Chapter 5

Alpha-Actinin-mCherry fusion protein
Building a paramount new tool for cardiomyocyte analysis

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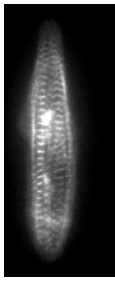
Abstract

Sarcomeric organization is crucial for function of cardiomyocytes and is used as a hallmark of maturation and cardiac diseases such as hypertrophic cardiomyopathies. One approach to determine sarcomeric organization is through visualization by immunofluorescent staining. This requires cell fixation and does therefore not allow assessment of sarcomere dynamics. Another approach is visualization in live cardiomyocytes by phase contrast imaging, which is only suitable for high degrees of sarcomeric organization, and therefore not suitable for immature cardiomycoytes such as those from human pluripotent stem cells (hPSC-CM). In order to visualize sarcomerogenesis and sarcomere dynamics in living cells, we generated a fusion protein, consisting of the sarcomeric α-actinin and a red fluorescent protein, mCherry. When overexpressed in hPSC-CM, the α-actinin-mCherry was incorporated into the sarcomeres, enabling the assessment and analysis of sarcomere movement during contraction. This new tool is potentially very useful for studies related to cardiomyocyte differentiation, maturation and cardiac disease modeling.

Introduction

The sarcomere is the smallest contractile unit of striated muscle. The concatenation of sarcomere units forms myofibers, which are the constituents of skeletal and cardiac muscle. Sarcomerogenesis occurs during early embryonic muscle development and following postnatal maturation controls sarcomeric organization[1], as shown in chapter 3 in relation to cardiomyocyte function. Besides during development from fetal to adult, sarcomeric organization also changes in cardiac disease, such as in hypertrophic cardiomyopathy[2] (either sarcomeric hypertrophy or disorganization). Myogenesis can thus be considered a hallmark of hPSC derived cardiomyocyte maturation[3] and hypertrophic cardiomyopathy. The sarcomeres are composed of different sets of proteins with a high degree of organization. The Z-disks mark the borders of the sarcomeric units, of which α-actinin is a major component. Alpha-actinin is a 104kDa protein member of the spectrin superfamily and consists of an N-terminal actin-binding domain, multiple spectrin repeats and a C-terminal calmodulin (CaM)-like domain[4]. This protein is functional as an anti-parallel homodimer that crosslinks actin filaments of adjacent sarcomere units forming a lattice-like structure that stabilizes the muscle contractile machinary[5]. This structure is evident by immunofluorescence microscopy as anti-parallel bands along the cell, providing cardiomyocytes with their characteristic striated appearance. Nevertheless, these immunofluorescent images only allow analysis of cardiomyocytes under static conditions. Therefore, we genetically engineered α-actinin fused with a fluorescent protein to be able to assess sarcomere structure and length in living cardiomyocytes, thus creating a useful tool to study sarcomere dynamics during maturation and disease. This fusion approach has been employed in studies of whole-cell stiffness in human osteoblasts[6].



To combine the sarcomeric reporter with the cardiac hESC-NKX2-5-eGFP reporter line, we fused alpha-actinin with mCherry that has an excitation/emission spectrum (587/610nm) distinct from GFP and has high photo stability[7]. The expression of fusion proteins is generally driven by constitutive promoters, from which the cytomegalovirus immediate-early promoter (CMV), mouse phosphoglycerate kinase 1 promoter (PGK) and human elongation factor 1a promoter (EF1A) are among the most commonly used. Despite their ubiquitous expression, activity of these promoters differs depending on the cell type in which they are expressed[8]. Furthermore, although these constitutively active promoters can be used for multiple purposes, they do not allow protein expression in time-controlled manner. In 1992 the tetracycline-controlled transcriptional activation (tetO) was described which induced coupled gene expression in the presence of tetracycline or its derivative[9,10]. This technology enables the production of clonally stable hESC lines carrying the α-actinin-mCherry in a repressed state, which can later be induced in differentiated cardiomyocytes.

Materials and methods

RNA isolation and cDNA synthesis

RNA was isolated from hESC derived cardiomyocytes using TRIzol Reagent (Invitrogen) and cleaned up with DNA free kit (Ambion). iScript kit (BioRad) was used to make cDNA.

α-Actinin and mCherry amplification

Primers were designed to amplify hACTN2 from cDNA template isolated from hESC-CM using PCR. The PCR reaction mix was prepared using Phusion Taq following manufacturer's guidelines (ThermoScientific) (Table S1). The PCR cycle was performed as shown in Table S2, where the annealing time was changed depending on the length of the DNA sequence being amplified. The samples were cleaned using the PCR cleaning kit (Qiagen). Afterwards, the samples were digested with the appropriate enzymes in conditions provided by manufacturer (O/N, usually 37ºC; Table S3), and separated on agarose gel. The right band was excised from the gel (QIAquick Gel Extraction Kit, QIAGEN).

Ligation

The vector was digested with the specific enzymes (Table S3) and then cleaned with the PCR cleaning kit (Qiagen). If second digestion was needed it was conducted after cleaning. The open vector was excised from agarose gel, and isolated using QIAquick Gel Extraction Kit (Qiagen). Vector and insert were ligated in the following Equation 1 with the molecular ratios (insert:vector): 3:1, 1:1 in standard protocol. In some cases more than two fragments were assembled, what is referred as multi-ligation. This method was conducted as all-in-one reaction using similar molecular ratio (insert1:insert2:vector=3:3:1, 1:1:1). The ligation was kept at 14ºC, O/N.

([ng of vector] x [size of insert in kb])/([size of vector in kb])=(3 or 1x insert)/(1x vector)
Equation 1

Transformation

Ligation mix used to transform Escherichia coli One Shot® TOP10 chemically competent cells for each transformation. 1-5 μl of DNA (10pg-100ng) was added to a vial of One Shot® cells, mixed gently and incubated on ice for 30 minutes. The samples were heated to 42°C for 30 seconds without shaking followed by 2mins on ice. 250 μl of pre-warmed S.O.C medium was added to the vial without antibiotics and incubated for 1 hour at 37°C shaking. The transformed bacteria were spread on Luria-Bertani (LB) + selection antibiotic (ampicilin - Amp or carbanicilin - Carb) plates and incubated O/N, 37ºC. Colonies (usually 24) were picked up and cultured in 3.0ml LB + Amp/Carb (O/N, 37ºC, 225rpm). DNA was isolated (PureLink Quick Plasmid Miniprep, Invitrogen) and evaluated by restriction analysis (Appendix III).. Clones were sequenced following Sanger method (Appendix X, XI). Correct clones were further expanded in larger volumes. DNA was isolated using the NucleBond Xtra Midi Kit (Macherey-Nagel).

Transfection

pcDNA3.1-hACTN2-mCherry was transfected into hESC-derived cardiomyocytes and human embryonic kidney (HEK) using transfection agents – FuGENE and GJ reagents (Appendix VIII). The control wells contained a pcDNA3.1-mCherry construct (purified or, purified and gel extracted). The amount of DNA was 1, 2, and 3μg. Cells were observed for 48hrs under the fluorescent microscope.

Immunocytochemistry

The cells were fixed with 4% paraformaldehyde for 20 mins and washed three times with PBS. Cells were permeabilized with 0.1% Triton for 8mins and blocked with 4% swine serum for 1h at room temperature (RT). For identification of sarcomeres the samples were incubated with the primary antibody Rabbit anti Troponin I (Santa Cruz sc-15368) diluted 1:500 for 1h at RT. The samples were washed three times 10 mins with PBS with 0.05% tween20 and incubated with the secondary antibodies donkey anti rabbit IgG A488 (Invitrogen A-21206) both diluted 1:200 for 1h at RT.

Lentivirus production

On day 1 HEK 293T cells were plated on 10cm dishes at density of 6 million per dish in DMEM (Gibco) plus 10% FBS (Invitrogen) plus antibiotics. Cells were cultured at 10% CO2 at 37 C overnight. On day 2 the cells were transfected in OptiMEM (Gibco) with the Lentiviral vector and the packaging constructs VSV-G, RRE and REV (added to a total of 40μg per dish) together with 50ul of Lipofectamine 2000. The medium was changed on day 3 with DMEM plus 10% FBS plus penicilline and streptavidin. On day 5 the medium was collected, combined and centrifuge at 1000 x g for 15 mins at 4°C. The supernatant was filtered through a 0.45 μm filter into sterile polyallomer centrifuge tube (Beckman Coulter). The tubes were inserted into a SW28 rotor (cat. #326823) and centrifuge at 20 000 RPM for 120 mins at 4°C. After centrifugation the supernatant was aspirated and the pellet resuspend in PBS. The final solution was aliquoted and stored at -80°C.

Mammalian expression vectors

pcDNA3.1 is a commercially available cloning/expression vector (Invitrogen). It was developed from pcDNA3 to permit high level of stable expression of recombinant protein in mammalian hosts using a CMV promoter. Despite of its small size (5.4kb), the vector has sequences of two selection markers: bacterial ampicillin and mammalian neomycin. Features of this vector are described **Figure S1** (Invitrogen).

pENTR1A is a Gateway® entry vector from Life Technologies (**Figure S2**). This vector contains multiple restriction sites flanking a ccdB gene (control of cell death), allowing multiple cloning possibilities and selection for positive clones. The multiple cloning sites are flanked by the attL1 and attL2 sites, which are used for site-specific recombination with a Gateway® destination vector, ensuring a fast and simple cloning step of the gene of interest in the correct orientation for expression.

pLenti CMVTRE3G Neo is a 3rd generation lentiviral vector containing a minimal CMV promoter preceded by TRE (Tetracycline Response Element), which is activated by tTA (Tetracycline-controlled activator) protein in presence of doxycycline. This promoter requires a co-expression of a vector encoding for the tTA protein. The attR1 and attR2 sites are present in front of the promoter for easy cloning by recombination with the entry vectors (see above). In addition a neomycin selection cassette for positive selection is included.

pLenti CMV rtTA3 Blast is a 3rd generation lentiviral vector expressing the reverse tetra-

cycline-controlled transactivator 3 (rtTA3) driven by a CMV promoter. This vector has a blasticidin selection cassette.

pLenti CMV Puro DEST is a 3rd generation lentiviral expression vector driven by a CMV promoter. The attR1 and attR2 sites are present in front of the promoter for easy cloning by recombination with the entry vectors. This vector has a puromycin selection cassette.

pLV-H1TetO-RFP-Puro (Biosettia) is a 3rd generation lentiviral vector for inducible expression of shRNA. The vector contains an EF1α promoter driving the expression of tTA protein followed by and IRES (Internal ribosome entry site) inducing the translation of a RFP fused to a puromycin. The shRNA expression is induced via a H1 promoter that can only be active in presence of doxycycline (TetON).

Human embryonic stem cell culture and differentiation

The human embryonic stem cell (hESC) line HES3-NKX2-5-eGFP was used for cardiac differentiation. This cardiac reporter line allows visualization and sorting of hESC-derived cardiomyocytes on the basis of GFP expression[11]. The cell line was maintained on irradiated mouse embryonic fibroblasts (MEFs). Differentiation was performed by two different methods. Monolayer differentiation was performed in a Matrigel coated plastic 6-well format where 300.000 hESC were seeded at day 0 in a BSA polyvinylalcohol essential lipids medium (BPEL)[12] together with 20ng/ml BMP4 (R&D), 20ng/ml Activin A (Miltenyi), 1.5μM chir99021 (Axon Medchem). The medium was refreshed after 3 days with BPEL supplemented with 5μM XAV-939. Alternatively, cardiomyocytes were generated using a "spin embryoid body (spin-EB)" method of differentiation[11]. Briefly, spin-EBs were formed by centrifugation of 30.000 hESCs in a 96-well format (Greiner) with BSA polyvinylalcohol essential lipids medium (BPEL) together with 35ng/ml BMP4 (R&D), 30ng/ml Activin A (Miltenyi), 30ng/ml VEGF (Miltenyi), 40ng/ml SCF (Miltenyi), 1.5μM chir99021 (Axon Medchem). The medium was refreshed on day 3. GFP positive spin-EBs started beating between day 7 and 10 of differentiation. Dissociation of both monolayers and spin EB derived cardiomyocytes was performed at day 20 with 1x TryplE select for 10 mins at 37C.

Single cell sorting

Four hours before start the sort Rock inhibitor (Fasudil, 5mM) was added, 1:1000 = 5uM. Undifferentiated hESCs were dissociated into single cells using TryplE (Gibco), resuspended in FACS buffer and passed through a cell strainer. The cells were pelleted and resuspended in FACS buffer, 2% FCS (Gibco), 1% normal goat serum and either primary SSEA4 (sc59368) antibody or isotype control. After 30mins on ice cells were washed 1x with FACS buffer containing 2% FCS and resuspended in FACS buffer, 2% FCS plus normal goat serum 1% and secondary goat anti-mouse IgG1 (H+L)-APC. After 30mins on ice, cells were washed and resuspended in FACS buffer plus 2% FCS and PI (1:1000). The cells were sorted to single well in a 96 well plate containing 9000 MEF/well plus Rock inhibitor 1:1000.

Analysis of sarcomere dynamics

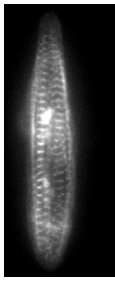
Image series of transduced cardiomyocytes were recorded with a Leica AF 6000 image

workstation (Leica Microsystems GmbH Wetzlar, Germany). A 40x dry objective with an internal 1.6x magnification extra was used to record images of 500x500 pixels during at least 6 contractions with a time resolution of 17 milliseconds. After export to uncompressed TIF format, Image stacks were constructed with the program of ImageJ or Fiji (National Institute of Health NIH U.S.A). The intrinsic function Z-profile of the program was used to construct a line-profile exactly perpendicular to the sarcomeres over the length of one cardiomyocyte. A plugin for ImageJ was written to export the profiles to a time-Z data set. A second program was developed for the interactive analysis using LabVIEW (National Instruments Corporation Austin, U.S.A.) - Power Spectral Analysis. A power spectral analysis per frame (Z-profile) was computed using a Hanning window output. Visual inspection of the power spectra always revealed a single peak and its successive second harmonics. The individual power spectral profiles were then fitted according a Gaussian function using the Levenberg–Marquardt algorithm with a ten times increased time resolution. The center frequency of this fitting (first peak in the spectrum) was used to compute the actual spacing (1/frequency) between the sarcomeres. Finally a graph was made of all computed points against time. From this graph speed of contraction, the speed of relaxation, the contraction duration and the repetition frequency was extracted. Requests for the ImageJ plugin and the Power Spectral.

Results

We have followed several strategies for introducing α-actinin-mCherry in human stem cell-derived cardiomyocytes for live imaging of sarcomeres during differentiation, assembly of sarcomeres and contractility. As a first step we cloned α-actinin into a mammalian expression vector pcDNA3.1 driven by the CMV promoter (**Figure S1**). The coding sequence of mCherry was has been previously incorporated into this vector. Human α-actinin was amplified by PCR from cDNA generated from hESC derived cardiomyocytes in a PCR reaction using primers FWamp1 + RVamp1 (**Figure 1A**, **Table S4**), followed by digestion with KpnI and subsequent ligation at the N-terminal side of mcherry. Positive clones (3 out of 24) were verified by sequence analysis (**Figure 1B** and **C**). In order to test whether α-actinin-mCherry was expressed and correctly incorporated in sarcomeres, we transfected the expression construct into HEK293T cells and hESC derived cardiomyocytes (hESC-CM), respectively. Although α-actinin-mCherry was clearly expressed in HEK 293T cells, expression was not observed in hESC-CM (**Figure 1D** and **E**). Lack of α-actinin-mCherry expression in hESC-CM could be due to multiple reasons, nonetheless, since it is expressed in the HEK 293T cells we assumed that the main issue was related to the lack of efficiency of the transfection procedure in cardiomyocytes.

In a next step we generated lentiviral vectors incorporating α-actinin-mCherry under control of the CMV promoter in order to obtain a higher transduction efficiency in hESC-CMs. For this, α-actinin and mCherry coding sequences were subcloned into a pENTR1A vector (**Figure S2**), which enables efficient recombination in the destination vector of choice using Gateway® technology. Alpha-actinin and mCherry were amplified using the FWamp2 and RVamp1 and FWamp3 and RVamp2 primer pair, respectively (**Figure 2A** and **Table**



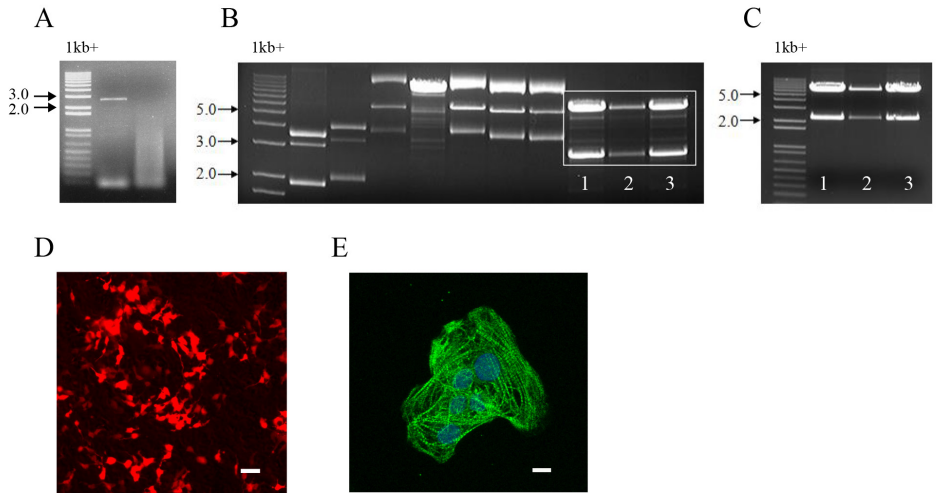


Figure 1- Cloning α-actinin-mCherry into pcDNA3.1. **A.** Band of the amplified α-actinin (2.7kb). **B.** Clone check after ligation with KpnI digestion with predicted bands at 2.7 and 5.4kb. **C.** Confirmation of correct clones with SalI digestion with predicted bands at 2.2 and 6.2kb. D. Expression of α-actinin-mCherry in HEK 293T cells in red (bar-50μm). **E.** Troponin I immunostaining in hESC-CM (green), nuclei (blue) with no expression of α-actinin-mCherry (red) (bar-10μm).

S4). PCR products were extracted from agarose gel and digested with either SalI and KpnI (α-actinin) or KpnI and XhoI (mCherry). The pENTR1A was digested with SalI and XhoI and ligated to both inserts in one reaction. Correct insertions (5 out of 6 clones, **Figure 2B** and **C**) were verified by sequence analysis and recombined into the pLenti CMVTRE3G Neo destination vector (**Figure S3**) using the LR Gateway® reaction. This vector allows inducible expression of α-actinin-mCherry in the presence of doxycycline (TetON system), avoiding a possible interference of this fusion protein during the process of cardiomyocyte differentiation. All clones recombined correctly (**Figure 2D**). Once clone was selected for further expansion and virus production.

Additionally, virus was produced for the pLenti CMV rtTA3 Blast expression vector. hESC-CM were transduced with both viruses in a glass bottom plate and doxycycline was added 48h post transduction. The expression and sarcomeric localization of α-actinin-mCherry was clearly visible by fluorescence microscopy. The movement of sarcomeres from contracting cardiomyocytes was recorded, demonstrating the feasibility to use this fusion protein for functional analysis of cardiomyocytes (**Figure 2E**). In order to generate a stable hESC line with the inducible α-actinin-mCherry expression both pLenti CMVTRE3G α-actinin-mCherry and pLenti CMV rtTA3 vectors (**Figure S3** and **S4**) were transduced into the HES3-NKX2-5-eGFP line and cultured for a week in the presence of antibiotics (Blasticidin and G418), followed by treatment with doxycycline (Dox). After 72h of Dox treatment only about ≈20% of the cells were expressing α-actinin-mCherry (**Figure 2F**), indicating silencing of the promoters. After differentiation to cardiomyocytes no overlap between GFP and mCherry expression was observed, indicating further silencing of expression in cardiomyocytes. In order to select for clonally derived cell lines displaying inducible mCherry expression, we performed FACS sorting based on mCherry and SSEA4 (pluripotency marker)

expression and seeded single cells in a 96 well plate (containing mouse feeders). Clonal expansion was successful in 51% (46 out of 90) of the wells, from which the cells were transferred by cut and paste both to a new well of feeders for expansion and to a matrigel-coated plate for cardiac differentiation. At day 10 of differentiation Dox was added to all wells and after 72h only 36% (18 out of 50) of the wells displayed mCherry positive cells. These positive wells displayed approximately 10% to 50% of mCherry positive cells. Unfortunately, we did not observe overlap between GFP and mCherry expression in the same cell (data not shown), suggesting silencing of α-actinin-mCherry expression, in particular in cardiomyocytes. Therefore, we decided to perform transient lentiviral-transduced expression of hESC-CMs in future experiments.

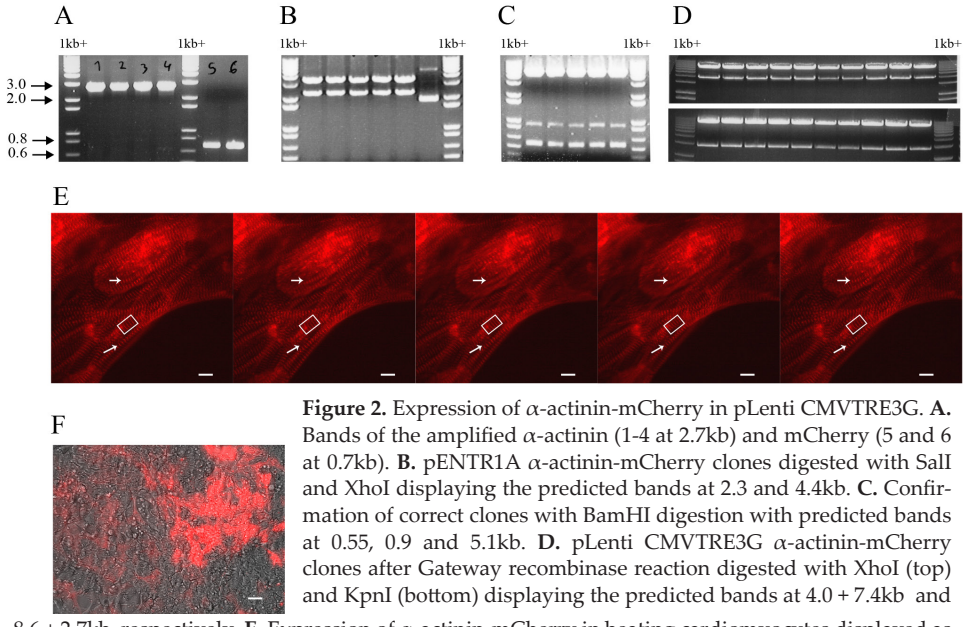


Figure 2. Expression of α-actinin-mCherry in pLenti CMVTRE3G. **A.** Bands of the amplified α-actinin (1-4 at 2.7kb) and mCherry (5 and 6 at 0.7kb). **B.** pENTR1A α-actinin-mCherry clones digested with SalI and XhoI displaying the predicted bands at 2.3 and 4.4kb. **C.** Confirmation of correct clones with BamHI digestion with predicted bands at 0.55, 0.9 and 5.1kb. **D.** pLenti CMVTRE3G α-actinin-mCherry clones after Gateway recombinase reaction digested with XhoI (top) and KpnI (bottom) displaying the predicted bands at 4.0 + 7.4kb and 8.6 + 2.7kb, respectively. **E.** Expression of α-actinin-mCherry in beating cardiomyocytes displayed as different frames of one contraction (bar-10μm). **F.** Merge between brightfield and fluorescent images of hESC expressing of α-actinin-mCherry after antibiotic selection (bar-50μm).

For this approach we generated a pLenti CMV α-actinin-mCherry viral vector following LR Gateway® recombination of pENTR1-α-actinin-mCherry and pLenti CMV Neo vectors (**Figure S5** and **3A**). HESC-CMs were transduced at day 15 of differentiation. Transduced cardiomyocytes were cultured in defined maturation medium during 7 days for phenotypical improvement (see Chapter 4), followed by dissociation and seeding on 1% gelatine 20x20 lines printed on a polyacrylamide gel. After four days beating single cardiomyocytes were imaged at 37°C - 5%CO2 at a speed of 56 frames per second, represented in selected frames in **Figure 8B**. In order to analyse the sarcomere movement during contraction, the frame sequence was loaded into the software Image J as a stack and a pixel intensity profile was made across the sarcomere length for each frame (**Figure 3C**). Subsequently the pixel intensity plot of every frame was uploaded into the Power Spectral Analysis software (**Figure 8D** left panel) and the most frequent distance between peaks was calculated using

a power spectrum analysis (Hanning) fitted with a Gaussian distribution (**Figure 3D** right panel) (see materials and methods). The distance corresponding to the peak of the power spectrum of each frame was plotted in function of time (**Figure 3E**). This data format enables the assessment of the sarcomere spacing in its relaxed (y0- 1.79μm) and contracted (y1- 1.51μm) form, the distance covered by the sarcomeres during contraction (shortening amplitude) (Δy- 0.27μm) and relaxation (elongation amplitude) (Δy2- 0.27μm) as well as the time taken by the sarcomeres during both actions (Δt- 0.11s and Δt2- 0.27s, respectively), which allow us to analyse kinetics of sarcomere movement during contraction and relaxation with a shortening speed of - 2.45μm/s and a elongation speed of - 1.01μm/s (**Figure 3E**).

Discussion

Here we generated an α-actinin-mCherry fusion protein following various cloning strategies and demonstrated proof of concept for its use as a tool to accurately identify the dynamic changes of Z-disks in live hPSC-CM. We further developed power spectral analysis software to calculate the length of each sarcomere during contraction, which we used to assess the kinetics of sarcomere movement during contraction.

Currently sarcomere length measurements are mostly based on observations made on fixed cells, which obviously interferes with assessment of dynamic changes of the sarcomeres. Other approaches such as laser diffraction techniques[13] or the use of 2-photon microscopes[14] have been applied to measure dynamics of sarcomere length in single cells or live tissue. Nevertheless, these techniques are rooted on the extremely well defined periodicity of the sarcomeres as they are based on a constant light diffraction pattern or on the presence of T-tubules, both traits of fully matured cardiomyocytes, which are currently non-existent or at least difficult to achieve in hPSC-CM. On the other hand, our approach of genetic labelling sarcomeres with a fluorescent reporter not only allows the assessment of sarcomeres kinetics but also of sarcomerogenesis during hPSC-CM maturation or disease modelling. With this approach we intended to develop a stable hESC line with temporal control of the α-actinin-mCherry expression by the use of a doxycycline inducible CMV promoter (TetON). Although the expression of the α-actinin-mCherry was present initially in undifferentiated hESC, its level decreased over time during cardiomyocyte differentiation due to an event of vector transcriptional silencing as previously reported in these cell types[8]. Most interestingly, even after clonal selection of undifferentiated hESC expressing α-actinin-mCherry, upon cardiac differentiation mCherry expression could no longer be detected in a large percentage of the cell population. This observation has been previously reported in another study[8] and can be explained by chromatin remodelling-induced silencing occurring during differentiation[15]. An alternative strategy to generate a stable reporter line is to genetically modify, for example by CRISPR/Cas9 system[16], the genomic locus of α-actinin by inserting a fluorescent reporter in frame in order to create a fluorescent sarcomeric fusion protein, which is regulated by endogenous genomic regulatory elements. This avoids not only transcriptional silencing of exogenous inserted DNA, but also non-specific over-expression of α-actinin in both cardiomyocytes and non-cardiomyocytes.

To circumvent the silencing problems that we encountered with generating stable cell lines,

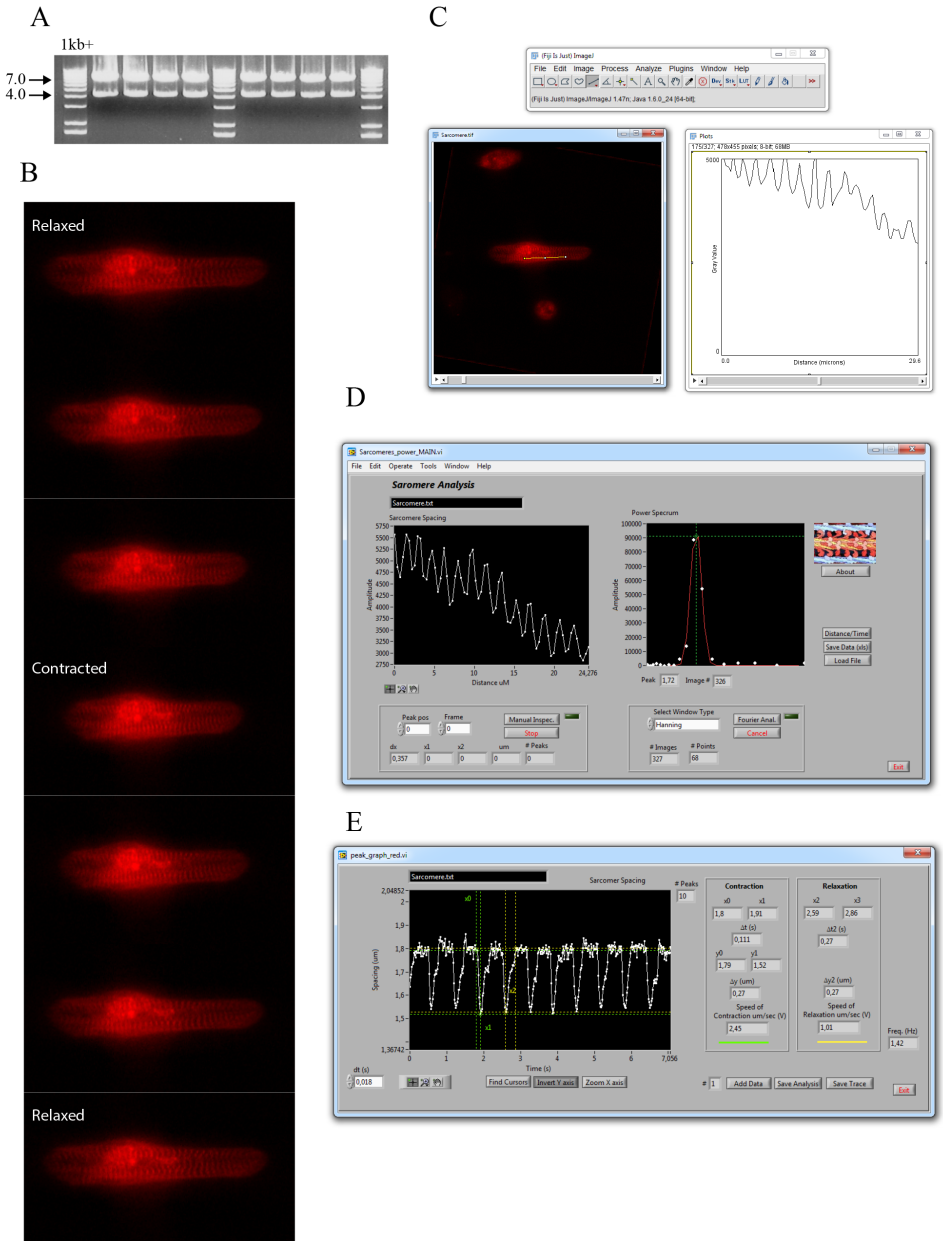


Figure 3- Sarcomere analysis. **A.** pLenti CMV α-actinin-mCherry clones after Gateway recombinase reaction digested with EcoRI displaying the predicted bands 4.2 and 7.4kb. **B.** Representative image sequence of a single cardiomyocyte contraction cycle (top-Relaxed, middle-Contracted and bottom-Relaxed form) – α-actinin-mCherry in Red. **C.** Image sequence loaded to software Image J as image stack (left); Pixel intensity profile of the line crossing the sarcomere length plotted over distance for each frame (right). **D.** Power Spectral Analysis software displaying on the left panel the pixel intensity profile with the user input of pixel size (dx) and on the right panel the power spectrum analysis output (white dots) fitted with a Gaussian distribution (red line), displaying the most frequent spacing observed (Peak) for each image and the possibility to change the window type. **E.** Sarcomere spacing of each frame plotted over time with user input of time resolution. Green lines - x0 and x1 are cursors that allow manual picking the interval between relaxed and contracted sarcomere form during active contraction; Yellow lines – x2 and x3 are cursors that allow manual picking the interval between contracted and relaxed sarcomere form during active relaxation.

we studied the use of a sarcomeric fluorescent reporter in hESC-CMs by transient over-expression of the sarcomeric reporter construct. Although the cells were selected for transduction based on the neomycin cassette, not every cell visibly expressed the α-actinin-mCherry. Moreover, there was a clear variation of sarcomeric-mCherry intensity between cardiomyocytes of the same well. Of note, regardless of the sarcomeric-mCherry intensity, all cardiomyocytes displayed no or low non-sarcomeric expression of α-actinin-mCherry, mostly localized around the nucleus as shown in **Figure 8b**, indicating an immediate degradation of the non-assembled α-actinin-mCherry inside the cardiomyocytes. This observation suggests that the variation in sarcomeric-mCherry intensity between each cardiomyocyte may not be caused by differences in expression of the α-actinin-mCherry but by the different amount of α-actinin-mCherry assembled onto the sarcomeres versus the endogenous α-actinin. Although the number of clearly visible and measurable α-actinin-mCherry positive cardiomyocytes is enough to conduct a single cell study, the variation of sarcomeric-mCherry intensity increases the number of wells and time needed to obtain enough measurable cells, especially when used in combination with other vectors (e.g. shRNA or protein over-expression). A possible solution to increase signal-to-noise ratio of the sarcomeric-mCherry intensity in this approach is to replace the mCherry by a more recently developed mRuby2 or TagRFP-T fluorescent proteins, which are 2.5x and 3x, respectively, brighter than mCherry[17,18].

After capturing good quality image sequence of contracting α-actinin-mCherry sarcomeres the most intuitive method to analyse sarcomere distances would be to measure the average distance between the consecutive sarcomeres using the intensity peak of each sarcomeric-mcherry as reference. However, the output of this method was extremely noisy due to the background created by the movement of the sarcomeres in each frame (data not shown), impairing the analysis of sarcomere kinetics. Different studies have employed the Fourier transformation to decompose the signal derived from pixel intensity plot made across the sarcomere length into a frequency spectrum, which can be used further to assess the most dense wavelength corresponding to the sarcomere distance[14,19,20]. The use of this tool fitted with a Gaussian distribution and enabled the assessment hESC-CM sarcomere velocity of shortening and relaxation. The preliminary data suggest that hESC-CM sarcomeres contract 5x slower than the observed velocities for Cat and Rat cardiac sarcomeres[13], most probably due to hESC-CM immature stage and/or to physiologic differences between species.

Conclusion

This chapter describes the development of α-actinin-mCherry fusion protein as a tool for sarcomere kinetics analysis in hPSC-CM. The use of the α-actinin-mCherry as a sarcomeric reporter tool was accomplished by lentiviral transiently delivery directly into hESC-CM, establishing a proof of concept of α-actinin-mCherry fusion assembly into functional sarcomeres. Power spectra analysis software was developed to determine the sarcomere spacing of each frame in a high-speed movie of a contracting cardiomyocyte, enabling the assessment of movement kinetics of single sarcomeres. In conclusion, we successfully developed a multifunctional tool, which will enable to study hPSC-CM contractile function in healthy and diseased conditions, responses to drugs and sarcomerogenesis and maturation.

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Supplementary figures

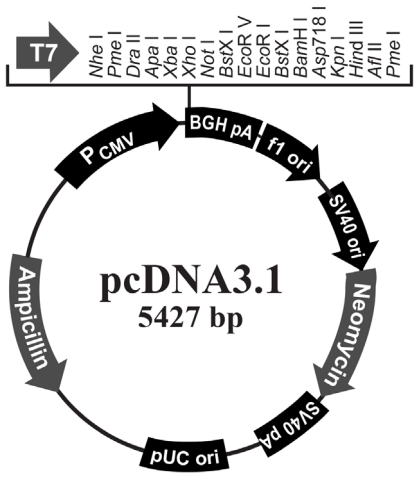

Figure S1. Vector map of pcDNA3.1 (Authorized by Thermo Fisher Scientific).

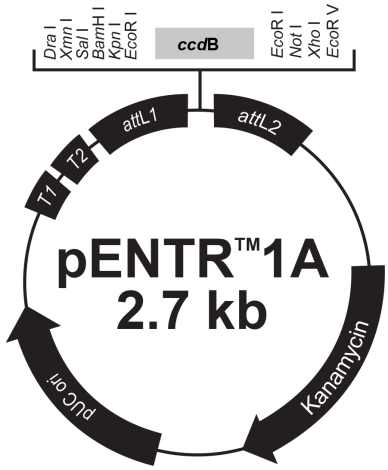

Figure S2. Vector map of pENTR1A (Authorized by Thermo Fisher Scientific).

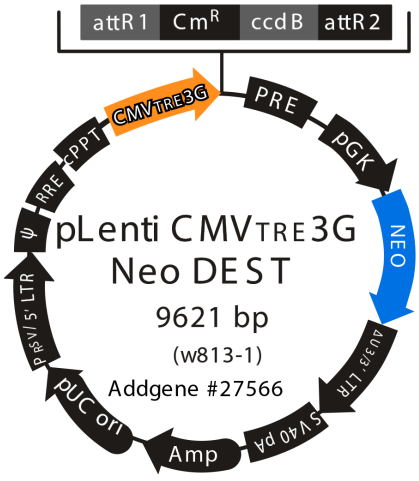

Figure S3. Vector map of pLenti CMVTRE3G Neo destination (provided by Dr. Eric Campeau and authorized by Addgene).

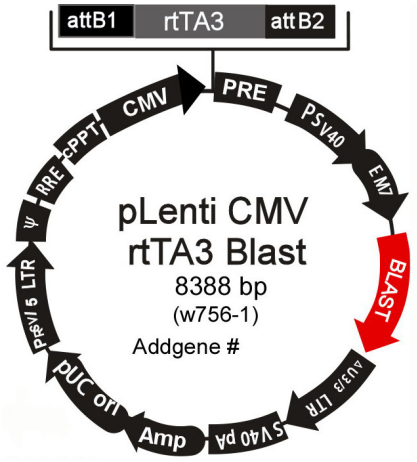

Figure S4. Vector map of pLenti CMV rtTA3 Blast (provided by Dr. Eric Campeau and authorized by Addgene).

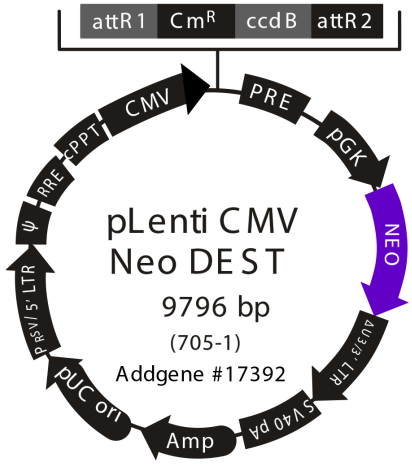

Figure S5. Vector map of pLenti CMV Neo (provided by Dr. Eric Campeau and authorized by Addgene).

Table S1 - Polymerase Chain Reaction

	Final concentration
Phusion Taq (ThermoScientific)	0.02U/µl
5x Phusion HF Buffer	1x
DMSO	3%
dNTP	200.0µM each
Fw primer	0.5µM
Rv primer	0.5µM
DNA	1.0pg-10.0ng (≥1.0 µl)
MQ	to 50.0µl
Total	50.0µl

Table S2 - PCR cycle program

Cycle step	*Temperature*	*Time*	*Cycles*
Initial denaturation	98°C	02:00	1x
Denaturation	98°C	00:30	25x
Annealing	X°C	00:30	
Extension	72°C	0:15-0:30s/kb	
Final extension	72°C	10:00	1x
Hold	12°C	∞	

Table S3 - Digestion reaction

Enzyme*	1.0-1.5µl
DNA	2-10µg
Buffer 10x**	1x
BSA 10x***	1x
MQ	to final volume
Total	20.0-50.0µl

*Enzyme's volume should not exceed 10% of the final volume. It is possible to use two enzymes in one reaction, if they are active in similar conditions (reaction buffer, temperature)

**Reaction buffer should be chosen following a producer's table of enzymes' activity

***BSA intensifies the reaction, but is not recommended for all enzymes (check producer's table of enzymes' activity)

Table S4 – Amplification primers

Name	Sequence	Restriction site	binding end
FWamp1	CGGGTACCACCATGAACCAGATAGAGCCC	KpnI	5'
RVamp1	CGGGTACCGCCAGATCGCTCTCCCCGTA	KpnI	3'
FWamp2	CAGTCGACCACCATGAACCAGATAGAGC	SalI	5'
FWamp3	TGGGTACCATGGTGAGCAAGGGCGAGGA	KpnI	5'
RVamp2	ATCTCGAGTTACTTGTACAGCTCGTCCA	XhoI	3'