Compound effects on calcium transients in Pluricyte® Cardiomyocytes using the Molecular Devices FLIPR Tetra® High-Throughput Cellular Screening System
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**Getting Started**

Please make sure to read the entire user guide carefully before you start thawing and culturing Pluricyte® Cardiomyocytes.

Pluricyte® Cardiomyocytes are for *in vitro* life science research use only.

A Material Safety Data Sheet (MSDS) for Pluricyte® Cardiomyocytes is available on our [website](http://www.ncardia.com).

**Technical support and training**

Our scientists are ready to help you with any questions you may have regarding this user guide or our Pluricyte® Cardiomyocytes. In addition, in-lab training is available upon request. For further information please visit our website [www.ncardia.com](http://www.ncardia.com), or contact us directly by e-mail (sci-support@ncardia).
1. Introduction

Pluricyte® Cardiomyocytes in combination with the Molecular Devices FLIPR Tetra® High-Throughput Cellular Screening System

Pluricyte® Cardiomyocytes are fully functional human induced pluripotent stem cell (hiPSC) derived cardiomyocytes that are highly suitable for fluorescence-based calcium transient assays for cardiac safety screening. The Molecular Devices FLIPR Tetra® High-Throughput Cellular Screening System (FLIPR system) represents the next generation plate reader technology, providing access to high throughput detection of fluorescent and luminescent signals. This system is capable of simultaneous fluid dispensing and signal detection, and is therefore ideal for the fast detection of acute compound effects on calcium transients in Pluricyte® Cardiomyocytes. The combination of Pluricyte® Cardiomyocytes and the Molecular Devices FLIPR system contributes to the detection of potential cardiotoxic effects of compounds at high-throughput level (up to 384-well), which will support scientists in making decisions regarding the cardiac safety profile of drug candidates at early stages of preclinical drug development.

Pluricyte® Cardiomyocytes strengths and characteristics

Pluricyte® Cardiomyocytes exhibit a relatively high level of maturity and present the following unique characteristics:

• High purity of ventricular cardiomyocytes
• Low resting membrane potentials (~-78 mV)
• Fast upstroke velocities and action potential amplitudes
• Organized sarcomeric structures
• Well-pronounced depolarization and repolarization peaks, enabling easy detection of field potential durations in MEA assays
• Strong contraction force
• Clear presence of calcium transients, as shown by patch clamp and Ca2+-flux assays

This user guide describes a protocol for the analysis of fluorescent dye-based calcium transients in Pluricyte® Cardiomyocytes using the Molecular Devices FLIPR system. In addition, a case study describing the assessment of the effects of a set of known cardioactive compounds in Pluricyte® Cardiomyocytes, showing the expected pharmacological responses can be downloaded at www.ncardia.com. Pluricyte® Cardiomyocytes, cultured in Pluricyte® Cardiomyocyte Medium, in combination with the Molecular Devices FLIPR system provide a highly relevant in vitro model to study the cardiac safety profile of compounds at an early stage of drug development.
2. **Workflow**

| Day 0 | • Coat clear bottom black culture plate (96- or 384-well plate formats)
|       | • Thaw and plate Pluricyte® Cardiomyocytes |
| Day 1 | • Refresh Pluricyte® Cardiomyocyte Medium |
| Day 3 | • Refresh Pluricyte® Cardiomyocyte Medium |
| Day 5 | • Refresh Pluricyte® Cardiomyocyte Medium |
| Day 7 | • Refresh Pluricyte® Cardiomyocyte Medium |
| Day 8-12 | • Load Pluricyte® Cardiomyocytes with calcium dye and incubate up to 2 hours
|       | • Prepare compound dilutions and record baseline
|       | • Add compounds and record compound effects |

Optional: in order to monitor the condition of the Pluricyte® Cardiomyocyte monolayer it is advised to perform daily measurements of spontaneous activity (≥ 1h after refreshment).
3. **Important Recommendations**

- Carefully follow the thawing and plating instructions, this step is essential for optimal cell survival and attachment to the plate (Section 4.2).
- We strongly recommend to use Matrigel® or fibronectin as coating substrate for the black wall, clear bottom culture plates.
- Always refresh the Pluricyte® Cardiomyocyte Medium of the cells the day after plating the cells (day 1) (Section 4.3). Subsequently, refresh the Pluricyte® Cardiomyocyte Medium of the cells every 2 days. The Pluricyte® Cardiomyocyte Medium could be refreshed on Friday afternoon and Monday morning to prevent weekend work.
- Refresh Pluricyte® Cardiomyocyte Medium preferably using an automated liquid handler.
- Suppliers of calcium dyes improve their products frequently, use the latest version of the calcium dye to benefit from improved sensitivity and reduced toxicity.
- First contractions of Pluricyte® Cardiomyocytes appear between 24-48 hours post-thawing. It will take 3-4 days before the cells have formed an electrically coupled monolayer. Stable beating monolayers can be observed 7-8 days post-thawing. The optimal time window to perform calcium-based assays with Pluricyte® Cardiomyocytes is between 8-12 days after plating the cardiomyocytes.
## 4. Equipment, Materials and Reagents

Equipment, materials and reagents are described in Tables 4.1, 4.2, 4.3 and 4.4., respectively.

### Equipment

<table>
<thead>
<tr>
<th>Equipment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Devices FLIPR Tetra&lt;sup&gt;®&lt;/sup&gt; High-Throughput Cellular Screening System</td>
</tr>
<tr>
<td>Flow cabinet</td>
</tr>
<tr>
<td>Incubator at 37˚C, 5% CO₂, with humidified air</td>
</tr>
<tr>
<td>P10, P20, P200 and P1000 pipettes</td>
</tr>
<tr>
<td>8/12-channel multichannel pipette</td>
</tr>
<tr>
<td>Hemocytometer</td>
</tr>
<tr>
<td>Optical light microscope</td>
</tr>
<tr>
<td>Optional: Automated liquid handling system</td>
</tr>
</tbody>
</table>

**Table 4.1: Equipment**

### Materials

<table>
<thead>
<tr>
<th>Materials</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile black wall clear bottom culture 96- or 384-well plate (e.g. Greiner Bio-One, Cat# 655090; Cat# 781091, respectively)</td>
</tr>
<tr>
<td>Sterile microtiter 96- or 384-well plate for compounds (e.g. Greiner Bio-One, Cat# 651161)</td>
</tr>
<tr>
<td>Cat#781270 , respectively</td>
</tr>
<tr>
<td>Sterile disposable 5ml pipettes</td>
</tr>
<tr>
<td>Sterile disposable 10ml pipettes</td>
</tr>
<tr>
<td>Sterile disposable 25ml pipettes</td>
</tr>
<tr>
<td>Sterile 15ml conical tubes</td>
</tr>
<tr>
<td>Sterile 50ml conical tubes</td>
</tr>
<tr>
<td>Sterile 20µl filter pipette tips</td>
</tr>
<tr>
<td>Sterile 200µl filter pipette tips</td>
</tr>
<tr>
<td>Sterile 1000µl filter pipette tips</td>
</tr>
<tr>
<td>Sterile multichannel reservoirs</td>
</tr>
</tbody>
</table>

**Table 4.2: Materials**
### Reagents

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Manufacturer</th>
<th>Catalog number</th>
</tr>
</thead>
<tbody>
<tr>
<td>For coating with Matrigel®:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Matrigel®, hESC qualified Matrix, LDEV-Free, 5ml</td>
<td>Corning</td>
<td>354277</td>
</tr>
<tr>
<td>DMEM/F12, Glutamax supplemented</td>
<td>e.g. Life technologies</td>
<td>Gibco 31331-028</td>
</tr>
<tr>
<td>For coating with fibronectin:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibronectin from bovine plasma</td>
<td>Sigma Aldrich</td>
<td>F1141</td>
</tr>
<tr>
<td>Dulbecco’s PBS (1x) (D-PBS) with calcium and magnesium</td>
<td>e.g. Life Technologies</td>
<td>14040</td>
</tr>
<tr>
<td>Pluricyte® Cardiomyocyte kit* containing</td>
<td>NCardia</td>
<td>PCK-1.5</td>
</tr>
<tr>
<td>• 1.5M Pluricyte® Cardiomyocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• 100ml Pluricyte® Cardiomyocyte Medium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium dye kit - FLIPR Calcium 6 Assay kit</td>
<td>Molecular Devices</td>
<td>R8191</td>
</tr>
</tbody>
</table>

**Table 4.3: Reagents**

* For a full 96-well plate, two Pluricyte® Cardiomyocyte Kits are needed.
* For a full 384-well plate, three Pluricyte® Cardiomyocyte Kits are needed.

<table>
<thead>
<tr>
<th>Component</th>
<th>Per well (384 well)</th>
<th>Per well (96 well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>For coating with Matrigel® (1:100)</td>
<td>25µl</td>
<td>100µl</td>
</tr>
<tr>
<td>For coating with fibronectin (1:100)</td>
<td>25µl</td>
<td>100µl</td>
</tr>
<tr>
<td>Pluricyte® Cardiomyocytes</td>
<td>10,000 cells</td>
<td>30,000 cells</td>
</tr>
<tr>
<td>Pluricyte® Cardiomyocyte Medium</td>
<td>25µl</td>
<td>100µl</td>
</tr>
<tr>
<td>Fibronectin from bovine plasma</td>
<td>Sigma Aldrich</td>
<td>F1141</td>
</tr>
</tbody>
</table>

**Table 4.4: Reagent amounts needed per well**
5. Methods

Experimental procedures in this method section are based on a 384-well format. Directions for using a 96-well format can be found in Appendix I.

5.1 Coating of the black, wall clear bottom 384-well culture plate

The black wall, clear bottom 384-well culture plate(s) should be coated on the day of plating the Pluricyte® Cardiomyocytes. This can be done either with Matrigel® (5.1.1) or fibronectin (5.1.2).

5.1.1 Coating of plates with Matrigel®

Matrigel® is a biological matrix preparation used to coat plastic ware in order to enable cardiomyocytes to adhere to the surface.

1. Dilute Matrigel® 1:100 in DMEM/F12. Mix the solution carefully.
   **Note:** Matrigel® polymerizes above 10°C. Keep all reagents and final coating solution on ice until use.
2. Add 25µl coating solution to each well. Incubate the plate at room temperature for at least 45 minutes.
   **Note:** longer incubation times are acceptable, however, the Matrigel® coating solution should not dry out; this causes irreversible loss of extracellular matrix properties.
3. Aspirate excess Matrigel® coating solution right before plating the cells (see Section 5.2 for thawing and plating the Pluricyte® Cardiomyocytes).

5.1.2 Coating of plastic ware with fibronectin

Fibronectin is a biological matrix preparation used to coat plastic ware in order to enable cardiomyocytes to adhere to the surface.

1. Dilute fibronectin 1:100 in D-PBS (incl. Ca2+/Mg2+) to get a 10µg/ml fibronectin coating solution. Mix the solution carefully.
   **Note:** fibronectin is susceptible to shear stress, do not vortex or spin the solution, and avoid harsh pipetting.
2. Add 25µl coating solution to each well. Incubate the fibronectin-coated plate in a cell culture incubator at 37°C, with 5% CO2 for at least 3 hours.
   **Note:** longer incubation times are acceptable, however, the fibronectin coating solution should not dry out; this causes irreversible loss of extracellular matrix properties.
3. Aspirate excess of fibronectin coating solution right before plating the cells (see Section 5.2 for thawing and plating the Pluricyte® Cardiomyocytes).
5.2 Thawing Pluricyte® Cardiomyocytes and plating onto the black wall, clear bottom 384-well culture plate

The volumes used below are calculated for three vials each containing 1.5 million Pluricyte® Cardiomyocytes per vial, to be plated at a density of 10,000 cells/well on one 384-well plate. For plating more than one 384-well plate, multiply the number of vials and the volumes used by the number of plates needed. We recommend to thaw a maximum of 3 vials per operator and thereby preparing one plate at a time.

4. Coat the black wall, clear bottom 384-well culture plate with Matrigel® or fibronectin coating solution as described in Section 5.1.
5. Warm 30ml Pluricyte® Cardiomyocyte Medium to room temperature (RT).
Note: make sure to mix the medium in the bottle by inverting before use.
6. Take 3 vials of Pluricyte® Cardiomyocytes from the liquid nitrogen (LN2) storage (optional: if needed, transport the vials on dry ice).
7. Place the vials in an incubator at 37°C for exactly 4 minutes.
Note: we recommend not to use a water bath for thawing due to potential infection hazard.
8. Gently transfer the content of all three vials (0.3ml per vial) to one 50ml tube using a P1000 pipette. Avoid pipetting up and down.
9. Rinse all vials with 1ml Pluricyte® Cardiomyocyte Medium and add the 1ml Pluricyte® Cardiomyocyte Medium drop-wise to the 50ml tube containing the cells, 1 drop every 5 seconds using a P1000 pipette under gentle continuous swirling.
10. Add another 1ml Pluricyte® Cardiomyocyte Medium drop-wise to the 50ml tube, 1 drop every 5 seconds under gentle continuous swirling.
11. Add 15.1ml Pluricyte® Cardiomyocyte Medium drop-wise to the 50ml tube, 1 drop every 2 seconds using a 10ml pipette (in two steps) under gentle continuous swirling.
Note: the total volume of the cell suspension is now 18ml.
12. Take a 20µl sample of the homogenous cell suspension and add to a micro centrifuge tube.
13. Spin down the cell suspension for 3 minutes at 250xg.
14. Aspirate the medium and gently resuspend the cells in 1ml Pluricyte® Cardiomyocyte Medium.
15. Determine the total cell number and cell viability as follows:

We highly recommend to perform the cell counting manually using a hemocytometer. For instance, by using the Fuchs Rosenthal Counting Chamber (Figure 1):

a. Add 20µl Trypan blue solution to the 20µl cell sample (collected in step 1), mix carefully.

b. Add 20µl of the Trypan blue/cell suspension mix to the counting chamber.

c. Calculate the total number of cells according to equation 1.
**Equation 1. Cell counting**

Count 4 #2 squares according to Figure 5.2

Viable cells:  
____ + ___ + ___ + ___ = _____ (#vc)

Non-viable (blue) cells:  
___ + ___ + ___ + ___ = _____(#nvc)

____ / 4 x 2 x 5000 = ___________ cells/ml

[#vc] 

_______ x _____________ = __________ (cells in total)

[# of cells/ml] [volume after step 13]

Viability = ___: (___ + ___) x 100 = ______ %

[#vc] [#vc] [#nvc]

---

16. Calculate the dilution factor to reach 400,000 cells/ml and add Pluricyte® Cardiomyocyte Medium to the cell suspension accordingly.

17. Transfer the cell suspension to a multichannel reservoir.

18. Gently add 25µl of the cell suspension to each well using a multichannel pipette.

*Note: to prevent a gradient in cell density, gently resuspend the cell suspension frequently.*

19. Transfer the plate to a cell culture incubator (37°C, 5% CO2).
5.3 Maintenance of the Pluricyte® Cardiomyocytes culture

It is crucial to always refresh the Pluricyte® Cardiomyocyte Medium one day after seeding the cells (day 1), and subsequently every 2 days (see Work flow in Section 2). The Pluricyte® Cardiomyocytes could be refreshed on Friday afternoon and Monday morning to prevent weekend-work. Monitor the cell culture using an optical light microscope and scan multiple wells. First contractions of Pluricyte® Cardiomyocytes appear between 24-48 hours post-thawing. It will take 3-4 days before the cells have formed an electrically coupled monolayer. Stably beating monolayers can be observed 7-8 days post-thawing.

Per black wall, clear bottom 384-well culture plate:

20. Pipette 12ml Pluricyte® Cardiomyocyte Medium into a sterile 15ml conical tube and warm the medium to 37˚C for 20-30 minutes.

21. Directly before use, transfer the warm medium into a multichannel reservoir and transfer the black wall, clear bottom 384-well culture plate from the incubator into the flow cabinet.

22. Aspirate the medium from each well using a multichannel aspirator or a liquid handling system. Avoid touching the bottom of the plate with the pipette tips to not disturb the cell monolayer.

23. Add 25µl Pluricyte® Cardiomyocyte Medium per well using a multichannel pipette. Avoid disturbing the cell monolayer by gently pipetting to the side of each well.

24. Transfer the black wall, clear bottom 384-well culture plate back into the incubator (37°C, 5% CO2).

5.4 Compound assay

The optimal time window to perform a calcium assay with Pluricyte® Cardiomyocytes is between 8-12 days after plating the cardiomyocytes.

25. Switch on the Molecular Devices FLIPR system to pre-cool the camera. Set the stage temperature to 37˚C (Figure 2).

26. Prepare the calcium dye solution according to the manual of the supplier (e.g. Molecular Devices FLIPR Calcium 6 assay kit).

27. Add 25µl of the dye solution per well to the Pluricyte® Cardiomyocytes in the 384-well plate (total volume per well is now 50µl).

Note: addition of the calcium dyes can cause a reduction in the spontaneous beat rate of cardiomyocytes.

28. Incubate cells with the calcium dye according to manufacturers’ manual (e.g. for the Molecular Devices FLIPR Calcium 6 assay kit: incubate at 37°C, 5% CO2 for 2 hours).

Note: while incubating the dye, define the instrument settings (Figure 3) and prepare compound dilution plate(s).

29. We recommend to prepare the test compounds in Pluricyte® Cardiomyocyte Medium at ≥10x final concentration. Transfer the compound dilutions to a 384-well microtiter plate.

Note: If test compounds are dissolved in DMSO, the 10x compound dilutions should not exceed 1% DMSO. As a result, the final DMSO concentration will not exceed 0.1%.
30. Compound dilution plates should be warmed for at least 15 minutes to 37°C prior to addition to minimize temperature effects.

31. Transfer the black wall, clear bottom 384-well culture plate containing cells and calcium dye to the FLIPR system.

32. Record a baseline measurement according to FLIPR Tetra® High-Throughput Cellular Screening System manual and the calcium dye kit instruction (e.g. interval time 0.1 seconds and 1200 reads, resulting in 2 minutes measurement time).

33. Program the software to record a measurement while a transfer of compound solutions is performed (e.g. for 10x compound solutions transfer 5.5µl/well. The final volume in the assay plate will be 55.5µl/well).

Note1: Aspirate/dispense (i.e. height, speed) settings may depend on compound viscosity, plate type and other parameters and thus should be optimized empirically. We recommend to use a low dispense speed (e.g. 5.5µl/second for an addition of 5.5µl) to reduce the chance of possible cell detachment.

Note2: Long-term compound effects can be measured i.e. after 30 minutes or 60 minutes post compound treatment. Due to potential toxicity of calcium dyes, we recommend not to measure more than 4 hours after dye addition.

Note3: Place the black wall, clear bottom 384-well culture plate in the incubator (37°C, 5% CO2) in between measurements.
5.5 Data analysis

34. Use the ScreenWorks Peak Pro software to analyze the acquired data. This software is able to export a variety of parameters (e.g. peak frequency, average peak amplitude, irregular spacing).

Note: See the FLIPR Tetra® High-Throughput Cellular Screening System user guide for specific instructions on using the software for data analysis.

Acknowledgement

The completion of this application note could not have been possible without the support of the Pivot Park Screening Centre (Oss, The Netherlands) and the access to their facilities. We would like to thank them for the fruitful collaboration.
6. **Appendix I: directions for using a 96-well plate platform**

6.1 **Coating of the black wall, clear bottom 96-well culture plate**

The black wall, clear bottom 96-well culture plate should be coated on the day of plating the Pluri- cyte® Cardiomyocytes.

### 6.1.1 Coating of plastic ware with Matrigel®

Matrigel® is a biological matrix preparation used to coat plastic ware in order to enable cardiomyocytes to adhere to the surface.

1. Dilute Matrigel® 1:100 in DMEM/F12. Mix the solution carefully.  
   *Note: Matrigel® polymerizes above 10°C. Keep all reagents and final coating solution on ice until use.*
2. Add 100µl coating solution to each well. Incubate the plate at room temperature for at least 45 minutes.  
   *Note: longer incubation times are acceptable, however, the Matrigel® coating solution should not dry out; this causes irreversible loss of extracellular matrix properties.*
3. Aspirate excess Matrigel® coating solution right before plating the cells (see Section 5.2 for thawing and plating the Pluricyte® Cardiomyocytes).

### 6.1.2 Coating of plastic ware with fibronectin

Fibronectin is a biological matrix preparation used to coat plastic ware in order to enable cardiomyocytes adhere to the surface.

1. Dilute fibronectin 1:100 in D-PBS (incl. Ca2+/Mg2+) to get a 10µg/ml fibronectin coating solution. Mix the solution carefully.  
   *Note: fibronectin is susceptible to shear stress, do not vortex or spin the solution, and avoid harsh pipetting.*
2. Add 100µl coating solution to each well. Incubate the fibronectin-coated plate in a cell culture incubator at 37°C, with 5% CO2 for 3 hours.  
   *Note: longer incubation times are acceptable, however, the fibronectin coating solution should not dry out; this causes irreversible loss of extracellular matrix properties.*
3. Aspirate excess fibronectin coating solution right before plating the cells (see Section 5.2 for thawing and plating the Pluricyte® Cardiomyocytes).
6.2 Thawing Pluricyte® Cardiomyocytes and plating onto the black wall, clear bottom 96-well culture plate

The volumes used below are calculated for two vials each containing 1.5 million Pluricyte® Cardiomyocytes per vial, to be plated at a density of 30,000 cells/well on one 96-well plate. For plating more than one 96-well plate, multiply the number of vials and the volumes used by the number of plates needed. We recommend to thaw a maximum of 3 vials per operator at a time.

4. Coat the black wall, clear bottom 96-well culture plate with fibronectin or Matrigel® coating solution as described in Section 5.1.
5. Warm 24ml Pluricyte® Cardiomyocyte Medium to room temperature (RT).
   Note: mix the medium before use by inverting.
6. Take 2 vials of Pluricyte® Cardiomyocytes from the LN2 storage (optional: if needed, transport the vials on dry ice).
7. Place the vials in an incubator at 37˚C for exactly 4 minutes.
8. Gently transfer the contents of both vials (0.3ml/vial) to one 50ml tube using a P1000 pipette. Avoid pipetting up and down.
9. Rinse the vials with 1ml Pluricyte® Cardiomyocyte Medium and add the 1ml Pluricyte® Cardiomyocyte Medium drop-wise to the 50ml tube containing the cells, 1 drop every 5 seconds, using a P1000 pipette under gentle continuous swirling.
10. Add another 1ml Pluricyte® Cardiomyocyte Medium drop-wise to the 50ml tube, 1 drop every 5 seconds under gentle continuous swirling.
11. Add 9.4ml Pluricyte® Cardiomyocyte Medium (in 2 steps) drop-wise to the 50ml tube, 1 drop every 2 seconds using a 10ml pipette under gentle continuous swirling.
   Note: the total volume of the cell suspension is now 12ml.
12. Take a 20µl sample of the homogenous cell suspension and add to a micro centrifuge tube.
13. Spin down the cell suspension for 3 minutes at 250xg.
14. Aspirate the medium and gently resuspend the cells in 1ml Pluricyte® Cardiomyocyte Medium.
15. Determine the total cell number and cell viability as follows:

We highly recommend to perform the cell counting manually using a hemocytometer. For instance, by using the Fuchs Rosenthal Counting Chamber (Figure 1):
   a. Add 20µl Trypan blue solution to the 20µl cell sample (collected in step 1), mix carefully.
   b. Add 20µl of the Trypan blue/cell suspension mix to the counting chamber.
   c. Calculate the total number of cells according to equation 1.
**Equation 1. Cell counting**

Count 4 #2 squares according to Figure 5.2

Viable cells:  \[ ___ + ___ + ___ + ___ = _____ (#vc) \]

Non-viable (blue) cells:  \[ ___ + ___ + ___ + ___ = _____ (#nvc) \]

\[ \frac{____}{4 \times 2 \times 5000} = _______ \text{cells/ml} \]

\[ \frac{____}{\text{[#vc]}} \times \frac{____}{\text{[volume after step 13]}} = _______ \text{(cells in total)} \]

Viability = \[ \frac{____}{\text{[#vc]}} : (____ + ____ \times 100 = _______ \% \]

16. Calculate the dilution factor to reach 300,000 cells/ml and add Pluricyte® Cardiomyocyte Medium to the cell suspension accordingly.

17. Transfer the cell suspension to a multichannel reservoir.

18. Gently add 100µl of the cell suspension to each well (30,000 cells/well) using a multichannel pipette.  

*Note: to prevent a gradient in cell density, gently resuspend the cells suspension frequently.*

19. Transfer the plate to an incubator (37°C, 5% CO2).

### 6.3 Maintenance of the Pluricyte® Cardiomyocytes culture

It is crucial to always refresh the Pluricyte® Cardiomyocyte Medium one day after seeding the cells (day 1), and subsequently every 2 days (see Workflow in Section 2). The Pluricyte® Cardiomyocytes Medium could be refreshed on Friday afternoon and Monday morning to prevent weekend-work. Monitor the cell culture using an optical light microscope and scan multiple wells.

Per black wall, clear bottom 96-well culture plate:

20. Pipette 12ml Pluricyte® Cardiomyocyte Medium into a sterile 15ml conical tube and warm the medium to 37°C for 20-30 minutes.

21. Directly before use, transfer the warm medium into a multichannel reservoir and transfer the black wall clear bottom 96-well culture plate from the incubator into the flow cabinet.

22. Aspirate the medium from each well using a multichannel aspirator. Avoid touching the bottom of the plate with the pipette tips to not disturb the cell monolayer.

23. Add 100µl Pluricyte® Cardiomyocyte Medium per well using a multichannel pipette. Avoid disturbing the cell monolayer by gently pipetting to the side of each well.

24. Transfer the black wall clear bottom 96-well culture plate back into the incubator (37°C, 5% CO2).
6.4 Compound assay

First contractions of Pluricyte® Cardiomyocytes appear between 24-48 hours post-thawing. It will take 3-4 days before the cells have formed an electrically coupled monolayer. Stable beating monolayers can be observed 7-8 days post-thawing. The optimal time window to perform a calcium assay with Pluricyte® Cardiomyocytes is between 8-12 days after plating the cardiomyocytes.

6.4.1 Loading the cells with calcium dye

25. Switch on the Molecular Devices FLIPR system to pre-cool the camera. Set the stage temperature to 37°C (Figure 2).
26. Prepare the calcium dye solution according to the manual of the supplier (e.g. Molecular Devices FLIPR Calcium 6 assay kit).
27. Add 100µl dye solution per well to the Pluricyte® Cardiomyocytes in the 96-well plate (total volume per well is now 200µl).

Note: calcium dyes could cause a reduction in the spontaneous beat rate of cardiomyocytes.

28. Incubate cells with the calcium dye according to manufacturers’ manual (e.g. for the Molecular Devices FLIPR Calcium 6 assay kit: incubate at 37°C, 5% CO2 for 2 hours).

Note: while incubating the dye, define the instrument settings (Figure 2) and prepare compound dilution plate(s).

29. We recommend to prepare the test compounds in Pluricyte® Cardiomyocyte Medium at ≥ 10x final concentration. Transfer the compound dilutions to a 96-well microtiter plate.

Note: If test compounds are dissolved in DMSO, the 10x compound solutions should not exceed 1% DMSO. Therefore, the final DMSO concentration will not exceed 0.1%.

30. Compound dilution plates should be warmed for at least 15 minutes to 37˚C prior to addition to minimize temperature effects.

31. Transfer the black wall, clear bottom 96-well culture plate containing cells and calcium dye to the FLIPR system.

32. Record a baseline measurement according to the FLIPR Tetra® system manual and the FLIPR calcium 6 assay kit instruction (e.g. interval time 0.1 seconds and 1200 reads, resulting in 2 minutes measurement time).

33. Program the software to record a measurement while a transfer of compound solutions is performed (e.g. for 10x compound solutions transfer 22µl/well. The final volume in the assay plate will be 222µl/well).

Note1: Aspirate/ dispense (i.e. height, speed) settings may depend on compound viscosity, plate type and other parameters and thus should be optimized empirically. We recommend to use a low dispense speed (e.g. 22µl/second for an addition of 22µl) to reduce the possible detachment of cells.

Note2: Long term compound effects can be measured i.e. after 30 minutes or 60 minutes post compound treatment. Due to potential toxicity of calcium dyes, we recommend not to measure more than 4 hours after dye addition.

Note3: Place the assay plate in the incubator (37°C, 5% CO2) in between measurements.
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