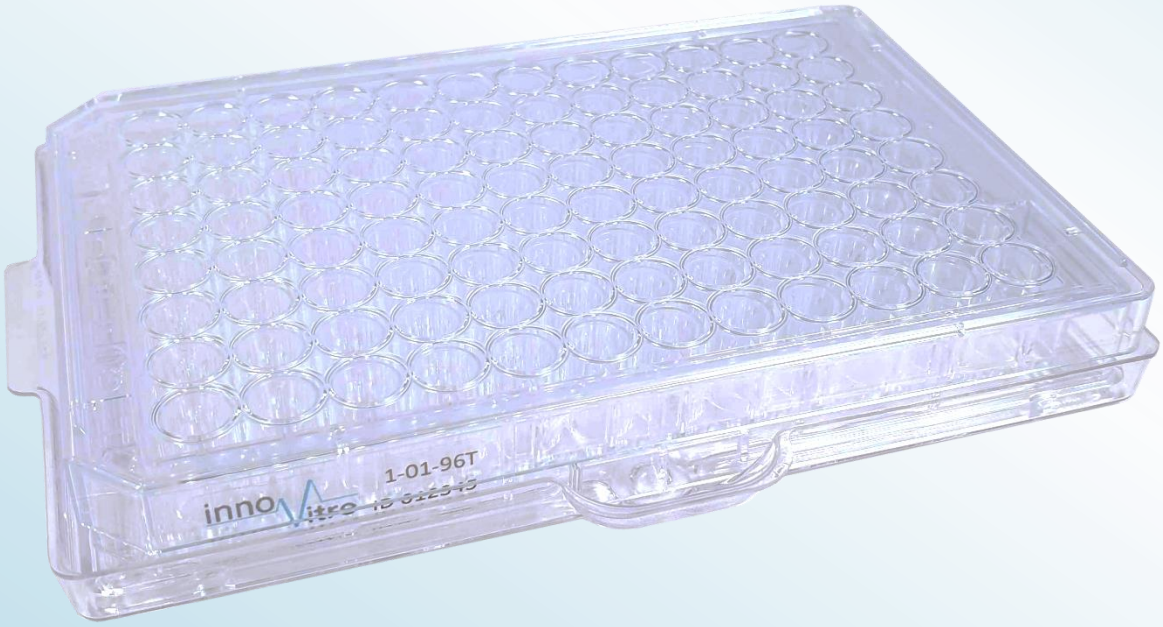
Handling Guide

FLX-96 Plate

iCell® Cardiomyocytes²



TOC

1. Introduction 3
2. Workflow 4
3. Day 0 - Handling of FLX-96 plates upon arrival and before use 5
   - 3.1 Important hints upon arrival of FLX-96 plates 5
   - 3.2 Preparation of FLX-96 plates before use 6
4. Day 0 - FLX-96 plate coating 7
   - 4.1 Supplementary material for FLX-96 plate coating 7
   - 4.2 Procedure 8
5. Day 0 - Seeding of iCell® Cardiomyocyte^2 into FLX-96 plates 8
   - 5.1 Important hints before seeding of FLX-96 plates 8
   - 5.2 Supplementary material for FLX-96 plate seeding 9
   - 5.3 Procedure 11
6. Day 1 - Medium exchange of FLX-96 plates 11
   - 6.1 Important hints before medium exchange of FLX-96 plates 11
   - 6.2 Supplementary material for FLX-96 plate medium exchange 12
   - 6.3 Procedure 13
7. Day 5 - Day 7 - Final medium exchange before compound addition 14
   - 7.1 Important hints before final medium exchange. 14
   - 7.2 Supplementary material before final medium exchange before compound addition 14
   - 7.3 Procedure 15
8. Day 5 – Day 7 - Compound addition and analysis 16
   - 8.1 Important hints before compound addition to FLX-96 plates. 16
   - 8.2 Supplementary material for compound addition and analysis 17
   - 8.3 Procedure 18
10. Appendix 19

1. Introduction

This designated protocol is for use of iCell® Cardiomyocyte^2 from Fujifilm CDI, please follow the detailed procedure with care for successful FLEXcyte cardiac contractility assessment.

The FLEXcyte 96 (FLX-96) plates are designed for high-throughput cardiac contractility measurements using the FLEXcyte 96 system, an add-on system for the CardioExcyte 96 device provided by Nanion Technologies.

FLX-96 plates are ready-to-use and should always be handled with care. Each of the wells contains a flexible membrane as substrate for the cells, disruption of the membrane will eliminate the well from performance in the FLEXcyte 96 system. Please read the entire user guide before you start your experiment and contact info@innovitro.de in case of questions regarding handling of the FLX-96 plate or support.cellular.networks@nanion.de for questions regarding the FLEXcyte 96 software.

The cardiomyocytes used for FLX-96 plates are from human induced pluripotent stem cell (iPSC)-derived origin. Please read the manufacturer's cell handling guide carefully before you start your experiment with the FLX-96 plate.

Helpful clues for every step are provided at the beginning of each chapter, read these hints carefully to obtain optimal results with FLX-96 plates.

You will find a PDF and a video version of this Handling Guide on www.innovitro.de.

2. Workflow

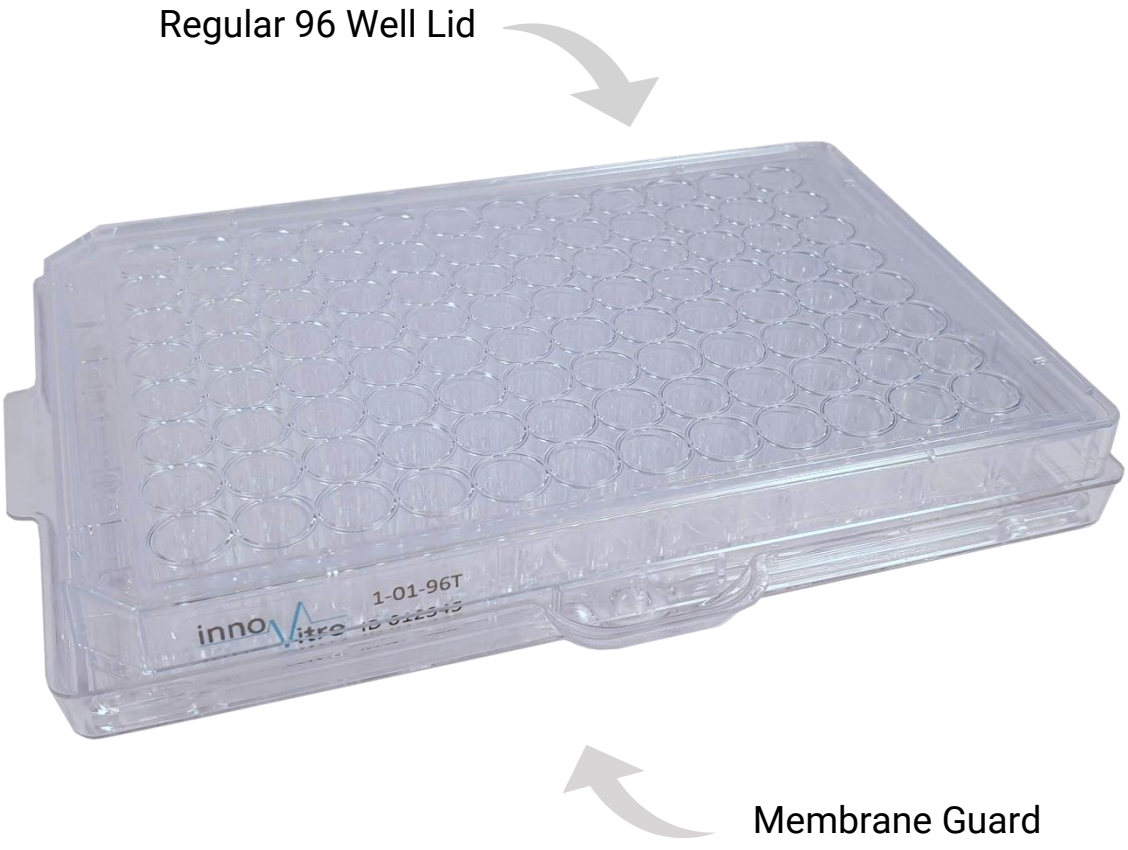
Day	Morning	Afternoon
0	Coating	Plating
1	Media Change: Maintenance Medium	
2		
3	Media Change: Maintenance Medium	
4		
5 -7	Media Change: Maintenance Medium or alternative Assay Medium	Compound Addition (4x) & Direct measurement

Notes

3. Day 0 - Handling of FLX-96 plates upon arrival and before use

3.1 Important hints upon arrival of FLX-96 plates

- FLX-96 plates are sterile and covered in vacuum-sealed plastic upon arrival. Please transport the sealed FLX-96 plate to a sterile environment (e.g. laminar flow hood) before removal of the plastic to avoid contamination before use. If the plates are not used straight away, do not remove the vacuum-sealed plastic and store the plates in a dry and dark environment at room temperature.
- The bottom of each FLX-96 well is covered with a very thin membrane which may be damaged if touched from inside or outside. The FLX-96 plate is protected from above with a regular 96 plate lid and from below with a membrane guard. Ensure that the membrane guard is attached to the bottom of the FLX-96 in order to protect the membranes throughout the procedure. Carefully remove the membrane guard when you want to check the cells under the microsocope or perform a medium change with the VIAFLO ASSIST and before you start your measurement in the FLEXcyte device.



3.2 Preparation of FLX-96 plates before use

1. Transport the sterile and vacuum-sealed FLX-96 plate into a sterile environment, e.g. a laminar flow hood.
2. Unpack the additionally supplied lid and place it on a flat surface.
3. Open the vacuum-sealed packaging and remove it from the FLX-96 plate.
4. Put the FLX-96 plate together with the membrane guard on a flat surface.
5. Keep the FLX-96 plate stack onto the membrane guard until measurements are performed in the FLEXcyte 96 device.

4. Day 0 - FLX-96 plate coating

4.1 Supplementary material for FLX-96 plate coating

Reagents:

- Geltrex™ hESC-Qualified, Ready-To-Use, Reduced Growth Factor Basement Membrane Matrix (ThermoFischer Scientific A1569601)
- *Optional to* Geltrex™*: Fibronectin stock solution (1 mg/mL, e.g. Sigma Aldrich F1141)*
- DPBS with Ca^{2+} and Mg^{2+} (e.g. GE Healthcare HyClone SH304264.01)

Disposable:

- FLX-96 plates
- Centrifuge tubes (50 mL)
- Serological pipettes (25mL)
- Reagent reservoirs (Integra CAT 4311)
- Pipette tips (1000 µL & 1250 µL)

Devices:

- Laminar flow hood
- Single channel adjustable pipette (e.g. 100-1000 µL)
- 12-channel adjustable pipette (100-1250 µL)
- Centrifuge (50 mL tubes)
- Water bath (37 °C)
- Incubator (37 °C, 5% CO_2)
- Vacuum aspiration system
- *Optional: VIAFLO ASSIST (Integra 4500)*

  VIAFLO 12 Channel Pipette (Integra 4634)

4.2 Procedure

1. For seeding iCell® Cardiomyocyte^2 into FLX-96 plates, prepare a diluted Geltrex™ coating solution by transfering 2,75 mL Geltrex™ Ready-To-Use solution in a sterile centrifuge tube. Then add 8,25 mL DPBS with Ca^{2+} and Mg^{2+} to receive the final Geltrex™ working solution. Mix the solution carefully.

   1.(optional) Prepare 11 mL fibronectin coating solution in a sterile centrifuge tube by diluting 110 µL fibronectin stock solution (1mg/mL) in 11 mL of DPBS with Ca^{2+} and Mg^{2+}, resulting in a 10 µg/mL working solution. Mix the solution carefully)

2. Transfer the coating solution into a sterile reagent reservoir. Pipette 100 µL of the coating solution to each well of the FLEXcyte Plate using a 12-channel pipette.

   2. (optional) When using a VIAFLO ASSIST with a 12-channel pipette, transfer the coating solution into a sterile reagent reservoir placed in the VIAFLO ASSIST, use program "ADD100µL" and start the coating procedure.

3. Place the lid back on to the FLX-96 and incubate the plate for 3h at 37°C.

5. Day 0 - Seeding of iCell® Cardiomyocyte^2 into FLX-96 plates

5.1 Important hints before seeding of FLX-96 plates

➔ Please read the cell manufacturer instructions carefully and follow recommendations for cell handling to ensure an optimal performance of the cells on the FLX-96 plate.

➔ Please refer to table 1 for seeding densities to achieve a synchronically beating syncytium.

➔ We recommend a manual counting chamber for consistent cell counting results.

➔ Depending on the number of compounds tested per FLX-96 plate, you may not need to seed the entire FLX-96 plate.

5.2 Supplementary material for FLX-96 plate seeding

Reagents:

- iCell® Cardiomyocyte Plating Medium
- iCell® Cardiomyocytes[2]

Disposables:

- Geltrex™-coated FLEXcyte 96 plate (from Step 4)
- Centrifuge tubes (50 mL)
- Reagent reservoir
- Pipette tips (1000 µL & 1250 µL)

Devices:

- Laminar flow hood
- Single channel adjustable pipette (e.g. 100-1000 µL)
- 12-channel adjustable pipette (100-1250 µL)
- Centrifuge (50 mL tubes)
- Water bath (37 °C)
- Incubator (37 °C, 5% CO_2)
- Vacuum aspiration system
- *Optional: VIAFLO ASSIST (Integra 4500)*
  VIAFLO 12 Channel Pipette (Integra 4634)

Notes

Table 1. Cell numbers for optimal cardiomyocyte seeding densities

Cell Type Manufacturer	**Cell state before seeding**	**Cell number / well**	**Plating volume / well**	**Total cell number / volume per FLX-96 plate**
iCell Cardiomyocyte² FujiFilm CDI	cryopreserved	1 x 10^5	100 µL	11 Mio / 11mL

To ensure full plating efficiency, we recommend to prepare the cell suspension with an excess of 15 % both for the total cell number as well as the total volume. This results in a total volume of the cell suspension of 11 mL.

Notes

5.3 Procedure

1. Thaw the cells according to manufacturer's guidelines.
2. Count the cells with a manual counting chamber and adjust the cells in iCell® Cardiomyocyte Plating Medium according to table 1 (right column).
3. Transfer the cell suspension (11mL total) into a sterile reagent reservoir.
4. Remove the coating solution from the wells with a vacuum aspiration system.
5. Pipette 100 µL of the cell suspension to each well of the FLX-96 plate using a 12-channel pipette.

   5.(optional) When using a VIAFLO ASSIST with a 12-channel pipette, transfer the cell suspension into a sterile reagent reservoir placed in the ViaFLO Assist, use program "CELLS_ADD100µL" and start the seeding procedure.

6. Immediately after cell seeding, transfer the FLX-96 plate into the incubator and let the cells settle over night.

6. Day 1 - Medium exchange of FLX-96 plates

6.1 Important hints before medium exchange of FLX-96 plates

- ➔ We recommend a complete medium exchange with iCell® Cardiomyocyte Maintenance Medium 18-24h after seeding.
- ➔ When medium change is performed manually: Remove the medium carefully row-wise with a single channel vacuum aspiration system. Four rows can be aspirated at a time before adding fresh medium. Do not remove the medium of the entire FLX-96 plate at once before adding fresh medium to avoid drying of the cells. Leave the FLX-96 plate on a flat surface when adding fresh medium and hold the 12-channel pipette in a 45° angle to avoid direct stress for the cell layer.
- ➔ When medium change is performed automatically with a pipette robot (e.g. INTEGRA VIAFLO ASSIST): The medium change can be performed at once for the entire plate, due to the high speed of the pipette robot the cells will not dry out during the procedure.
- ➔ After medium change, each well should contain a final volume of 200 µL.
- ➔ Recommendations regarding the number of medium changes per week should be obtained from the cell manufacturer.

6.2 Supplementary material for FLX-96 plate medium exchange

Reagents:

- Minimum of 22 mL iCell® Cardiomyocyte Maintenance Medium

Disposables:

- Serological pipette (e.g. 25 mL)
- Pipette tips (1250 µL)
- Reagent reservoir

Devices:

- Laminar flow hood
- 12-channel pipette (100-1250 µL)
- Water bath (37 °C)
- Incubator (37 °C, 5% CO_2)
- *Optional: VIAFLO ASSIST (Integra 4500)*

  VIAFLO 12 Channel Pipette (Integra 4634)

Notes

6.3 Procedure

1. 18-24 h after seeding, warm at least 22 mL of iCell® Cardiomyocyte Maintenance Medium for one FLX-96 plate.
2. Transfer the warm medium into a sterile reagent reservoir.
3. Hold the FLX-96 plate in a 45°angle and aspirate four rows of the FLX-96 plate (A-H) with a single channel vacuum aspiration system.

    3.(optional) When using a VIAFLO ASSIST with a 12-channel pipette, transfer the fresh medium into a sterile reagent reservoir and leave it right next to the VIAFLO ASSIST. Place an empty reagent reservoir in the VIAFLO ASSIST, use program "REMOVE100µL" and perform medium removal twice. Afterwards exchange the reagent reservoir containing the waste medium with the reagent reservoir containg the fresh medium and dispense the fresh medium with program "ADD100µL". Perform this step twice again to reach the final volume of 200µL per well. Skip step 4 and 5 and proceed with step 6.

4. Dispense 200 µL fresh medium into each well using a 12-channel pipette. Leave the FLX-96 plate on a flat surface and hold the 12-channel pipette in a 45° angle to avoid maximum stress for the cell layer.
5. Proceed with the next four rows of the FLX-96 plate (E – H) and repeat steps 3 and 4.
6. Immediately after medium exchange transfer the FLX-96 plate back into the incubator.

Notes

7. Day 5 - Day 7 – Final medium exchange before compound addition

7.1 Important hints before final medium exchange

➔ Perform a last medium change 4-6 h before starting your measurements to keep nutrition levels stable for best cardiomyocyte performance.

➔ For analysis of positive inotropic effects, we recommend serum-free medium during compound measurements.

➔ For long-term analysis over several days, please follow cell manufacturers guidelines regarding medium recommendation.

7.2 Supplementary material before final medium exchange before compound addition

Reagents:

- 22+ mL complete culture medium *or* alternative assay medium provided by cell manufacturer

Disposables:

- Serological pipette (e.g. 25 mL)
- Pipette tips (1250 µL)
- Reagent reservoir

Devices:

- Laminar flow hood
- 12-channel pipette (100-1250 µL)
- Water bath (37 °C)
- Incubator (37 °C, 5% CO_2)
- *Optional: VIAFLO ASSIST (Integra 4500)*

  VIAFLO 12 Channel Pipette (Integra 4634)

7.3 Procedure

1. Perform a final medium change 4 – 6 h before compound addition.
2. Warm at least 22 mL of iCell® Complete Culture Medium or alternative assay medium for one FLX-96 plate.
3. Transfer the warm medium into a sterile reagent reservoir.
4. Hold the FLX-96 plate in a 45°angle and aspirate four rows of the FLX-96 plate (A-H) with a single channel vacuum aspiration system.

   3.(optional) When using a VIAFLO ASSIST with a 12-channel pipette, transfer the fresh medium into a sterile reagent reservoir and leave it right next to the VIAFLO ASSIST. Place an empty reagent reservoir in the VIAFLO ASSIST, use program "REMOVE100µL" and perform medium removal twice. Afterwards exchange the reagent reservoir containing the waste medium with the reagent reservoir containg the fresh medium and dispense the fresh medium with program "ADD100µL". Perform this step twice again to reach the final volume of 200µL per well. Skip step 4 and 5 and proceed with step 6.

5. Dispense 200 µL fresh medium into each well using a 12-channel pipette. Leave the FLX-96 plate on a flat surface and hold the 12-channel pipette in a 45° angle to avoid maximum stress for the cell layer.
6. Proceed with the next four rows of the FLX-96 plate (E – H) and repeat steps 3 and 4.
7. Immediately after medium exchange transfer the FLX-96 plate back into the incubator.

Notes

8. Day 5 – Day 7 - Compound addition and analysis

8.1 Important hints before compound addition to FLX-96 plates.

- ➔ Have your measurement plan ready before you start your experiment.
- ➔ Prepare compounds in a 4x concentrated manner. Compound addition is performed with ¼ (50 µL) of the total medium per well, hence the final concentration will dilute to 1x concentrated per well.
- ➔ Perform a baseline measurement of the FLX-96 plate in the FLEXcyte 96 device, right BEFORE you add the compounds. We recommend 3 sweeps for a good reference baseline.
- ➔ Compound addition should be performed quickly to avoid temperature decrease of the plate. When using a FLEXcyte 96 benchtop device: compound addition is performed in the device.

Notes

8.2 Supplementary material for compound addition and analysis

Reagents:

- 6+ mL iCell Cardiomycoyte Maintenance medium or alternative assay medium for compound preparation
- Compounds for your analysis

Disposables:

- Serological pipette (e.g. 25 mL)
- Pipette tips (1250 µL)
- Reagent reservoir
- 96 deep well plate (for compound preparation)

Devices:

- Laminar flow hood
- FLEXcyte 96 device (Nanion Technologies)
- 12-channel pipette (100-1250 µL)
- Water bath (37 °C)
- Incubator (37 °C, 5% CO_2)
- Vacuum aspiration system
- *Optional: VIAFLO ASSIST (Integra 4500)*

  VIAFLO 12 Channel Pipette (Integra 4634)

Notes

8.3 Procedure

1. Prepare your compound working solution in the laminar flow hood using a sterile regular 96 deep well plate. The working solution per compound should be 4x concentrated. Transfer the 96 deep well plate containing the compound solution for at least 1h into the incubator to adjust it to the same condition as the FLX-96 plate.
2. Transfer the FLX-96 plate into the FLEXcyte 96 device 1h before you perform a baseline measurement. We recommend 3 baseline measurements (sweeps) in 5 min intervals shortly before compound addition.

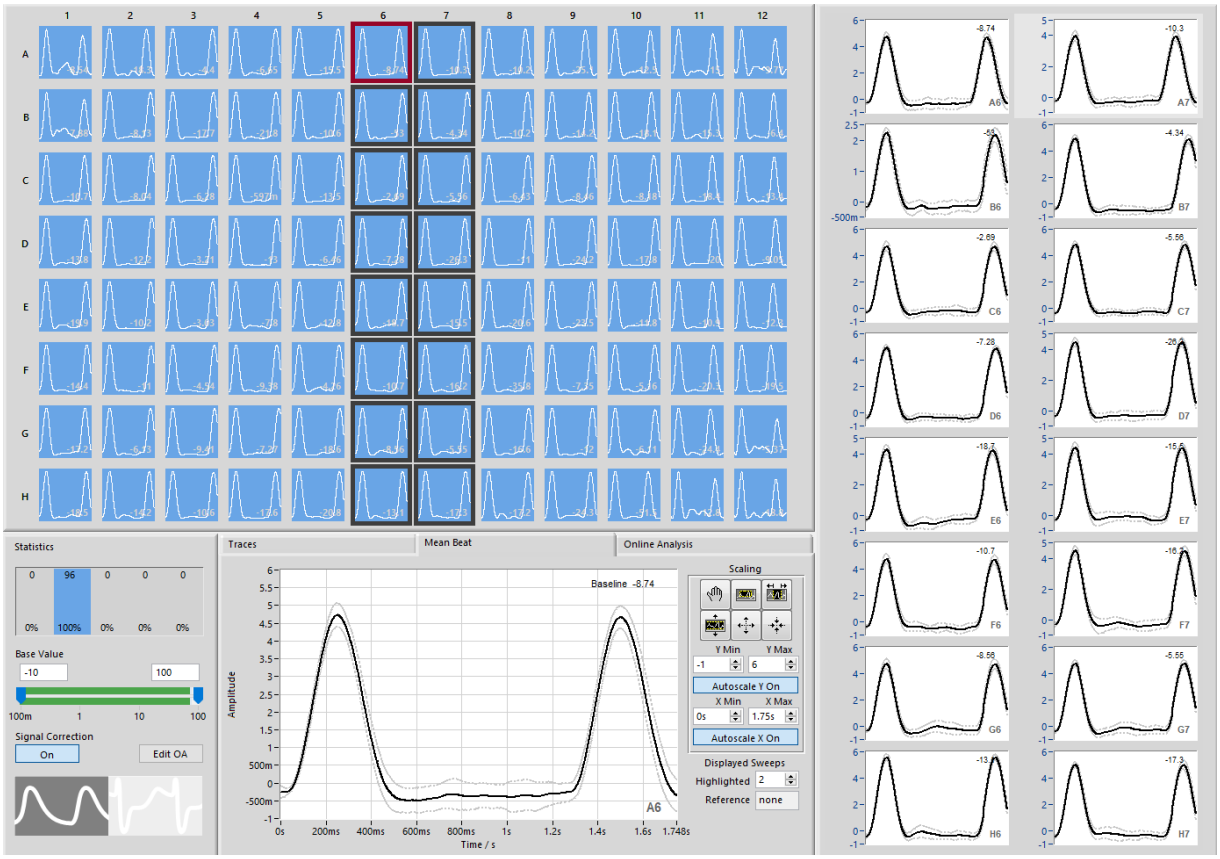


Figure 1. Reference baseline measurement before compound application

3. Remove 50 µL medium of each well of the FLX-96 plate.
4. Add 50 µL of the 4x concentrated compound solution into the FLX-96 plate, according to your measurement plan.
5. Start your measurement in the FLEXcyte 96. The number and intervals of measurements may differ dependent on acute or chronic analysis.

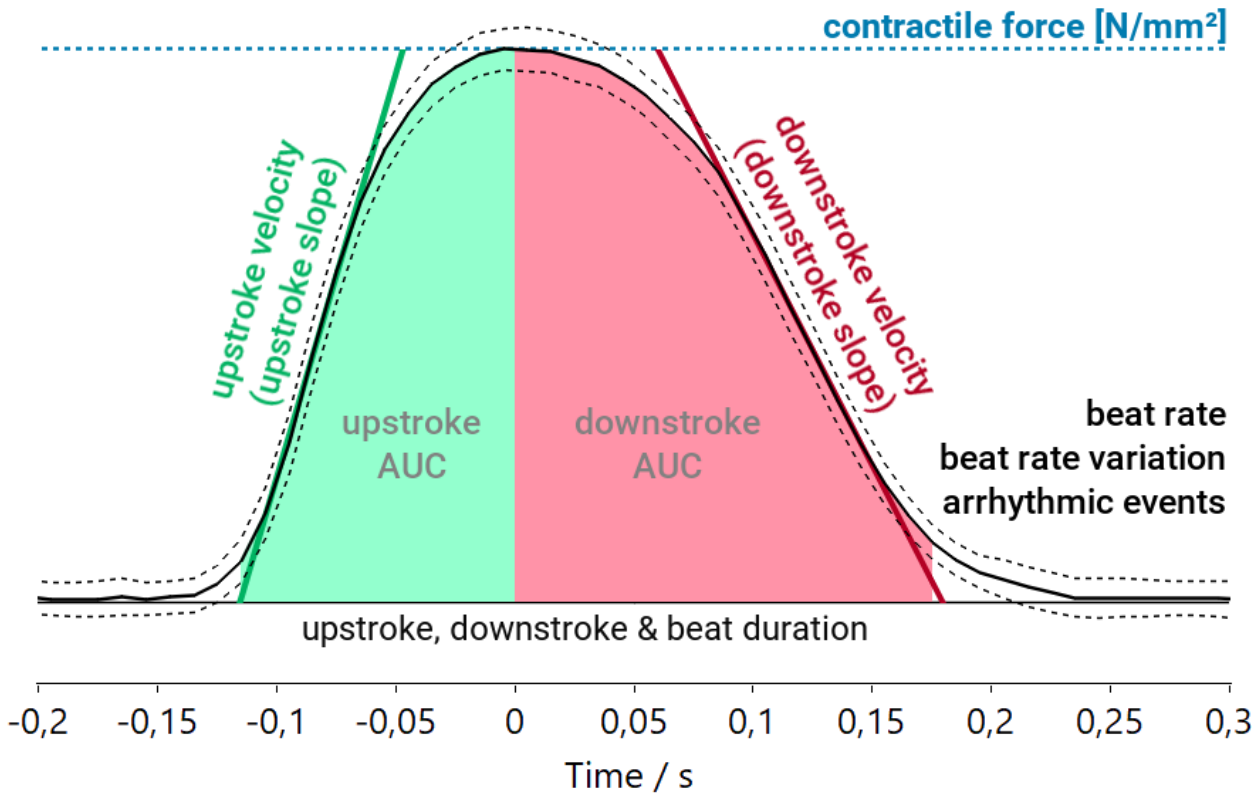
Please refer to info@innovitro.de for support regarding the cell and plate handling or support.cellular.networks@nanion.de for support regarding FLEXcyte 96 software and analysis.

10. Appendix

Figure 1. Multiparametric analysis of cardiac contraction with the FLEXcyte 96 device and FLX-96 plates

FLEXcyte 96 Device

FLX-96 Plate



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