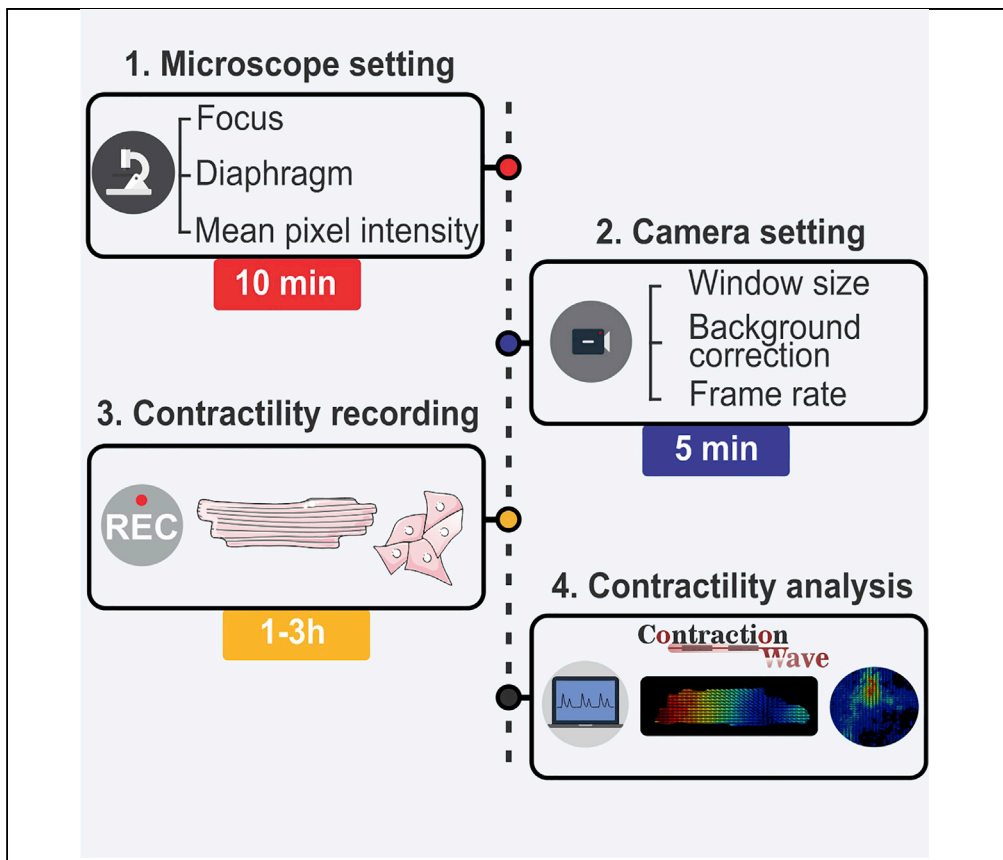


Protocol

Microscopy-based cellular contractility assay for adult, neonatal, and hiPSC cardiomyocytes



This protocol provides instructions to acquire high-quality cellular contractility data from adult, neonatal, and human induced pluripotent stem cell-derived cardiomyocytes. Contractility parameters are key to unravel mechanisms underlying cardiac pathologies, yet difficulties in acquiring data can compromise measurement accuracy and reproducibility. We provide optimized steps for microscope and camera setup, as well as cellular selection criteria for different cardiomyocyte cell types, aiming to obtain robust and reliable data. Moreover, we use CONTRACTIONWAVE software to analyze and show the optimized results.

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Highlights
High-quality
contractility data
requires specific
settings for different
cardiac cells

Distinct cell types
require specific
selection criteria for
proper contractility
assay

High-quality
recordings require
adjustments to focus,
diaphragm, and
lighting

Non-optimized data
acquisition settings
can influence cell
contractility speed

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Protocol

Microscopy-based cellular contractility assay for adult, neonatal, and hiPSC cardiomyocytes

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SUMMARY

This protocol provides instructions to acquire high-quality cellular contractility data from adult, neonatal, and human induced pluripotent stem cell-derived cardiomyocytes. Contractility parameters are key to unravel mechanisms underlying cardiac pathologies, yet difficulties in acquiring data can compromise measurement accuracy and reproducibility. We provide optimized steps for microscope and camera setup, as well as cellular selection criteria for different cardiomyocyte cell types, aiming to obtain robust and reliable data. Moreover, we use CONTRACTIONWAVE software to analyze and show the optimized results. For complete details on the use and execution of this profile, please refer to Scalzo et al. (2021).

BEFORE YOU BEGIN

This optimized protocol includes methods for different types of cardiomyocytes (adult, neonatal, and human induced pluripotent stem cells), each with their own experimental particularities. The steps were written as a guide for both beginners and expert researchers to acquire robust and reproducible cellular contractility data.

Cardiomyocyte cellular dissociation

The protocol described below has been tested using freshly dissociated adult ventricular myocytes (adult-CM) as well as cultured neonatal (neonatal-CM) and human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM). The studies with both adult and neonatal animals were conducted in accordance with the National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals*, and approved by the Institutional Animal Care and Use Committee at UFMG (CEUA 138/2018). As each laboratory likely follows its protocols, the methods cited below are for reference only: for freshly adult cardiomyocytes (Guatimosim et al., 2001; Tian et al., 2020), for neonatal cardiomyocytes (Guatimosim et al., 2008), for human induced pluripotent stem cell-derived cardiomyocytes (Cruvinel et al., 2020).

Cell imaging setup optimization

Kohler illumination

⌚ Timing: 10–15 min



For robust image acquisition and analysis, the microscope and camera should provide uniform illumination, reasonable contrast, and clear delimitation of the cell boundary. These criteria can be satisfied by setting up the microscope for brightfield examination and Köhler illumination (set up instructions can be usually found in the microscope manual).

Note: Even modern microscopes can have compromised image quality due to issues with the light source. An optimal cell illumination should be glare-free and dispersed uniformly in the field of view.

Microscope cleanliness

⌚ **Timing: 10–30 min**

For high-quality cell imaging, it is important to certify that no dust or dirt appears in the captured images, as it can substantially affect the contractility analysis. Therefore, dirt along the optical pathway or on the camera lens needs to be removed. There are few steps to identify if the dirt is on the specimen, microscopy optical system, or in the camera:

1. Dirt on the specimen can be identified by moving the specimen stage. If that is the case, the dirt will have the same relative movement as the specimen.
2. Dirt on the objective can be identified by comparison with images using a different objective, if available. If the dirt is on the objective, it will disappear when the objective is changed. Alternatively, dirt on the objective can also be tested by moving the objective slightly and observing if the dirt also moves or remains stationary.

Note: Remember also that the front of the objective is not in the focal plane and therefore a bit of dirt on the front of the objective might appear out of focus and may just cause an overall bad image.

3. If the dirt stays static when the stage moves and is not on the objective, it is probably located in the optical system, either on the intermediate lens or in the camera sensor, and can be identified by rotating the camera. If the dirt moves with the camera rotation, the dirt is on the camera. If it remains stationary, by process of elimination, the dirt is on an intermediate lens. Once the dirt is found, the following steps need to be taken:
 - a. Place the objective lens on a dust-free surface.
 - b. Use an air blower to gently remove the loose dust from the optical glass surface. To avoid damaging the lens, the air must be blown across the lens surface.
 - c. Use a cotton swab or a low-lint lens tissue for cleaning optics. Do not rub the lens with dry cotton.
 - d. To remove water-soluble dirt, dip a cotton swab or lens wipe into distilled water or lens cleaning solution, remove the excess liquid, and then wipe the lens in a spiral motion.
 - e. To remove oily dirt, a lens cleaning liquid or absolute ethanol should be used following the same procedure as for distilled water. In some cases, the dirt may require several passes to be completely removed.

⚠ CRITICAL: Do not use an excessive amount of liquid. A single drop or immersion of the cotton swab or lens wipe should be sufficient. Remember to never apply the liquid directly to the lens.

Calibration of image pixel size

⌚ **Timing: 10 min**

Normally, a microscope with a built-in camera has a scale bar in its system that provides a convertible value for the image pixels into a known unit, such as micrometers. However, if the camera is added as

a peripheral, the user needs to find out what a pixel unit corresponds to in the desired unit. One way to acquire the image pixel size value is by a simple calculation of the ratio between the camera pixel size and the objective magnification (Equation 1).

$$\text{Image pixel size} = \frac{\text{Camera pixel size}}{\text{Objective magnification}} \quad (\text{Equation 1})$$

△ CRITICAL: Each camera has a specific image pixel size. Make sure to obtain the right value in the camera manual.

The image pixel size value can also be obtained experimentally. Calibration of image pixel size should be performed by imaging a stage micrometer and calculating the X- and Y-axis pixel size in ImageJ software (Schneider et al., 2012). If the microscope has built-in calibration, it is recommended to verify the calibration using the same procedure. For that, a stage micrometer calibration slide can be used with an image capture program. For ImageJ, follow these steps:

4. Put a stage micrometer on the microscope and acquire the image.
5. Use ImageJ to find the pixel value:
 - a. Open the image file on ImageJ.
 - b. On the scale bar, draw a straight line to define a known distance.
 - c. Go to the menu and select 'Analyze' > 'Set Scale'.
 - d. In the dialog window, type in the 'Known distance' box, the distance value in micrometers (from the stage micrometer).
 - e. The scale will show the relationship between the pixel and micrometers.
 - f. Use the following formula to find the image pixel size in micrometers:

$$\text{Image pixel size} = \frac{\text{distance}}{\text{Number of pixels}} \quad (\text{Equation 2})$$

To add the scale bars to the images using ImageJ, please follow the steps below: <http://www.swarthmore.edu/NatSci/nkaplin1/scalebar.htm>

Microscope resolution

The microscope resolution defines the sensitivity for measurements of cell contractility. The microscope spatial resolution can be estimated by the formula:

$$\text{Resolution}_{x,y} = \frac{1.22\lambda}{\text{NA}_{\text{obj}} + \text{NA}_{\text{con}}} \quad (\text{Equation 3})$$

where, λ is the imaging wavelength of light, and NA_{obj} and NA_{con} are the objective and the condenser numerical aperture, respectively. In our experiments, we used an objective with 40× magnification ($\text{NA}_{\text{obj}} = 0.55$) and the Long Working Distance (LWD) condenser ($\text{NA}_{\text{con}} = 0.8$). Using an average wavelength for visible light = 0.55 μm , we have:

$$\text{Resolution}_{x,y} = \frac{1.22 \times 0.55}{0.55 + 0.8} = \sim 0.5 \mu\text{m} \quad (\text{Equation 4})$$

To obtain robust data, the pixel size should be below the microscope resolution. In our setup, the pixel size was 0.25 μm .

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---------------|------------|
| Chemicals, peptides, and recombinant proteins | | |
| Penicillin-Streptomycin | Sigma-Aldrich | Cat#P4333 |
| NaCl | Sigma-Aldrich | Cat#746398 |

(Continued on next page)

Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Glucose | Sigma-Aldrich | Cat#G7528 |
| MgCl ₂ | Sigma-Aldrich | Cat# M1028 |
| HEPES | Sigma-Aldrich | Cat#H3375 |
| Dulbecco's Modified Eagle's Medium | Sigma-Aldrich | Cat#D1152 |
| Absolute ethanol | Sigma-Aldrich | Cat#24102 |
| Fetal Bovine Serum | Thermo Fisher Scientific | Cat#16000044 |
| CaCl ₂ | Thermo Fisher Scientific | Cat#CX01561 |
| B27 supplement | Thermo Fisher Scientific | Cat#17504044 |
| RPMI 1640 | Thermo Fisher Scientific | Cat#11875085 |
| NaH ₂ PO ₄ | Dinâmica Química LTDA | Cat#1160 |
| KCl | Vetec | Cat#109 |
| Y27632 | Cayman Chemical | Cat#10005583 |
| Experimental models: Cell lines | | |
| Human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM) | Pluricell Biotech® | https://www.pluricellbiotech.com |
| Experimental models: Organisms/strains | | |
| Mouse - C57BL/6J, (Male: 4 weeks and older) | Animal Facility UFMG | https://www.jax.org/strain/000664 |
| Rat - Wistar (Male: 1–3 days) | Animal Facility of the Department of Physiology and Biophysics ICB-UFMG | N/A |
| Software and algorithms | | |
| CONTRACTIONWAVE executable file | Scalzo et al., 2021 | https://doi.org/10.1016/j.crmeth.2021.100044 |
| ImageJ | Schneider et al., 2012 | https://imagej.nih.gov/ij/ |
| PIXI® V3.8 Software | EPIX, Inc. | https://www.epixinc.com/support/files.php |
| Other | | |
| CMOS camera | EPIX, Inc | SILICON VIDEO ® 642M |
| Nikon Eclipse Ti-E inverted microscope | Nikon | Ti-E |
| Chamlide C-CU Incubator | Live Cell Instrument, Nowan-gu, Korea | 109 |
| Square Pulse Stimulator | Grass Instruments | SD9 |
| Platinum electrodes | N/A | N/A |
| Stericup-GP Sterile Vacuum Filtration System 0.22 µm | Millipore | SCGPU11RE |
| ZEISS Lens Cleaning KIT (Air blower, dust brush, lens cleaning solution (30 ml), microfibre cloth (18 x 8 cm), 10 moist cleaning wipes) | Zeiss | N/A |
| Lens Wipes | Zeiss | N/A |
| 1 mm Stage Micrometer with 10 µm Divisions | Thorlabs | R1L3S2P |
| CPU - Intel Celeron 800 MHz (Intel Core 2 Duo 2 GHz – RAM 4GB - HD 500GB) | N/A | N/A |
| CPU Monitor 19" | N/A | N/A |

MATERIALS AND EQUIPMENT

To perform this protocol, the following technical equipment are necessary:

- CPU, monitor, a microscope and a high-speed digital camera with software for image acquisition.
- A mini incubator coupled to a microscope stage is necessary for experiments that require temperature and/or CO₂ control.
- Lens cleaning kit (if required).
- Stage micrometer (if required).
- Square Pulse Stimulator and platinum electrodes for adult-CM stimulation.

Tyrode's solution for adult-CM

| Reagent | Final concentration (mmol/L) | Amount |
|--------------------------------------|------------------------------|---------------|
| NaCl | 140 | 818.1 mg |
| KCl | 5 | 37.27 mg |
| Glucose | 10 | 180.1 mg |
| HEPES | 5 | 119.1 mg |
| CaCl ₂ | 1.8 | 20 mg |
| NaH ₂ PO ₄ | 0.4 | 4.8 mg |
| MgCl ₂ solution (1 mol/L) | 1 | 0.1 mL |
| NaOH (5 mol/L) | – | pH adjustment |
| Milli-Q water | – | Up to 100 mL |
| Total | – | 100 mL |

Add constituents above to 90 mL Milli-Q grade water. Adjust the pH with NaOH to 7.4 and complete the volume to 100 mL. Keep the solution at room temperature for use on the same day.

△ **CRITICAL:** Warm up the solution in a water bath according to the desired experimental temperature condition.

Different types of experiments may require a specific solution.

Dulbecco's modified Eagle's medium for neonatal-CM

| Reagent | Final concentration | Amount |
|------------------------------------|---------------------|----------------|
| Dulbecco's Modified Eagle's Medium | – | 4.5 g |
| Penicillin-Streptomycin | 100 U/mL–100 µg/mL | 10 mL |
| NaOH (5 mol/L) | – | pH adjustment |
| Milli-Q water | – | Up to 1000 mL |
| Total | n/a | 1000 mL |
| Fetal Bovine Serum | 10% | 100 mL |

Add the medium and Penicillin-Streptomycin to 950 mL Milli-Q grade water. Adjust the pH with NaOH to 7.4, and complete the volume to 1000 mL. Filter the solution with a 0.2 µm filter/vacuum for sterilization. Add the Fetal Bovine Serum. This step has to be performed with sterile material in a laminar flow cabinet.

Store at 4°C for up to 1 month.

△ **CRITICAL:** Warm up the solution in a water bath according to the desired experimental temperature condition. Different types of experiments may require a specific culture medium.

RPMI 1640 medium for hiPSC-CMs

| Reagent | Final concentration | Amount |
|--------------------|---------------------|---------------|
| RPMI 1640 | n/a | 97.99 mL |
| Y27632 (0.1 mol/L) | 10 µmol/L | 0.01 mL |
| B27 supplement | 2% | 2 mL |
| Total | n/a | 100 mL |

These steps have to be performed with sterile materials in a laminar flow cabinet.

Store the medium at 4°C for up to 1 week.

△ **CRITICAL:** Warm up the solution in a water bath according to the desired experimental temperature condition. Different types of experiments may require a specific culture medium.

STEP-BY-STEP METHOD DETAILS

Microscope and image setting for the different cell types

⌚ Timing: 5–10 min

To perform cellular contractility experiments, a standard microscope coupled with a digital camera is necessary. The microscope contrast needs to be sufficient to identify the cellular membrane. Importantly, the magnification must allow the user to visualize the whole cell, typically requiring between 20×–40× magnification with a pixel size of 0.2–0.5 μm. In order to capture a high-quality image and fully detect the entire cell movement, both microscope resolution and image pixel size should be smaller than the sarcomere length, which is around 1.8 μm (Li et al., 2016; Pollack and Huntsman, 1974). Due to differences in cell contractility speed within the different cell types, a camera should have a frame rate ranging from 25 to 200 fps (see Table 1 for details).

Different cell types may require specific microscope settings, and to achieve the best contrast, some adjustments to diaphragm aperture, focus, and light intensity may be necessary. The mature sarcomere structure found in adult cardiomyocytes provides robust contrast for contraction measurements. In comparison, neonatal-CMs and hiPSC-CMs have intrinsically lower contrast due to the absence of an organized sarcomere structure and lack of a t-tubule system (Bedada et al., 2016; Khan et al., 2015; Li et al., 2017). In addition, contractility can be significantly affected by differences in cardiomyocyte structural maturity. Neonatal-CMs and hiPSC-CMs not only have weaker contraction compared to adult-CMs, but their syncytium self-organization can also contract spontaneously (Bedada et al., 2016; Bezzerides et al., 2019; Li et al., 2017). These characteristics require slightly different microscope settings between these types of cells.

△ **CRITICAL:** An inappropriate microscope setting will directly influence the acquisition of contractility that is obtained through cell membrane movement, reflecting in a poor measurement of the contractility parameters.

1. Focus: the focus should be adjusted until the cell border is delimited with minimum edge brightness (see Focus on Figures 1, 2, and 3). Small adjustments should be made to the fine focus control until the cell border is sharp and without any optical bloom.

Note: For adult-CMs, the focus should be optimized to obtain the greatest amount of black and white variation longitudinally, providing a clear visualization of sarcomere striations (see Focus on Figure 1A). For neonatal-CM or hiPSC-CM, intracellular vesicles or cell nuclei

Table 1. Contractility recording settings

| Parameters | Adult-CM | Neonatal-CM | hiPSC-CM |
|-----------------------------------|-----------|-------------|-----------|
| Microscope setting | | | |
| Objective magnification | 40× | 20×–40× | 20×–40× |
| Microscope diaphragm aperture (%) | 30 | 30 | 30 |
| Mean gray value (8-bit image) | ~127 | ~127 | ~127 |
| Minimum microscope resolution | ~ 0.5 μm | ~ 0.5 μm | ~ 0.5 μm |
| Camera settings | | | |
| Recording speed (fps) | 200 | 25–100 | 25–100 |
| Camera suggestion | CMOS | CMOS | CMOS |
| Image Type | 8-bit | 8-bit | 8-bit |
| Window size (px) | 640 × 200 | 640 × 480 | 640 × 480 |

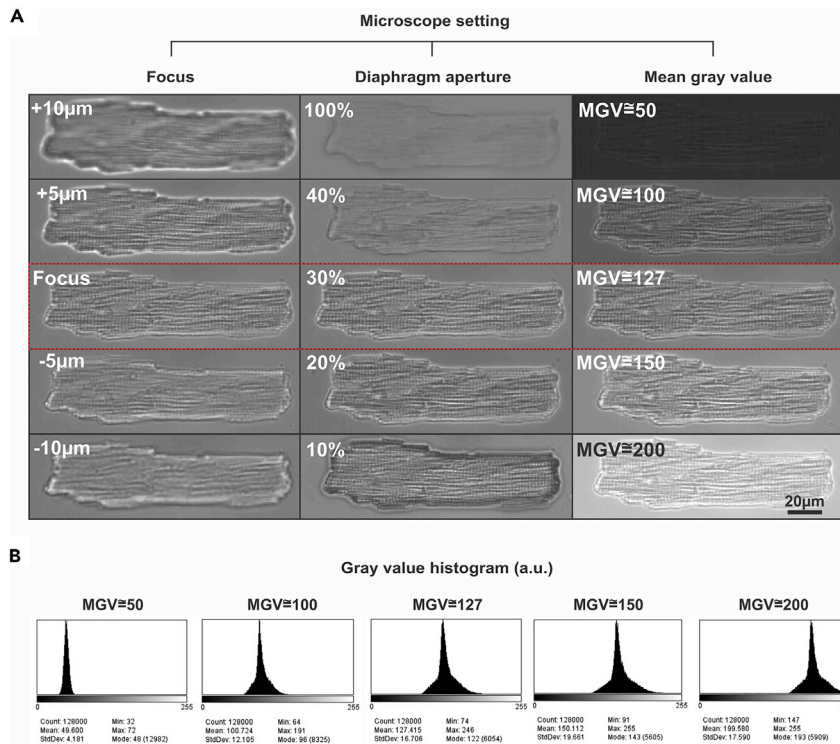


Figure 1. Optimal brightfield microscope settings for adult-CMs

(A) Focus: Out-of-focus cells present a bright halo around the cell border and poor resolution of intracellular structures. Use the fine focus control to minimize border brightness and to bring intracellular structures into sharp focus. Diaphragm: changes in the diaphragm drastically alter cell visualization. A fully-opened diaphragm (100%) results in low contrast whereas a closed-down diaphragm (10%) produces sufficient contrast but an overall-all dark image. Mean gray value (MGV): Lighting and camera settings should be adjusted so that the gray value histogram is centered on the half-maximal range (127 for an 8-bit image), with no pixel intensities close to zero or to saturation. Ideal settings are highlighted in the red rectangle.

(B) Gray value histogram acquired for each condition presented in (A). For greater precision, the variation of illumination should be observed from the gray value histogram, generated by the camera's application program or the image can be processed by ImageJ software.

can be used as a reference to find the best focus. Well-focused vesicles usually will have a black interior and both vesicles and nuclei should have a well-defined border (see Focus on Figures 2A and 3A).

2. Diaphragm aperture (Condenser lens): Start with the microscope setup for Kohler illumination. Open the diaphragm fully, which results in a cell image with low contrast and with little cell border delineation (see panel Diaphragm 100% in Figures 1A, 2A, and 3A). Slowly close the diaphragm until the delineation of the cell edge and nucleus appear and contrast is optimized.

Note: For adult-CMs, good contrast can generally be obtained with the diaphragm 20%–30% open (see Diaphragm 30% Figure 1A), and for neonatal-CM or hiPSC-CM, 30% (see Diaphragm 30% Figure 3A).

3. Mean gray value (MGV): Pixel brightness is converted into a grayscale value that is the primary information in an image. Usually, an 8-bit image is sufficient to acquire the cell contractility parameters. This kind of image has a grayscale that ranges from 0 to 255, and the ideal illumination intensity will nearly fill this range, leaving a margin of error to guarantee that no pixels go below camera sensitivity or above saturation. Thus, the best approach is to adjust lighting and camera

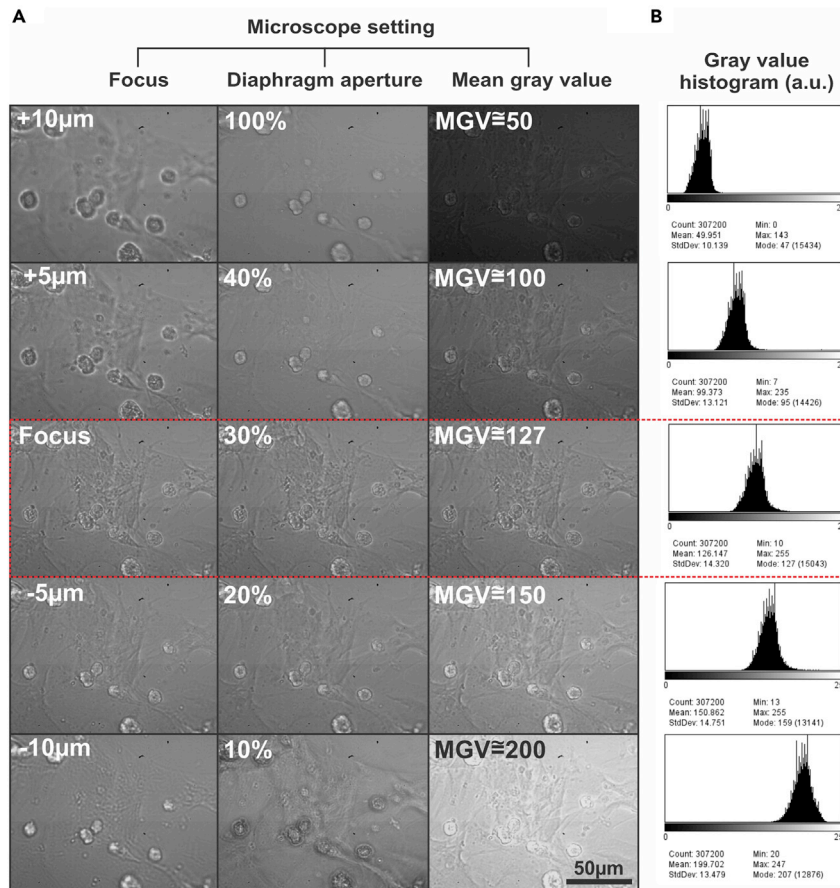


Figure 2. Optimal brightfield microscope settings for neonatal-CMs

(A) Focus: Out-of-focus cells present a bright halo around the cell border and poor resolution of intracellular structures. Use the fine focus control to minimize border brightness and to bring intracellular structures into sharp focus. Diaphragm: changes in the diaphragm drastically alter cell visualization, when it is fully open (100%) the cell does not have a clear delimitation and contrast. When it is partially 10% opened the cell has a dark border, indicating poor lighting of the cell. Therefore, normally in a brightfield microscope, the diaphragm should be 30% opened to get the best contrast. Mean gray value (MGV): In an 8-bit image, the best illumination will be proportional to the average total pixels of the image, which in this case will be 127. Smaller values do not produce good lighting, and larger values produce an excess of illumination and can reach exaggerated pixel intensity. Ideal settings are highlighted in the red rectangle.

(B) Gray value histogram acquired for each condition presented in (A). For greater precision, the variation of illumination should be observed from the gray value histogram, generated by the camera's own application program or the image can be processed by the ImageJ software.

settings to set the background close to 127, which is the middle of this range. This procedure can be carried out using a grayscale value histogram plot (Figures 1B, 2B, and 3B).

Note: The histogram plot can be usually acquired in real time through the software camera acquisition, or the image can be captured, stored, and processed by ImageJ software ('Analyze' > 'Histogram' or Ctrl + h).

△ CRITICAL: Make sure that the combination of these steps (focus, diaphragm and mean gray value) leads to the best contrast image.

Camera setting and image collection for the different cell types

⌚ Timing: 5 min

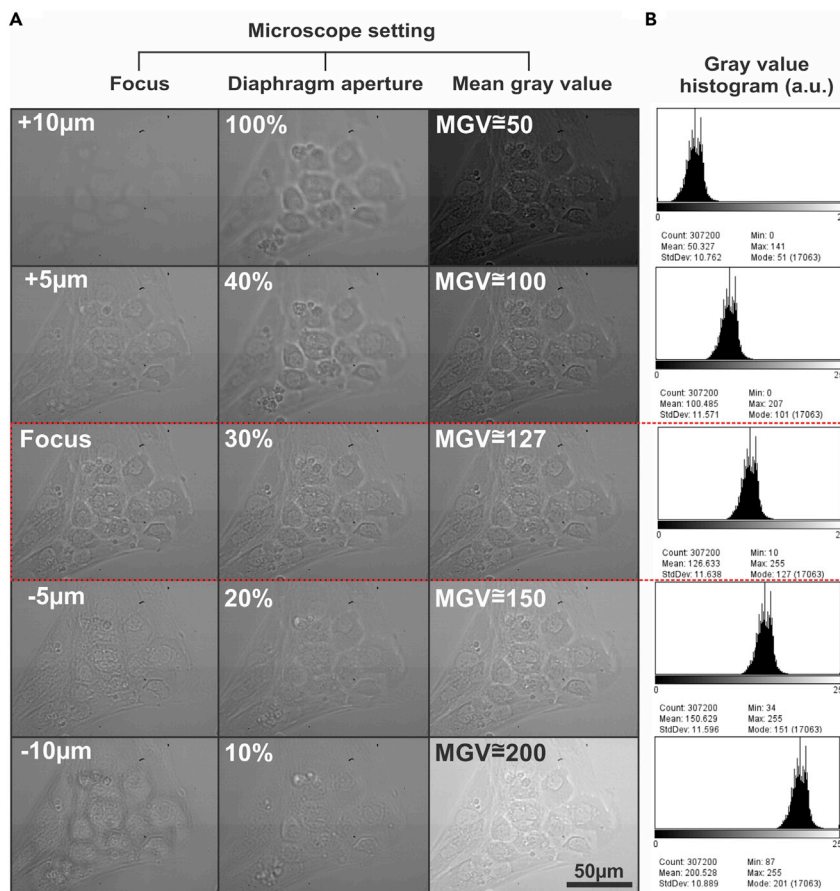


Figure 3. Optimal brightfield microscope settings for hiPSC-CMs

(A) Focus: Out-of-focus cells present a bright halo around the cell border and poor resolution of intracellular structures. Use the fine focus control to minimize border brightness and to bring intracellular structures into sharp focus. Diaphragm: changes in the diaphragm drastically alter cell visualization, when it is fully open (100%) the cell does not have a clear delimitation and contrast. When it is 10% opened the cell has a dark border, indicating poor lighting of the cell. Therefore, normally in a brightfield microscope, the diaphragm should be 30% opened to get the best contrast. Mean gray value (MGV): In an 8-bit image, the best illumination will be proportional to the average total pixels of the image, which in this case will be 127. Smaller values do not produce good lighting, and larger values produce an excess of illumination and can reach exaggerated pixel intensity. Ideal settings are highlighted in the red rectangle.

(B) Gray value histogram acquired for each condition presented in (A). For greater precision, the variation of illumination should be observed from the gray value histogram, generated by the camera's own application program or the image can be processed by the ImageJ software.

The first step to cell imaging acquisition is to set up the camera for image collection. Importantly, different cell types require specific parameters, such as image size and capture rate, and they must be set before starting the experiment.

In the camera software:

4. Select the image window size to record just the area of interest around the cell. For adult-CMs experiments, using the magnification (40 \times) and pixel size (0.25 μ m/pixel) of our microscope (see Microscope resolution), an image window size of 640 horizontal \times 200 vertical pixels allows most cells to fit within the image. Neonatal-CM or and hiPSC-CM require a field of view, and an image window size of 640 \times 480 pixels is our standard configuration for these cells.

Note: If in the experiment more than 5% of the adult-CMs does not fit within the image window, increase the height or width window size. For many cameras, variation of the window height does not affect the maximum frame rate that can be acquired, which is limited by the window width. Therefore, it is easier to change the height size to find the best window size.

△ CRITICAL: The resolution window size must be the same within and across experimental groups to provide the best comparison.

5. Background correction

To acquire a clearer image without background interference, we recommend the use of background correction (for details, see Troubleshooting - [problem 1](#)).

6. Select the capture frame rate

The capture frame rate is an important parameter to acquire a complete and robust contraction-relaxation cycle wave. To do so, it is important to keep in mind that mature cells have a greater contraction speed and contraction area compared to immature cells, and therefore, require higher frame rates. To find the best rate, note that the minimum acquisition period to reliably reconstruct a signal needs to be smaller than half the time of the fastest component ([Nyquist, 1928](#)). For more details see [Scalzo et al. \(2021\)](#) (User Manual provided as Methods S1, section 2.2 Requirements, subsection: Minimum frame rate).

Note: The frame rate and the intrinsic noise produced by the cell movement can influence the way the data is visualized (for more details, see Troubleshooting - [problem 2](#)).

7. Save images

The camera should be connected to the computer through a USB 3.0 interface, to provide high frame rate acquisition. Most of the camera software allows capturing and storing the image in different ways. The best way to avoid variation in the capture rate value is to choose the option that first stores the images in the RAM, and then the user saves manually in computer HD.

△ CRITICAL: Make sure that throughout image acquisition the capture rate remains at the desired speed during all the capture time. Sometimes, there is a slight variation in the camera capture rate. Be aware that the variation must not exceed 1% of the desired value.

Contractility assay in cardiomyocytes

Adult-CM experiments

⌚ Timing: 1–4 h

The adult-CM usually has a robust contraction and a good cell image contrast ([Methods video S1](#)), allowing an easier image acquisition compared to more immature neonatal cells ([Methods video S2](#)) and hiPSC-CMs ([Methods video S3](#)). However, since healthy adult-CM require electrical stimulation to contract, and exhibit a higher degree of heterogeneity compared to immature cells, some steps are essential to acquire the contraction properly:

8. Assemble the cell-stimulation chamber and fix it onto the stage of the microscope.

Note: In our cellular contractility experiments, isolated adult-CMs must be plated on a suitable chamber. Our laboratory utilizes a commercial chamber (model ChamlideIC-CU: 109, Live Cell Instrument, Nowan-gu, Korea) (see [key resources table](#)). However, any mini-incubator with

temperature and CO₂ control (when required) should work. Usually, the chamber can be attached to the microscope through a specific stage provided with the incubation system.

9. Set the temperature to 37°C. Position the recording chamber and platinum electrodes that will provide the field stimulation to generate the cell action potential.
10. Turn on the stimulator and set up the parameters (frequency, voltage, and pulse width duration).
11. Resuspend the dissociated adult-CM in the Tyrode solution.

Note: High cellular density can impair single cell visualization, so dilution is recommended (for more details, see Troubleshooting - [problem 3](#)).

12. Gently homogenize the cell solution and add a few drops of cells in the chamber at 37°C until the experiment starts. Wait at least 1 min for the cells to settle. Usually, the cells attach to the surface without any coated substrates. However, different types of experiments may require the surface to be coated with laminin or another matrix solution (e.g., cell perfusion experiments).
13. Select a healthy cardiomyocyte, position the cell in the center of the field of view, and rotate the camera as necessary to align the long axis of the cell with the horizontal axis of the image window. To distinguish between healthy and unhealthy cells, please see Troubleshooting - [problem 4](#). Due to the strength of their contractions, adult-CMs can occasionally detach from the surface, changing the alignment. However, even in this case, the analysis can be performed provided that no part of the cell leaves the image field of view.
14. Adjust the adult-CM image focus as described in the “[microscope and image setting for the different cell types](#)” section.
15. Start the camera recorder.
16. Turn on the stimulator. Choose the pacing condition which will depend on the experiment goal.
17. Acquire cell contractility.

For a healthy control cardiomyocyte, starting with the cell at rest, the first contraction will be the strongest one, as the sarcoplasmic reticulum calcium store is full. As the stimulation sequence proceeds, there is a decay in the cell shortening of each subsequent contraction through calcium homeostasis. Control cells generally reach a steady state after 10–15 contractions, although this can vary from animal to animal and experimental conditions, such as the temperature setting. After the cell contraction amplitude reaches steady-state, record relevant contractility data.

△ CRITICAL: In healthy cardiomyocytes, the cells must contract in synchrony with the stimulation. Moreover, before electrical pacing begins, the cells should be quiescent. Cells that contract in the absence of stimulation or that present arrhythmic events ([Methods videos S4 and S5](#)) should be excluded from the analysis.

Note: To obtain the CM fractional shortening, the cell border must be well defined.

Neonatal-CM and hiPSC-CM experiments

⌚ **Timing:** 1–4 h

Make a preparation of neonatal-CM or hiPSC-CM that has several separated syncytium and spontaneously contracting cells.

△ CRITICAL: A culture that has not formed a connection between cells to form a syncytium usually does not contract, or if it does, shows a lower contraction area.

18. Turn on the chamber at 37°C and 5% CO₂.
19. Put the recipient containing the culture cells in the microscope chamber.

△ **CRITICAL:** The temperature and the culture medium can strongly impact cardiomyocyte contractility. Hence, subtle variations in these parameters can drastically alter cellular contractility results. In our experiment, we use Dulbecco's modified Eagle's culture medium (DMEM) supplemented with 10% fetal bovine serum (FBS) for neonatal-CM and RPMI 1640 supplemented with 10 μ M of Y27632 for hiPSC-CM (for more details, see [key resources table](#) and Materials and equipment). However, different types of experiments may require a specific culture medium. Wait at least 5 min to stabilize the cell temperature at 37°C.

20. Select the cell in the region of interest (ROI) in the camera image field according to the steps described in the "[microscope and image setting for the different cell types](#)" section.

Note: It is recommended to acquire the data in a syncytium where all cell contractions are synchronized and that have similar areas and number of cells (that can be estimated by counting cell nuclei). Images of multiple syncytia or an unsynchronized syncytium will not show a typical signal to be analyzed. In neonatal-CM culture select the cell area without debris or fibroblasts (for details see Troubleshooting - [problem 5](#)).

Importantly, the size of the pre-set window should be adjusted accordingly and be kept the same throughout the entire experiment. If the syncytium is too big and does not fit in the image, a tip is to look for and acquire the contraction at its center of convergence. Acquire the cell contractility for at least 15 s.

Saving images

Images should be saved at a depth of at least 8-bit. There are three standard ways to store contractility images:

Image sequence: a folder containing multiple images in sequence.

Multipage image: one TIFF file.

Video file data: one video with .avi extension.

It is recommended that images are saved using the TIFF file format. Many other common image formats, such as JPEG, use image compression resulting in loss of information. However, if HD space limitations require the use of compressed file formats, it is important to be aware of the possibility of information loss and to consistently use the same file format in all experimental groups that will be compared.

Contractility analysis using CONTRACTIONWAVE

Once high-quality data is acquired, the analysis of contractility parameters can be performed using a variety of available software programs (IonWizard Ionoptix; [Boudaoud et al., 2014](#); [Grune et al., 2019](#); [Maddah et al., 2015](#); [Pasqualin et al., 2016](#); [Sala et al., 2018](#)). The authors have developed CONTRACTIONWAVE ([Scalzo et al., 2021](#)), a high-performance Python program that processes video micrograph films using dense optical flow image analysis. This program has been validated for large-scale analysis of different cell types (adult-, neonatal- and hiPSC-CMs), treated or not with drugs, including both healthy cells and cells isolated from animal models of human disease. [Figure 4](#) illustrates the CONTRACTIONWAVE window that shows the average speed of contraction-relaxation cycle results of an adult-CM that was electrically stimulated at 1 Hz and processed using the software.

In the wave detection window, a time interval can be selected by the user (top panel, orange area, [Figure 4A](#)). Once an interval is chosen, the program displays a zoomed plot of the selection in the window below (bottom panel, [Figure 4A](#)). An innovative feature of CONTRACTIONWAVE compared

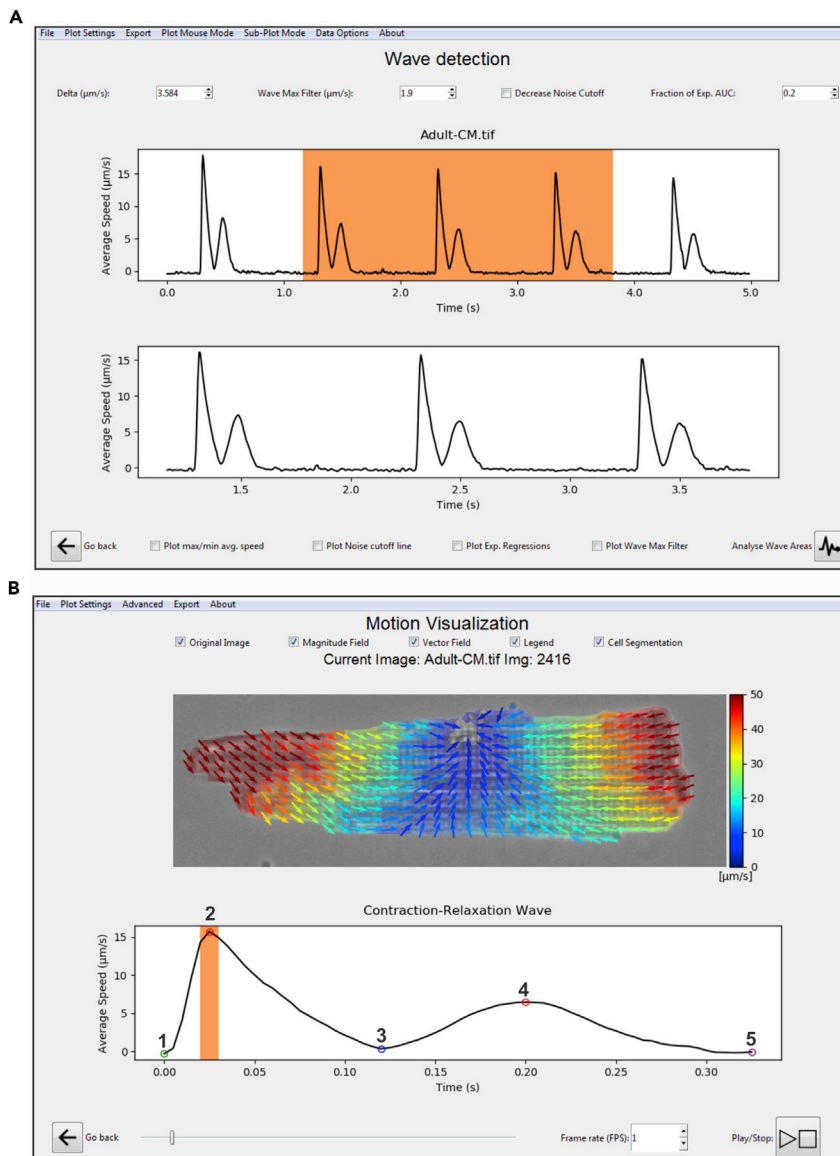


Figure 4. Analysis of cellular contractility using CONTRACTIONWAVE

(A) CONTRACTIONWAVE data analysis window allows interval selections of the contraction-relaxation waves. The software automatically detects the average speed of the contraction-relaxation waves and allows for fine adjustments to improve peak detections (top panel). To assist single or multiple peak selection for data analysis, a zoomed version of the selected interval is displayed on the panel below (bottom panel).

(B) CONTRACTIONWAVE simultaneously displays both the cell image (top panel) and the contraction-relaxation waves (bottom panel). The top panel shows the cardiomyocyte image with the magnitude and motions vectors of the contractility movement acquired during step (2) represented on the bottom panel (the orange area highlights the time in which the upper cell image was acquired). Note that the average speed corresponds to a single contraction-relaxation cycle of an electrically stimulated cardiomyocyte. Colored circles presented in the contraction-relaxation wave (bottom panel) represent (1) contraction initiation; (2) maximum contraction speed (MCS); (3) minimum contraction speed; (4) maximum relaxation speed (MRS); (5) return to baseline. Figure adapted from Scalzo et al. (2021)

to other available software is its ability to display simultaneous views of the cell image and the derived contraction-relaxation wave (Figure 4B). Contractility analysis using CONTRACTIONWAVE allows for the acquisition of three distinct types of parameters of the contraction-relaxation cycle: speed of contraction at different points along the cycle, time intervals between several important phases of contraction-relaxation cycle, and shortening area, in appropriate calibrated units. For a

complete description and definition of all contractility parameters, please see [Figure 3](#) in the original description of CONTRACTIONWAVE ([Scalzo et al., 2021](#)).

Note: CONTRACTIONWAVE is open-source software and all details for download, installation, and optimized use are fully described in [Scalzo et al. \(2021\)](#).

EXPECTED OUTCOMES

On a successful acquisition of cellular contractility, CONTRACTIONWAVE will properly detect the contraction-relaxation cycle through the dense optical flow. The software can obtain the magnitude and vector field of the membrane movement ([Figures 5A, 6A, and 7A](#) show only the maximum contraction speeds), and their respective cycle graphs ([Figures 5B, 6B, and 7B](#)), to analyze the

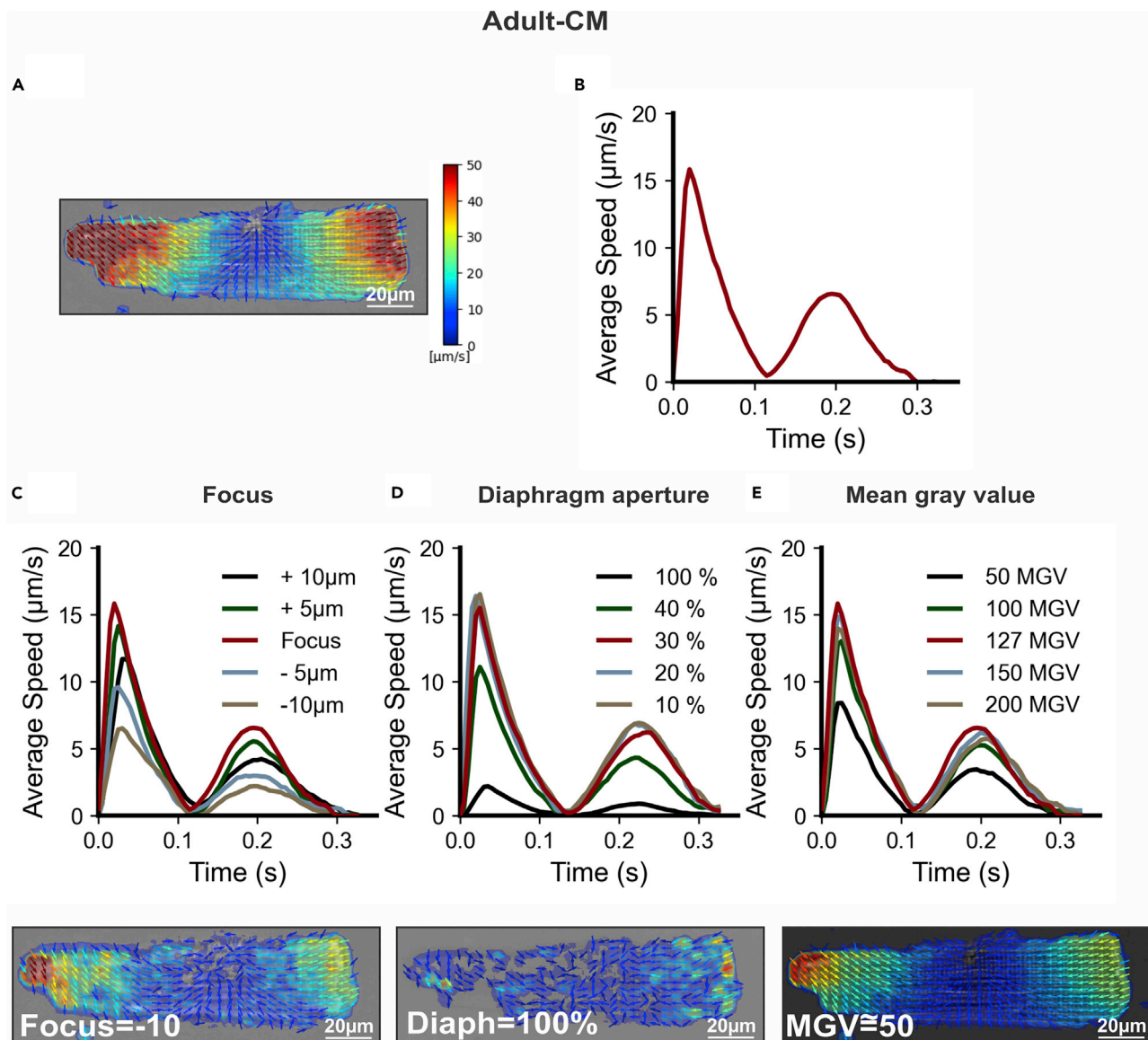


Figure 5. Sub-optimal microscope settings for image acquisition of adult-CMs can impact contraction-relaxation average speed
(A–E) Representative image of magnitude and vector field from the maximum contraction speed, and (B) the respective cell contraction-relaxation cycle processed by CONTRACTIONWAVE using the optimal acquisition parameters (Cell in focus; Diaphragm = 30% and MGv = 127). Sub-optimal microscope settings such as poor focus (C), improper diaphragm opening (D), or low mean gray values (MGV) (E) could result in an underestimate of the contraction-relaxation speed. Below each graph is a representative image of magnitude and vector field from the maximum contraction speed acquired in a specific setting.

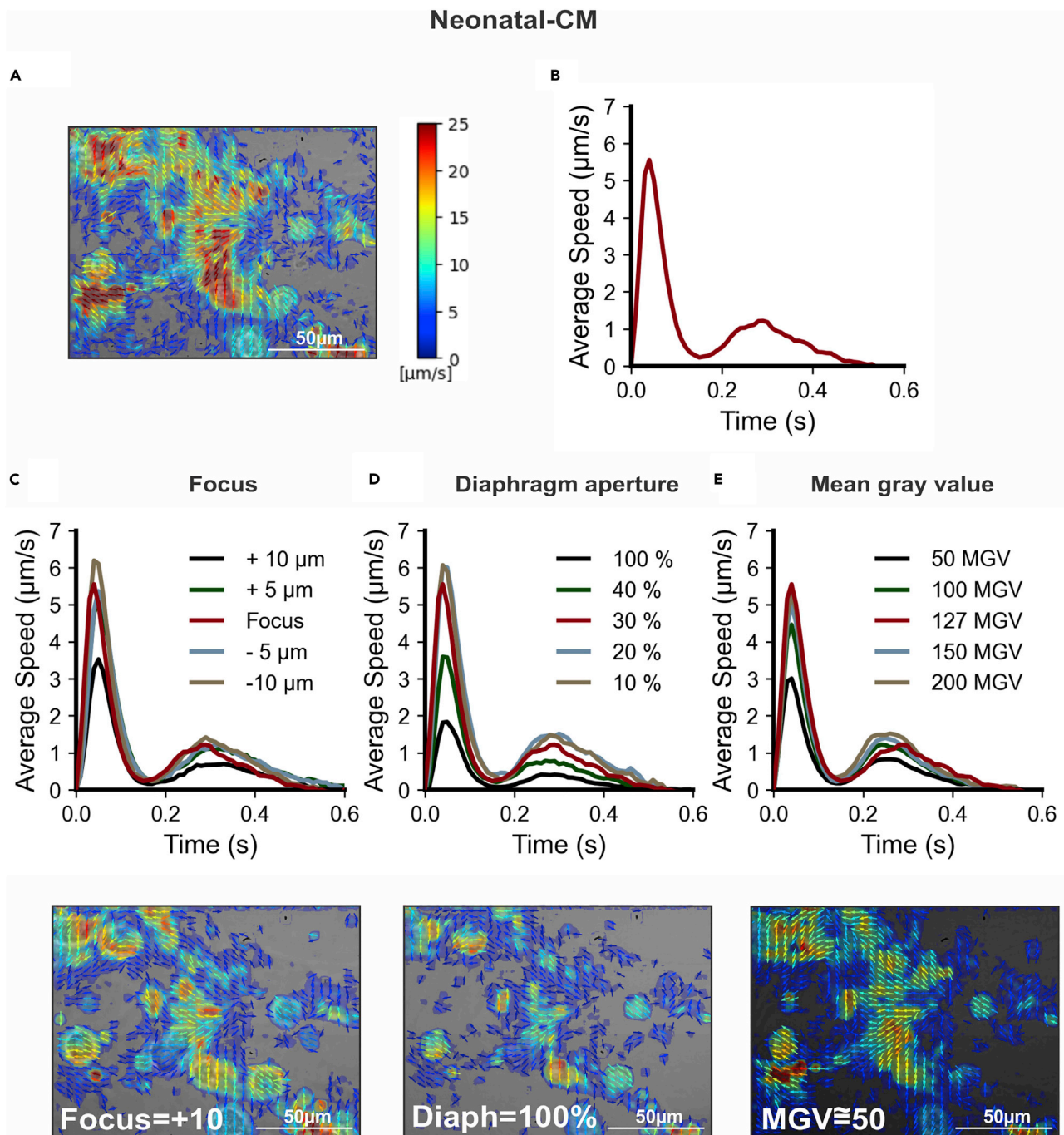


Figure 6. Sub-optimal microscope settings for image acquisition of neonatal-CMs can impact contraction-relaxation average speed

(A–E) Representative image of magnitude and vector field from the maximum contraction speed, and (B) the respective cell contraction-relaxation cycle processed by CONTRACTIONWAVE using the optimal acquisition parameters (Cell on Focus; Diaphragma = 30% opened and MGV = 127). Sub-optimal microscope settings such as poor focus (C), improper diaphragm opening (D), or low mean gray values (MGV) (E) could result in an underestimate of the contraction-relaxation speed. Below each graph is a representative image of magnitude and vector field from the maximum contraction speed acquired under a specific setting.

contractility parameters. In order to demonstrate how the microscope settings can influence data acquisition, contraction-relaxation data were acquired and analyzed for different settings (Figures 5C–5E, 6C–6E, and 7C–7E)

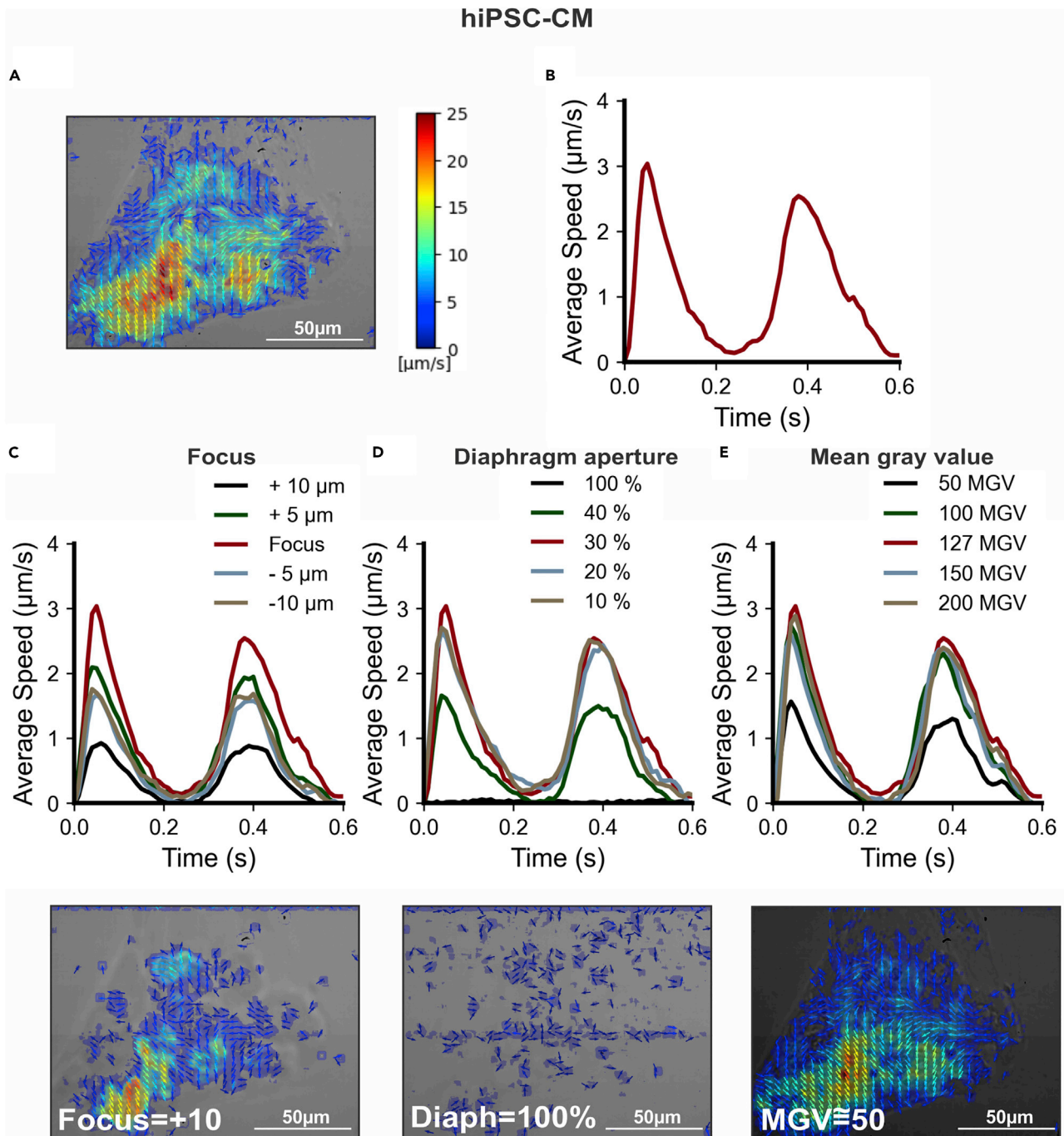


Figure 7. Sub-optimal microscope settings for image acquisition of hiPSC-CMs can impact contraction-relaxation average speed

(A–E) Representative image of magnitude and vector field from the maximum contraction speed, and (B) the respective cell contraction-relaxation cycle processed by CONTRACTIONWAVE using the optimal acquisition parameters (Cell on Focus; Diaphragma = 30% opened and MGV = 127). Sub-optimal microscope settings such as poor focus (C), improper diaphragm opening (D), or low mean gray values (MGV) (E) could result in an underestimate of the contraction-relaxation speed. Below each graph is a representative image of magnitude and vector field from the maximum contraction speed acquired under a specific setting.

All three parameters analyzed (focus, diaphragm, and MGV) showed an influence in the contraction-relaxation speed in the three cell types (Figures 5, 6, and 7). It is interesting to note that the contractility wave of cardiac cells is influenced by the focus. In the example shown in Figures 5C and 6C, the

contraction-relaxation speed is greatly altered when the focus is below $-5\ \mu\text{m}$ for adult-CM and at or above $+10\ \mu\text{m}$ for neonatal-CM compared to the focused image. Measurement of contraction-relaxation speed of hiPSC-CM is significantly influenced by the quality of focus (Figure 7C), partly due to lower contrast and cell thickness. Of note, the optimal focus value may need to be adjusted for each cell.

The diaphragm is a vital microscope component when ensuring image quality for contractility analysis. With diaphragm values higher than 40% opened, the cell contrast is not properly obtained, significantly influencing the contraction-relaxation waves (Figures 5D, 6D, and 7D). Although diaphragm settings can influence the movement detection for all cell types, it is important to point out that hiPSC-CM cells are the most influenced by this type of setting. Diaphragm aperture higher than 40% will not allow for a proper detection of the coordinated movements to generate a typical wave of hiPSC-CMs (Figure 7D). Mean gray values (MGV) of 50 and lower show a decrease in the contraction-relaxation speed (Figures 5E, 6E, and 7E). This is probably due to the fact that the camera loses information from small gray level values. Therefore, for an 8-bit image, MGV should be set to the middle of the camera's dynamic range, close to 127. For data analysis using CONTRACTIONWAVE, the brightness does not significantly affect the contraction-relaxation results. With this said, good results can be achieved with an illumination that provides sufficient light to visualize the cell with no difficulty. This value should be higher than 100 MGV to avoid the camera's influence.

In summary, all these parameters will influence the final contraction-relaxation speed, since they provide a variation in the detection sensitivity of the magnitude and motion vectors of the cell membrane (see sample images provided below panels C-E in Figures 5, 6 and 7). See Table 1 for a summary of all contractility recording settings.

LIMITATIONS

A high-quality cardiomyocyte contractility analysis is largely dependent on the condition of the isolated cells and how they were prepared. For instance, adult-CM samples with a large number of dead cells or inadequate morphology (see Troubleshooting - problem 4) will not provide robust contractility data. The small shortening area of neonatal-CM and hiPSC-CM can also impact contractility data acquisition. For instance, more subtle contractions can sometimes be interpreted as noise, and because of that, they cannot be measured (see Scalzo et al., 2021).

TROUBLESHOOTING

Problem 1: Background correction for non-uniformity of illumination

Non-uniform illumination in a brightfield imaging experiment does not significantly impact cellular contractility analysis using CONTRACTIONWAVE. Nonetheless, it can affect the image background (Figure 8), and the user may choose to correct it.

Some camera software allows the user to automatically set up the background correction during the recording step. When that option is not available, the background correction can also be done after images are acquired (see potential solution below).

Potential solution 1

A standard way of doing the background correction after data acquisition is by using the ImageJ software (download and installation instructions: <https://imagej.nih.gov/ij/> or <https://fiji.sc/>). First, an image of the empty chamber without the cell is acquired to show the non-uniform illumination of the background in a brightfield image (Figure 8i). The correction consists in dividing the cell image by the background image and multiplying the result by the average value of the original image (Equation 5).

$$\text{Background correction} = \frac{\text{Cell image sequence}}{\text{Image background}} \times \text{Background mean gray value} \quad (\text{Equation 5})$$

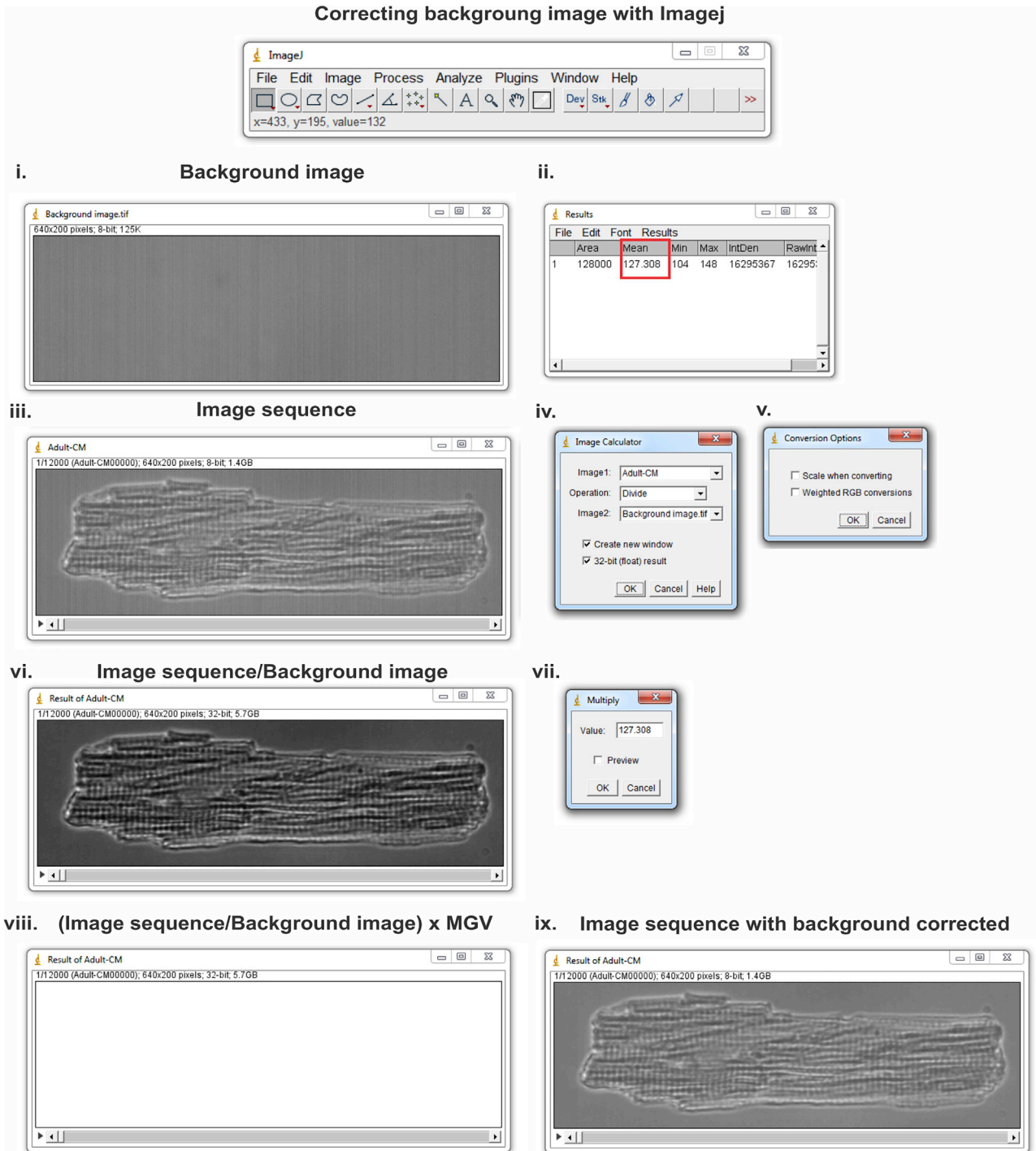


Figure 8. Background correction for non-uniformity of illumination using ImageJ

i. Import one “background-image” taken on a surface with no cell and ii. Obtain the background mean gray value (red square value). iii. Open the cell “image sequence”. iv. Select Image 1 with the “image sequence stack”, Operation “Divide” and Image 2 with the “background-image”. Click the boxes “Create a new window” and “32-bit (float) result”, then click “OK” and select “Yes” to process all images. v. Go to Edit > Options > Conversions to make sure no option is selected. Multiply the resulting image (vi) by the background mean gray value obtained in step ii as shown in step vii. A white image will appear (viii). ix. Convert the 32-bit image (viii) into 8-bit and save the correction image.

The steps to process the background correction using ImageJ goes as follows:

- a. In ImageJ software, import the “background-image” (captured from a surface with no cell, [Figure 8i](#)) and go to Analyze > Measure, to obtain the background mean gray value parameter ([Figure 8ii](#)).
- b. Select File > Import > Image Sequence to open the “Image sequence” ([Figure 8iii](#)).
- c. Go to Process > Image Calculator and select the previous imported “Image sequence” (in Image 1), “Divide” (in operation), and “background-image” (in image 2) ([Figure 8iv](#)). Click “Create a new window” and “32-bit (float) result”, and then hit the “OK” button and select “Yes” to process all images.

Go to Edit > Options > Conversions to make sure no option is selected in the window ([Figure 8v](#)).

d. In the resulting image ([Figure 8vi](#)) go to Process > Math > Multiply and put the background mean gray value ([Figure 8vii](#)), which was obtained in [Figure 8ii](#). A white image will appear ([Figure 8viii](#)), representing the brightness adjustment. Then, go to Image > Type > 8-bit to convert the 32-bit image into 8-bit.

e. The new window will automatically show the converted final image with the corrected background ([Figure 8ix](#)).

f. Save image as multipageTIFF (File > Save as > TIFF), or as image sequence (File > Save as > Image Sequence).

Note: To resolve other background problems such as hot pixels and banding, please see the following ImageJ tutorial at: https://imagejdocu.tudor.lu/howto/working/how_to_correct_background_illumination_in_brightfield_microscopy

Problem 2: Noisy contractility data

Commonly, contraction-relaxation recordings from immature cells have a lower signal-to-noise ratio compared to recordings from adult cardiomyocytes. In particular, neonatal-CMs and hiPSC-CMs often show an increased signal noise during the relaxation phase ([Figures 9A](#) and [9B](#)). For these cellular types, it is important to minimize the noise to obtain a reliable and robust contractility analysis.

Potential solution 2: Change the capture rate or apply a filter

The image capture rate can influence the signal noise. Essentially, the higher the capture rate, the shorter the displacement collected for each pixel between two frames. A short cell displacement can result in a calculated speed that is close to the limit of detection, increasing the amount of captured noise. Adjustments in the frame rate can be made to minimize this effect. Reducing the frame rate to an optimal value can significantly reduce noise with little or no impact on the derived contraction-relaxation wave parameters (maximum contraction speed (MCS) and maximum relaxation speed (MRS)). Our experiments demonstrate that hiPSC-CMs have a low average speed (<10 $\mu\text{m/s}$) and a cell recording performed at 100 fps captures noisy data that is difficult to analyze ([Figure 9A](#)). By simply reducing the camera frame rate, the noise gradually decreases, while the contractility speed parameters are maintained ([Figures 9B](#) and [9C](#)). Importantly, an excessive frame rate decrease can directly impact the MCS and MRS parameters ([Figures 9D](#) and [9E](#)), so a careful calibration analysis must be done to identify the optimal frame rate value for the specific cell type.

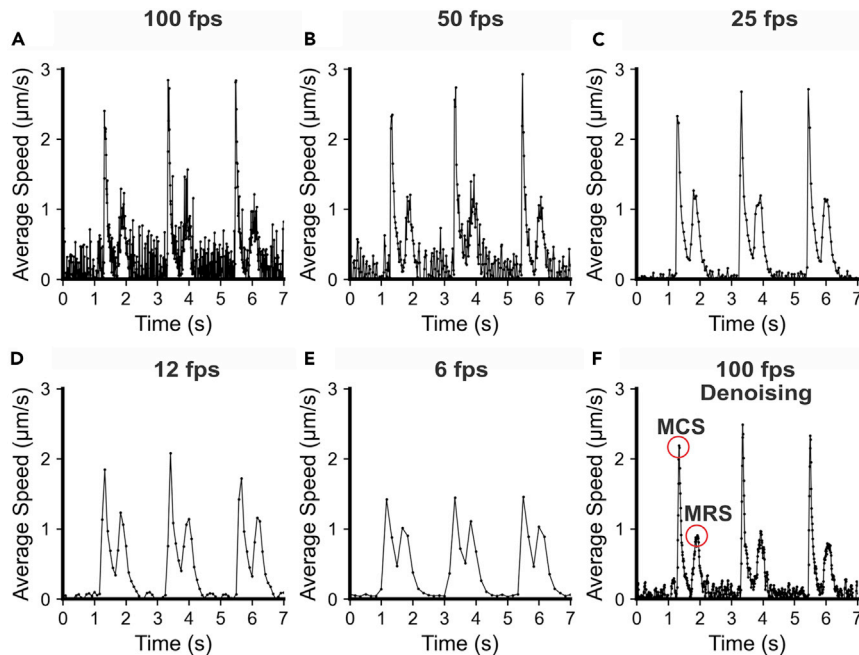


Figure 9. Reducing noise by adjusting frame rate and using filters

(A and B) Contraction-relaxation data acquired at 100 and 50 frames per second (fps) from hiPSC-CM show a high noise content that can contribute to errors in the data analysis.

(C) Contraction-relaxation data acquired at 25 fps shows an optimal balance between noise minimization and data integrity.

(D and E) Contraction-relaxation data acquired at 12 and 6 fps show significant reductions in the signal noise, but significantly affect maximum contraction speed (MCS) and maximum relaxation speed (MRS).

(F) Contraction-relaxation data acquired at 100 fps and analyzed using the denoising filter provided by CONTRACTIONWAVE shows significant reductions in the signal noise, not compromising MCS and MRS values.

Alternatively, CONTRACTIONWAVE provides denoising filters that can also minimize the influence of background noise, with no requirement of a specific frame rate (Figure 9F). All details for filter use can be found in Scalzo et al. (2021) (Method S1- User Manual).

Problem 3: Adult-CM cellular density for image acquisition

When acquiring adult-CM contractility data, it is important that there be only a single cell in the field of view, as the presence of neighboring cells can impact the results. Thus, the density of dissociated cells in the suspension is a key parameter for clear cell image acquisition.

Potential solution 3

The cell image should be as clean as possible. For this, be careful with the adult-CM final cell density. The best way to obtain a clean image is by adjusting the volume that is plated so that the settled cells will have a confluence of about 40%. The greater the confluence, the more nearby cells will be acquired in the same image window, and the more difficult it will be to acquire a single-cell image. This type of dilution will also decrease the amount of cell debris and dirt that may remain throughout the solution.

Problem 4: Adult-CM cell variability

When choosing an adult-CM cell to acquire the image data, be aware that bubbles and cell debris can dramatically impact the contractility results. It is very important to identify these undesirable components to guarantee that the best cell image will be captured and then analyzed. In addition, the shape of the cell can help identify healthy cardiomyocytes (see details below).

Potential solution 4

Bubbles or cell debris that enters the field of view can be observed directly or by the shadow they cast. If this happens, stop recording since their presence will cause the analysis to underestimate contraction speed. The experiment can be restarted after clearing the bubbles or debris. When adding the cell solution, avoid generating bubbles by the pipette, since during the experiment they can burst, provoking spontaneous contraction.

To obtain the best contraction-relaxation data, it is necessary to be aware of the cell viability, contraction pattern, and cell morphology. A good adult-CM preparation usually has at least 60% viable cells. To ensure that quality data is acquired, rod-shaped cells with a clear striation pattern and well-defined edges should be selected (Figure 10Ai). Make sure not to record from cells with “cauliflower”-shaped ends, unclear striation patterns, or contaminated with debris or dead cells (Figure 10Aii-iv). Blebs on the cell membrane are an indication that the cells are unhealthy (Figure 10Bi). Another issue is that cells may settle to the chamber resting on their narrow edge rather than resting flat (Figure 10Bii). These cells should be avoided, as they may impact data analysis by CONTRACTIONWAVE, as the software analyzes cellular contractility throughout the entire cell membrane.

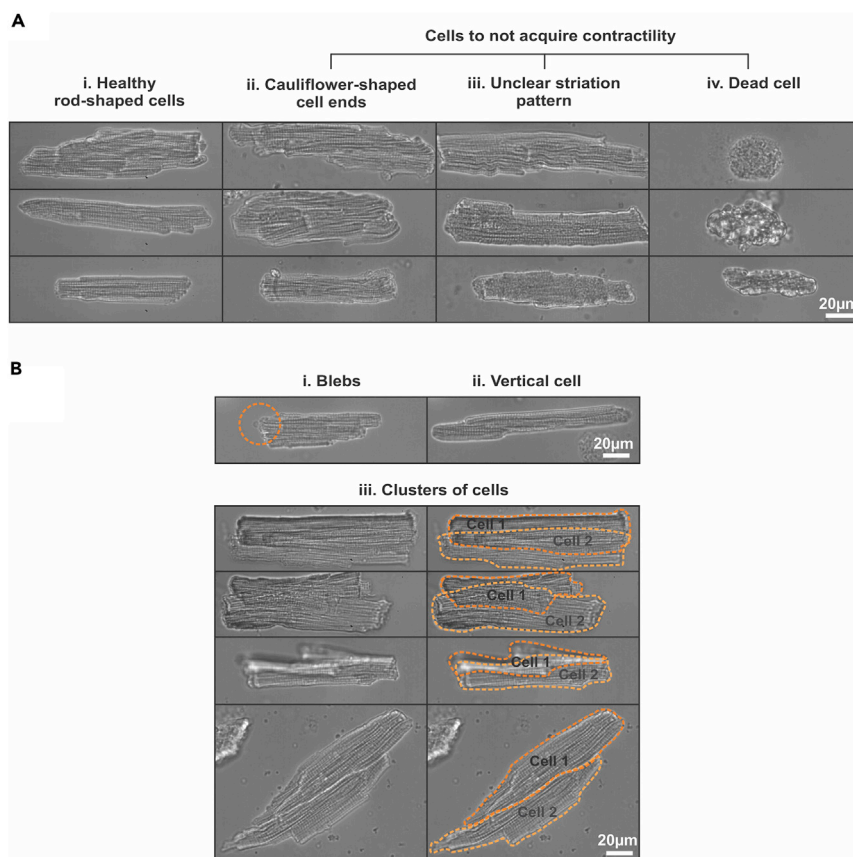


Figure 10. Cell selection is an important step to get the best contractility results

(A) Ai. Healthy cells are rod-shaped and have a well-defined border and linear sarcomere striations. Aii. Cells with cauliflower-shaped ends should not be used to study contractility as they are likely in the process of cell death. Aiii. The unclear striation pattern will affect the potency of cell contraction-relaxation, therefore should also be avoided. Aiv. Dead cells are easy to identify, having a more rounded shape and little or no contraction-relaxation. (B) Bi. Membrane blebs can be an indication of apoptosis and cell damage. Bii. Cells can sediment and be attached to the substrate on their thin edge, rather than laying flat. Biii. Clusters of two or more cells either on top of each other (Biii - top) or beside each other (Biii - below) should be avoided.

Adult cardiomyocytes from healthy animals should not present spontaneous contraction. In this case, cells that do present spontaneous contraction should be avoided. However, for some disease models or drug treatments, spontaneous or lack of contractions can occur.

△ **CRITICAL:** Sometimes one cell may lie on top of another (Figure 10Biii). Analyzing the cell border or through stimulation, it is possible to identify the two cells. If the cell clings to another cell or to debris, the contractility acquisition will be underestimated. In cells from normal healthy animals, thinner cells must be examined carefully to be sure that they are not cells that settled on its side.

Problem 5: Neonatal-CM and hiPSC-CM cell variations

Obtaining a good image of the cell syncytium for neonatal-CMs and hiPSC-CMs is crucial to provide high-quality data of cell contractility. The number of cells, presence of cell debris, and fibroblasts must be carefully analyzed before choosing the best cell area to perform the experiment (Figure 11).

Potential solution 5

Cell debris and dead cells are commonly found in neonatal-CM cultures, usually due to cells that did not attach to the plate or problems with the dissociation process (Figure 11A). Always try to acquire an image with the least amount of debris overlaying the cells. Normally, the amount of debris in hiPSC-CM is much lower. Additionally, caution should be taken with contamination by fibroblasts in the neonatal-CM culture, as they can indirectly influence cardiomyocyte contractility through chemical signaling or even physically preventing cardiomyocytes from contracting properly.

Note: Fibroblasts have a more transparent appearance with a trapezoidal shape, presenting a larger area and nucleus compared to cardiomyocytes, and do not contract (Figure 11B).

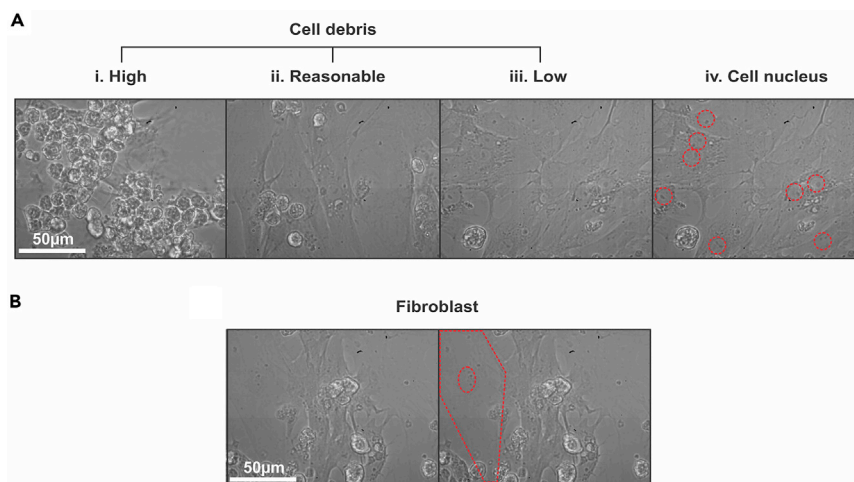


Figure 11. Cell debris and fibroblasts could impact cell membrane movement and influence the contractility results

(A) Ai. The image labeled “high” is an example of preparation with a large amount of dead rounded cells loosely tethered above the viable stellate cells fixed to the substrate. Aii. The image labeled “reasonable” shows somewhat less debris. Aiii. The image labeled “Low” is an example of preparation with very little debris, a situation that is ideal for contractility acquisition. Aiv. Red circles identify the cell nucleus (outlined in red) of the images that contain low cell debris.

(B) Example of a culture contaminated by a large fibroblast (outlined in red in the right-hand image).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contacts, Prof. Silvia Guatimosim (guatimosim@icb.ufmg.br) and Prof. Ubirajara Agüero (bira@fisica.ufmg.br)

Materials availability

This study did not generate new materials.

Data and code availability

This study did not generate new data and code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2022.101144>.

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AUTHOR CONTRIBUTIONS

S.G., U.A., S.S., and C.A.T.F.M. conceived and led the project. S.S. performed the experiments and analyzed data. S.G., U.A., S.S., C.A.T.F.M., and C.K. wrote and edited the manuscript. All authors critically reviewed and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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