Cell Calcium 47 (2010) 224–233

Contents lists available at ScienceDirect

Cell Calcium

journal homepage: www.elsevier.com/locate/ceca

A system for optical high resolution screening of electrical excitable cells

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ARTICLE INFO

Article history:
Received 22 June 2009
Received in revised form 15 October 2009
Accepted 27 November 2009
Available online 24 December 2009

Keywords:
High content screening
Calcium imaging
Cardiac myocytes
Multi-well plates
TIRF
Elastic coating

ABSTRACT

The application of primary excitable cells for high content screening (HCS) requires a multitude of novel developments including cell culture and multi-well plates. Here we introduce a novel system combining optimised culture conditions of primary adult cardiomyocytes with the particular needs of excitable cells for arbitrary field stimulation of individual wells. The major advancements of our design were tested in calcium imaging experiments and comprise (i) each well of the plate can be subjected to individual pulse protocols, (ii) the software driving electrical stimulation can run as a stand-alone application but also as a plug-in in HCS software packages, (iii) the optical properties of the plastic substrate (foil) resemble those of glass coverslips fostering high resolution immersion-based microscopy, (iv) the bottom of the foil is coated with an oleophobic layer that prevents immersion oil from sticking, (v) the top of the foil is coated with an elastic film. The latter enables cardiomyocytes to display loaded contractions by mimicking the physiologically occurring local elastic network (e.g. extracellular matrix) and results in significantly increased contractions (with identical calcium transients) when compared to non-elastic substrates. Thus, our novel design and culture conditions represent an essential further step towards the application of primary cultured adult cardiomyocytes for HCS applications.

1. Introduction

Optical measurements of cellular functions have been established on the laboratory level for many years (e.g. [1–3]). They were always driven by the technological developments towards real-time high resolution imaging devices throughout the recent two decades [4]. These low-throughput techniques have proven great value and have fostered our understanding of a great variety of physiological but also pathophysiological processes in living cells [5,6]. One of the areas of cell physiology that has benefited greatly from optical advancements was our understanding of cellular and sub-cellular calcium signalling [7].

Such investigations include imaging molecular events such as calcium blips [8] and quarks [9] and cellular calcium transients [10,11] but also in vivo calcium imaging by means of genetically encoded biosensors (GEBs) expressed in transgenic mouse lines [12,13]. Recently, these developments were complemented by major advancements in the genetic manipulation of primary cells with viral gene transfer [14]. The establishment of a large variety of animal models for human pathologies [15] can be seen along the same line. In addition to in vivo analysis that will always represent ultra-low throughput experimental series, cellular and/or sub-cellular studies of the physiology and pathophysiology of individual cells complement the in vivo data. These high resolution characterisations of cellular responses are often highly repetitive and laborious tasks.

It would thus be desirable to transfer such single-cell experiments to a screening environment allowing for higher throughput and higher reproducibility of the measurements. However, so far optical high content screening (oHCS) has largely been restricted to cultured cell lines [16]. Such specimens are relatively easy to handle and genetic manipulation is straightforward. They have represented and will most likely be the prime tool in ultra-high throughput screening (uHTS) since they allow for extremely reproducible experimental results, a requirement for screening large chemical libraries.

In contrast, the utilisation of primary cells in oHCS does not only appear desirable, instead it seems almost mandatory in the further development of pharmacological targets and safety screens. This appears essential because the optimal proximity to the in vivo situation is a prime prerequisite and goal. In particular screening based measurements of excitable cells are very demanding and have thus not made it into automised screening applications. The major reasons are: (i) limited reproducibility in the yield and quality of cell isolation and the following primary culture; (ii) lack of controllable trigger devices to excite cells in multi-well plates; (iii) low photon detection efficiency due to the use of low numerical aperture (NA) air objectives. Especially high NA oil objectives were excluded due to thick plastic well bottoms and insufficient oil handling on multi-well plates, but they are necessary for high resolution sub-

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doi:10.1016/j.ceca.2009.11.012

cellular imaging. Taken together, up to now these challenges have been largely unsolved and have thus limited the use of primary cells, especially primary electrical excitable cells such as cardiac myocytes or neurones, for the application in oHCS.

Recently, we introduced an improved cell isolation and primary culture method that resolved the issue mentioned in (i) for cardiac myocytes [17,18]. Here we present a complementary system that overcomes the restrictions mentioned above. Going even further it adds more benefits especially for screening of primary isolated cardiomyocytes, through mimicking a native-like extracellular environment. We will demonstrate the use of such a system for global calcium and contraction measurements but also for high resolution calcium imaging such as total internal reflection fluorescence (TIRF) microscopy.

2. Materials and methods

2.1. Cell isolation and culture

Isolation and culture of adult rat cardiomyocytes was performed as described previously [18]. Adult male Wistar rats (6–12 weeks old, 200–400 g) were handled and sacrificed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Animals received an intraperitoneal injection of a mixture of ketaminehydrochloride (Ursotamin, Serumwerk, Bernburg, Germany) and xylazinhydrochloride (Rompun, Bayer Health Care, Leverkusen, Germany) at a final dose of 137 mg/kg body weight and 6.6 mg/kg body weight, respectively. When anaesthetised the rat was killed by decapitation. The heart was flushed with 10 ml of ice-cold Ca^{2+}-free solution (CFS) containing (in mM): NaCl 134, glucose 11, KCl 4, $MgSO_4$ 1.2, Na_2HPO_4 1.2, HEPES (Merck, Darmstadt, Germany) 10 (pH adjusted to 7.35 with NaOH). After that, the heart was removed, attached to a Langendorff apparatus and perfused retrogradely with O_2 saturated CFS containing 200 μM EGTA at a rate of 4 ml/min for 5 min. The perfusate was then changed to O_2 saturated CFS containing Liberase Blendzym IV (Roche Diagnostics Corp., Indianapolis, USA) at a final concentration of 335 μg/ml for 25 min.

The ventricles were removed, minced and placed in O_2 saturated CFS containing 335 μg/ml Liberase Blendzym IV (at 37 °C in a water bath for 2 min). After sedimentation, the resulting supernatant was discarded and the pellet was mixed and resuspended in 20–25 ml of O_2 saturated CFS and incubated as above. The supernatant was discarded again and the pellet was mixed and resuspended in 20–25 ml of O_2 saturated low-Ca^{2+} solution containing 50% of CFS and 50% of Ca^{2+} containing solution (CCS) and incubated as above. CCS is composed of CFS supplemented with 0.09% of DNAse and 200 μM of Ca^{2+}. Furthermore, the supernatant was discarded, the pellet was resuspended in 20–25 ml of O_2 saturated CCS and incubated as above. Now, rat ventricular myocytes were released from the soft tissue by gentle trituration. The cell suspension was plated into the measuring cavities, the internal bottom surface of which was coated with extracellular matrix proteins (ECM gel from Engelbreth-Holm-Swarm mouse sarcoma, Sigma–Aldrich, St. Louis, USA), they were allowed to settle down for approximately 1 h in medium M199 with Earle's modified salts, glutamine (Biowest, Nuaillé, France), 100 μg/ml Penicillin/Streptomycin and 50 μg/ml Kanamycin (PAA Laboratories, Linz, Austria). The medium was supplemented with 870 nM insulin, 65 nM transferrin and 29 nM Na-selenite (Sigma–Aldrich, St. Louis, USA) (ITS supplemented medium). Myocytes were cultured in an incubator at 37 °C with a 5% CO_2 atmosphere. One hour after plating, the medium was changed for fresh medium supplemented with ITS. This procedure was repeated at the first and third day in vitro (DIV).

For TIRF microscopy and related measurements DIV1 cells were loaded with 1 μM Fluo-4 AM and for photometric measurements cardiomyocytes were loaded with 5 μM Indo-1 AM (both dyes, Molecular Probes, Eugene, USA). In all instances loading time was 30 min and 10 min were allowed for deesterification. All experiments were carried out at room temperature (23 °C).

2.2. Imaging, photometry and cell length measurements

Experiments involving TIRF microscopy were conducted as previously described [19], in short: cells were placed on an inverted microscope (IX70, Olympus, Tokyo, Japan) equipped with a 100× TIRF objective (Plan Apo 1.45 NA, Olympus, Tokyo, Japan). Excitation of Fluo-4 was achieved using a 20 mW 488 nm laser (Cyan Scientific, Spectra Physics, Mountain View, USA) and a monochromator (VisiChrome, VisiTron Systems GmbH, Puchheim, Germany) for TIRF and epi-fluorescence imaging, respectively. In both cases fluorescence was recorded with a back-thinned electron multiplying charge coupled device (CCD) camera (QuantEM:512SC, Photometrics, Tucson, USA). Acquisition was performed with MetaMorph software (Molecular Devices, Downingtown, USA). Image processing was carried out with ImageJ (Wayne Rasband, National Institute of Mental Health, Bethesda, USA).

Documentation of the cells in phase contrast images was performed on an inverted microscope (TS100) equipped with a 20× air objective and a CCD-camera (DN100; all Nikon, Tokyo, Japan).

Global Ca^{2+} transients were measured using Indo-1. For this, the myocytes were transferred to an inverted microscope (TE2000U, Nikon, Tokyo, Japan) attached to a combined fluorescence/cell length imaging system using a 40× oil-immersion objective (S Fluor 1.4 NA, Nikon, Tokyo, Japan). The system comprised two avalanche photo diodes (APD) and a monochromator (Polychrome IV) for fluorescence acquisition (both: TILL Photonics, Gräfelfing, Germany). The cells were excited at 360 nm while simultaneously recording the fluorescence signal at 415 ± 30 nm and 470 nm longpass, respectively (sampling rate 1 kHz). The ratio and further semi-automatic peak detection was determined in Igor Pro software (WaveMetrics, Inc., Lake Oswego, USA) running custom-made macros.

Real-time cell length changes were monitored with a fast camera (sampling rate 240 Hz, MyoCam, IonOptix Corp., Milton, USA) from electrically stimulated cells maintained on coverslips or elastic surfaces by using an edge-detection approach by the Ion Wizard software (IonOptix Corp., Milton, USA). The system directly stores cell length changes that were further analysed in Igor Pro software with custom-made macros.

The measuring cavities referred to above are chambers of a 24-well plate. Since the design of this multi-well plate was a major aim of this paper, it is described in great detail in Section 3.

2.3. Measurements of plate movement

In order to investigate the interaction between the culture substrate, immersion oil and objective we utilised a fully motorised and software controlled microscope (uiMic, TILL Photonics GmbH, Gräfelfing, Germany) equipped with oil-immersion objectives (UPLSAPO 20×oil 0.85 and SPLANAPO 100×oil 1.4, Olympus, Tokyo, Japan) which both displayed flat top surfaces (area around 35 mm^2). We tested two different oils: Type N immersion liquid (Leica Microsystems CMS GmbH, Wetzlar, Germany) with a viscosity of 600 cSt at 23 °C and Nikon immersion oil NF (Nikon, Tokyo, Japan) with a viscosity of 800 cSt at 23 °C. For our experiments we used bottles immediately after initial opening. The volume of the oil drops applied was 60 μl for both types of oil.