FLECS Technology for High-Throughput Single-Cell Force Biology and Screening

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ABSTRACT

Dr. Ivan Pushkarsky from the Department of Bioengineering at UCLA and Forcyte Biotechnologies, Inc. was awarded The President's Innovation Award at the Annual Society of Biomolecular Imaging and Informatics meeting held in San Diego, September 2017. All cell types produce mechanical forces to serve important physiological roles. Since aberrant force-generating phenotypes directly lead to disease, cellular force-generation mechanisms are high-value targets for new therapies. Despite knowledge of their significance in disease, drug developers have had limited access to force-generating cellular phenotypes, especially in the context of high-throughput screening. To serve this valuable need, we have developed a platform microtechnology called "FLECS" that can acquire robust contractility data from 1000s of uniformly patterned single cells simultaneously and seamlessly integrates with the 96- and 384-well plate formats to facilitate large-scale drug screens. This perspective discusses the challenges facing existing laboratory methods for measuring cellular force in the context of drug discovery. It then provides an overview of the FLECS platform, describes how it was designed to overcome many of these challenges, and discusses some exciting work already accomplished with FLECS. It concludes by highlighting the platform nature of the technology and the potential value that it promises for a myriad of drug development efforts.

Keywords: cellular force, contractility, force generation, smooth muscle cells, asthma, hypertension

INTRODUCTION

Mechanical force generation is a key function performed, in some capacity, by virtually all cell types as they fulfill their physiological roles. At the single-cell level, contractile forces are used for mechanotransduction,¹ migration,² cytokinesis,³ and immune processes.^{4,5} At the multicellular level, mechanical forces are utilized by the three types of muscle tissue,⁶ for both structural and functional purposes, including the production of

cardiac contractions (cardiac muscle), gross motor movements (skeletal muscle), and vital involuntary processes (smooth muscle) such as regulation of vascular resistance, maintenance of airway tone, peristalsis, bladder control, and others. As a result, if left uncontrolled, aberrant cellular force generation gives rise to serious diseases at the organism level, and, in many cases, is the direct upstream cause of symptoms.

Specifically, excessive cellular force generation is implicated in the pathogenesis of asthma,⁷ hypertension, fibrosis,⁸ stroke,⁹ and cancer,¹⁰ whereas insufficient force generation lies at the root of lifestyle diseases such as cardiac insufficiency and congenital defects such as X-linked neutropenia and muscular dystrophy. Uncontrolled cellular relaxation has also been linked to migraine. Because of direct functional roles of force in health physiology, force biology disorders place a significant burden on patient health. Unfortunately, there is a paucity of effective drugs for many of these conditions, arguably due to a lack of adequate tools for evaluating cellular force in early drug discovery. Functional phenotypic assays that directly and quantitatively report on the force generation of cells—the physiological endpoint underlying these indications—and that can be miniaturized and automated to achieve high throughput have the potential to revolutionize drug discovery efforts, and to provide more efficient and lower-cost pre-clinical selectivity and safety profiles for existing candidates.

LIMITATIONS OF EXISTING ASSAYS

Although existing cellular force assays used in biological and biophysics research have been useful for answering specific questions using relatively small numbers of cells, these methods were not designed with large-scale implementation in mind and are unsuitable for automation and high-throughput screening. The most widely used assay for cellular force is traction force microscopy (TFM),^{11,12} which uses a polyacrylamide (PA) gel with embedded particles to map the forces applied by adherent cells. Although TFM is ideal for detailed traction fields around individual irregularly adhering cells, high-resolution imaging needs, complex image analysis, and an extra re-imaging step after cell removal have all contributed to limiting the throughput of this approach to only a few single cells per experiment. A recent implementation of TFM in a microtiter plate format achieved increased

throughput but could report only a noisy bulk-averaged “force response-ratio.”¹³ This readout is confounded by a heterogeneous response among individual cells in primary cell populations and has greatly limited sensitivity to force variations. Micropost arrays and atomic force microscopy have also been used to assay cellular force generation, but the complexity and low-throughput manual operation has prevented use in high-throughput drug screening.^{14,15}

For drug developers, an ideal measurement system would allow analysis of precise force magnitude over short and long timeframes for hundreds to thousands of single cells per condition in a multi-well plate format with tightly controlled parameters. These single-cell datasets would then lead to high levels of statistical confidence in even small changes between cell states or drug effects (larger Z' and z scores) as well as identification and screening of multiple cell types or sub-populations in a multiplexed manner with reduced noise. A high-throughput and quantitative contractility screening platform, which offers cell-by-cell data and only uses small numbers of precious primary cells per condition, is needed to accelerate drug development for a wide range of contractile cell populations.

FLECS: A HIGH-THROUGHPUT METHOD OF MEASURING SINGLE-CELL FORCE GENERATION

Advances in miniaturization have allowed us to develop a new approach to quantitatively assay cellular force generation in a highly parallel manner and at the single-cell level to allow for quantization, normalization of results, and multiplexing. In our approach based on Fluorescently-Labeled Elastomeric Contractile Surfaces (“FLECS”), precise arrays of fluorescent and adhesive micro-patterns are covalently embedded into soft substrates with controlled elasticity by using a breakthrough microfabrication process.¹⁶ Single cells adhere to, spread over, and ultimately apply contractile forces onto these micro-patterns, producing observable displacements that are used to infer relative force generation independently for each adhered cell. The use of micro-patterns, which we have previously found to have no detrimental effects on viability,¹⁶ ensures uniform control over cell mor-

phology and identically orients cellular force application, thereby permitting direct comparisons to be made between all cells. In addition, the shapes of these micro-patterns are strategically designed to concentrate cell forces in a way that they may be characterized by simple scalar measurements, rather than more complex force field measurements that are necessary for randomly spread cells.

For example, the “X” pattern shown in *Figure 1* guides stress fiber formation between adjacent terminals, and it concentrates cumulative forces along two perpendicular axes, causing predictable one-dimensional displacements along those axes toward the micro-pattern centers. Subsequent imaging of these micro-patterns, rather than of the cells themselves, yields simple image-based data that can be processed by using straightforward and highly parallelized algorithms. Importantly, these measurements are self-controlled since cell-occupied micro-pattern dimensions are compared with a collection of nonoccupied micro-patterns present in the same images, eliminating the need to remove cells to obtain a reference measurement. Further, since precise locations and footprints are known for all cells in this system, live cell probes may be used to distinguish the inhibitory effects of compounds from any potential compound toxicity independently for each single cell.

Another key advantage of monitoring micro-patterns adhered to by individual cells, rather than the cells themselves is the added ease of registering a series of time-course images

Fig. 1. Image of single 384-well plate from a FLECSplate seeded with primary HASM. **(A)** Entire 384-well plate captured at 4× magnification. Arrays of micro-patterns embedded directly into the elastomer at the well bottom are adhered to and contracted by individual HASM cells. More than 1,200 micropatterns are present in each well. **(B)** Blow-up of **(A)** captured at 10× for more detail. Relative to the unoccupied micro-patterns located top-left and middle-right, micro-patterns that are adhered to by cells are displaced inward, and they provide information on the contractile capacity of those cells. Although the image in **(B)** was captured by using 10× magnification, 4× magnification is sufficient for resolving effects of contractile agonist on the cells. Cells were fixed in their contracted state, permeabilized, and stained for F-actin for visualization. **(C)** Blow-up of selected cell. The bright bands of actin between adjacent terminals illustrate the orientation of the force generation specifically as dictated by the “X” shape. White arrows indicated the direction of net force application and micro-pattern displacement. HASM, human airway smooth muscle.

Fig. 2. FLECS-based assay to characterize bronchodilator activity in test compounds. Figure and caption reprinted with permission as they appear in Koziol-White *et al.*¹⁷ PI3K inhibition reverses bradykinin-induced shortening in isolated HASM cells. Cells were stimulated to contract with bradykinin (10⁻⁶ M) before treatment with 0.5% v/v DMSO control, or formoterol or CAL-101 (10⁻¹⁰, 10⁻⁷, and 10⁻⁴ M). **(A)** Representative images of fibronectin micro-patterns on ultra-soft silicone elastomer films. *Top:* typical uncontracted and contracted micro-patterns. HASM F-actin is shown in green. *Bottom:* averaged patterns for each treatment case generated from one pattern taken from each of the nine imaging sites used per sample. **(B)** Quantification of cell relaxation to formoterol or CAL-101 (10⁻¹⁰, 10⁻⁷, and 10⁻⁴ M) after bradykinin contraction. Bars represent mean of the triplicates + SEM, with each column representing ≥720 cells analyzed per triplicate well measurement from a single donor. *P* < 0.05 was considered significant.

and tracking the contractile trajectories of individual cells, based on how each micro-pattern changes with time. Doing so significantly simplifies the task of tracking a cell's contractility from one time-point to another. Moreover, since the micro-patterns are elastically (*i.e.*, reversibly) deformed by cells, FLECS can report on basal contractility (*i.e.*, cell tone) as well as on the effects of both pro-contractile agonists and relaxing agents. By evaluating time-resolved single-cell data, nonresponsive cells can be excluded from analysis, ensuring that only cells exhibiting a positive phenotype are evaluated for a response to a test compound.

Critically, we have shown that such single-cell force measurements accurately reflect *in vivo* biology in the cell types for which we performed extensive studies. Specifically, we found that human airway smooth muscle (HASM) cells isolated from donors, under certain culture conditions, exhibit very robust contraction after exposure to asthmatic pro-contractile agonists such as bradykinin,¹⁷ carbachol,¹⁸ as well as to endothelin-1 and histamine—behaviors that are congruent to their responses within intact airways. As an additional parallel to *in vivo* responsiveness, we observed a standard asthma treatment (*i.e.*, the β₂-adrenoceptor agonist formoterol) to produce robust rescue effects on agonist-stimulated HASM single cells, by using FLECS¹⁷ (Fig. 2). Overall, we find the FLECS method to be well suited to measure several force-generating behaviors for a variety of primary cell types,

including smooth muscle cells,^{17,18} mesenchymal stem cells,¹⁹ macrophages,²⁰ and others, typically achieving throughputs of >1,000 cells per experiment.

FLECSPLATES FOR HIGH-THROUGHPUT SCREENING

To provide a complete solution that facilitates automation as well as enables high-throughput screening by using the technique, we have produced a fully integrated multi-well plate embodiment of FLECS, known as the *FLECSplate*. Our sacrificial micro-patterning technique enables the creation of FLECS substrates of any size, for example, as large as the standard SLAS footprint, by using a fixed process and a number of fabrication steps. As a result, both a 96-well and a 384-well FLECSplate are achieved without added difficulty. These formats, which increase experimental throughput by up to an additional 100-fold, are immediately compatible with laboratory automation equipment and we have shown that they can be used to perform parallelized studies as well as drug screens. As part of one such demonstration, we titrated a tool compound with known antagonism of cell contractility, and we were able to calculate an IC₅₀ with excellent agreement with previously published value.²⁰ This demonstration also shows that the FLECS platform provides nuanced quantitative data on the level of force generation, which provides value when comparing between effects of multiple compounds.

FLECS AS A PLATFORM FOR PHENOTYPIC ASSAY DEVELOPMENT

As a platform technology, FLECS provides users with independent control over micropattern shape, biomolecular composition, and substrate stiffness. By tuning these parameters, a variety of force-generating phenotypes can be addressed in a screening format. For example, the traditional “X” micro-pattern is particularly useful for assaying contractility in tissue cells such as smooth muscle cells and fibroblasts, whereas a more polarized rod-like pattern is better suited for patterning and measuring myotube and cardiomyocyte forces. Modification of the biomolecular composition also allows users to recapitulate different micro-environments to optimize cell interaction and even guide behaviors. For instance, we found that fibronectin is a preferred adhesion scaffold for many tissue cells, whereas collagens are better suited for cancer cells, and Matrigel promotes optimal sustained adhesion and beating in cardiomyocytes. Moreover, functionalization of the micro-patterns with antigen-antibody immune complexes has been shown to promote phagocytosis by primary human macrophages, whereas patterns lacking antibody only weakly promoted adhesion in the same cells.¹⁹ This “plug-and-play” aspect stands to enable investigators to design very detailed assays focusing on unique aspects of force generation at large scales.

As an illustration of the platform nature of this technology, we developed an antagonist assay in which cells that are stimulated to contract by using a disease-specific agonist are screened against a library of compounds with potential “rescue” properties. Specifically, using the HASM data discussed earlier, we developed a FLECS-based bronchodilator screen in which formoterol served as a positive control and used it to show that CAL-101, a PI3K inhibitor, potently reversed agonist-induced bronchoconstriction.¹⁷ Using this concept, ongoing efforts are now focusing on developing similar assays for hypertension as well as migraine therapeutic discovery. Overall, we show that FLECS technology provides the technological means to facilitate development for a variety of novel assays in which the force-generating phenotype is the target. As an enabling technology that measures a previously inaccessible phenotype at large scales, we expect FLECS to aid in the discovery of altogether new classes of therapeutics for all diseases caused by uncontrolled cellular force generation. A more thorough study describing FLECS technology and demonstrating its application can be found elsewhere.²¹

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DISCLOSURE STATEMENT

The author is a co-founder and senior employee at Forcyte Biotechnologies, Inc., an early-stage biotech company aiming at commercial FLECS technology for drug discovery, innovative consumables, and assay development services.

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Abbreviations Used

FLECS = Fluorescently-Labeled Elastomeric Contractible Surfaces
 HASM = human airway smooth muscle
 PA = polyacrylamide
 TFM = traction force microscopy