Application Note

Cardiomyocyte isolation using Cellenion's cellenONE® instrument

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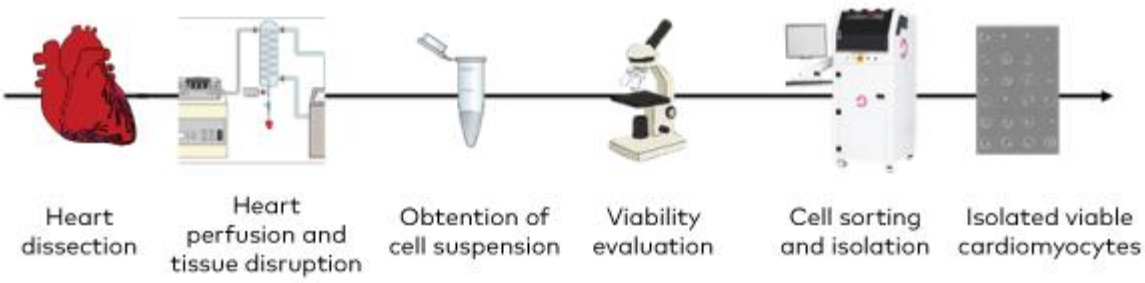

Figure 1: Graphical summary of the cardiomyocyte isolation workflow using the cellenONE® platform.

Introduction

Cardiovascular diseases (CVDs) are the leading cause of death among adults worldwide[1]. Improved clinical care and novel therapeutic interventions have dramatically reduced all-time mortality and morbidity in both adult and pediatric patients with CVDs[1]. Despite these advancements, heart failure imposes a major clinical and economic burden worldwide[2]. Increased understanding of the clinicopathologic mechanisms of heart failure have improved outcomes, but current therapeutic approaches are predominately focusing on modulating neuroendocrine functions, heart rate and ventricular unloading while novel targets modulating metabolic and immune function are limited[3-4]. Drug development is further complicated by the complexity of comorbidities in patients that create unique clinical profiles. Here, novel analytical techniques at the single cell level hold the promise to advance our understanding of fundamental mechanisms that drive cellular adaptation and disease development.

Within the heart, cardiomyocytes are specialized cells responsible for the heart's ability to beat. While the electrophysiology and contractility properties of isolated cardiomyocytes have been under investigation for decades, the isolation methods used in these studies do not provide the throughput required for downstream single cell applications, such as single cell transcriptomics and proteomics, highlighting a need for improved single cell cardiomyocyte isolation. Furthermore, single cell transcriptomic and proteomic studies utilizing cardiomyocytes have traditionally been difficult to perform due to the large size, elongated shape, and reduced viability of isolated cardiomyocytes[5]. Single cell mRNA sequencing of cardiomyocytes allows for profiling of disease phenotypes for both mechanistic preclinical studies and personalized medicine. However, transcriptomic studies alone are limited by the inherent disconnect between gene and protein expression which drive cellular functions. Detailed proteomic information at the single cell level is required to provide insights into cellular processes and shed light on cardiac cellular heterogeneity. Here, we report an optimized protocol for the isolation and sorting of viable cardiomyocytes from the whole heart and tissue samples, which allows for integration of cell suspensions into downstream single cell workflows. Our protocol utilizes the cellenONE (Cellenion, Lyon, France) system that enables the isolation of individual cardiomyocytes, ranging from 35 µm to over 180 µm in a sub-nanoliter aqueous droplet, which are then suitable for downstream single cell transcriptomics and proteomics, among other applications. Furthermore, our method is also amenable to scaling, such that hundreds to thousands of cells from one or more heart can be analyzed.

The cellenONE platform is ideal for the isolation of fragile and heterogeneous cell size populations, such as cardiomyocytes, because it provides gentle cell isolation without the risk of sample loss due to instrument clogging associated with the long hydraulic pipeline used in traditional fluorescence-activated cell sorting (FACS). The cellenONE enables processing of samples from 1 µL to 50 µL at a time in dispensed volumes ranging from 150 pL to 600 pL utilizing its piezo-acoustic dispensing technology, providing the versatility required for miniaturizing single cell experiments. Importantly, the cellenONE captures images of every cell prior to isolation, enabling users to visually confirm single cell isolation, and correlative analytics relating to cell size and morphology, including the presence of an intact plasma membrane and sarcomere patterning.

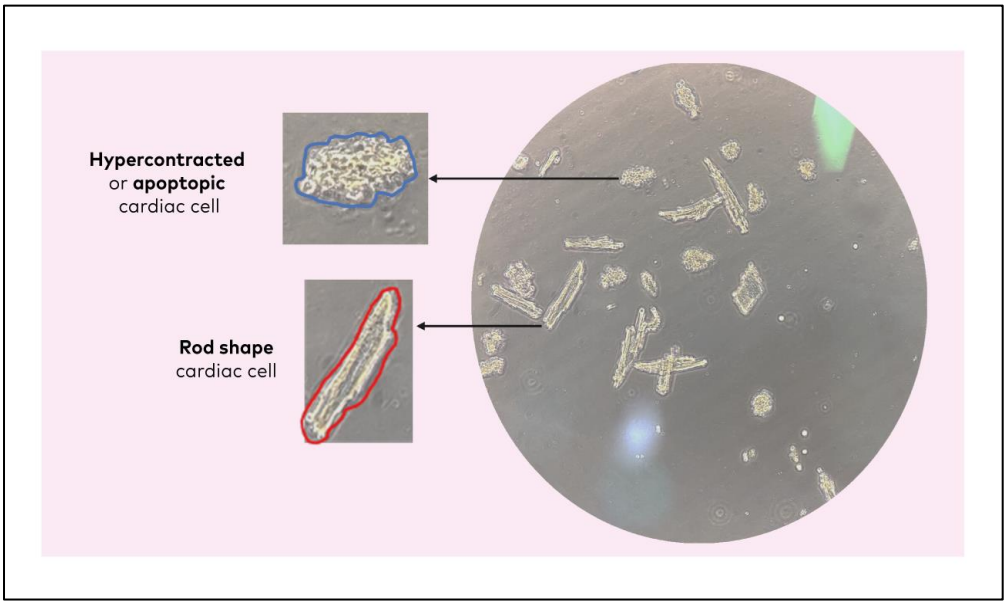

Figure 2: **Representative cardiomyocyte suspension obtained from a mouse heart.** Observation of 10 µL of cardiac cell suspension using optical microscopy. Two cardiac cell shapes can be distinguished: hypercontracted or apoptotic cells (blue, top panel) and rod shape cardiac cells (red, bottom panel).

Cardiomyocyte suspension preparation for cellenONE isolation

a. Preparation of cell suspensions from mouse or human heart tissue

Cardiomyocytes can be isolated by retrograde heart perfusion techniques, such as the Langendorff method[6], or by dicing and mincing of tissue sample[7] followed by enzymatic dissociation.

A prerequisite for single cell proteomic analysis is an optimal isolation of cardiomyocytes to allow for a high degree of viability. The digestion and total perfusion time are critical determinants of isolation quality. Furthermore, the composition of the isolation buffer can help to limit osmotic stress and hypercontractility of isolated cardiomyocytes. For example, 2,3-Butanedione monoxime (BDM) can be added during the perfusion step, which prevents hypercontraction of the isolated cardiomyocytes. BDM binds to myosin and blocks its interaction with actin-tropomyosin, allowing the cardiomyocytes to remain in a relaxed state[8]. Further, BDM has been shown to reduce cellular damage[9-10]. It is ideal to obtain relaxed cardiomyocytes to allow for ease in the differentiation of myocytes from other cell types due to the presence of their sarcomere structure. Additionally, relaxed cardiomyocytes are less likely to become damaged during single cell isolation using the cellenONE.

b. Observations of the cell suspension using an optical microscope

Enrichment of viable cells can be achieved by low speed (40 to 80 *g*) and short duration (5 minutes) centrifugation as apoptotic, hypercontracted cardiomyocytes or cellular debris remain in the supernatant compared to viable cardiomyocytes which pellet at the bottom of the tube. Using a 5 mL serological pipette at a low speed, cells can be gently resuspended with minimal cellular damage. At this stage, cell viability can be determined using an optical microscope and a hematocytometer. The number of rod-shaped cells compared to the total number of cells will provide an approximation of cell viability (**Figure 2**), which can be complemented by staining using trypan blue or similar stains. The viability percentage helps to determine the quality of the initial cardiac cell suspension. The higher the percentage of hypercontracted or apoptotic cells, the longer the isolation of rod-shaped cells (i.e., healthy, viable cardiomyocytes) will take.

Preparation of the cellenONE system for single cell cardiomyocyte isolation

We recommend starting with intact cardiomyocytes at approximately 60k-100k cells/mL and with a cell viability greater than 45%. While keeping the cells at room temperature, begin by mounting a PDC (piezo dispensing capillary)- L, which is required for handling the large size range of cardiomyocytes (i.e., cells over 120 µm in length), in the cellenONE. Then, proceed with priming the system and the PDC, ensuring proper nozzle alignment, and drop stability (drop volume standard deviation should be <1% with system liquid). Next, prepare the target substrate in which

you intend to isolate cells to and place a new cellenVIAL, with the cap removed, in position A of the cellenWASH station. To prepare the cardiomyocytes, resuspend the cell suspension using a 5 mL serological pipette. Once the cell suspension is resuspended, use a p1000 micropipette to add 200 µL of the cell suspension to the cellenVIAL. Take care to avoid generating air bubbles in the cellenVIAL or allowing enough time to pass for cells to settle at the bottom of the cellenVIAL prior to aspiration with the cellenONE. If necessary, the cells can be manually resuspended in the cellenVIAL prior to uptake using the cellenONE.

Parameters for isolation of mouse cardiomyocytes in cellenONE mode

To begin analysis of the cardiomyocyte sample in the cellenONE, aspirate 10 µL of the cell sample into the PDC and begin the mapping process. This process identifies the ejection zone for single cell isolation and produces a histogram displaying cell size and elongation.

Detection or Isolation	Parameter	Mouse	Human
Detection	Diameter (min., um)	20	20
	Diameter (max., um)	400	400
	Elongation (max.)	8	8
Isolation	Diameter (min., um)	35	45
	Diameter (max., um)	150	180
	Elongation (min.)	2.0	2.6

Table 1: Optimized settings for isolation of mouse and human cardiomyocytes with the cellenONE.

a. Classic settings (map population)

Once the sample has been characterized, the population of interest can be selected by adjusting the isolation diameter and elongation to ranges that encompass the desired population. Here, parameters were optimized for the isolation of mouse and human cardiomyocytes[11] and are presented in **Table 1**.

b. Specific settings optimized for adult mouse and human cardiomyocytes

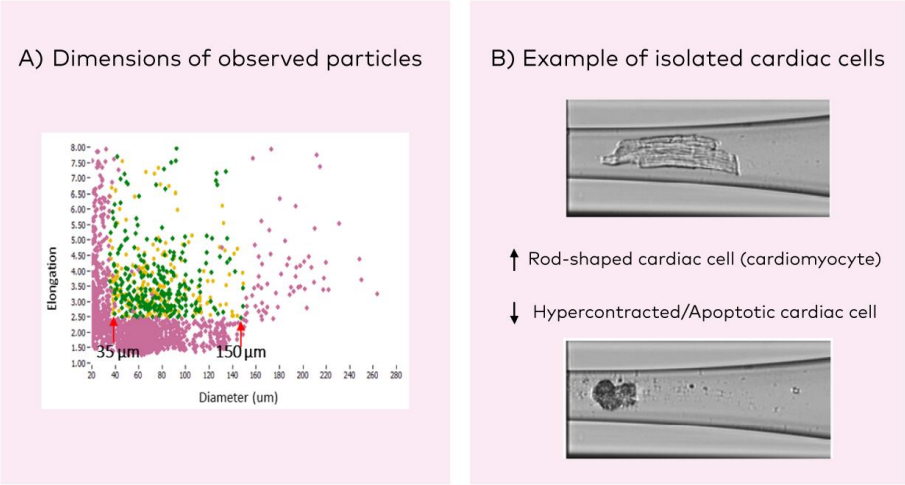

Figure 3: **A. Example of a scatterplot automatically generated by the cellenONE after mouse cardiac cell isolation.** The color code represents the status of the cell: Green: the cell was isolated, Yellow: the cell met the isolation requirements but was not isolated because the nozzle contained multiple cells, Pink: the cell was not isolated because it did not meet the isolation requirements. **B. Examples of pictures acquired by the cellenONE instrument for a rod-shaped cardiomyocyte (top) and a hypercontracted or apoptotic cardiomyocyte (bottom).**

The cellenONE instrument automatically collects an image of each cell (**Figure 3B**) before dispensing into a well. During this isolation step, cell diameter and elongation (µm) are collected, which are then used in the automatic generation of a scatterplot showing the distribution of cell diameter and elongation (**Figure 3A**). As depicted in **Figure 3A**, a wide range of cell diameters are observed from a single mouse heart, but only a subset met the *a priori*-defined selection criteria (**Table 1**) and were therefore isolated into a 384-well plate (**Figure 3A**, green dots). These isolated cardiomyocytes ranged in size from 35 µm to 150 µm and from 45 µm to 180 µm in diameter for mouse and human cardiomyocytes, respectively.

Conclusion and future directions

Due to the large size range and fragile nature of cardiomyocytes, single cell isolation has traditionally been a challenging endeavor, especially for single cell applications which require hundreds to thousands of cells. The cellenONE enables efficient isolation of cardiomyocytes, while maintaining viability, due to its gentle dispensing technology and automation capabilities. Additionally, the cellenONE records images of isolated cells immediately prior to isolation, providing researchers the opportunity to verify single cell isolation, characterize phenotypic diversity, and select cardiomyocytes based on morphology for further processing.

The method described here for single, viable cardiomyocyte isolation using the cellenONE is ideally suited for a variety of downstream applications, including single cell transcriptomic and proteomic workflows. The rising prevalence of heart failure despite advances in the detection and management of CVDs highlights a gap in our understanding of the fundamental mechanisms that drive cellular adaptation and disease development in the heart. Confounding factors include patient-specific complexities in disease subtypes and comorbidities in which characterization at the single cell level would be of benefit. The cellenONE platform is an invaluable tool for single cardiomyocyte characterization studies aimed at elucidating cellular processes and cellular heterogeneity, which are critical for the development of novel therapeutic drugs to treat CVDs.

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