**MS ID:  JoVE59052**

**MS TITLE:  Analyzing Oxygen Consumption Rate in Primary Cultured Mouse Neonatal Cardiomyocytes Using an Extracellular Flux Analyzer**

We were pleased that the reviewers found the manuscript was well written and acknowledged that we have developed a detailed protocol to culture mouse neonatal cardiomyocytes and assess mitochondrial respiration in these cells using an extracellular flux analyzer. We have carefully considered all their comments, performed some additional analysis address some of the concerns, and revised the manuscript to address the issues that were raised. Please find below a summary of the modifications to the manuscript and a detailed point-by-point response to the reviewers’ comments.

**Summary of modifications: (added figures and tables)**

1) Added new Table 1, showing how to make reagents and buffers.

2) Added Figure 2C, presenting positive immunostaining of cardiomyocyte specific maker sarcomeric -Actinin in isolated neonatal cardiomyocytes.

**Point-by-point response to the reviewer’s comments (reviewer’s comments are in italics):**

Reviewer #1:  
  
Manuscript Summary:  
The authors have developed a detailed protocol to culture mouse neonatal cardiomyocytes and assess mitochondrial respiration in these cells using an extracellular flux analyzer. They have conducted various experiments to show the potential use of these cells for research purposes  
  
Major Concerns:  
*1] Authors need to discuss how this protocol is different from already published protocols.*We appreciate reviewer’s comments. Please see our revised discussion in lines 383-390.

2] *Authors mention that these cells have characteristics of cardiomyocytes. They need to characterize these cells using cardiomyocyte specific antibodies.*  
A. We have immunostained the cells with an antibody against sarcomeric -Actinin, which is a cardiomyocyte-specific marker. As shown in Figure 2C, almost all cells show positive immunostaining for sarcomeric -Actinin. These results demonstrate that the majority of isolated cells are cardiomyocytes. Please see lines 288-291, 354-357, and Figure 2C.

Minor Concerns:  
*3] Authors have used same determinants for maximal and basal respiration in the result section for figure 4. They need to correct that.*A. We would like to thank the reviewer for this important comment. The manuscript has been revised accordingly. Please see line 314.

Reviewer #2:  
Manuscript Summary:  
The manuscript by Tachibana et al., describes the protocol for rapid isolation of neonatal mouse cardiomyocytes and assess their mitochondrial function via oxygen consumption rate of these cardiomyocytes using an extracellular flux analyzer 96 (XFe96). The manuscript was well written and easy to follow. The reviewer has no concerns on the manuscript.