INTRODUCTION

Within bone, the mechanotransduction pathway is recognized as one of the primary pathways determining bone strength. However, despite an intense research effort over the last 30 years seeking to identify potential mechanosensing molecules in bone cells, only a handful of candidates have been identified. This is due in large part to the low throughput of existing mechanotransduction assays, which preclude the development of systems that integrate with automation robotics. While appreciable research efforts have been focused on the former, little consideration has been paid to the latter. Importantly, the development of systems that combine the ability to mechanically stimulate cells with automated liquid handling would unlock a powerful new avenue of large-scale mechanobiological investigations in which tens of thousands of different cell populations may be subjected to distinct mechanical (e.g., different frequencies or levels of shear) and/or biochemical (e.g., different small molecules or siRNAs) factors. Given the potential of such large-scale investigations to accelerate mechanobiological discovery, in this study our objective was to develop an assay for assessing flow-induced bone cell activity that could be integrated into automated liquid handling systems.

RESULTS AND DISCUSSION

FDG-Based Assays: Feasibility Analysis

We assessed the potential to generate well-defined shear stresses within 96-, 384-, or 1536-well plates (common formats for high-throughput screening) using Fluid Dynamic Gauging (FDG)-based assays. FDG is a recently developed technique for measuring the thickness and mechanical properties of soft materials deposited on a surface [1]. In FDG, a nozzle is brought close to the surface of the deposit, and fluid surrounding the deposit is withdrawn through the nozzle. This gives rise to well-defined flow adjacent to the sample surface (Fig. 1A). Using recent findings by Peralta et al. [2], we assessed the feasibility of adapting FDG to stimulate cells seeded in high-density multi-well plate format. In particular, these authors demonstrated that for an annular nozzle of inner radius \( r_1 \) and outer radius \( r_o \), flow underneath the nozzle rim (i.e., \( r_1 < r < r_o \)) can be accurately modeled as radial flow between two parallel disks:

\[
\tau(r) = \frac{3\mu Q}{2\pi h^3} r \quad \text{for} \quad r_1 < r < r_o \tag{Eq. 1}
\]

where \( \tau(r) \) is the shear stress, \( r \) is the radial coordinate, \( \mu \) is the dynamic viscosity, \( Q \) is the volumetric flow rate, and \( h \) is the clearance between the nozzle tip and the measured surface. For \( r < r_1 \), shear stress was found to increase in an approximately linear manner, while for \( r > r_o \), shear stress was negligible [2]. In this case, if the nozzle is located within a well of radius \( R \) seeded with cells (Fig. 2B), the average shear stress experienced by the cells is:

\[
\langle \tau \rangle = \frac{2\mu Q (3r_1 - 2r)}{\pi R^2 h^2} \tag{Eq. 2}
\]

Liquid handling systems can readily achieve flow rates of ~100µL/s. However, for multi-well plates with very small well volumes (such as 384- and 1536-well plates), the flow rate must be small enough such that the total volume of fluid dispensed does not exceed the total volume of the well. In particular, if the height of the well is \( H \), and the fluid is repeatedly dispensed and aspirated with frequency \( f \), the peak flow rate that does not give rise to well overflow is approximately...
where we have assumed that \( h < \frac{1}{2}H \) and that the flow rate during dispense/aspiration is constant. Assuming \( f = 1 \text{Hz}, h = 0.3 \text{mm}, \) and common pipette tip and multi-well plate geometries, we estimated that an average shear stress of \( \tau = 0.03 \text{Pa}, \) \( 0.15 \text{Pa}, \) and \( 0.07 \text{Pa} \) could be achieved within 96-, 384-, and 1536-well plates, respectively. Given that bone cells can be stimulated by as little as 0.1 Pa [3], these studies suggest that common pipette tips can be used to stimulate cells in 384-well plate format, but not 96- and 1536-well plate formats. The low shear in 96-well plates stems from the large radius of the well relative to the pipette tip, while the low shear in 1536-well plates is due to the need to use low flow rates to prevent well overflow.

FDG-Based Flow Induces ERK1/2 Activation in Bone Cells

While the large dimensions of 96-well plates are not amenable to stimulating cells using common pipette tips, this larger format has the advantage of enabling a greater quantity of RNA and/or protein to be collected for assessment of cell activation. In this case, we assessed the potential to use custom pipette tips to stimulate cells in 96-well plate format (Fig. 2A). We fabricated a pipette tip that had a thin annulus attached at the tip end, resulting in a large effective outer radius \( r_e = 0.85 R \). To assess bone cell activation, we quantified p-ERK1/2 levels. Our choice to focus on the ERK1/2 pathway was motivated by our recent studies indicating that recurring ERK1/2 activation may mediate enhanced bone anabolism arising from rest-inserted mechanical loading in vivo [3].

We performed a set of experiments to determine a) the capacity of FDG-based flow to stimulate ERK1/2 activity, and b) the similarity of this response to that induced using an established model of fluid flow. For the former, we exposed bone cells to FDG-based oscillatory fluid flow \( (1 \text{Hz}, 0.15 \text{Pa}, \) 5min) and measured p-ERK1/2 levels. As a positive control for ERK1/2 phosphorylation, we also subjected cells to human EGF \((10 \text{ng/ml})\). In separate experiments, we established a “gold standard” to compare our FDG-based assay to by assessing ERK1/2 activity in cells subjected to orbital shaking \( (2.2 \text{Hz}, 0.1 \text{Pa}, \) 5min) [3]. Notably, we found that ERK1/2 activation following FDG-based flow and orbital shaking was nearly equivalent (Fig. 2B), with orbital flow and FDG-based flow inducing an approximately 14% and 33% increase in p-ERK1/2, respectively. Interestingly, in the same plates as exposed to FDG-based flow we could also detect a high degree of ERK1/2 activation in positive control (EGF-treated) cells. This suggests that the assay has a robust dynamic range that is sufficient to detect not only inhibitors of mechanically-induced ERK1/2 activation, but enhancers as well.

Collectively, these experiments suggest that FDG-based flow assays, when integrated with automated liquid handling instruments, are a viable approach for realizing high-throughput mechanotransduction investigations. Importantly, these assays do not require specialized microfabrication techniques, and can be integrated into commercially available liquid handling instruments. Thus, these studies serve as a promising starting point for enabling broad efforts directed at de novo mechanobiological discovery through high-throughput explorations.

METHODS

MC3T3-E1 cells were cultured in α-MEM containing 10% FBS at 37°C and 5% CO₂. For orbital flow experiments, cells were cultured in 6-well plates and placed on an orbital shaker resulting in a 2.2Hz waveform and \(~0.1 \text{Pa}\) shear stress [3]. p-ERK1/2 was assessed via Western blot, and normalized to ERK1/2. For FDG-based flow experiments, cells were cultured in 96-well plates, and flow within the well was generated using a manually actuated pipette. The pipette was mounted to a custom apparatus that controlled the clearance between the pipette tip and the bottom of the well. To enable detection of the low protein levels within 96-well plates, p-ERK1/2 was assessed using the AlphaScreen p-ERK1/2 fluorescence-based assay (Perkin Elmer). Results were normalized to controls, and log2 transformed.

REFERENCES