Webinar Q&A Report:
Understanding the Heart's Intracellular Orchestra: Functional Characterization of Excitation-Contraction Coupling in Cardiomyocytes

1. **Can you elaborate further on the algorithm behind CytoMotion?**

   CytoMotion quantifies the pixel intensity at each pixel in the region of interest. Initially, a reference image is captured while the myocyte is in diastole. Every subsequent image captured in time is compared to the reference. The difference at each pixel is then subtracted from the reference over time and plotted in real time.

2. **What is the throughput for CytoMotion/induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs)?**

   This will depend on the system, the configuration of the setup, and the maturity of your myocytes. If you're using our MultiCell with a consistent, synchronous monolayer of myocytes paced at 1Hz, and you're measuring each region for 10 seconds, then you could measure over 50 regions in 10 minutes.

3. **In the myosin II ATPase inhibitor dose response, why do you have different numbers of cells in different doses?**

   Rather than continually measuring from the same myocytes with increasing concentrations, pairwise experiments were done measuring each myocyte before and after a single dose. This was done to prevent any confounding effects that might arise from pre-treating the myocytes with lower concentrations followed by higher ones.

4. **Regarding hardware, is Fura plus FluoVolt possible to use at the same time?**

   Unfortunately, no. FluoVolt and Fura share similar emissions, and FluoVolt can be excited with UV light. So, there would be too much spectral overlap to separate them. Ideally you would want to collect FluoVolt at higher sampling rates (depends on cell type but 500Hz-1kHz), so the best configuration would be to collect from both FluoVolt and the calcium (Ca) indicator at the same time. You can do this with FluoVolt and Rhod-2.

5. **If you are using electrical stimulation for the cells, what intensity or frequency would be the best?**

   This will depend on the myocytes. Regarding intensity and the voltage required to 'capture' them: the electrical field strength will depend on the voltage as well as the resistance and the distance between the electrodes. A better approach is to treat it empirically. Slowly increase the voltage while watching a field of myocytes. When most are captured (contraction), increase the voltage by 10-20% above the capture threshold. Regarding frequency, that will depend on the type and species of cardiomyocyte. Mouse myocytes are typically stimulated at or above 3Hz, while rat myocytes can be paced at 1Hz or ever slower.

6. **Any success with measuring Ca in differentiated iPS cells?**

   Absolutely. Check out the app note on our website:
   https://www.ionoptix.com/resource/measuring-ipsc-cm-ca-contractility/

7. **Do iPS myocytes need to be plated sparsely to assess Ca or can useful data be acquired from multiple cells at the same time such as the contrast detection of motion.**

   They would need to be plated sparsely if you were attempting to collect calcium from one myocyte at a time. We prefer to capture from a monolayer where we are averaging calcium from several at a time.

8. **Have you tried evaluating sarcomere length in iPS-CMs or engineered tissues?**

   We have not, but our customers have. The issue is maturation. If the sarcomeres are well organized and visible by eye, then it is easy to do. But this is rare. One or our customers has ~20% success with their myocytes. That's the best I've heard.

9. **What facility/equipment is needed to keep those cells alive in the lab? How much does it cost?**

   We don't know if this question referenced isolated adult cardiomyocytes or iPSC-CMS, so we'll try to answer both. For isolated adult myocytes, those myocytes are typically used fresh. They can be maintained in a culture incubator. So, the costs to keep them alive would be on par with any immortalized cell line (incubator and media). Stem cell-derived myocytes don't require special equipment, but they often require proprietary media which can be costly. The exact price will depend on the cell source, however. Both adult and stem cell-derived cardiomyocytes can benefit from other stimuli as well, including electrical and mechanical stimulation. Systems that provide either electrical or mechanical stimulation, including ours, range from ~$8k USD upwards depending on the functionality of the system and the type and quantity of tissue culture plates required.

10. **Is FluoVlot applicable to the isolated skeletal muscle fibers?**

    Definitely. They tolerate it better than cardiomyocytes.

11. Is there any possibility that the dyes like Fura influences the sarcomere length contractility?

Absolutely. In theory, you could load enough Fura to completely blunt downstream signaling. This is very rare. The most common issue is that dye buffering prolongs relaxation.

12. Can we measure contractility from frozen left ventricular cardiomyocytes (eg. skinned cardiac myocytes typically used in Aurora Scientific)?

Skinned preparations can be measured but it's less straightforward and the system typically needs to be modified to accommodate different applications. For example, a common measure of skinned myocytes is force-pCa, where the myocyte tension is measured at specific calcium concentrations. This requires a very fast solution switching system. Ours uses superfusion and would need to be modified with a different chamber that allows faster solution switching.

13. How many days of iPSC contractility were recorded?

hiPSC-CMs (human induced pluripotent stem cell-derived cardiomyocytes) were measured on day 40 after start of differentiation. The entire protocol can be found on our website: https://www.ionoptix.com/resource/measuring-ipsc-cm-ca-contractility/

14. How to measure Ca and contractility in hiPSC-derived cardiomyocytes? Can they measure in culture, or does it also need to be a single cell?

The white paper describing the methods is posted on our website here: https://www.ionoptix.com/resource/measuring-ipsc-cm-ca-contractility/. In short, plated myocytes were grown in monolayers on treated glass-bottom plates. On the day of data acquisition, cells were loaded with Fura-2 and then mounted on a MultiCell System. Both calcium and contractility (via CytoMotion) were acquired simultaneously in 100x100 micron regions for 10s pre- and post-isoprenaline. Data was uploaded to CytoSolver for batch analysis and visualization of beat frequency (Hz), contraction time or time to peak (s), relaxation time or time to baseline 50% (s), and 50% peak width (s).

15. Is possible to use the calcium and contractility system in heart slices?

Yes. We're currently working on a system to do just that. We have a working prototype in beta and plan to release the system in the summer/fall.

16. The calcium and contractility system allow you to manually modulate the basal level of the recording (calcium or contractility). If this level is adjusted manually the amplitude of the record changes (in the same cell). This could give an artifact in getting results. Can you confirm?

No. The amplitude of ratiometric calcium (Fura-2) should not change even if the user manually changes settings (unless they do so in an unprescribed manner, such as increasing the intensity of excitation light disproportionately for one channel). Similarly, adjusting contractility measurements during acquisition to get better signal-to-noise will not influence the cell or

sarcomere length. It could influence the amplitude using CytoMotion, but the amplitude is not a viable parameter for that algorithm.

17. What starts relaxation and role, if any, of “funny current"?

Relaxation is initiated by calcium sequestration in the SR and extrusion through the sarcolemma. For the former, SERCA pump activity is regulated by phospholamban which is, in turn, regulated by PKA and cAMP levels. For the latter, the sodium-calcium exchanger (NCX) is responsible for calcium efflux, although mitochondria and PM calcium ATPase also play a role in controlling calcium. With respect to funny currents, these are associated with pacemaker cells and initiation of beating at the whole heart level. During isolated ventricular myocyte experiments, pacemakers are replaced by electrical field stimulation, so they won't play a role. In spontaneously beating iPSC-CMs with pacemaker activity, funny currents have been identified and do influence beat rate.

18. What are your sarcomere length quality and exclusion criteria?

In general, a resting sarcomere length below 1.7um suggests that the myocyte's membrane is compromised and calcium is leaking in. This only applies to wild type (WT) myocytes. Exclusionary criteria for sarcomere length is also species-dependent.

19. What method are you using for isolating adult cardiomyocytes, and do you see negative effects of these enzymes on certain ion channels?

We use Langendorff/Liberase isolations for small animals. We also use wedge preparations for large animals. Over digestion using any methodology will negatively affect ion channels.

20. Can you expand on the pros and cons of multi-cell contractility compared to single cell contractility?

Both systems use the same algorithms to measure contractility. MultiCell, however, marks cell position and orientation so that you can measure from the same cells repeatedly. There are few other distinctions, but the end result is that you can make more measurements from the same myocytes, improving throughput and getting more data.

21. What temperature do you recommend to be used for Ca and contractility measurements?

37°C but never above. 35°C is common as well.

22. Could you please detail a little bit of the C-Stim superfusion chamber functionality?

All of the chambers we offer provide both stimulation and perfusion. The advantage of the C-Stim chamber is its flow properties (it is laminar even at higher flow rates). Its disadvantage is that it's a little fussy. It needs to be primed to get solution to flow to the outlet and it's more prone to clogging.

23. Can I have higher calcium transient value if I put a higher concentration of Fura-2?

No. Fura-2's response does not depend on dye concentration, which is one of its most significant advantages. This is why you can compare the signal amplitude from one myocyte to another.

24. When accessing data, we need to keep the cells in a physical environment, so can you go into detail about your additional devices that can achieve this?

If you're using a bicarbonate-based buffer, you would need a system to maintain 5% CO_2 (not necessary if you're using a HEPES-based buffer). Other than that, you would want temperature control and perfusion if you plan to measure for any length of time. For temperature, you could use an inline preheater to heat the perfusate, or you could keep the microscope in a temperature-controlled environment if you're not.

25. Does the system give a surrogate measure of cardiomyocyte viability? Does it require a confluent monolayer of cardiomyocytes?

With respect to adult cardiomyocytes, certain parameters are often used to identify viability. Resting sarcomere length is commonly used as well as performance metrics such as percent shortening. For iPSC-CMs, both spontaneous beat frequency and kinetic information are sometimes used as a measure of viability. More often, parameters are associated with maturation. For example, if iPSCs do not respond to electrical field stimulation, they are likely too immature. Regarding confluence, no, a monolayer is not required. We have measured from isolated iPSC-CMs, embryonic bodies, monolayers, etc.

26. It appears to measure sarcomere length of one cell with well-defined sarcomere structure. Does it then take the mean of many cells measured within a well to give an average? Does it automatically select "well-defined" cardiomyocytes for measuring?

Typically, you'd average transients from a single myocyte, analyze that averaged transient, then average the analysis outputs and compare against myocytes under different conditions. We do not automatically select myocytes based on sarcomere length, but our MultiCell will select based on myocyte morphology.

27. Can atrial myocytes also be isolated/assayed? What additional considerations are involved?

Yes. Any contractile cell can be measured. Regarding calcium, the system uses general fluorometry. We focus on speed and sensitivity, so the most appropriate applications are transient changes that require high sampling rates. Regarding contractility, there are several algorithms that can be used for atrial myocytes including sarcomore length, cell length, and cytomotion.

28. How technically challenging is cardiomyocyte isolation? Do less technically demanding isolation techniques exist?

It is challenging, but there are many resources available, including on our website. Traditionally researchers use retrograde perfusion enzymatic digestion. More recently, labs are successfully using a simpler Langendorff-free technique described by Ackers-Johnson and Pavlovic.

29. In a couple of videos from the first half of the webinar, you showed a myocyte physically attached. What is that and why would you use it?

That is our MyoStretcher. It is used to tension myocytes, provide a restorative force, measure force directly, and measure work output (work loops). It is far more difficult to use but it provides a wealth of information that exceeds what can be done in unloaded myocytes.

30. In terms of Fura-2-dependent calcium buffering, what are the recommended concentrations and load times to minimize myocyte contractility diminution?

Cardiomyocytes are inherently easy to load with Fura. We recommend starting at 1uM Fura loading for 15-min at room temperature. If myocyte relengthening is affected (slowed), then decrease the loading time.

31. You mentioned that CytoMotion could be used for amorphous cell types. Can you expand on that? Do you have any other examples of cells whose contractility could be measured?

If you can see the cell move, it can be measured. We've measured from neonatal myocytes, C2C12 myocytes, and various types of stem cell-derived myocytes. We've yet to try HL-1 myocytes, but it is likely that those would work as well.

32. In your iPSC-CM experiment with isoproterenol treatment, were you anticipating an increase in contractility (pixel correlation) with treatment in cells incubated with maturation media?

Yes. In the data shown, we did not observe an increase in contractility amplitude, but we did observe an increase in calcium. Contractility kinetics (shortening and relaxation times as well as transient duration) were affected by iso. But it is clear from our own recordings as well as those by an independent group in Amsterdam that contractility amplitude can be measured in a relativistic manner (pre- and post-treatment from the same region of cells). It may be that the myocytes shown grown in maturation media were more mature than those grown in RPMI, but not enough to demonstrate an increase in contraction amplitude.

33. How do you attribute cardiomyocytes function to poor isolation or the drug not working?

Poor isolations are typically fairly evident (poor morphology, low resting sarcomere length <1.7um, etc.). In terms of function, you'll want to establish a set of performance metrics that are indicative of healthy, normal cells. For example, several labs use shortening percentage as a barometer, ruling out cells that don't shorten enough or shorten too much.

34. Can the contractility assay be used in cardio organoids?

Yes. Three dimensional constructs can also be measured, provided that their contractions are observable. For larger preparations, you might also consider measuring force directly.

35. Do you have a protocol to measure sarcomere length that you could share, and any advice/recommendations?

We have several resources (linked below) on our website that describe the algorithm in detail, including the manual itself (in the section entitled Sarcomere Spacing Recording Task). While none of these provide a stepwise guide to using the algorithm, they are informative. If you're unfamiliar with the algorithm and would like instruction, please let us know at info@ionoptix.com and we'll set up an online tutorial.
Resources:
https://www.ionoptix.com/resource/sarclen-algorithm/;
https://www.ionoptix.com/resource/sarclen-ffts/;
https://www.ionoptix.com/resource/ionwizard-acquisition-manual/

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