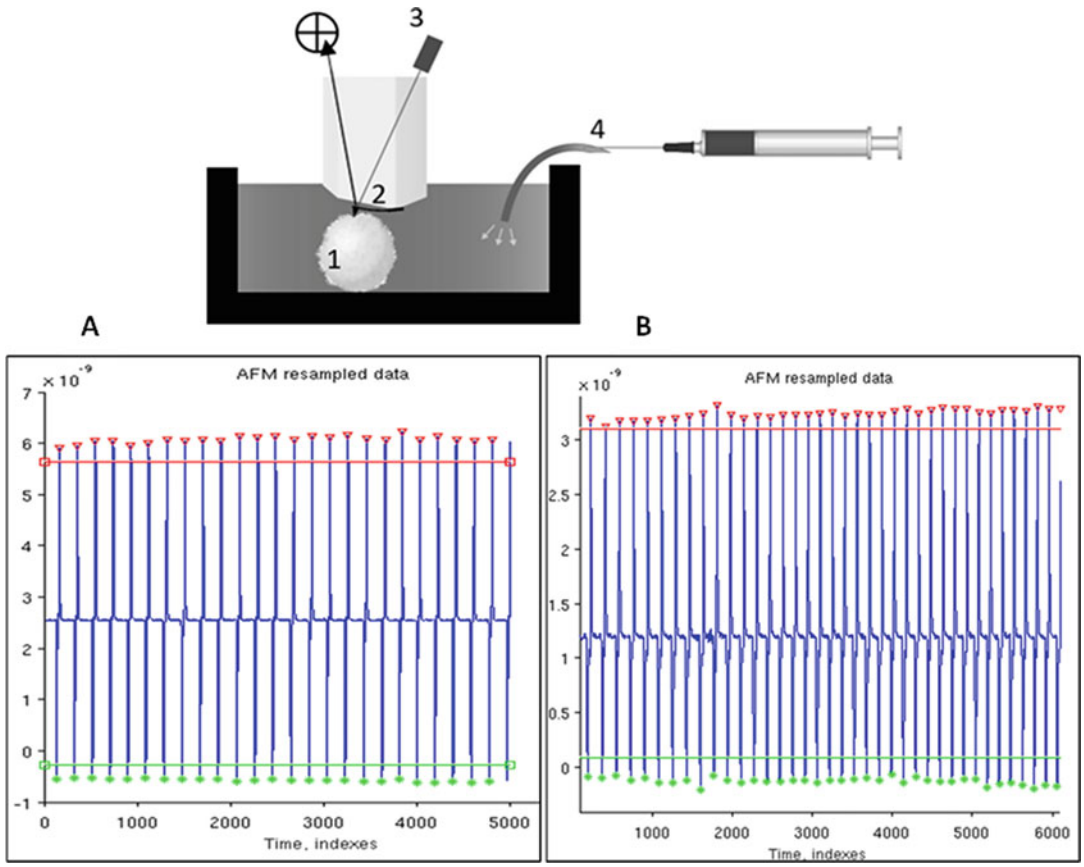

Fig. 2 Scheme of the setup used for biomechanical characterization of the EB clusters, and recorded MCGs. The setup (top) consists of the following main parts: *1* EB cell cluster, *2* AFM cantilever fixed to a glass block, *3* laser detection system, *4* injection system composed of plastic syringe and PP plastic tube with stainless steel tube (i.d. 0.5 mm). Examples of basal (**a**) and 1 μM isoproterenol stimulated (**b**) MCG curves are plotted and analyzed in MATLAB environment: the median values of R–S amplitude were found to be 7.3 ± 0.2 nN (**a**) and 3.5 ± 0.4 nN (**b**); the median values of beat rate were calculated as 32.1 ± 0.7 bpm (**a**) and 72.3 ± 1.4 bpm (**b**)

height above the sample surface. The passive cantilever displacement caused by EB vertical contraction is registered. Isometric contraction force can be evaluated by subtracting from this the cantilever deflection value, similarly to the conversion algorithm of FD curves into tip-sample separation curves. From the practical point of view, the force spectroscopy mode (constant interaction) of the JPK measurement software is employed. A FD curve is initiated and the cantilever is kept to a constant height on the EB surface for typical periods of 300 s, during which the force-time curve is recorded. The following parameters are used for stable measures: $I_{\mathrm{Gain}} = 50$, P_{Gain} 0.001, set point 2 nN, relative set point value 2 nN, Z-length 15 μm, Z position closed-loop feedback on, extension time

0.5 s, extension delay 300 s, and retraction delay 0 s. The MCG curve is recorded in the oscilloscope windows similarly as in the active mode (sampling frequency 1 kHz) for the time interval of FD curve duration.

The MCG curves are saved as standard ASCII text file containing two (or three—in case of height channel recording) columns of data. The first column is filled with time stamps, the second one with recorded force values (in Newtons), and the third column with reference values of height (in meters). The evaluation of the recorded curves can be found in Subheading 3.6.

3.5 Drug Testing Protocol

The main aim of the constructed biosensor setup is to study the effect of various drugs on the biomechanical properties of cardiomyocytes presented in the form of clusters— so-called pharmacology (or drug response) studies. The sequential increase of the concentration of a single compound, or a sequence of different cardioactive drugs, is used and its effects studied. Typical drugs affecting biomechanical properties of CMs, used also in clinical treatment of heart diseases, are isoproterenol and metoprolol, typically applied in the micro-molar range (solutions prepared as described in Subheading 2.2). The general protocol is then extended by investigating the cardioactive drug effect. The protocol presented here was found to be valid for all the tested drug solutions.

1. Bio-AFM-based setup needs to be prepared, preheated, and calibrated as described in Subheading 3.2. The uncoated silicon nitride cantilever Hydra2R-100N is used to find optimal position on the EB surface, with the modalities described in Subheading 3.4.
2. The optimized position is kept during the recording of MCG curves, when different drugs are applied to the measurement chamber—an injection system integrated in the sample holder ring is used to deliver the appropriate drug to Petri dish.
3. The drug solution aliquot is preheated and equilibrated in cell incubator atmosphere.
4. The whole internal volume is first gently sucked from the dish by a standard medical syringe (2 mL volume, 15 s for suction process). After wasting of the solution, the same syringe is used to inject another solution in the dish with the same gentle speed. Tip-EB contact is kept during this process.
5. MCG curves are recorded in 2 × 10 min intervals following the injection of the drug solution to the measurement chamber. The first interval is called stabilizing period, when diffusion, membrane penetration, and temperature stabilization are taking place. The kinetics of the processes following injection can

be monitored. Equilibrium phase (another 10 min) comes after, when stabilized biomechanical properties can be recorded as a separated MCG curve. The pharmacology testing protocol consists usually of several consecutive stabilizing and equilibrium phases.

3.6 Evaluation of Signal Traces

Time-series data from the Nanowizard AFM are preprocessed and analyzed by an in-house developed algorithm using MATLAB 2015a (ver. 8.5, The MathWorks Inc., Natwick, MA, USA). After resampling (noise and dataset size reduction), the local maximum and its corresponding local minimum (R and S, respectively) for each contraction are identified and processed to obtain the force of each individual contraction (R–S amplitude, as in the maximum upstroke and minimum downstroke of a QRS complex in ECG, combination of the Q wave, R wave, and S wave) and beat rate of EB.

3.7 Statistical Evaluation of Data

Quantitative data are presented as mean ± standard error of the mean, calculated over three to six experiments. Extracted parameters are statistically evaluated in Origin 7.0 software (Microcal, Northampton, MA, USA). The normality of data distribution is evaluated by the Shapiro-Wilk test, with 95% confidence interval. One-tailed Student's *t* test is used to test statistical difference of the two measured data sets means, for $\alpha < 0.05$. One way ANOVA method implementing the Bonferroni test is employed to compare statistical significance of measured signals (force or beat rate) at different concentrations of various drugs or compounds at different significance levels ($p < 0.05$, $p < 0.01$, $p < 0.005$).

4 Notes

1. $CaCl_2$ and glucose monohydrate can be added to the stock Tyrode's solution, provided that the solution is then kept in −20 °C freezer and thawed just before the experiments.
2. Full sterile environment for AFM is not necessary, and may be hard to achieve in most of AFM premises. Nevertheless, UV light and possible ethanol sterilization of the cantilever mounted on its holder have been found to give longer reliable measurement periods up to 48–72 h, or repeated procedures possibilities, with culture maintenance on AFM workplace. The cell sample should not be moved back to original cultivation site before the end of measures, which can take several days, to prevent dislodgment or contamination.
3. Constant force as well as constant height modes could be used to record MCG curves. The active feedback loop employed in the constant force mode allows long-lasting monitoring of the

interaction between tip and EB surface, with no observed sample damage. On the other hand, force feedback loop parameters misrepresent the real values of the measured force. Therefore, for relative comparisons of force, feedback loop gains should remain constant between experiments. Constant height mode does not guarantee constant interaction for long time periods (sample may creep or be damaged); however, it offers truly quantitative force values.

Acknowledgments

We wish to thank Anton Salykin for providing assistance on data evaluation, Stanislava Koskova, Tereza Jurakova, and Aleksandra Vilotic for kind assistance and reliable preparation of cardiomyocyte clusters.

This work was supported by the Ministry of Education, Youth and Sports of the Czech Republic under the projects CEITEC 2020 (LQ1601) and FNUSA-ICRC (LQ1605), FNUSA- ICRC no. CZ.1.05/1.1.00/02.0123 (OP VaVpI), as well as CIISB research infrastructure project LM2015043, project MSM0021622430 and Grant Agency of the Czech Republic (Grant No. P302/12/G157). This work was also supported by the European Regional Development Fund Project "CIISB4-HEALTH" no.CZ.02.1.01/0.0/0.0/16_013/0001776. Martin Pešl and Guido Caluori were supported by Grant Agency of the Masaryk University (MUNI/A/1010/2016).

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