Dear Dr. Mukherjee,

Thank you for considering our manuscript titled: “**A Pragmatic Workflow for Gene Expression Analysis of Endothelial Cells Exposed to Shear Stress Using Multiple Parallel Plate Flow Chambers.”** This manuscript includes our detailed protocols for experiments that involve the application of fluid shear stress used in our recent publication “Angiogenic Patterning by STEEL, an Endothelial-Enriched long noncoding RNA”. These protocols were developed to minimize variability between experiments and we believe that strategies used in these protocols will be applicable in many settings.

We present unique elements of our workflow to increase accessibility of these experiments to labs less experienced in this field. The flow circuit is assembly is based on the excellent protocol by Lane W. et al in a previous issue of Jove. In this manuscript, we present several key adaptations that reflect differences in our experimental systems. Our experiments involved multiple conditions in endothelial cells exposed to fluid shear stress. To accommodate multiple concurrent experiments, we use a large heated unit that provides a standard environment for the operation of four or more simultaneous flow experiments. To standardize fluid shear stress between experimental conditions, we continuously monitor flow rates to each flow chamber. We include details of tubing size and luers that we have optimized to reduce leakage from the flow circuit. Similarly, we present details of our cell seeding procedures that we have found important for fluid shear stress experiments involving early passage endothelial cells. In our own experience, we have found that the implementation of these, and other steps in our protocols, crucial for the success of our experiments involving fluid shear stress.

For experiments that involve gene expression analysis with reverse-transcription quantitative PCR, we use an exogenous reference RNA to account for inter-sample differences in efficiency of RNA extraction and cDNA synthesis. In particular, cDNA synthesis is a process with quantitative variability. In this manuscript, we present our entire workflow for the implementation of an exogenous reference RNA from synthesis to analysis. This workflow is easy to implement and cost-effective. These protocols were used in our published fluid shear stress experiments and were particularly important because of the complex nature of these experiments. Nevertheless, this element of our manuscript will be widely applicable to gene expression analyses in all settings.

Overall, these protocols have facilitated experiments that have yielded significant insight into the regulatory mechanisms that control endothelial cell angiogenic potential in response to shear stress conditions. We hope that this pragmatic workflow, in whole or in part, can facilitate other such significant findings.

Thank you for your time and consideration.

Warmly,

Philip A. Marsden