Journal of Visualized Experiments

# Assessing cardiac reprogramming using high content imaging analysis

--Manuscript Draft--

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|---|---|
| **Article Type:** | Invited Methods Article - JoVE Produced Video |
| **Manuscript Number:** | JoVE61859R1 |
| **Full Title:** | Assessing cardiac reprogramming using high content imaging analysis |
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| **Additional Information:** | |
| **Question** | **Response** |
| Please indicate whether this article will be Standard Access or Open Access. | Standard Access (US$2,400) |
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**TITLE:**

# Assessing Cardiac Reprogramming Using High Content Imaging Analysis

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**KEYWORDS:**

reprogramming, cardiomyocyte, high content imaging, fibroblast, iCMs, in vitro

**SUMMARY:**

We present a protocol to quantify directly reprogrammed induced cardiomyocyte-like cells (iCMs) in vitro using high content imaging analysis. This method allows us to quantify the efficiency of cardiac reprogramming in an automated manner and to directly visualize iCMs.

**ABSTRACT:**

The goal of this protocol is to describe a method for quantifying induced cardiomyocyte-like cells (iCMs), which are directly reprogrammed in vitro by a reprogramming technique. Cardiac reprogramming provides a strategy to generate new cardiomyocytes. By introducing core cardiogenic transcription factors into fibroblasts; fibroblasts can be converted to iCMs without transition through the pluripotent stem cell state. However, the conversion rate of fibroblasts to iCMs still remains low. Accordingly, there have been numerous additional approaches to enhance cardiac reprogramming efficiency. Most of these studies assessed cardiac reprogramming efficiency using flow cytometry, while at the same time performed immunocytochemistry to visualize iCMs. Thus, at least two separate sets of reprogramming experiments are required to demonstrate the success of iCM reprogramming. In contrast, automated high content imaging analysis will provide both quantification and qualification of iCM reprogramming with a relatively small number of cells. With this method, it is possible to directly assess the quantity and quality of iCMs with a single reprogramming experiment. This approach will be able to facilitate future cardiac reprogramming studies that require large-scale reprogramming experiments such as screening genetic or pharmacological factors for enhancing reprogramming efficiency. In addition, the application of high content imaging analysis protocol is not limited to cardiac reprogramming. It can be applied to reprogramming of other cell lineages as well as any

immunostaining experiments which need both quantification and visualization of immunostained cells.

## INTRODUCTION:

Cardiac reprogramming has been developed as an alternative approach to stem cell mediated approaches to generate new cardiomyocytes. Given that it does not transition through stem cell state, it has a high potential to bypass some inherited limitations in stem cell mediated approaches. It has been shown that viral infection of at least three or four cardiogenic transcription factors into fibroblasts can convert fibroblasts toward a cardiac fate by eliminating fibroblast gene programs and rebuilding cardiogenic transcriptional networks in fibroblasts[1–17].

Since the first landmark study demonstrating cardiac reprogramming in vitro[1], the cardiac reprogramming protocol has been optimized by numerous studies[3,5–7,9,11–16,18]. Common technical approaches to assess cardiac phenotypes in fibroblasts following cardiac reprogramming have been flow cytometry analysis for quantifying cells expressing specific cardiomyocyte markers and immunocytochemistry for visualizing those cells at a single cell level. Although both experiments (i.e., flow cytometry and immunocytochemistry) are to demonstrate expression of cardiomyocyte markers using the same antibodies, they have to be performed separately. In addition, flow cytometry needs a relatively larger number of cells, thereby increasing the amount of reagents needed for the experiment. Alternatively, cardiomyocyte marker positive cells can be quantified by manual counting following immunocytochemistry. However, it is very labor intensive and tends to be less accurate.

The purpose of this protocol is to describe the method that can quantify and visualize iCMs by a single immunostaining experiment using automated high content imaging analysis. It requires a relatively small number of starting cells because this protocol is performed in a well of 24-well plate. As many as three different markers can be used at the same time. Single, double, and triple positive cells can be automatically quantified. In addition to quantification of immunostained cells, high content imaging analysis provides high quality 10x objective images. If necessary, the same immunostained cells used in high content imaging analysis can be re-used for further imaging studies, such as confocal microscopy. The main advantage of this protocol is that it provides not only unbiased quantification of iCMs with a much smaller number of cells, but also visualization of iCMs. Furthermore, this protocol can be utilized for assessing non-cardiac lineage reprogramming (e.g., iPSC, neuron, and hepatocyte reprogramming).

## PROTOCOL:

All animal procedures were performed with the approval of Vanderbilt University Medical Center Institutional Animal Care and Use Committee.

### 1) Retrovirus generation and in vitro cardiac reprogramming

1.1) Culture Platinum E cells in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 1 µg/mL puromycin, and 10 µg/mL blasticidin until Platinum E cell confluency reaches 70%–80%.

1.2) On Day 1, seed ~0.55 x $10^6$ cells (first well) and ~0.18 x $10^6$ cells (second well) into two separate wells of 12-well plate with 1 mL of DMEM supplemented with 10% FBS and 1% penicillin/streptomycin a day prior to the first transfection.

NOTE: Different sizes of culture dishes can be used depending on the amount of viral media needed for the experiments. Refer to **Table 1** for other scales of experiments.

1.3) On Day 2, transfect the quad-cistronic M-G-T-H retroviral construct encoding Mef2c, Gata4, Tbx5, and Hand2 described in the previous study[9] or empty vector into Platinum E cells of the first well. Add 3 µL of transfection reagent to 30 µL of reduced serum media. Five minutes later, add 1 µg retroviral construct or empty the vector into the mixture of transfection reagent and reduced serum media. After 20 min incubation at room temperature, add the mixture to Platinum E cells.

1.4) On Day 3, 16–20 h after transfection, remove the media and replenish fresh DMEM supplemented with 10% FBS and 1% penicillin/streptomycin.

1.5) On Day 3, 24 h after the first transfection, perform the second transfection into the second well in which Platinum E cells were plated on Day 1 as described in step 1.3).

1.6) On Day 3, seed ~5 x $10^4$ frozen mouse embryonic fibroblasts (MEFs) isolated from Titin-GFP reporter knock-in mice[19] into a well of 24-well plate. A total of two wells of 24-well plate are needed (i.e., uninfected control and M-G-T-H infection).

NOTE: The number of MEFs plated may need to be adjusted, because the recovery rate of frozen MEFs can vary depending on cell freezing conditions. About 10% confluency a day after seeding cells is adequate for reprogramming. Reprogramming efficiency could be enhanced using fresh unfrozen MEFs.

1.7) On Day 4, 16–20 h after the second transfection, remove the media and replenish fresh DMEM supplemented with 10% FBS and 1% penicillin/streptomycin.

1.8) On Day 4, 48 h after the first transfection, collect the viral media in the first well of Platinum E cells using a 5 mL syringe and filter them through a 0.45 µm polyethersulfone (PES) membrane filter. Remove the fibroblast growth media on MEFs, and replace them with the viral media supplemented with polybrene at 6 µg/mL (first infection).

1.9) On Day 5, 48 h after the second transfection, perform the second infection using the viral media in the second well as described in step 1.8).

1.10) On Day 6, 24 h after the second infection, the viral media are replaced with cardiac induction media composed of DMEM/199 (4:1), 10% FBS, 5% horse serum, 1% penicillin/streptomycin, 1% non-essential amino acids, 1% essential amino acids, 1% B-27, 1%

insulin-selenium-transferrin, 1% vitamin mixture, and 1% sodium pyruvate, 1 µM SB431542, and 0.5 µm A83-01. Change cardiac induction media every three days until the cells are harvested.

**2) Immunostaining**

2.1) At 14–15 days post infection, fix the cells on a 24-well plate with 2% paraformaldehyde for 15 min.

2.2) Permeabilize the fixed cells with permeabilization buffer (0.05% Triton-X in PBS) by washing the cells three times with permeabilization buffer every 5 min.

2.3) Incubate the cells with universal blocking buffer for 45 min.

2.4) Incubate the cells with mouse α-actinin (1:400 dilution) and chicken GFP antibodies (1:400 dilution) for 1.5 h at room temperature.

NOTE: About 150 µL of antibody solution can cover the whole area of a well of 24-well plate.

2.5) Wash the cells with permeabilization buffer for 5 min three times.

2.6) Incubate the cells with anti-mouse Alexa-555 and anti-chicken Alexa-488 secondary antibodies (1:400 dilution) for 1 h at room temperature

2.7) Wash the cells with permeabilization buffer for 5 min three times.

2.8) Add 2.5 µL of DAPI solution into 250 µL of permeabilization buffer.

**3) High content imaging**

3.1) Turn on the imaging system.

3.2) Open the associated software.

3.3) Log into the system.

3.4) On the taskbar, select **Run a Plate**.

3.5) Click on **Open Door-Eject Plate** to open the door on the machine and put the plate in. Make sure the plate is in the right direction. Click on **Close Door-Load Plate** to close the door.

3.6) Click on **Load Plate Settings**. Select a template protocol, and then click on **Load From DB**.

3.7) Click on **Acquisition Setup**.

3.8) In the **Plate Acquisition Setup** dialog, click on **Configure**.

3.9) In the **Objective and Camera** tab, select 10x objective.

3.10) In the **Plate** tab, select a 24-well plate.

3.11) In the **Sites to Visit** tab, determine the number of imaging sites by choosing the number in columns and rows. A total of 36 imaging sites are used by selecting 6 in columns and 6 in rows. Select the distance between each imaging site (i.e., 500 um).

3.12) In the **Acquisition** tab, set the number of wavelength as **3**.

3.13) In the **Wavelengths** tab, select **DAPI**, **FITC**, and **Texas Red**.

3.14) Click on the **Run** tab and then on **Calculate** to set up the focus offset for each wavelength. Set up the foucs offset for DAPI first. Click on **Auto Expose** to set up the exposure time automatically for each wavelength. Then, click on **Acquire Plate** to start imaging the selected sites.

**4) Analysis of high content imaging**

4.1) Once high content imaging is completed, click on the **Screening** menu, and select **Review Plate Data** to select a plate for analysis.

4.2) Click on **Select Plate**. In the **Select Plate for Review** dialog, open the folder and select the plate saved in the database and then click on Select.

NOTE: To display the images, select **DAPI**, **FITC**, and **Texas Red** in the **Wavelengths** field. In the **Sites** field, select **All Sites**. Click on a site among the selected 36 sites to display it in each wavelength image window, and then click on **Look-Up Table** to select a color for the wavelength. Use the **Print Screen** key on the keyboard and paste the image in a photo editing software (e.g., Paint and Photoshop) to save it.

4.3) Click on **Run Analysis**, and select a template setting.

4.4) Click on **Configure Settings** and then on **Number of Wavelengths**. Select three wavelengths (i.e., **DAPI** for nuclei staining, **Teaxs Red** for α-actinin, and **FITC** for Titin-GFP).

4.5) Using the **Line** tool in the tool bar, measure the width across the short axis of a cell. Based on the measured widths of cells, set the “Approximate min width” and “Approximate max width” to include most of cells in the selected imaging site.

4.6) Click on **Preview** to check whether nearly all nuclei are selected. The selected nuclei exhibit white color, while the unselected nuclei remain blue (DAPI stained). If necessary, make adjustments for "Approximate min width" and "Approximate max width".

4.7) To set **Intensity Above Local Background**, place a mouse cursor inside and outside of a cell. The intensity value appears at the bottom of the window. Slightly reduce the intensity of a dim cell to evenly exhibit the intensity throughout the whole area of each cell. Define this intensity value as **Intensity Above Local Background** value. This value needs to be set separately for each channel.

4.8) Click on the **Screening** menu and select **Plate Data Utilities**. In the **Plate Data Utilities** dialog, click on **Run Analysis** to select the plate.

4.9) In the **Settings** field, select the saved setting for analysis; select **Add to Auto Run List**, and then click on **OK** to run the analysis.

4.10) After the analysis is completed, click on **Screening** menu and select **Plate Data Utilities**. Then, in the **Plate Data Utilities** dialog, click on **Export Measurements** to export the analysis results.

4.11) Click on **Cell and Image Measurements** and then on **OK**.

4.12) On the **Export Measurements Wizard - Step 1** page, select the plate and click on **Next**.

4.13) On the **Export Measurements Wizard - Step 2** page, click on **Finish**.

4.14) On the **Configure Data Export** page, select the data types (i.e., well name, total cell number, subtotal cell number for $DAPI^+$, subtotal cell number for Texas $Red^+$, subtotal cell number for $FITC^+$, subtotal cell number for $DAPI^+$Texas $Red^+$, subtotal cell number for $DAPI^+FITC^+$, subtotal cell number for $DAPI^+FITC^+$Texas $Red^+$, percentage of $DAPI^+$ cells, percentage of $DAPI^+$Texas $Red^+$ cells, percentage of $DAPI^+FITC^+$ cells, and percentage of $DAPI^+$Texas $Red^+FITC^+$ cells) and then click on **OK**.

4.16) On the **Export as text file** page, select the destination to save the file and click on **OK**.

4.17) Open the file in spreadsheet. The results will be presented by each site (**Table 2**). There are 36 sites per well. Using the analysis tool, **Pivot Table**, summarize the data of the 36 sites (i.e., the sums of cell numbers and the averages of the indicated cell percentage in all the 36 sites) (**Table 3**).

**REPRESENTATIVE RESULTS:**

Following reprogramming experiments, we quantified iCMs using high content imaging analysis as described above. Composite images of 36 imaging sites that were used for high content imaging analysis were shown in **Figure 1**. iCMs are defined as double-positive cells (α-

actinin$^+$Titin-eGFP$^+$) in these experiments. High content imaging analysis shows that ~26% of cells exhibited both cardiac markers following M-G-T-H transduction, while ~1% of empty vector transduced control cells show double-positive cells. Individual iCMs were visualized in 10x images taken by high content imaging system (**Figure 2**).

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Representative composite images of automated high content imaging analysis. MEFs isolated from Titin-GFP knock-in mice were transduced with M-G-T-H construct or empty vector.** Empty vector transduced cells were used as control. Fifteen days after transduction, the MEFs were immunostained for GFP and α-actinin. The immunostained cells were analyzed by automated high content imaging system to quantify DAPI$^+$GFP$^+$, DAPI$^+$α-actinin$^+$, or DAPI$^+$GFP$^+$α-actinin$^+$ cells. The graph shows summary of high content imaging analyses. Three independent experiments are presented as mean ± S.D. *$p$ < 0.005. Scale bar = 2.5 mm.

**Figure 2: Representative images of 10x objective pictures used for high content imaging analysis.** These images were chosen from the composite images of 36 imaging sites as shown in **Figure 1**. Each image represents an individual imaging site per each channel. Scale bar = 400 μm.

**Table 1: Transfection scale depending on the size of a cell plate.**

**Table 2: Raw data set analyzed by high content imaging system.**

**Table 3: Summarized data set**.

**DISCUSSION:**

The previous reprogramming studies assessed reprogramming efficiency using flow cytometry and demonstrated the structural quality of iCMs using immunocytochemistry in two separate experiments. Flow cytometry analysis requires a much larger number of starting cells, thereby increasing the scale of experiments. In contrast, high content imaging analysis can evaluate both quality and quantity of iCM reprogramming by a single experiment with a relatively small number of cells. Therefore, this new method can provide an efficient new technical platform for future reprogramming studies. In particular, this new method will be useful for the reprogramming experiments for screening new genetic or pharmacological factors. For testing a large number of factors, the format of analysis can be down-scaled to a 384-well format.

Transduction efficiency is directly correlated to reprogramming efficiency. In previous studies, we have shown that ensuring the expression of all reprogramming factors into fibroblasts is important to achieve high reprogramming efficiency[8,9]. Since a relatively small number of fibroblasts expressed all reprogramming factors by transducing individual viral vectors, using a single polycistronic vector harboring all reprogramming factors is beneficial for enhancing reprogramming efficiency[7,9]. Additionally, to increase transduction efficiency, we performed two sequential transductions using two separate transfections of reprogramming vectors into

Platinum E cells. We found that this method is superior to the previous sequential transduction with a single transfection by re-using transfected Platinum E cells for the second transduction.

Success of cardiac reprogramming can be defined in different ways. The most commonly used method was to quantify the percentage of cells expressing a cardiac structural protein, which is absent in starting fibroblasts. In this study, we used cardiac muscle specific α-actinin as well as striated muscle specific Titin as markers for cardiac structural protein induction during cardiac reprogramming. The reason for using Titin was that we were able to more clearly demonstrate sarcomere formation using fibroblasts isolated from Titin-GFP knock-in mouse line[8,9]. Regardless, we define iCMs as a double-positive cell (actinin$^{+}$Titin$^{+}$) to exclude Titin expressing non-cardiac muscle cells, if any. Importantly, any combination of cardiac structural proteins can be used for high content imaging analysis as long as there are specific antibodies available against those proteins. Although the method we described here is solely based on structural protein expression, it is important to recognize that the cells expressing a cardiac structural protein are not necessarily functional cardiomyocytes. In our previous studies, we have shown that sarcomere assembly is required for iCMs to be functional[4,8,9]. Importantly, the structural development of iCMs can be directly examined through high content imaging analysis. In addition, further structural evaluation (e.g., confocal microscopy) can be subsequently performed using the same immunostained cells for high content imaging analysis. However, the true functionality of iCMs should be determined by recording spontaneous contraction and electrophysiological properties (i.e., calcium transients and action potentials) of iCMs. In addition, demonstrating whole transcriptomic changes from fibroblast to cardiomyocyte would be necessary for defining successful iCM reprogramming (e.g., single cell RNA-seq). Therefore, the major goal of high content imaging analysis for assessing cardiac reprogramming is not to quantify functional iCMs, but to evaluate overall structural progresses of iCM reprogramming as an initial assessment.

**ACKNOWLEDGMENTS:**

High content imaging analysis was performed in the Vanderbilt High-Throughput Screening (HTS) Core Facility with assistance provided by David Westover and Joshua Bauer. The HTS Core receives support from the Vanderbilt Institute of Chemical Biology and the Vanderbilt Ingram Cancer Center (P30 CA68485). This work was supported by AHA Innovative Project Award 18IPA34110341 and NIH R01 HL146524 (Y-.J. N.), and AHA post-doctoral fellowship award 20POST35210170 (Z.Z).

**DISCLOSURES:**

The authors have nothing to disclose.

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Figure 1



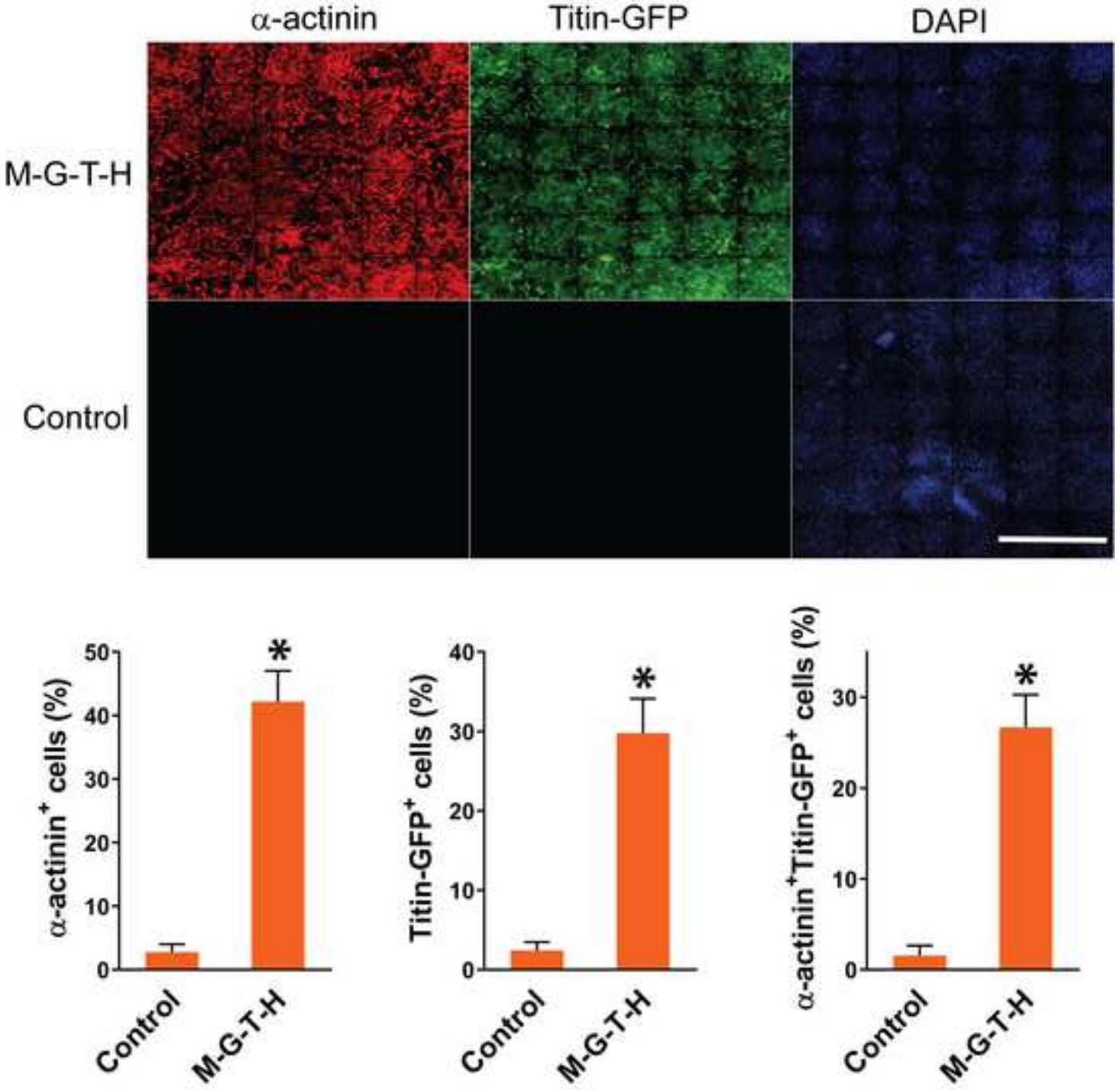

Figure 2



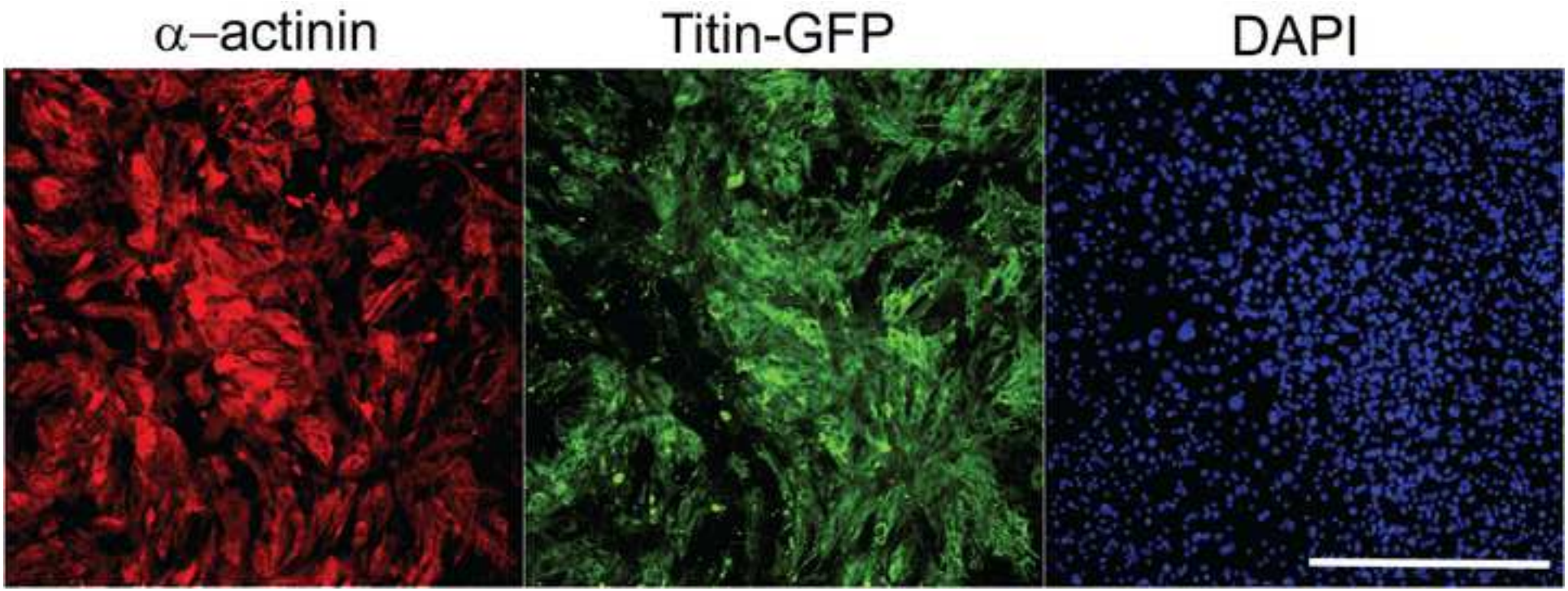

Table 1



| Cell culture dish | Platinum E cells (miilion/plate) | Media (ml) | Fugene 6 (µl) | Opti-MEM (µl) | DNA (µg) |
|---|---|---|---|---|---|
| 15 cm | 13.75 | 25 | 75 | 750 | 25 |
| 10 cm | 5.5 | 10 | 30 | 300 | 10 |
| 6 cm | 2.2 | 4 | 12 | 120 | 4 |
| 35 mm (6 well) | 1.1 | 2 | 6 | 60 | 2 |
| 12 well | 0.55 | 1 | 3 | 30 | 1 |
| 24 well | 0.275 | 0.5 | 1.5 | 15 | 0.5 |

Table 2

Click here to access/download;Table;Table 2 (raw data set).xlsx

| Well Name | Total Cells (MultiWaveScoring) | Subtotal Profile 1xxxxxx (MultiWaveScoring) | Subtotal Profile 12xxxxx (MultiWaveScoring) | Subtotal Profile 1x3xxxx (MultiWaveScoring) | Subtotal Profile 123xxxx (MultiWaveScoring) | Percentage Subtotal Profile 1xxxxxx (MultiWaveScoring) | Percentage Subtotal Profile 12xxxxx (MultiWaveScoring) | Percentage Subtotal Profile 1x3xxxx (MultiWaveScoring) | Percentage Subtotal Profile 123xxxx (MultiWaveScoring) |
|---|---|---|---|---|---|---|---|---|---|
| A02 | 128 | 128 | 10 | 9 | 3 | 100 | 7.8125 | 7.03125 | 2.34375 |
| A02 | 37 | 37 | 7 | 1 | 1 | 100 | 18.91892 | 2.702703 | 2.702703 |
| A02 | 89 | 89 | 16 | 18 | 12 | 100 | 17.97753 | 20.22472 | 13.48315 |
| A02 | 336 | 336 | 74 | 138 | 66 | 100 | 22.02381 | 41.07143 | 19.64286 |
| A02 | 789 | 789 | 173 | 333 | 159 | 100 | 21.92649 | 42.20532 | 20.15209 |
| A02 | 1224 | 1224 | 669 | 911 | 636 | 100 | 54.65686 | 74.42811 | 51.96079 |
| A02 | 51 | 51 | 14 | 3 | 3 | 100 | 27.45098 | 5.882353 | 5.882353 |
| A02 | 95 | 95 | 22 | 26 | 17 | 100 | 23.15789 | 27.36842 | 17.89474 |
| A02 | 488 | 488 | 135 | 278 | 134 | 100 | 27.66393 | 56.96721 | 27.45902 |
| A02 | 1765 | 1765 | 845 | 1171 | 788 | 100 | 47.87535 | 66.34561 | 44.64589 |
| A02 | 1713 | 1713 | 1069 | 1350 | 1019 | 100 | 62.40514 | 78.80911 | 59.48628 |
| A02 | 1801 | 1801 | 1389 | 1373 | 1257 | 100 | 77.12382 | 76.23543 | 69.79456 |
| A02 | 32 | 32 | 7 | 2 | 2 | 100 | 21.875 | 6.25 | 6.25 |
| A02 | 187 | 187 | 66 | 76 | 57 | 100 | 35.29412 | 40.64171 | 30.48128 |
| A02 | 1046 | 1046 | 496 | 661 | 447 | 100 | 47.41874 | 63.19311 | 42.73423 |
| A02 | 1120 | 1120 | 451 | 728 | 418 | 100 | 40.26786 | 65 | 37.32143 |
| A02 | 1709 | 1709 | 1042 | 1261 | 961 | 100 | 60.97133 | 73.78584 | 56.23172 |
| A02 | 1904 | 1904 | 1378 | 1341 | 1171 | 100 | 72.37395 | 70.43067 | 61.5021 |
| A02 | 8 | 8 | 1 | 0 | 0 | 100 | 12.5 | 0 | 0 |
| A02 | 101 | 101 | 26 | 23 | 15 | 100 | 25.74257 | 22.77228 | 14.85149 |
| A02 | 1134 | 1134 | 461 | 597 | 393 | 100 | 40.65256 | 52.6455 | 34.65609 |
| A02 | 731 | 731 | 226 | 470 | 205 | 100 | 30.91655 | 64.29549 | 28.04378 |
| A02 | 1017 | 1017 | 523 | 754 | 505 | 100 | 51.42576 | 74.13963 | 49.65585 |
| A02 | 1585 | 1585 | 1038 | 1057 | 871 | 100 | 65.48896 | 66.6877 | 54.95268 |
| A02 | 14 | 14 | 0 | 0 | 0 | 100 | 0 | 0 | 0 |
| A02 | 67 | 67 | 7 | 5 | 2 | 100 | 10.44776 | 7.462687 | 2.985075 |
| A02 | 393 | 393 | 158 | 193 | 144 | 100 | 40.20356 | 49.10941 | 36.64122 |
| A02 | 1042 | 1042 | 591 | 713 | 554 | 100 | 56.71785 | 68.4261 | 53.16699 |
| A02 | 725 | 725 | 275 | 431 | 253 | 100 | 37.93103 | 59.44828 | 34.89655 |
| A02 | 1618 | 1618 | 806 | 920 | 740 | 100 | 49.81459 | 56.86032 | 45.73548 |
| A02 | 22 | 22 | 11 | 7 | 7 | 100 | 50 | 31.81818 | 31.81818 |
| A02 | 35 | 35 | 10 | 7 | 5 | 100 | 28.57143 | 20 | 14.28571 |
| A02 | 160 | 160 | 29 | 34 | 22 | 100 | 18.125 | 21.25 | 13.75 |
| A02 | 728 | 728 | 332 | 381 | 299 | 100 | 45.6044 | 52.33516 | 41.07143 |
| A02 | 846 | 846 | 464 | 572 | 419 | 100 | 54.84634 | 67.61229 | 49.52719 |
| A02 | 852 | 852 | 539 | 649 | 498 | 100 | 63.26291 | 76.17371 | 58.4507 |
| D04 | 753 | 753 | 0 | 0 | 0 | 100 | 0 | 0 | 0 |
| D04 | 813 | 813 | 0 | 0 | 0 | 100 | 0 | 0 | 0 |
| D04 | 1363 | 1363 | 3 | 0 | 0 | 100 | 0.220103 | 0 | 0 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| D04 | 1168 | 1168 | 1 | 2 | 1 | 100 | 8.56E-02 | 0.171233 | 8.56E-02 |
| D04 | 746 | 746 | 0 | 0 | 0 | 100 | 0 | 0 | 0 |
| D04 | 780 | 780 | 0 | 0 | 0 | 100 | 0 | 0 | 0 |
| D04 | 719 | 719 | 3 | 0 | 0 | 100 | 0.417246 | 0 | 0 |
| D04 | 854 | 854 | 10 | 0 | 0 | 100 | 1.17096 | 0 | 0 |
| D04 | 886 | 886 | 8 | 0 | 0 | 100 | 0.902935 | 0 | 0 |
| D04 | 966 | 966 | 28 | 10 | 10 | 100 | 2.898551 | 1.035197 | 1.035197 |
| D04 | 775 | 775 | 14 | 4 | 4 | 100 | 1.806452 | 0.516129 | 0.516129 |
| D04 | 632 | 632 | 1 | 0 | 0 | 100 | 0.158228 | 0 | 0 |
| D04 | 782 | 782 | 0 | 0 | 0 | 100 | 0 | 0 | 0 |
| D04 | 631 | 631 | 0 | 0 | 0 | 100 | 0 | 0 | 0 |
| D04 | 863 | 863 | 0 | 0 | 0 | 100 | 0 | 0 | 0 |
| D04 | 652 | 652 | 39 | 6 | 6 | 100 | 5.981595 | 0.920245 | 0.920245 |
| D04 | 1080 | 1080 | 37 | 13 | 12 | 100 | 3.425926 | 1.203704 | 1.111111 |
| D04 | 888 | 888 | 0 | 1 | 0 | 100 | 0 | 0.112613 | 0 |
| D04 | 926 | 926 | 0 | 0 | 0 | 100 | 0 | 0 | 0 |
| D04 | 1138 | 1138 | 3 | 0 | 0 | 100 | 0.26362 | 0 | 0 |
| D04 | 1498 | 1498 | 0 | 0 | 0 | 100 | 0 | 0 | 0 |
| D04 | 1262 | 1262 | 0 | 0 | 0 | 100 | 0 | 0 | 0 |
| D04 | 1030 | 1030 | 0 | 0 | 0 | 100 | 0 | 0 | 0 |
| D04 | 1022 | 1022 | 90 | 35 | 35 | 100 | 8.806262 | 3.424658 | 3.424658 |
| D04 | 916 | 916 | 0 | 0 | 0 | 100 | 0 | 0 | 0 |
| D04 | 1556 | 1556 | 0 | 0 | 0 | 100 | 0 | 0 | 0 |
| D04 | 1788 | 1788 | 0 | 0 | 0 | 100 | 0 | 0 | 0 |
| D04 | 1651 | 1651 | 8 | 5 | 5 | 100 | 0.484555 | 0.302847 | 0.302847 |
| D04 | 902 | 902 | 3 | 0 | 0 | 100 | 0.332594 | 0 | 0 |
| D04 | 993 | 993 | 34 | 13 | 13 | 100 | 3.423968 | 1.309164 | 1.309164 |
| D04 | 839 | 839 | 3 | 0 | 0 | 100 | 0.357569 | 0 | 0 |
| D04 | 936 | 936 | 0 | 0 | 0 | 100 | 0 | 0 | 0 |
| D04 | 942 | 942 | 46 | 0 | 0 | 100 | 4.883227 | 0 | 0 |
| D04 | 790 | 790 | 0 | 0 | 0 | 100 | 0 | 0 | 0 |
| D04 | 467 | 467 | 4 | 0 | 0 | 100 | 0.856531 | 0 | 0 |
| D04 | 1000 | 1000 | 0 | 0 | 0 | 100 | 0 | 0 | 0 |

Table 3

Click here to access/download;Table;Table 3 (summarized data set).xlsx

| Well Name | Total Cells (MultiWaveScoring) | DAPI+ | DAPI+/Titin-GFP+ | DAPI+/actinin+ | DAPI+/actinin+/Titin-GFP+ | Percentage DAPI+ | Percentage DAPI+/Titin-GFP+ | Percentage DAPI+/actinin+ | Percentage DAPI+/actinin+/Titin-GFP+ |
|---|---|---|---|---|---|---|---|---|---|
| | Total Cells (MultiWaveScoring) | Subtotal Profile 1xxxxxx (MultiWaveScoring) | Subtotal Profile 12xxxxx (MultiWaveScoring) | Subtotal Profile 1x3xxxx (MultiWaveScoring) | Subtotal Profile 123xxxx (MultiWaveScoring) | Percentage Subtotal Profile 1xxxxxx (MultiWaveScoring) | Percentage Subtotal Profile 12xxxxx (MultiWaveScoring) | Percentage Subtotal Profile 1x3xxxx (MultiWaveScoring) | Percentage Subtotal Profile 123xxxx (MultiWaveScoring) |
| A02 | 25592 | 25592 | 13360 | 16493 | 12083 | 100 | 38.04015 | 44.71138 | 31.5127 |
| D04 | 35007 | 35007 | 335 | 89 | 86 | 100 | 1.013221 | 0.249883 | 0.241805 |

| Name of Material/ Equipment | Company | Catalog Number | Comments/Description |
|---|---|---|---|
| A83-01 | Tocris | 2939 | |
| anti-chicken Alexa 488 | Thermofisher | A11039 | |
| anti-GFP antibody | Invitrogen | A10262 | |
| anti-mouse Alexa 555 | Thermofisher | A21422 | |
| anti-α-actinin antibody | Sigma | A7811 | |
| DAPI solution | Vector labs | H1200 | |
| Fugene 6 | Promega | E2691 | |
| Insulin-Transferrin-SeleniumG supplement | Invitrogen | 41400-045 | |
| Medium 199 | Invitrogen | 11150059 | |
| MEM vitamin solution | Invitrogen | 11120-052 | |
| MetaXpress software | Molecular device | | |
| Micro XL automated cell imagining system | Molecular device | | |
| Minimal essential amino acid solution | Sigma | M7145 | |
| Opti-MEM | Gibco | 31905-070 | |
| PES filter (0.45 µm) | Thomas scientific | 1159T84 | |
| Platninum E cells | Cell Biolabs | RV-101 | |
| Polybrene | Sigma | H9268 | |
| SB431542 | Sigma | S4317 | |
| Universal blocking buffer | BiogeneX | HK083-50K | |

We appreciate the constructive comments provided by the Reviewers, and we have attempted to address them on a point-by-point basis below. The changes that we made were tracked in red in the main text.

**Reviewer #1**

1) In step 1.6, the author used frozen MEF as the starting material for reprogramming. Is the usage of frozen MEF essential for the success of this reprogramming protocol? Or how is the frozen MEF compared to the MEF in culture? Does fresh cardiac fibroblast work with this protocol? It would be helpful if the author could provide more detailed guidance on the selection of starting cell material.

**The usage of frozen MEFs is not essential for the success of the reprogramming protocol. As shown previously (Qian et. al. Nature protocol 2013), using fresh fibroblasts is more effective for cardiac reprogramming. However, isolating fresh fibroblasts every time is inconvenient. We were able to achieve good reprogramming efficiency using the MEFs which was frozen without expansion (P1). We added this comment in the text.**

2) In the image acquisition and analysis step, adding essential screenshots or even a short video demonstrating how the analysis is performed within the MetaXpress is recommended. For example, in step 3.14, showing a screenshot for setting the focus offset would be helpful to readers with zero experience in metaXpress software.

**We do agree on this suggestion. We plan to film this part in detail.**

**Reviewer #2**

1) For figure 1, starlike symbols could be marked for the groups to have statistically signification.

**Added.**

2) For the discussion part. The protocol described here is an improvement for measurement reprogramming efficiency in the protein level. Other levels of measurement like single-cell seq in mRNA level and contraction in functional level also should be discussed and whether those protocols can be combined.

**We discussed this issue in the discussion.**

3) Also, in the method part, Titin and alpha-actinin were selected as the marker for cardiomyocyte. The generally used marker like Myh6 and Tnnt2 was not shown here. Therefore, it should be discussed why Titin and alpha-actinin were selected in the discussion.

**We discussed this issue in the discussion.**

**Reviewer #3**

I would also suggest to use more specific cardiac marker genes to assess the efficacy of reprogramming. Indeed authors performed a double immunofluorescence with a-actinin and titin, which cannot be properly considered cardiac-specific marker genes, instead of Cardiac Troponin C (cTnT), Myosin Light Chain 2V (MLC-2v), myosin light chain-2a (MLC2a), and HCN4.

**We discussed this issue in the discussion.**