



A complete workflow for the differentiation and the dissociation of hiPSC-derived cardiospheres

Benjamin Fischer^{a,1}, Anna Meier^{a,2,1}, Annika Dehne^a, Aseem Salhotra^{a,3}, Thao Anh Tran^a, Sascha Neumann^{a,4}, Katharina Schmidt^a, Ina Meiser^a, Julia C. Neubauer^{a,b}, Heiko Zimmermann^{a,b,c,d}, Luca Gentile^{a,e,f,*,5}

^a Fraunhofer Institute for Biomedical Engineering, Joseph-von-Fraunhofer-Weg 1, Sulzbach 66280, Germany

^b Fraunhofer Project Centre for Stem Cell Process Engineering, Neunerplatz 2, Würzburg 97082, Germany

^c Saarland University, Gebäude A, Saarbrücken 66123, Germany

^d Universidad Católica del Norte, Larrondo 1281, Coquimbo 1780000, Chile

^e University of Applied Sciences Kaiserslautern, Campus Zweibrücken, Building G, Amerikastr. 1, Zweibrücken 66482, Germany

^f Hasselt University, Campus Diepenbeek, Diepenbeek 3590, Belgium

ARTICLE INFO

Keywords:

hiPSCs
Cardiomyocytes
Cardiac induction
3D bioreactor
Papain dissociation

ABSTRACT

Cardiomyocytes derived from human induced pluripotent stem cells (hiPSC-CMs) are an invaluable tool for both basic and translational cardiovascular research. The potential that these cells hold for therapy, disease modeling and drug discovery is hampered by several bottlenecks that currently limit both the yield and the efficiency of cardiac induction. Here, we present a complete workflow for the production of ready-to-use hiPSC-CMs in a dynamic suspension bioreactor. This includes the efficient and highly reproducible differentiation of hiPSCs into cardiospheres, which display enhanced physiological maturation compared to static 3D induction in hanging drops, and a novel papain-based dissociation method that offers higher yield and viability than the broadly used dissociation reagents TrypLE and Accutase. Molecular and functional analyses of the cardiomyocytes reseeded after dissociation confirmed both the identity and the functionality of the cells, which can be used in downstream applications, either as monolayers or spheroids.

1. Introduction

Cardiomyocytes derived from human induced pluripotent stem cells (hiPSC-CMs) are an excellent source of patient-specific cardiac cells for pharmacological testing, disease modeling and cardiac tissue engineering. In recent years, remarkable work has been done to identify the conditions needed to recapitulate human cardiac development in vitro. The sequential activation and inhibition of the canonical Wnt pathway was shown to play a critical role for mesoderm specification and terminal cardiomyocyte differentiation, in turn (Burridge et al., 2015; Zhang et al., 2015). This has allowed the production of hiPSC-CMs to become a routine procedure in many cardiovascular

laboratories. However, conventional 2D or static 3D culture systems are not suitable for mid- to large-scale production of hiPSC-CMs. Another limitation is the efficiency of cardiac induction, which is subject to operator- and cell line-dependent inconsistencies. This has spurred efforts to move towards cardiac induction in scalable suspension culture systems amenable to a higher degree of automation (Chen et al., 2015; Kempf et al., 2014). Moreover, differentiation in dynamic suspension has the big advantage of providing the cells with a 3D microenvironment that promotes higher physiological maturation, as it allows better intercellular interactions and spatial organization (Pampaloni et al., 2007). It raises, however, the problem of generating spheroids, which are harder to dissociate for downstream applications than conventional

Abbreviations: hiPSC, human induced pluripotent stem cell; CM, cardiomyocyte; BL, BioLevigator; HD, hanging drop; NC, NucleoCounter; LT, LeviTube; Ca, Calcein

* Corresponding author at: University of Applied Sciences Kaiserslautern, Campus Zweibrücken, Building G, Amerikastr. 1, 66482 Zweibrücken, Germany.

E-mail addresses: mckind75@gmail.com, luca.gentile@uhasselt.be (L. Gentile).

¹ These authors contributed equally

² Current address: Klinikum Rechts der Isar, Technical University of Munich, Ismaningerstraße 22, 81,675, Munich, Germany

³ Current address: Department of Chemistry and Biomedical Sciences, Faculty of Health and Life Sciences, Linnaeus University, SE-391 82 Kalmar, Sweden

⁴ Current address: University Clinic Essen, Virchowstraße 171, 45147, Essen, Germany

⁵ Current address: University of Applied Sciences Kaiserslautern, Campus Zweibrücken, Building G, Amerikastr. 1, 66482, Zweibrücken, Germany.

<https://doi.org/10.1016/j.scr.2018.08.015>

Received 29 March 2018; Received in revised form 8 August 2018; Accepted 13 August 2018

Available online 24 August 2018

1873-5061/ © 2018 Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license

(<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

2D monolayers. In this study, we used a benchtop, small footprint suspension bioreactor (BioLevigator; BL) to produce hiPSC-derived cardiospheres. These displayed enhanced maturation compared to those obtained in hanging drops (HD). Combining the dynamic 3D cardiac induction with a novel papain-based dissociation protocol that consistently yields both higher cell number and viability than the broadly-used reagents TrypLE and Accutase, we established a complete workflow for the production of hiPSC-CMs for any downstream applications.

2. Materials and methods

2.1. hiPSCs maintenance

Two hiPSC lines were used: UKBi005, from the European Bank for induced pluripotent Stem Cells and IBMT1, reprogrammed in-house with mRNAs (Stemgent #00-0071) from BJ fibroblasts (ATCC #CRL-2522). Cells were maintained in mTeSR1 medium (Stem Cell Technologies #8580) in 60 mm tissue culture plates coated with growth factor-reduced Matrigel (Corning #356231), according to manufacturer's instructions. Cells were routinely passaged using 0.5 mM EDTA pH 8.0 (Invitrogen #AM9260G) in DPBS without Ca^{2+} and Mg^{2+} (DPBS^{-/-}; Gibco #14190144).

2.2. Cardiac induction in 3D

Cardiac fate was induced as previously described (Zhang et al., 2015). Briefly, hiPSCs at 70–80% confluence were harvested using TrypLE Select (Gibco #12563011). Digestion was quenched with 1 volume mTeSR1 (Stemcell Technologies #8580) with 10 μM ROCK inhibitor Y27632 (abcam #ab120129). The resulting cell clusters were broken up into single cells with the tip of a pipette. After counting (NucleoCounter NC-200; NC; ChemoMetec, Denmark), cells were diluted in day 0 medium (Table S1) to a concentration of 7.5×10^5 viable cells/mL. Up to 40 mL were transferred to a LeviTube (LT; OMNI Life Sciences #2800005) placed in the BioLevigator (BL; Hamilton, Switzerland). Cells were cultured at 37 °C and 5% CO_2 , under continuous rotation at 60 rpm in alternating directions (2 s/direction). After 24 h, the LT was placed on the benchtop to allow the small spheroids to sink to the bottom. Spent medium was replaced with an equal volume of day 1 medium (Table S1). On the following days, medium was in turn replaced with day 2 and day 3 media (Table S1). From day 4 onwards, medium was replaced with TS medium (Table S1) every other day. Cardiac induction in HD was performed as previously described (Zhang et al., 2015) in Perfecta3D 96-well hanging drop plates (Sigma #HDP1096). Medium was exchanged by replacing half of the drop volume twice.

2.3. Enzymatic dissociation of hiPSC-CMs

Cardiospheres were harvested for enzymatic dissociation after 8 or 15 days of cardiac induction. The spheroids from 1 to 2 LTs were washed twice with 2 mM EDTA in DPBS^{-/-}. After washing, the spheroids were enzymatically dissociated. Papain dissociation was carried out in a water bath at 37 °C for 30 min. Briefly, the spheroids were taken up in 500 μL DPBS^{-/-} and 500 μL freshly prepared 2 $\times$ papain solution (Table S2) was added. Digestion was stopped by adding 1 mL freshly prepared 2 $\times$ stop solution (Table S2). The spheroids were then gently disrupted into a homogeneous suspension by pipetting approximately 20 times with a wide borehole pipette tip. Cells were then washed by adding 5 mL KO-DMEM. For dissociation with Accutase or TrypLE, the spheroids were taken up in 1 mL Accutase (Sigma #A6964) or 1 mL TrypLE Select (Gibco #12563011) and incubated in a water bath at 37 °C for 15 min. The suspension was then homogenized by gently pipetting up and down 5–10 times with a wide borehole pipette tip and the reaction was quenched by adding 5 mL KO-DMEM. Adding the stop

solution used in the papain protocol before dilution with KO-DMEM did not have an effect on the dissociation results of Accutase and TrypLE (data not shown). After dissociation, the cells were used directly for analysis or re-seeded in TS medium, either on Matrigel-coated plates at a density of 2.4×10^5 cells/cm² or in the BL at a density of 7.5×10^5 cells/mL. The dissociation protocols described for cardiospheres also applied to hiPSC-CMs in monolayers, scaling down the volumes of reagents 5 times.

2.4. Measurement of cell concentration and viability at the NucleoCounter

Cell suspensions from freshly dissociated cardiospheres/monolayers were filtered through a 40 μm cell strainer placed on a 50 mL tube. Undissociated aggregates larger than 40 μm were collected from the strainer that was flipped over onto a new tube and carefully washed with at least 3 mL KO-DMEM. Cells/small clusters in the flow-through were resuspended in 1–3 mL KO-DMEM. The cell concentration in both the undissociated fraction and the flow-through was determined at the NC, following the manufacturer's instructions. To measure both cell concentration and viability, one volume was lysed to count the total number of nuclei and another volume was measured without lysis, allowing only non-viable cells to be stained with DAPI.

2.5. Flow cytometry

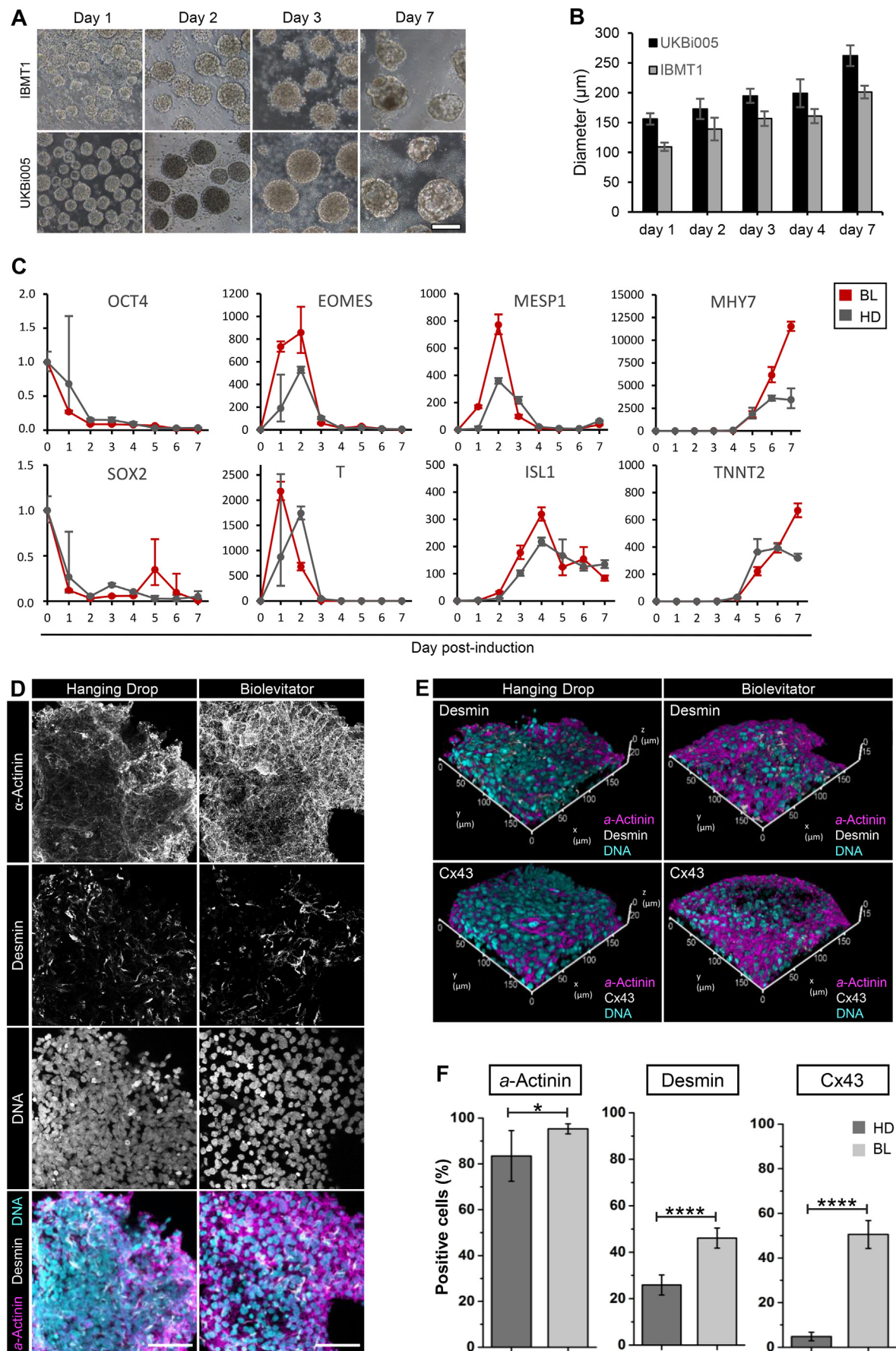
To label viable cells for flow cytometry analysis, 1×10^6 freshly dissociated hiPSC-CMs were incubated with 50 ng calcein acetoxymethyl (AM; Fisher Scientific #C1430) for 25 min at 37 °C. Propidium iodide (PI; Fisher Scientific #P1304MP) was added at a final concentration of 1 $\mu\text{g}/\text{mL}$ to label non-viable cells. Flow cytometry analysis was performed with a FACSaria III (BD).

2.6. Gene expression analysis

Samples were collected for gene expression analysis on days 1 to 7 of cardiac induction or after 2–3 weeks of culture as either cardiospheres or monolayers after dissociation. Briefly, RNA was extracted using the RNeasy Micro kit (Qiagen #74004) following the manufacturer's instructions. The RNA concentration was measured with a Nanodrop 2000 (Thermo Scientific). Reverse transcription was performed with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems #4368814), following the manufacturer's instructions. Gene expression was measured via the 5'-nuclease assay (TaqMan) quantitative PCR (qPCR) using a QuantStudio 7 Flex system (Applied Biosystems) and TaqMan assays (Fisher Scientific #4331182; Table S3). Relative quantification was calculated with the $2^{-\Delta\Delta\text{Ct}}$ method using *GAPDH*, *HPRT1* and *GUSB* as endogenous references for normalization.

2.7. Immunocytochemistry

Samples fixed for 15 min in 4.2% PFA (Cytofix, BD #554655) were permeabilized in 0.2% Triton-X-100 (Roth #3051.3) in DPBS^{-/-} for 20 (cell layers) or 30 (spheroids) minutes. After 30 min incubation in blocking solution (BS; Table S2) samples were incubated overnight at 4 °C with primary antibodies against sarcomeric α -actinin (1:200; Sigma #A7811), connexin 43 (1:400; Abcam #ab11370) or desmin (1:500; Abcam #15200). The appropriate Alexa-488 or Alexa-555-conjugated secondary antibodies (Life Technologies #A11034 and #A21422) were diluted 1:1000 in BS and incubated for 1 h at room temperature. DNA was counterstained with DAPI (Fisher Scientific #R37605). Specimens were imaged with a confocal fluorescence microscope (Leica TCS SP8); 200 cells/sample were scored for the expression of the markers tested.



(caption on next page)

Fig. 1. 3D cardiac induction of hiPSCs in static or dynamic conditions. Cardiospheres differentiated in the BL from two hiPSC lines (UKBi005 and IBMT1) were harvested on different days (A) and their diameter was measured (B; Mean $\pm$ SEM; $n = 20$ /day). The expression of pluripotency (*OCT4*, *SOX2*), mesoderm (*T*, *EOMES*), cardiovascular specification (*MESP1*, *ISL1*) and cardiomyocyte differentiation (*MYH7*, *TNNT2*) markers was assessed by qPCR in cardiospheres derived from UKBi005 hiPSCs and cultured either in the BL or in HD. Expression data are presented as relative quantification ($2^{-\Delta\Delta C_t}$ method) after normalization on the geometric average of the expression of *GAPDH*, *GUSB*, *HPRT1* (C; $n \geq 3$ per day and condition). Representative maximum projections (D) and 3D projections (E) of confocal images of α -actinin, desmin and connexin 43 immunostaining of cardiospheres at 7 dpi. Quantitative analysis of desmin-, connexin 43- and α -actinin-expressing cells based on the immunostaining presented in D and E (F; Mean $\pm$ SD; $n = 3$ –6; 200 nuclei scored for each replicate). Scale bar in A: 200 μ m. scale bar in D: 50 μ m.

2.8. Statistical analysis

Statistical analysis was carried out with GraphPad Prism 5.0. Counting data were analyzed by 1-way ANOVA with Tukey post-hoc test, Kruskal-Wallis test with Dunn's multiple comparison post-hoc test and two-tailed *t*-test. When variance was found different between sets of data, Welch's correction was applied. Linear dimensionality reduction was achieved via Principal Component Analysis, using the function *prcomp* in R (Team, 2008).

3. Results and discussion

3.1. Cardiac induction in a dynamic suspension bioreactor

We adapted the cardiac induction protocol established by Zhang and colleagues for static 3D culture (Zhang et al., 2015) to a dynamic suspension bioreactor, the BioLevigator (BL), with the goal of generating high numbers of hiPSC-CMs. Using two hiPSC lines seeded at a density of 7.5×10^5 cells/mL in up to 40 mL per tube, after a 7-day induction we obtained on average 1.3 ± 0.57 or 0.9 ± 0.43 CMs per UKBi005 or IBMT1 hiPSC input at day 0, respectively (Fig. 1A; Supplementary Movies S1–S2). At day 7, UKBi005 or IBMT1 cardiospheres reached a diameter of $262.1 \pm 17.3 \mu$ m and $215.4 \pm 10.7 \mu$ m, respectively (Fig. 1B). To evaluate the efficiency of the cardiac induction in the BL, we compared the gene expression profile of UKBi005 BL- and HD-spheroids (Fig. 1C). Both pluripotency markers considered, *OCT4* and *SOX2*, were downregulated 1 day post-induction (dpi; *OCT4*: 0.68- and 0.27-fold; *SOX2*: 0.27- and 0.12-fold in HD and BL, respectively). Mesodermal specification took place between 1 and 2 dpi in both conditions tested, as indicated by the expression of the transcription factors *Brachyury* (*T*) and *EOMES*. Interestingly, the peak of expression of *Brachyury* was delayed of 1 day in HD spheroids (peak at 2 dpi) compared to BL spheroids (peak at 1 dpi). The cardiogenic mesoderm marker *MESP1* and the cardiovascular progenitor marker *ISL1* showed a peak of expression at 2 and 4 dpi, respectively. A significant difference in the level of expression of these two markers was observed between BL and HD conditions (*MESP1*: 359- and 771-fold at 2 dpi, $p = .0044$; *ISL1*: 218- and 319-fold at 4 dpi, $p = .0025$, in HD and BL, respectively). At 7 dpi, the mature cardiomyocyte markers *MYH7* (β -myosin heavy chain) and *TNNT2* (cardiac troponin T) –two proteins essential for the assembly of the sarcomere– were both expressed at significantly higher levels in BL than in HD cardiospheres ($p = .0021$ for *MYH7*; $p = .0351$ for *TNNT2*). We then performed quantitative immunocytochemical analysis of spheroids at 7 dpi, targeting key structural components of the cardiac cells. We found that BL spheroids contained $95.3 \pm 2.2\%$ sarcomeric α -actinin⁺ cells, while HD spheroids contained $83.5 \pm 11\%$ ($p = 0.0470$; Fig. 1D, top panel; Fig. 1E; Fig. 1F, left panel, Fig. S1A,B). BL spheroids also had a significantly higher percentage of desmin⁺ cells ($46.1 \pm 4.3\%$ and $25.9 \pm 4.3\%$ in BL and HD spheroids, respectively; $p \leq 0.0001$) (Fig. 1D, mid panel; Fig. 1E, top panel; Fig. 1F, mid panel, Supplementary Fig. S1 A). This intermediate filament protein is important in the regulation of the sarcomere (Sequeira et al., 2014). In vivo, mature cardiomyocytes are electrically coupled into a functional syncytium through the formation of intercellular gap junctions (Scuderi and Butcher, 2017). We found that the percentage of cells expressing connexin 43, one of the main gap junction proteins, was ten times higher in BL spheroids ($50.5 \pm 6.2\%$) than

in HD spheroids ($4.8 \pm 1.9\%$; $p \leq .0001$; Fig. 1E, bottom panel; Fig. 1F, right panel, Supplementary Fig. S1 B).

Altogether, the cardiac induction worked in both static (HD) and dynamic (BL) conditions. However, the higher level of expression of both *MESP1* and *ISL1* during the differentiation process and of cardiomyocyte-specific markers at 7 dpi suggested that the dynamic induction is more efficient. Also, mesoderm specification was swifter in BL cardiospheres than in HD cardiospheres. Consistently with the gene expression data, the expression of proteins required for the organization of a functional sarcomere and for the electrical coupling of the cardiomyocytes was also markedly higher in BL cardiospheres, indicating that dynamic culture induced a higher level of physiological maturation. As both HD and BL culture methods involve the formation of a 3D structure, the better outcome of the BL probably owes to a better distribution of oxygen and nutrients, generated by the medium flow, which likely improves the cardiac differentiation of the hiPSCs, as shown for other differentiation pathways (Wang et al., 2005; Li et al., 2009). Remarkably, in order to produce the same number of cardiomyocytes obtained in one LT (40 mL), $> 100 \times 96$ -well HD plates or 10 $\times$ T75 flasks would be necessary.

3.2. Comparison of papain, TrypLE and Accutase for the dissociation of hiPSC-CMs

As the BL proved to be a suitable system for the cardiac induction in 3D, we looked for a way to effectively dissociate the spheroids into single cells. Three-dimensional cell structures are more challenging to dissociate than monolayers, where every cell is equally accessible to the protease used. The enzymatic treatment must be efficient enough to achieve complete dissociation without causing significant cell damage. We decided to test the efficiency of papain, a cysteine protease from *Carica papaya* shown to deliver higher yield and cell viability than other dissociation methods for brain tissues and planarian flatworms (Brewer, 1997; Moritz et al., 2012). We compared papain with two broadly used dissociation reagents, TrypLE and Accutase. These two reagents are commonly employed for the dissociation of hiPSC-CMs in monolayer or 3D cultures (Zhang et al., 2015; Chen et al., 2015; Piccini et al., 2015). To compare the performances of the three dissociation reagents, we assessed both yield and viability of the cell suspensions obtained after dissociation using two platforms, an accessible benchtop cell counting instrument (the Nucleocounter) and a flow cytometer (FACS Aria III).

After the enzymatic treatment, single cells and small clusters were separated from large aggregates by passing the cell suspensions through a 40 μ m cell strainer; the strainers were visually inspected prior to counting (Fig. 2A). Afterwards, the cells in the flow-through as well as those in the large aggregates were counted with the NC. Accutase yielded $57 \pm 12.1\%$ of single cells, TrypLE $76.1 \pm 11.6\%$ and papain $95.3 \pm 3.2\%$ (Fig. 2B; $p = 0.012$). By using the automated viability measurement of the NC as a quick readout, we calculated that Accutase yielded $41.5 \pm 8.5\%$ of single viable cells, TrypLE $70.1 \pm 10.8\%$ and papain $90.3 \pm 3.7\%$ (Fig. 2C; $p = 0.0003$).

We then analyzed the flow-through fractions by flow cytometry, to precisely quantify the proportion of single cells, small cell clusters and cell debris. These were distinguished based on their forward and side-ward scatter properties, using the gating strategy shown in Fig. 2D. We found that the percentage of single cells yielded by the treatments were $33.5 \pm 10\%$ for Accutase, $45.9 \pm 9\%$ for TrypLE and $74.8 \pm 9.8\%$

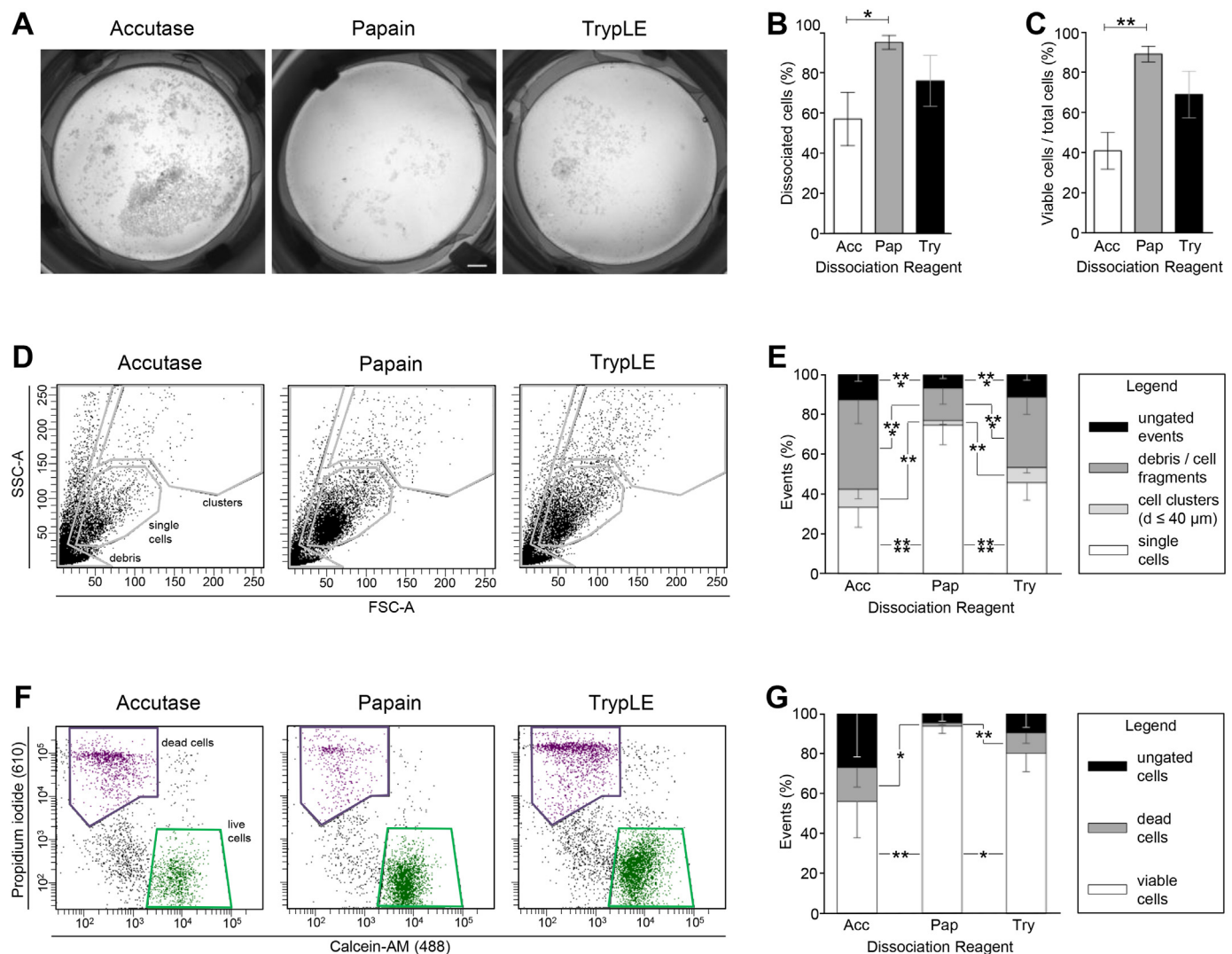


Fig. 2. Single viable cell number obtained from hiPSC-cardiospheres varies greatly depending on the dissociation reagent used. Representative images of undissociated aggregates collected on 40 μm cell strainers after filtration of the cell suspensions obtained with either Accutase, Papain or TrypLE from cardiospheres at 8 dpi (A). The number of single (B) and viable single (C) cells in the flow-through was assessed using a NucleoCounter (n = 6–7/condition); *: $p < 0.05$; ***: $p \leq 0.005$. Representative FSC/SSC scatter plots obtained following the use of the dissociation reagents considered (D). The gates define single cells, $\leq 40 \mu\text{m}$ cell clusters and debris/cell fragments. Events gated according to D were quantified as Mean $\pm$ SD (E; n = 6–7/condition). Cell viability was measured within the single cell gates defined in D, based on Ca/PI double staining (F). The number of viable, dead and ungated cells is presented as mean $\pm$ SD for each dissociation reagent used (G; n = 6–7/condition).

for papain (Fig. 2E; $p \leq 0.0001$). The flow-through fraction obtained with papain also contained a percentage of debris ($16.2 \pm 8\%$) and small cell clusters ($2.3 \pm 2\%$) lower than both TrypLE ($35.3 \pm 8.6\%$; $p \leq 0.0001$; $7.4 \pm 2.6\%$; $p = 0.017$) and Accutase ($45 \pm 12.1\%$; $p \leq 0.0001$; $8.9 \pm 4.7\%$; $p = 0.0095$) (Fig. 2E). The percentage of single cells is a direct measurement of the efficiency of the enzymatic digestion, but important is also the proportion of viable and dead cells. Therefore, we incubated the cells with both propidium iodide (PI) and non-fluorescent calcein AM –hydrolytically converted into green-fluorescent calcein (Ca) by viable cells. Using the gating shown in Fig. 2F, we found that, among the single cells digested with papain, $93.6 \pm 3.7\%$ were viable (Fig. 2G), significantly more than those obtained with either TrypLE ($80.4 \pm 9.3\%$; $p = 0.016$) or Accutase ($56 \pm 18.1\%$; $p = 0.0018$). To this regard, the advantage of TrypLE over Accutase was also significant ($p = 0.014$). Incidentally, we noticed that both the classic live/dead gating based on FSC/PI and the automated viability assay run by the NC significantly overestimated the number of viable cells compared to the flow cytometer data based on the mutually exclusive dyes Ca and PI (Supplementary Fig. S2). This

likely owes to the fact that dying cells may be negative for PI (or DAPI, used for NC counting). Since they are also weak/negative for Ca, the live-dead gating based on Ca/PI is more accurate than that based on PI (or DAPI) alone.

Having found that papain gave advantages over Accutase and TrypLE in dissociating cardiospheres into single, viable cardiomyocytes, we decided to assess experimentally whether this would hold true for other culture conditions. As reported (Burridge et al., 2015), hiPSC-CMs become more resistant to dissociation in prolonged cultures. Thus, we compared the dissociation efficiency of 8 dpi cardiospheres to that of 15 dpi cardiospheres (Fig. 3A, left panel). The prolonged culture did not significantly alter the composition of the cell suspension obtained after Accutase digestion; the additional time in culture, however, reduced the yield of single cells obtained using either papain ($54.4 \pm 2.7\%$; $p = 0.0101$) or TrypLE ($17.1 \pm 1.7\%$; $p = 0.0008$), compared to 8 dpi cardiospheres. This was associated with significantly more debris in the cell suspension obtained by TrypLE ($59.4 \pm 4.5\%$; $p = 0.0032$). Importantly, however, the relative efficiency of the three dissociation methods at 15 dpi remained consistent with that observed at 8 dpi. In

particular, the percentage of single cells obtained with papain was still significantly higher ($p \leq 0.0001$; Fig. 3A, left panel) and the percentage of debris significantly lower ($p \leq 0.0001$; Fig. 3A, center panel) than those obtained with TrypLE and Accutase. Looking at the percentage of viable single cells, no difference was found between cardiospheres at 8 and 15 dpi dissociated with either reagent (Fig. 3A, right panel). Next, we assessed the performance of papain in dissociating cardiomyocytes cultured in monolayers, as this is still the standard format of cardiac induction that most laboratories and commercial providers use. We performed the flow cytometry analysis on cells detached from 96-well plates and found that for the three dissociation methods considered, the cell suspensions obtained from cardiomyocyte monolayers did not differ from those obtained from cardiospheres, for any of the observed parameters (Fig. 3B). Papain dissociation of cardiomyocyte cultured in monolayer yielded $53.4 \pm 7.9\%$ single cells (Fig. 3B, left panel), which was higher than both TrypLE ($29.3 \pm 3.8\%$; $p = 0.0144$) and Accutase ($30.7 \pm 3.2\%$; $p = 0.0171$). It also produced a significantly lower percentage of debris ($26.1 \pm 4.7\%$; Fig. 3B, center panel) than TrypLE ($48.5 \pm 5.0\%$; $p = .0048$) and Accutase ($45.7 \pm 3.2\%$; $p = 0.0033$). Most importantly, the percentage of viable single cells was again markedly higher with papain ($92.2 \pm 2.0\%$; Fig. 3B, right panel) than

with TrypLE ($63.3 \pm 6.0\%$; $p = 0.0003$) and Accutase ($57.5 \pm 5.2\%$; $p \leq 0.0001$). Eventually, we assessed cell line-related differences. The results obtained when dissociating UKBi005- or IBMT1-derived cardiospheres showed that no line-specific bias affected the efficiency of the digestions, regardless of the dissociation reagent used (Supplementary Fig. S3 A,B).

These findings showed that papain is superior to other commonly used reagents for the dissociation of hiPSC-CMs, either as cardiospheres or monolayers.

3.3. Characterization of cardiomyocytes post-dissociation

In order to validate our papain-based dissociation protocol, we verified that it did not have undesirable effects on the seeding and culture of the dissociated hiPSC-CMs. To this end, hiPSC-CMs dissociated from cardiospheres with either papain, TrypLE or Accutase were re-seeded on Matrigel-coated cell culture plates (2D, 2.4×10^5 cells/cm²) or in a BL (3D, 7.5×10^5 cells/mL). We noticed that some of the cells obtained after either Accutase or TrypLE digestions died between counting and plating. Also, the freshly seeded cells after dissociation with papain had a well-defined shape, differently to those

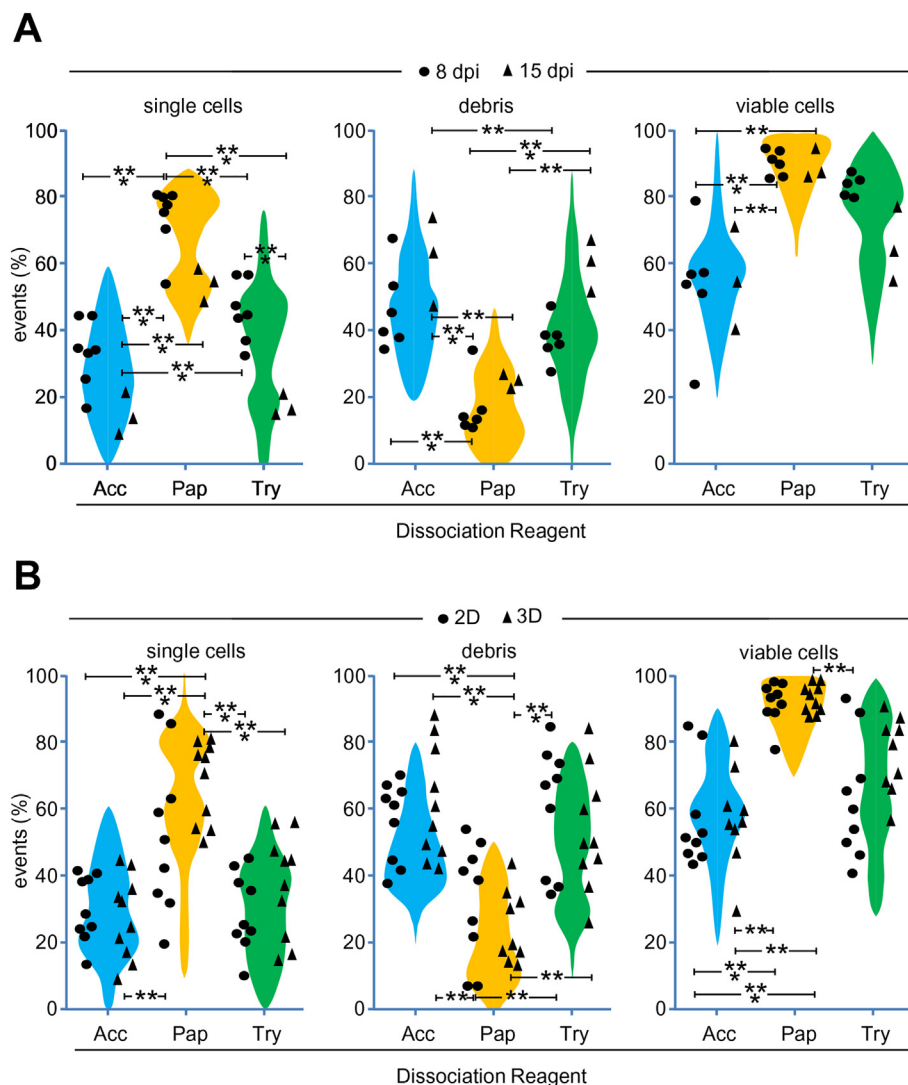


Fig. 3. Age- and culture condition-related effects on the dissociation of hiPSC-CMs. Cardiospheres at either 8 or 15 dpi were dissociated with either Accutase, papain or TrypLE and scored for the number of single viable cells obtained (A). The hybrid scatter plot/violin charts show both the individual data points as well as their uni- or bi-modal distribution. *: $p < 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.005$. The analysis presented in A was also conducted to show the dissociation efficiency of spheroids (3D) compared to monolayers (2D) (B). *: $p < 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.005$.

dissociated with the other two reagents (Fig. 4A, left panel). The attachment of hiPSC-CMs seeded in monolayer was assessed by time-lapse microscopy. Papain-digested CMs attached to the Matrigel-coated surface slower than Accutase- and TrypLE-digested CMs (Supplementary Movies S3–S5). This initial delay, however, did not produce significant differences in the abilities of the cells to adhere to the substrate and spread. (Fig. 4A). Spontaneous synchronous beating was promptly observed in all three groups, both in 2D (as early as 3 days after seeding; Supplementary Movies S6–S8) and 3D culture conditions (in U-bottom shape 96-well plate; Supplementary Movies S9–S11). This suggests that,

in spite of the different outcome in terms of viable cell number, the three dissociation reagents tested did not influence the functionality of the cells after reseeding, either in 2D or 3D. Nonetheless, in order to detect subtle differences in the cells' post-dissociation phenotype that could potentially interfere with the data generated in downstream applications, we looked at the expression of genes encoding the sarcomeric proteins cardiac troponin T (*TNNT2*), titin (*TTN*), myosin heavy chain (*MYH7*), myosin light chain 2 (*MYL2*), myosin light chain 7 (*MYL7*), desmin (*DES*) and the ion channels Kv7.1 (*KCNQ1*) and Nav1.5 (*SCN5A*). Quantitative PCR data acquired after 2 or 3 weeks in culture

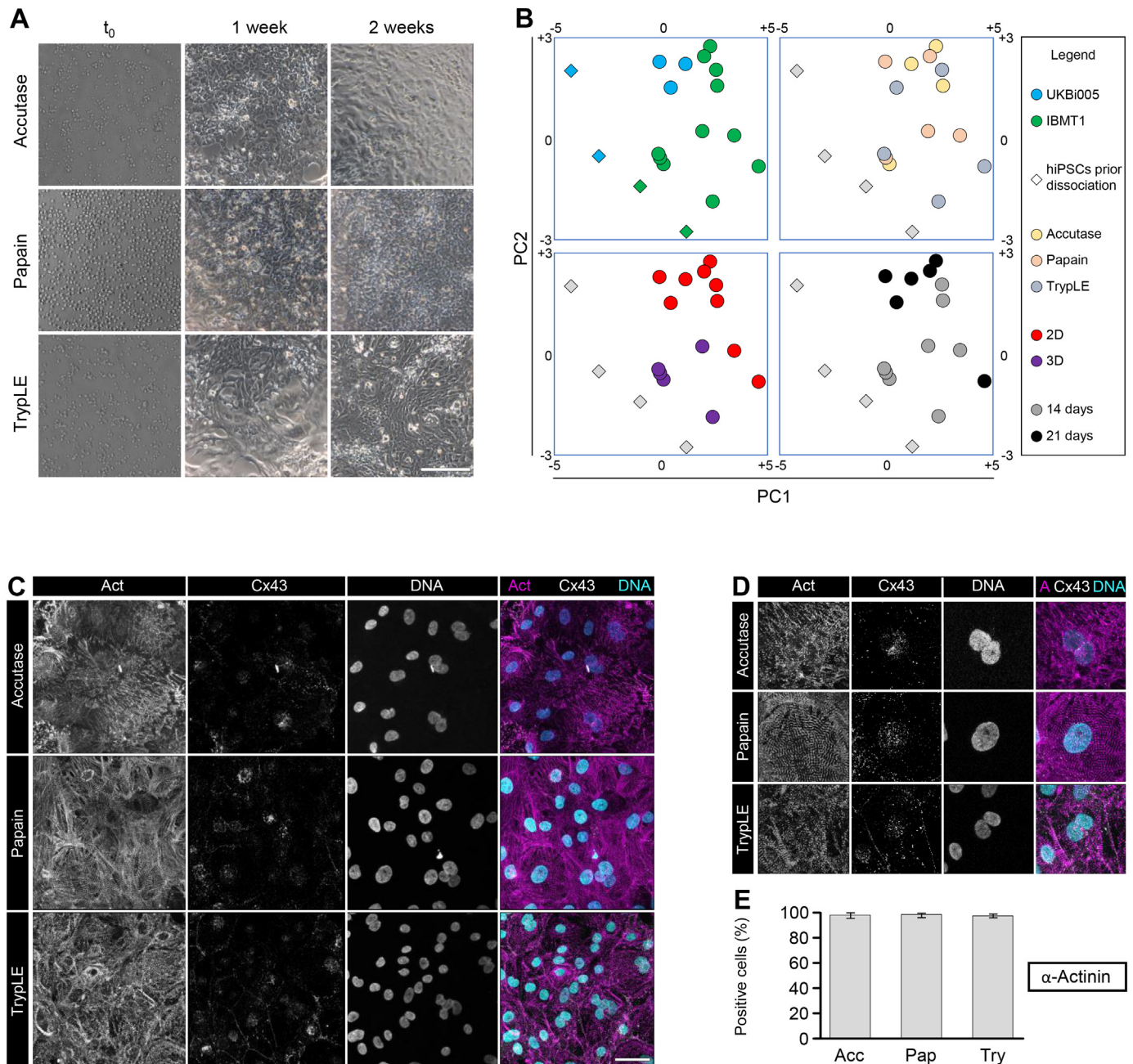


Fig. 4. Phenotype of hiPSC-cardiomyocytes cultured in after dissociation with Accutase, Papain or TrypLE. Representative images of 2.4×10^5 dissociated cardiomyocytes/cm² at t_0 and after 1 or 2 weeks in culture (A). After 2/3 weeks in culture, gene expression was assessed via qPCR. Data analyzed via principal component analysis (PCA) are plotted as individual data points in function of the first two PCs (B). In the 4 identical plots, the color codes highlight the 4 variables at play: cell line (top-left), culture conditions (bottom-left), time in culture (bottom-right) and dissociation reagent used (top-right). The diamond shape represents hiPSCs before dissociation, colored according to the cell line or left grey when the other variables were considered. Double immunocytochemistry against α -actinin and connexin 43 showing the sarcomere organization and the gap junctions of cardiomyocytes after 2 weeks of culture in monolayer (C). A magnification of the yellow squares in C is provided (D). Quantitative analysis of α -actinin-expressing cells presented as mean $\pm$ SD (E; $n = 3$, 200 nuclei scored for each replicate). Scale bars in A, C, D: 50 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

were analyzed via principal component analysis (PCA) to gain an unbiased and global view of how the many variables at play may influence the gene expression profile of the cells (Fig. 4B). We could observe clustering according to the cell line used (Fig. 4B, top-left panel), to the culture conditions (Fig. 4B, bottom-left panel) and to the time in culture (Fig. 4B, bottom-right panel) but not to the dissociation reagent used (Fig. 4B, top-right panel). We then looked at protein expression. Both sarcomeric α -actinin and connexin 43 were found abundantly expressed by all re-seeded CMs (Fig. 4C,D), suggesting that neither papain nor the other dissociation reagents used introduced biases in the phenotype of the hiPSC-CMs. From the quantitative point of view, > 97% of the re-seeded cells expressed sarcomeric α -actinin ($97.73 \pm 1.5\%$, $98.27 \pm 1.3\%$ and $97.32 \pm 1.6\%$ for Accutase, Papain and TrypLE treatment, respectively; Fig. 4E). From the qualitative point of view, however, we noticed that Accutase-dissociated CMs showed a poorer organization of the sarcomere after re-seeding in monolayers (Fig. 4C,D), compared to the other two dissociation reagents used. Similar results were seen when assessing the expression of sarcomeric α -actinin, connexin 43 and desmin proteins in cardiospheres after 2 weeks in culture in the BL (data not shown). These finding further confirmed that the cells obtained from papain dissociation preserved an intact functional phenotype and could be used in any downstream applications. On the other hand, gene expression data suggested that parameters others than the dissociation reagents should be regarded as potentially bias inducers (e.g. cell line, culture length and condition) and must be taken into careful consideration depending on the desired application.

3.4. Conclusions

Our data demonstrated that the cardiac induction in the BL, combined with papain-based cell harvesting is a powerful combination of techniques that reproducibly generate high number of viable, functional single hiPSC-CMs that can be effectively used for cardiovascular research.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scr.2018.08.015>.

Funding sources

BF and TAT were supported by Uni-Saar PhD fellowships; AS was supported by the Erasmus+ programme; this work was supported by the Fraunhofer Institute for Biomedical Engineering.

Competing interests

The authors declare no competing interests.

Acknowledgements

The authors are grateful to Sybille Richter and Susan Zöllner for the maintenance of hiPSCs, and to Dr. Sören Moritz for introducing us to the power of papain.

References

- Brewer, G.J., 1997. Isolation and culture of adult rat hippocampal neurons. *J. Neurosci. Methods* 71, 143–155.
- Burridge, P.W., Holmström, A., Wu, J.C., 2015. Chemically defined culture and cardiomyocyte differentiation of human pluripotent stem cells. *Curr. Protocols Human Genetics* 87, 21–25.
- Chen, V.C., Ye, J., Shukla, P., Hua, G., Chen, D., Lin, Z., Liu, J.-c., Chai, J., Gold, J., Wu, J., Hsu, D., Couture, L.A., 2015. Development of a scalable suspension culture for cardiac differentiation from human pluripotent stem cells. *Stem Cell Res.* 15, 365–375.
- Kempf, H., Olmer, R., Kropp, C., Rückert, M., Jara-Avaca, M., Robles-Diaz, D., Franke, A., Elliott, D.A., Wojciechowski, D., Fischer, M., Roa Lara, A., Kensah, G., Gruh, I., Haverich, A., Martin, U., Zweigerdt, R., 2014. Controlling expansion and cardiomyogenic differentiation of human pluripotent stem cells in scalable suspension culture. *Stem Cell Reports* 3, 1132–1146.
- Li, D., Tang, T., Lu, J., Dai, K., 2009. Effects of flow shear stress and mass transport on the construction of a large-scale tissue-engineered bone in a perfusion bioreactor. *Tissue Eng.* 15, 2773–2783.
- Moritz, S., Stöckle, F., Ortmeier, C., Schmitz, H., Rodríguez-Esteban, G., Key, G., Gentile, L., 2012. Heterogeneity of planarian stem cells in the S/G2/M phase. *Int. J. Dev. Biol.* 56, 117–125.
- Pampaloni, F., Reynaud, E.G., Stelzer, E.H.K., 2007. The third dimension bridges the gap between cell culture and live tissue. *Nat. Rev. Mol. Cell Biol.* 8, 839–845.
- Piccini, I., Rao, J., Seebohm, G., Greber, B., 2015. Human pluripotent stem cell-derived cardiomyocytes: Genome-wide expression profiling of long-term in vitro maturation in comparison to human heart tissue. *Genomics data* 4, 69–72.
- R.D.C. Team, 2008. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
- Scuderi, G.J., Butcher, J., 2017. Naturally engineered maturation of cardiomyocytes. *Front. Cell Dev. Biol.* 5 <https://doi.org/10.3389/fcell.2017.00050>. <https://www.frontiersin.org/article/10.3389/fcell.2017.00050> (50 pages, ISSN: 2296-634X).
- Sequeira, V., Nijenkamp, L.L.A.M., Regan, J.A., van der Velden, J., 2014. The physiological role of cardiac cytoskeleton and its alterations in heart failure. *Biochim. Biophys. Acta Biomembr.* 1838, 700–722.
- Wang, H., Riha, G.M., Yan, S., Li, M., Chai, H., Yang, H., Yao, Q., Chen, C., 2005. Shear stress induces endothelial differentiation from a murine embryonic mesenchymal progenitor cell line. *Arterioscler. Thromb. Vasc. Biol.* 25, 1817–1823.
- Zhang, M., Schulte, J.S., Heinick, A., Piccini, I., Rao, J., Quaranta, R., Zeuschner, D., Malan, D., Kim, K.-P., Röpke, A., Sasse, P., Araújo-Bravo, M., Seebohm, G., Schöler, H., Fabritz, L., Kirchhof, P., Müller, F.U., Greber, B., 2015. Universal cardiac induction of human pluripotent stem cells in two and three-dimensional formats: Implications for in vitro maturation. *Stem Cells* 33, 1456–1469.