Calcium & Contractility Best Practices

Best practices for measuring intracellular calcium using Fura-2 and contractility in primary cardiomyocytes and iPSC-CM

Introduction

Precise assessment of calcium handling and contractility in cardiomyocytes is essential to comprehend cardiac physiology and pathogenesis. To obtain dependable results, it is imperative to prepare samples properly and acquire accurate data. This application note presents suggested optimal techniques for these two aspects while utilizing IonOptix systems to evaluate calcium handling and contractility in primary cardiomyocytes and iPSC-CM.

Sample preparation

a. Cell preparation for the experiment

Primary Cardiomyocytes:

Standard protocols should be followed for primary cardiomyocyte isolation. There are two commonly used methods for isolating primary cardiomyocytes: Langendorff and non-Langendorff approaches. Selection of the preferred method depends on the experimental design and equipment availability. For detailed info and tutorials, please refer to the following in the IonOptix resources library:

- Techniques and Best Practices for Cardiomyocyte Isolation (https://www.ionoptix.com/resource/webinar-techniques-and-best-practices-for-cardiomyocyte-isolation/)
- IonOptix Video Tutorials: Murine Cardiomyocyte Isolation (https://www.ionoptix.com/resource/ionoptix-video-tutorials-murine-cardiomyocyte-isolation/)

Sample preparation (cont)

Prior to acquiring data, primary cardiomyocytes preferably should pass all three of the following check points:

- Cells should have a rod-shaped structure with well-defined sarcomeres.
- Baseline value for sarcomere length (area highlighted below) is highly recommended to be higher than or equal to 1.7µm.
- Cells should not exhibit spontaneous contractions.

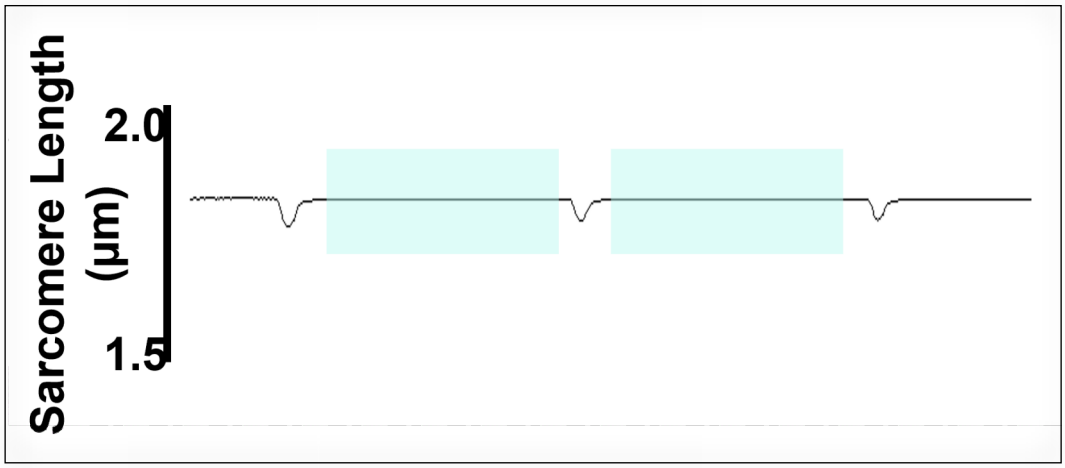

Figure 1: Recommended resting sarcomere length.

iPSC-CM:

When preparing iPSC-CM for calcium handling and contractility measurements, it is crucial to ensure that cells have differentiated properly into cardiomyocytes. Culturing cells for at least two weeks is recommended to allow for proper differentiation. During this time, it is important to regularly monitor cells for proper morphology and spontaneous beating activity.

Morphology:

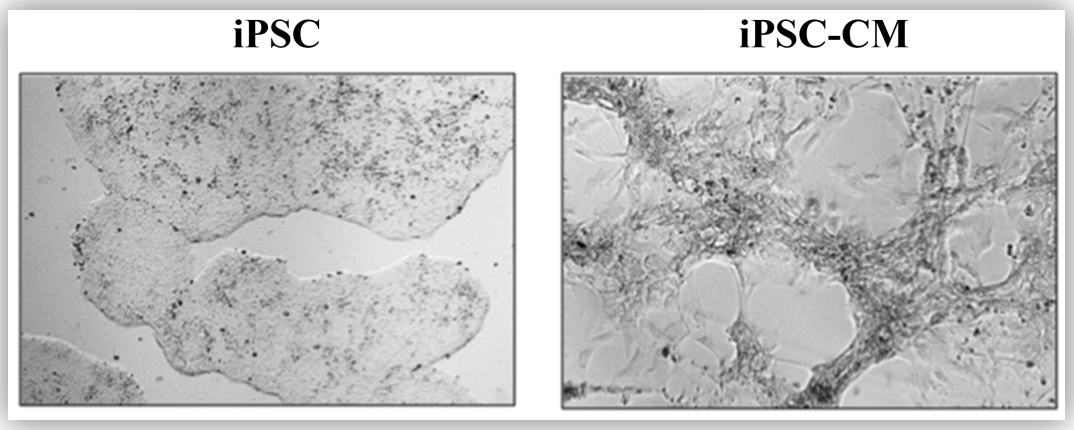

Figure 2: iPSC differentiation. Cells should be regularly monitored for morphology and spontaneous contractions.

Sample preparation (cont)

b. Fura-2 AM stock solution preparation

Fura-2 AM is a commonly used cell-permeable fluorescent dye for measuring intracellular calcium. Typically, Fura-2 AM is dissolved in a solution containing Pluronic F-127, a nonionic surfactant used to facilitate solubility of water-insoluble acetoxymethyl (AM) esters. However, Pluronic is unnecessary at the low concentration of Fura-2 AM used in our recommended protocols.

.To prepare a 1 mM stock solution of Fura-2 AM, add 50 µL of DMSO to a vial containing 50 µg of Fura-2 AM powder, ***vortex for 1 minute*** until Fura-2 is fully dissolved and the solution appears pale yellow to the eye.

Note: It is important to aliquot the stock solution for single use to avoid repeated freeze-thaw cycles that may affect the dye's stability.

Store aliquots at -20°C in the dark

Note: Ester bonds are highly labile and hydrolyze in the presence of water. Premature hydrolysis will render the dye membrane insoluble. Care should be taken to avoid introducing water into your 1 mM Fura-2 AM stock solutions. It is advisable to use anhydrous DMSO to solubilize lyophilized Fura-2 AM powder. It is also recommended to warm the stock solution to room temperature *before* opening the stock vial to avoid condensation.

c. Loading cells with Fura-2

Note: Cells should be loaded on the day of the experiment. While the dye will be de-esterified in the cell and rendered membrane impermeable, cells can actively transport the de-esterified dye out of the cell via anion transporters.

Primary Cardiomyocytes:

1. Take 1 ml of cell suspension and put it in an Eppendorf tube or add a few drops of cell suspension to 1 ml of Tyrode's solution in an Eppendorf tube, depending on the concentration of cells.
2. Add dissolved Fura-2 AM to the Eppendorf tube.
   Aim for a final concentration between 1-2 µM.
3. Mix the Fura and cell suspension by inverting the Eppendorf tube a few times.
4. Wrap the Eppendorf in aluminum foil to protect it from light.
5. Store the tube upright at room temperature*.

Sample preparation (cont)

6. After 20 minutes of incubation stop the loading by washing the cells. There should be a small pellet of cells at the bottom of the tube. Take off the supernatant and add fresh Tyrode's solution that does not contain Fura. Mix the cell with the solution by inverting the tube a few times (tap the bottom of the tube to dislodge the pellet).
7. Repeat this after 5 minutes.
8. It is safe to start the experiment 20 minutes after the loading to provide sufficient time for the de-esterification.

iPSC-CM:

1. Dilute Fura-2 stock solution to a final concentration of 0.5-1 µM in HBSS (Hank's Balanced Salt Solution) or Tyrode's solution buffer to prepare a working solution of Fura-2 AM.
2. Remove culture medium from the plated iPSC-CMs.
3. Add the 0.5-1 µM Fura-suspension to the dish or wells. Use ~1 ml for both 35mm and 24 well dishes.
4. Incubate at 37°C for 15 minutes*.
5. Stop the incubation by washing the cells. It is recommended to wash 2x or 3x with Tyrode's solution.
6. It is safe to start the experiment 10 minutes after the loading to provide sufficient time for the de-esterification.

*Note: Ideally you would load at room temperature to minimize compartmentalization of the dye during loading. While adult cardiomyocytes tolerate RT loading, typically iPSC-CMs cannot.

d. Temperature control

Primary cardiomyocytes and iPSC-CMs are highly sensitive to temperature fluctuations, making it crucial to maintain proper temperature control throughout data acquisition processes. Failing to maintain optimal temperature conditions at 37°C could result in altered cellular behavior and potential experimental artifacts.

Sample preparation (cont)

Temperature control can be attained by using our in-line preheater with the conventional calcium and contractility system or the MultiCell Lite system. Our MultiCell HTS has built-in stage temperature controls and users can monitor real time plate temperature in IonWizard.

e. Cell stimulation

Cardiomyocytes can be stimulated using electrical or chemical methods, both of which should be carefully optimized. When optimizing the stimulation protocol for cardiomyocytes, it's important to note that there may be differences between iPSC-CMs and primary cardiomyocytes. For example, iPSC-CMs are typically smaller and have different electrical properties compared to primary cardiomyocytes. As a result, the optimal stimulation parameters may differ between these two types of cells.

Electrical stimulation parameters such as frequency and voltage are easily adjusted on our MyoPacer electrical field stimulator. For detailed info, please refer to the manual (https://www.ionoptix.com/resource/myopacer-manual/). Usually, electrical stimulation of iPSC-CMs requires lower voltage and frequency which is more appropriate for their smaller size and slower electrical properties. In contrast, primary cardiomyocytes may require higher voltage and frequency for effective stimulation.

Similarly, the optimal drug concentrations and timing for chemical stimulation may also differ between iPSC-CMs and primary cardiomyocytes due to differences in their cellular behavior and response to external stimuli. It is advised to optimize chemical stimulation parameters such as drug concentration and timing by performing dose response curves, especially for genetically modified cells or drugs that are not commonly used.

To assist in conducting experiments, the following guidelines for voltages, frequencies, and commonly used pharmacological agents are suggested as a starting point. However, it's crucial to optimize these parameters for each experiment, considering factors such as cell type, species, age, and culture conditions, as well as the specific research question being addressed.

Stimulus	iPSC-CM	Primary Cardiomyocytes
Frequency (on MyoPacer)	Up to 4Hz (preferred below 2)	Up to 4Hz
Voltage (on MyoPacer)	Up to 15V	Up to 20V
Pharmacological Agents		
Isoproterenol	Up to 500nM	Up to 1μM
Caffeine	Up to 10mM	Up to 10mM

Sample preparation (cont)

f. Minimizing background noise

Background noise is an inevitable result of light scattering and internal reflections within the optical path of the data acquisition system. However, excessively high levels of noise can impede data analysis and lead to artifacts. Vibrations, electrical noise, and ambient light can cause such noise. It is crucial to control for these factors by taking the following measures when collecting data:

- Keeping the system in a dark room (this applies to all IonOptix systems except for the MultiCell HTS, which already is an enclosed microscope).
- Placing the system on a vibration control or sufficiently sturdy table.
- If cyclical noise is present in the data trace, check for nearby equipment that might cause electrical noise or excessive vibrations and relocate either the equipment or the IonOptix system. Often electrical noise is present on the mains (electrical supply lines). If so, a line conditioner may be necessary.

Data acquisition

a. Optimizing fluorescence light source

While IonOptix systems are calibrated upon installation, experiments with varying conditions may require adjustments, especially to the light source and MyoPacer (discussed earlier). This section will focus on optimizing the fluorescence light source.

To attain optimal results when handling a Fura-2 loaded plate, it is recommended to regulate light intensity of background fluorescence (a selected area within the plate that does not have cells in the field of view) at 200-500 au for both numerator (calcium-bound Fura-2) and denominator (calcium-free Fura-2) raw counts. Similarly, for Fura-2 loaded cells, it is advisable to sustain a baseline fluorescence value of 10X the background signal for both numerator and denominator raw counts. Ensuring that the numerator and denominator values are comparable at baseline (resting calcium levels) will yield a calcium ratio that is close to 1. Please refer to the provided image for an illustration of baseline (highlighted sections). For further instructions on how to adjust the fluorescence light intensity for optimal outcomes, please refer to our "Optimizing Fluorescence Signal Quality" application note in the resource library.

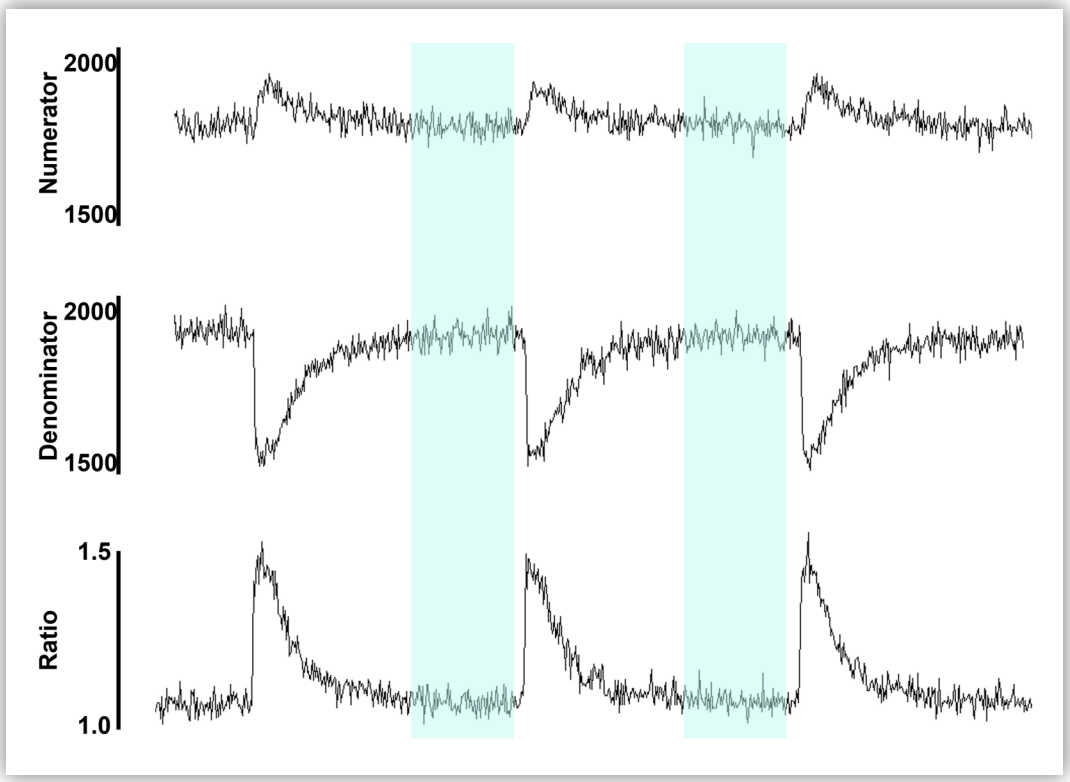

Figure 3: Optimizing fluorescence. Fura-2 loaded cells should exhibit approximately 10x baseline signal over background (baseline is highlighted in blue).

b. Selecting proper ROIs for contractility measurements

Primary Cardiomyocytes:

IonOptix uses two distinct methods for analyzing the contractility of primary cardiomyocytes: sarcomere length and edge detection algorithms. Sarcomere length provides a direct assessment of the length of cell's contractile units (sarcomeres), while edge detection tracks the movement of cell's edges during contraction. Although sarcomere length is a more accurate for evaluating contractility, both techniques require precise alignment of each software tool. To aid in the selection of appropriate ROIs in primary cardiomyocytes, it is recommended to plate them sparsely on precoated glass-bottomed plates.

Sarcomere length:

It is essential to position the region of interest (ROI) in a way that minimizes any distortion or curvature at the edges of the image. This can be accomplished by including as much of the cell as possible in the purple box on IonWizard Sarcomere Length window, or orange box on MultiCell window. Additionally, it is important to adjust the focus until a single red peak between the two green lines is visible and ensure that the left green line is positioned next to the single red peak.

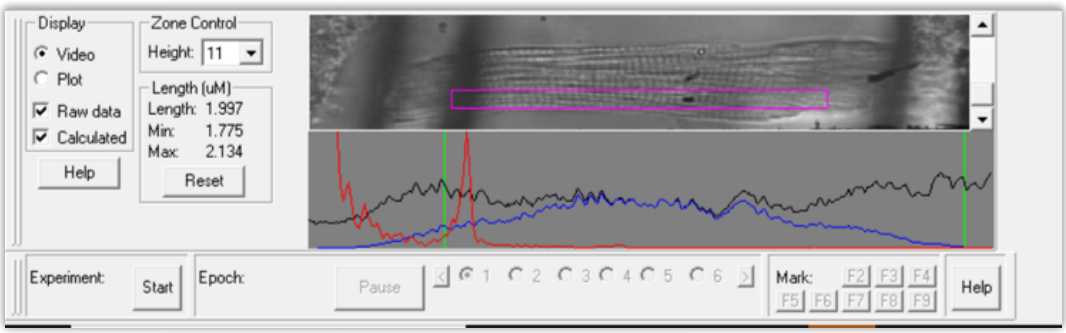

Figure 4: Sarcomere length measurements. IonWizard's Sarcomere Length interface allows users to position ROI and optimize FFT quality.

Collecting data from adjacent or overlapping cells, resulting in two cells sharing the same region of interest (ROI), is a **common mistake in data acquisition**. This can cause erratic and noisy signals during contraction as the fast Fourier transform (FFT) alternates between each myocyte. Data obtained from such cases should not be used. If you are using our automated cell analysis tool **(CytoSolver 3.0),** such data will be rejected and typically labeled with the error "Max Derivative Limit". Therefore, always ensure that the ROI and field of view contain only a single cell.

For detailed info about the FFT and sarcomere length algorithm, please refer to our application note "SarcLen & FFTs" (https://www.ionoptix.com/resource/sarclen-ffts/)

Edge detection:

Selecting appropriate cell boundaries requires adherence to certain general principles. These principles include ensuring that both edges are accurately captured to obtain accurate measurements of the cell's dimensions. Additionally, controlling the threshold of the left, right, or both edges is crucial to avoid regions containing background noise or artifacts that could impair the algorithm's accuracy. Lastly, any neighboring cells visible in the image should be excluded.

iPSC-CM:

CytoMotion software was developed to address difficulties in analyzing iPSC-CMs, particularly in detecting clear edges or sarcomeres. Similar to algorithms for detecting sarcomere length and edges, selecting the appropriate region of interest (ROI) is crucial when using CytoMotion.

- iPSC-CMs in clusters, select an area with clear center of contraction and include it in the middle of the purple box on IonWizard or orange box on MultiCell window.
- iPSC-CM monolayers, any area can suffice as the whole plate contracts simultaneously.

Data acquisition (cont)

In CytoMotion, iPSC-CMs contractility is measured through pixel intensity and pixel correlation. To ensure accurate measurement, a reference frame for each ROI must be captured when cells are at rest. This can be done manually by selecting "Capture" within IonWizard during acquisition when using CytoMotion Lite or our conventional calcium and contractility system.

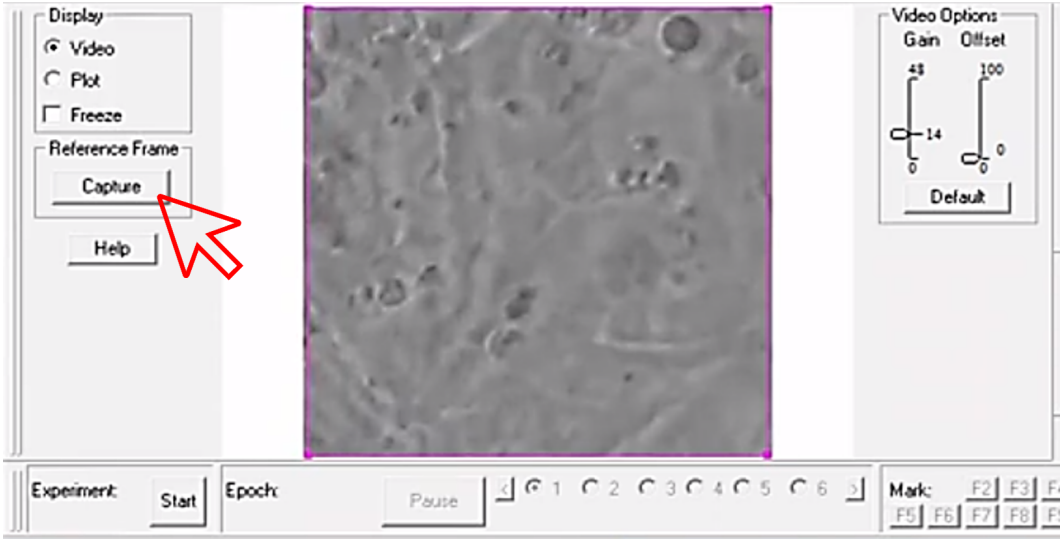

Figure 5: Reference frame capture. Users can manually capture the reference frame when cells are at rest.

With MultiCell Lite or MultiCell HTS, the reference frame is automatically selected.

One way to ensure that the correct reference frame is captured for the selected ROI is by observing the direction of pixel intensity and pixel correlation data. When iPSC-CM are contracting, you should expect to see an increase in pixel intensity and a decrease in pixel correlation from baseline.

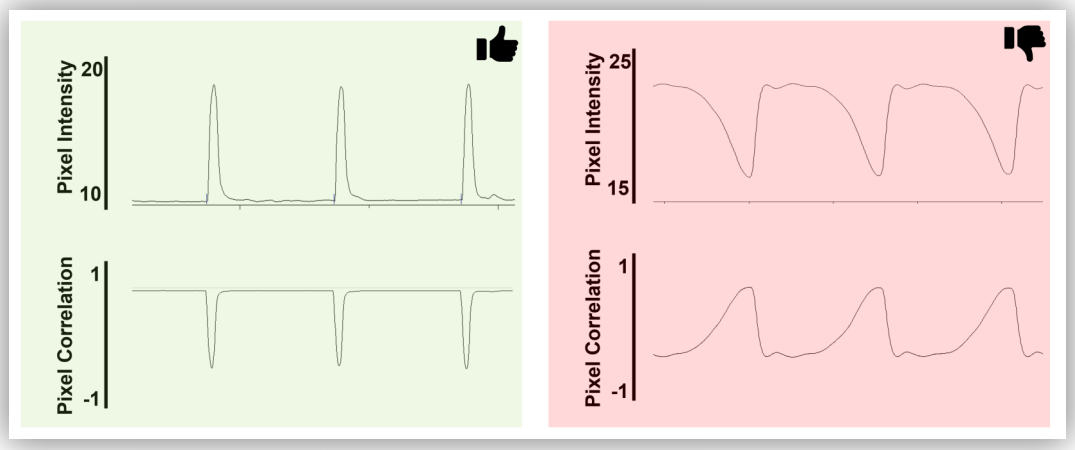

Figure 6: Correct v. incorrect reference frame capture. Pixel intensity data should rise during contractions, while pixel correlation data falls. Recapture the reference frame if these are reversed.

Data acquisition (cont)

c. Selecting proper aperture for calcium measurements

To begin the experiment, plates containing cells loaded with Fura-2 are placed on a microscope stage and stimulated with appropriate excitation wavelengths (340nm and 385nm). This excites all Fura-2 loaded cells in the entire plate. To prevent any artifacts, it is important to limit the aperture to a single cell with primary cardiomyocytes or single cluster in the case of iPSC-CMs*.

The method for controlling aperture size depends on the IonOptix system used. For the conventional calcium and contractility system, the aperture can be manually adjusted by moving aperture knobs located between the Cell Framing Adaptor (CFA) and microscope camera side port, pleases refer to the CFA manual for more info (https://www.ionoptix.com/resource/cfa-hardware-manual/).

In contrast, the aperture size is preconfigured during MultiCell HTS and MultiCell Lite installations. Aperture size can be seen on the MultiCell screen as a blue circle, refer to the image below. It is crucial to include only a single primary cardiomyocyte or iPSC-CM cluster in the aperture to collect calcium signals. Therefore, maintaining low-density cells on the plate is vital not only for proper contractility but also for accurate calcium measurements. If the aperture is not adjusted, it can result in an incorrect attribution of calcium signals from multiple cells to a single cell.

* If you are conducting experiments with monolayers, maintain a consistent size for the aperture in all comparable experiments.

Figure 7: MultiCell HTS and Lite fluorescence illumination. The aperture displaying illumination area is framed by the blue circle shown here. This region should contain only the cell of interest and no debris.

Frequently Asked Questions (FAQ)

1. **Is it necessary to discard cells that display a calcium ratio of 0.8 or 1.2?**

There is no need to discard cells that show slight deviations in calcium ratio as different cells can vary in response to stimulation. However, it is crucial to maintain a consistent background ratio for that particular experiment.

2. **Is it necessary to have background fluorescence measurement for each experiment?**

Having a background fluorescence measurement for each experiment is strongly recommended to guarantee the accuracy of collected signal and ensure that the data is quantitative. Both CytoSolver and IonWizard software subtract background measurement from all fluorescence traces for a particular experiment.

3. **Can the baseline sarcomere length be used as an exclusion criterion?**

Certainly, baseline sarcomere length can be utilized in real-time or after acquisition as a criterion for exclusion. During acquisition, it is advisable to avoid cells with a sarcomere length lower than 1.7 m. However, if the experiments have already been conducted, our analysis software, IonWizard and CytoSolver, can export baseline values for each cell, enabling post-acquisition data sorting. Bear in mind that certain treatments and genetic modifications can influence resting sarcomere length. Do not use sarcomere length as exclusion criteria if it could bias your data against a legitimate result.

4. **What is the maximum acceptable value for the fluorescence signal for Fura-2?**

To remain within the linear range of Fura-2, it is typically recommended to stay below 6000 counts for the raw numerator and denominator values. 6000 counts per millisecond is the linear range of the photomultiplier tube used to detect fluorescence emission.

5. **If CytoSolver 3.0 rejects a trace and designates it as "Peak Signal To Noise Ratio" error, what should be done?**

This implies that the distinction between noise and an authentic peak is minimal. Typically, this occurs when cells are inadequately loaded with Fura-2, or if they are not healthy enough to respond appropriately to stimulation and do not exhibit significant calcium peaks.

FAQ (cont)

6. **What are the variations in morphology between dead and healthy cardiomyocytes?**

See below.

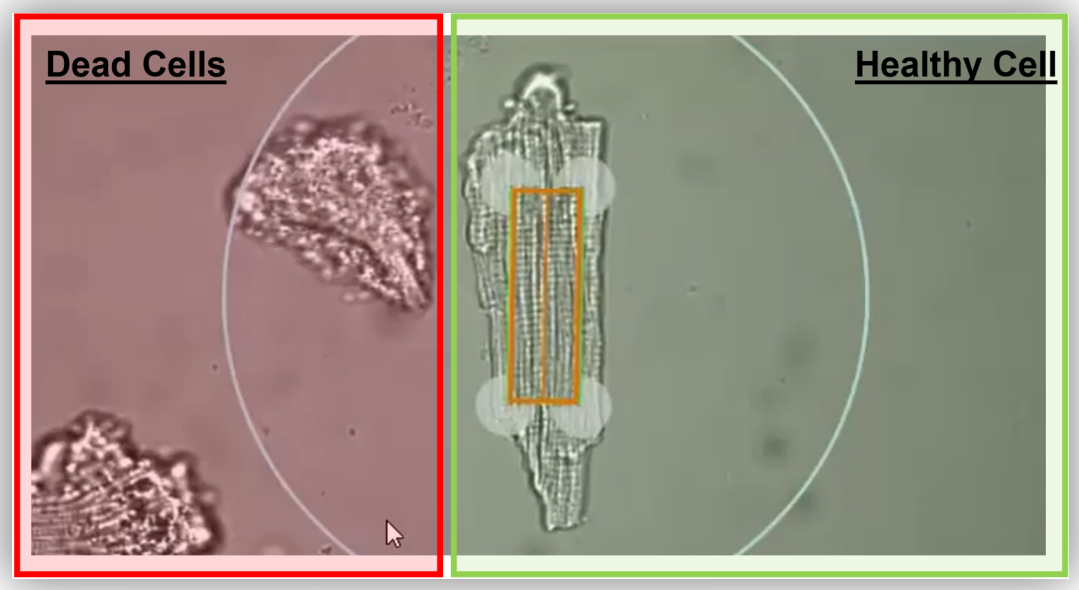

Figure 8: Dead vs. live cells.

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