Pulling it together in three dimensions

Xavier Trepat, Ben Fabry & Jeffrey J Fredberg

The most abundant proteins in our cells are there to generate mechanical forces, and measurement of these forces has just become possible.

The study of mechanobiology is not at all new, and we have known for quite a long time that physical force is central to biological form and function[1]. Throughout the vasculature, for example, the local diameters of blood vessels roughly match the local blood flows so as to keep fluid shear stresses at the vessel wall nearly constant[2]. Across species of widely varying body mass, from shrew to elephant, long bones thicken[3] and overall energy metabolism increases[4] in a manner that is attributable to avoidance of buckling under mechanical load[5]. Much more recently, we have come to learn that physical forces at the cellular level play pivotal roles in cancer biology[6], monolayer barrier disruption[7] and stem cell differentiation[8]. In this issue of *Nature Methods*, Legant *et al.*[9] use mathematical analysis of matrix deformations to make visible the physical forces at the interface between a cell and its three-dimensional microenvironment.

If some of the over-riding principles of mechanobiology at the organ level are now known, then our understanding of underlying mechanisms at the cellular level still remains unclear. For example, how does the individual cell manage to sense physical forces locally in its three-dimensional microenvironment, respond to those forces or even generate those forces? More specifically, how do physical forces in three dimensions modulate cell adhesion, cytoskeletal tension, the rate of cytoskeletal remodeling as well as chemotaxis, durotaxis and cellular responses to tissue stretch? Do local physical forces guide cellular migrations, or are forces a by-product of those migrations?

Except in highly unphysiological cell culture systems with a flat two-dimensional substrate, understanding of these basic questions has been held back because no method existed by which local physical forces themselves were accessible to quantitative measurement or direct visualization. We have come to learn that cell forces are inherently three dimensional and will cause a flat surface to wrinkle, ruffle and bulge[10] (**Fig. 1a**). But in regard to experimental investigation of physical forces in which the cell is fully embedded in a three-dimensional environment, it is fair to say that we have been navigating in the dark.

With the methodological advance of Legant *et al.*[9], however, these forces are now open to experimental attack. A transparent polyethylene glycol hydrogel with biodegradable domains and adhesive ligands serves as a three-dimensional matrix that cells can invade. Embedded in that matrix, the cell then spreads, adopts physiologically relevant morphologies and develops traction forces. Resulting matrix deformations are visualized by tracking fluorescent tracers with a confocal microscope. The traction forces that give rise to those deformations are then inferred using the laws of elasticity.

We are still far from our ultimate goal of studying the regulation of cell function by physical forces in the cell's native microenvironment, however. One central obstacle is the complexity of the native extracellular matrix, which is heterogeneous, viscoelastic and strongly nonlinear. Moreover, matrix varies with time: cells continuously degrade and synthesize matrix, which restricts force measurements to scales of time that are short compared with many important biological events. Therefore, obtaining reliable traction maps will require substantial improvements in experimental or modeling strategies.

Finally, traction forces reported by Legant *et al.*[9] represent only those forces generated by one cell acting on a passive matrix (**Fig. 1b**), thus leaving active forces of mutual cell-cell interactions (**Fig. 1c**) not only as a major unknown, but also, in all

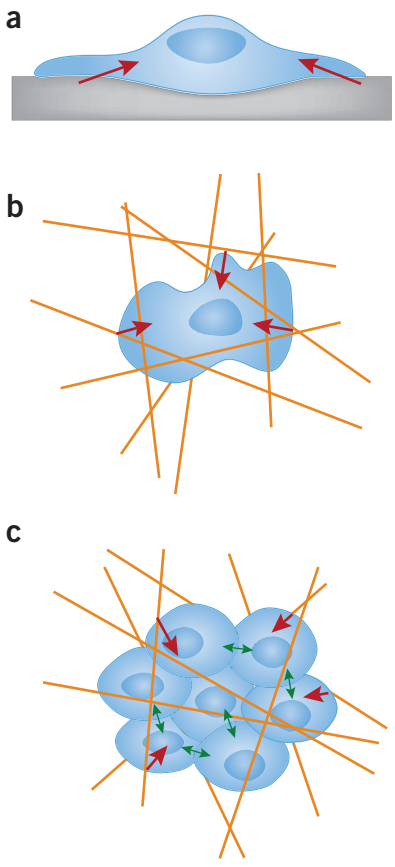

Figure 1 | Cell traction forces. (**a**) Traditional traction microscopy measures forces at the interface between the cell and the two-dimensional substrate. (**b**) Measurement of cell tractions across the entire three-dimensional cell-matrix interface. (**c**) Although cell-matrix forces (red) are now accessible[9], forces at the cell-cell interface (green) remain inaccessible to experimental observation.

Xavier Trepat is at the Institute for Bioengineering of Catalonia and at the University of Barcelona, Barcelona, Spain. Ben Fabry is at the Department of Physics at the University of Erlangen-Nuremberg, Erlangen, Germany. Jeffrey J. Fredberg is in the Program of Molecular and Integrative Pulmonary Sciences at the Harvard School of Public Health, Boston, Massachusetts, USA.
e-mail: jeffrey_fredberg@harvard.edu

likelihood, a dominant one. Without this information, mechanisms of force regulation during processes involving collective cellular behavior—as in development, collective cell migration or cancer cell invasion—will remain inaccessible experimentally. Despite these limitations, the method presented by Legant *et al.*[9] constitutes a dramatic advance on our way to understanding the interaction between a cell and its physical microenvironment in experimental models of increasing physiological relevance.

COMPETING FINANCIAL INTERESTS
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The RNA structurome: high-throughput probing

Eric Westhof & Pascale Romby

Novel deep-sequencing strategies are used to monitor, at the genomic scale, the structure of cellular RNAs using enzymatic probing.

Massively parallel sequencing techniques that allow fast and deep sequencing of genomes have changed genomics[1]. Underwood *et al.*[2], in this issue of *Nature Methods*, and Kertesz *et al.*[3], in *Nature*, exploit high-throughput sequencing techniques for determining RNA secondary structures in full transcriptomes.

Genome-sequencing technologies have been applied to characterize complexes formed between cellular machineries and either DNA regions or mRNAs[4–7]. Although these studies provided information on the composition of regulatory complexes, they could not resolve the structure of the RNAs.

Recently, the multiple roles of RNAs in regulation of gene expression and genome stability have become a topic of soaring interest. In these regulatory processes, RNA structure is a key element—either by monitoring external or internal signals directly or by presenting specific binding sites for *trans*-acting factors[8,9].

RNA transcripts are single-stranded molecules with a strong tendency to fold back on themselves and form Watson-Crick pairs, leading to hairpins of various lengths and complexities. The hairpins defining the secondary structure can further assemble into intricate three-dimensional architectures[10]. The knowledge of RNA secondary structure is the first necessary step toward understanding the activity of the RNA, describing the binding footprints of its protein partners and explaining how mutations affect it.

For structurally stable RNAs, the most efficient and secure method to determine secondary structures is phylogenetic comparisons of homologous sequences. Indeed, homologous sequences are expected to yield similar folds, maintaining the number and lengths of core helices. Such a conservation of Watson-Crick paired regions implies that, within a given Watson-Crick pair, both nucleotides vary so as to keep a usual Watson-Crick pair. This approach, however, breaks down when the sequences are highly conserved throughout phylogeny and do not display nucleotide co-variation.

When the RNA can adopt more than one stably folded state, however, phylogenetic comparison is extremely difficult. In such cases, one can resort to *in silico* approaches, which rely on experimentally derived energy sets of base-paired stacks to compute the minimum free energy of the secondary structure by maximizing the number of base pairs[11].

Experimentally, one can address such issues using chemical or enzymatic probing. These experiments can be performed *in vitro* or *in vivo*, in the absence or in the presence of protein or other ligands and at various temperatures or conditions. With both chemical and enzymatic probing, RNA accessibility is the major criterion for reactivity. In chemical probing, a defined chemical, chosen for its reactivity to a precise position on the RNA either on the base or the sugar-phosphate backbone, is allowed to react with the RNA. In enzymatic probing, an RNA-cleaving enzyme with a distinct preference either for unpaired or paired regions reacts with the RNA. The detection method (generally primer extension followed by gel electrophoresis of the fragments) will show those bases accessible to the chemical or the enzyme in the structure present in the solution. Chemical probing yields information at the atomic level, whereas enzymatic probing yields information on the helical and nonhelical regions. These experimental methods are labor-intensive and demand expertise during the various processes[12]. The data can be used as constraints in computer folding programs, facilitating the prediction of the RNA secondary structure inferred from the sequence[11].

The high-throughput methods of Underwood *et al.*[2] and Kertesz *et al.*[3] apply deep-sequencing strategies to obtain secondary structure information in a complex mixture of RNA transcripts extracted from a cell (**Fig. 1**). In both methods, RNAs are extracted from yeast[3] or mouse[2] cells, enzymatically hydrolyzed under defined experimental conditions and size-selected. In both methods, the enzymes leave a 5′-phosphate group enabling, by using adaptors, selection of only the cleaved fragments rather than hydrolytic degradation products that leave a 5′-OH fragment. After reverse transcription and amplification by PCR, the libraries are deep-sequenced.

The two methods differ in the enzymes used and in how the data are subsequently processed. Kertesz *et al.*[3] use two enzymes: RNase V1, which is specific to double-stranded regions, and nuclease S1, which is specific to single-stranded regions. The final score is the log-ratio of the number of reads for the fragments resulting from the RNase V1 and nuclease S1 cuts, starting at the nucleotide following the analyzed residue. Underwood *et al.*[2] use a single enzyme—nuclease P1, which is specific to single-stranded regions—with two controls, one without nuclease to estimate the amount of endogenous cleavages leaving a 5′ phosphate and one with the addition of T4 ligase to measure cleavages not leaving a

Eric Westhof and Pascale Romby are at Architecture et Réactivité de l'ARN, Université de Strasbourg, Institut de Biologie Moléculaire et Cellulaire du Centre National de la Recherche Scientifique, Strasbourg, France.
e-mail: e.westhof@ibmc-cnrs.unistra.fr