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Abstract

Living systems are fascinating sensing machines that outmatch all artificial machines. Our aim is to put a focus on the dynamics of mechanosensing in cellular systems through concepts and experimental approaches that have been developed during the past decades. By recognizing that a cellular system is not simply the intricate assembly of active and passive macromolecular actors but that it can also manifest scale-invariant and/or highly nonlinear global dynamics, biophysicists have opened a new domain of investigation of living systems. In this chapter, we review methods and techniques that have been implemented to decipher the cascade of temporal events which enable a cell to sense a mechanical stimulus and to elaborate a response to adapt or to counteract this perturbation. We mainly describe intrusive (mechanical probes) and nonintrusive (optical devices) experimental methods that have proved to be efficient for real-time characterization of stationary and nonstationary cellular dynamics. Finally, we discuss whether thermal fluctuations, which are inherent to living systems, are a source of coordination (e.g., synchronization) or randomization of the global dynamics of a cell.

Keywords: Mechanosensing, biosensing, mechanotransduction, cytoskeleton, multiscale, scale-invariant, nonlinear dynamics, focal adhesion, cellular rheology, coherent dynamics
1. Introduction

The concept of mechanical sensing dates back to the 19th century with the emergent theory of tone sensing proposed by H. Helmholtz and J. Muller [1, 2]. All living organisms have the ability to sense mechanical stress and/or hydrostatic pressure, either locally or globally. One of the most studied example is the touch perception of metazoa [3-6]. The first model of mechanosensing proposed by physiologists was constructed on the concept of mechanical receptors [3, 4, 7], i.e., mechanical machineries that are able to transform a mechanical information in another signal that will afterward be interpreted by the cell and potentially transformed into an adaptative response [8]. This concept of receptor or transducer is inspired from mechanical engineering methods. The term mechanotransduction [5] has been more recently introduced to explain how a single cell transforms a mechanical stress through signaling pathways down to nuclear molecular processes.

Mechanosensing [9-11] is a complex mechanism that involves not only a whole range of molecular actors with nanometer-scale sensibility but also a dynamical integration and regulation of these molecular actors that allow a much larger scale (μm to mm) response in amplitude, with strength and duration adapted to the perturbation. Our physical models of cellular nanomechanics still rarely consider active viscoelastic systems [12] and despite a recent increase of the rate of publications devoted to nanobiomechanics of cells, the concept of cellular biodynamics is still in its middle age. This relative slow progression comes from the necessity to introduce concepts of active matter [13-18] into biology. All cells interact physically with their surrounding tissue and they can establish their response on various timescales. To get a full understanding of cellular biodynamics, we actually need to master the most fundamental concepts of atomic and statistical physics, submicron-scale hydrodynamics and out-of-equilibrium nonlinear dynamics and to associate nanotechnologies with optogenetic, microfluidic tools and molecular and cellular biology methods to achieve a complete mechanogenetic characterization of living cells. Actually, a cell is able to combine biochemical submolecular and supramolecular active or passive interactions with micron-size mechanical and electromagnetic informations that we still have much difficulty to reproduce, with either our experimental devices or our huge computer machines.

There are three aspects that we would like to put forward in this chapter. The first one is the importance of molecular machines of living systems, also called molecular motors, which drive all the cell movements thanks to ATP consumption. These molecular machines are not fully deterministic motors but are in part driven by thermal fluctuations. The second aspect that seems important to us is the fact that fluctuations are predominant in biological systems, and that the fluctuation dissipation theorem [19] must not be taken as granted in all situations. This means that standard approaches in statistical physics and corresponding mathematical models must be used with caution. The third aspect is the importance of multiscale properties in mechanosensing: short-, middle-, and long-distance interactions contribute to the dynamics of cellular systems and more widely of living systems [20-29]. Because they are nonlinear and nonstationary, these interactions produce a global dynamics that each element could not achieve alone. However, the nature of these interactions is still the subject of current debate. We will illustrate different approaches that have been used so far to address this issue.
The most striking example of the interplay of mechanics and cell dynamics is illustrated in cell migration and adhesion. Actually, the mobility of a cell is a subtle combination of two counteracting mechanisms: on the one hand, adhesion which tends to immobilize the cell and, on the other hand, protrusion/retraction mechanisms which modify the cell shape and assist its movement. These processes also underlie the ability of a cell to deform under a mechanical perturbation. The cytoskeletal dynamics and mechanics are univocally linked to cellular tension in cell adhesion [30-33]. Cellular movement by membrane protrusion and formation of new adhesions at the cell front cannot occur without a tight link of the cell cortex to the whole cytoskeleton (CSK), allowing the settling of traction forces that drive the cell forward in motion in synchrony with the disassembly of the rear fibers. External membrane protrusions are important components of the ability of a cell to migrate or interact with other neighboring cells [34-36]. These protrusions can be viewed as local instabilities of the cell cortex. They are not independent of the internal dynamics of the CSK: the microtubule plus ends associate with F-actin via plus-tip proteins and act as a scaffolding complex [37] that recruits further down other protein effectors involved in the actin network remodeling [38].

When cells are placed in adherent conditions, they rapidly develop integrin-mediated adhesion complexes that link the extracellular matrix (ECM) to the actin CSK. These transmembrane proteins are associated with a complex of proteins (vinculin, actin, paxillin, tensin, etc.) which allows very fast and reversible connectivity of the intracellular CSK to the outer membrane complexes [39, 40]. The integrin-based molecular complexes concentrate in small domains with different size and shapes that focalize the cell traction force on the ECM. In addition to their function as adhesion sites, matrix adhesion foci also participate in the adhesion-dependent signaling pathways via tyrosine kinases, tyrosine phosphatases, etc. Focal adhesion (FA) centers function as both adhesion and signal transduction hubs that communicate the external stresses of the ECM to the cell interior [39]. The maturation of these FA complexes cannot exist if the cell does not have a contractile machinery, e.g., canceling the cell contractility by inhibiting the Rho GTPase or tyrosine kinase activity aborts this maturation. The higher the cell tension, the larger and more mature are the FA complexes. The FA complexes can therefore be considered as mechanosensing intermediates coupling the internal cell traction forces with the ECM rigidity [41-44].

The actin network can be viewed as a fluid-gel structure which plays both a passive (viscous) and an active (ATP-driven) role in the spatiotemporal dynamics of the cells. This network is dynamically intertwined with microtubules and interfilament networks in such a way that the leading edge of the cell undergoes retrograde flow away from this edge simultaneously to the cell migration. This retrograde flow occurs in two steps: (i) on short timescales, a fast flow in the most peripheral region of the cell, the lamellipodium (a 1-4 μm width extension filled with a dense network of branching F-actin filaments); and (ii) on longer timescales, a slower centripetal flow over the broad (more than 10 μm width) lamellum [45]. The fast lamellipodium flow principally involves an F-actin network. The slower lamellum flow involves all the CSK filaments (actin stress fibers, microtubules, and intermediate filaments) and a relatively more sparse actin network. These two types of flow are each characterized by a specific organization of the cytoskeletal network and a different turnover rate. In addition, they are
driven by distinct forces, namely actin assembly/disassembly in lamellipodium and actomyosin contraction in lamellum [46]. This indicates that the whole dynamics of a cell during protrusion, traction, and migration is a highly correlated, multiscale (in time and in space) process that entails long-range and short-range mechanisms that can only be tackled using multiscale experimental concepts and methods.

2. Physical probes to capture the mechanical response of living cells

In this section, we concentrate on the nano and micromechanical tools which have been designed in the past two decades to record in real time the mechanics and rheology of a living cell, with the specific purpose to understand its mechanosensing properties. These methods can be classified into two groups: (i) the methods that introduce mechanical tracers inside the cell and follow their spatiotemporal dynamics and (ii) the methods remaining external to the cell and that bring a mechanical device (nano- or microscale in size) close to the cell to follow its response in real time. In each case, a few examples will be described as regard to their ability and efficiency in extracting characteristic temporal and/or spatial scales in the dynamics of cell adaptation to a mechanical stress.

Cellular rheology from the outer membrane

Rheological properties of cells, their deformability under stress are key features of their ability to sense their environment. Recent studies of the microrheology of the intracellular medium have highlighted the fact that this viscoelastic medium is complex and cannot be modeled by the association of a finite set of elastic and viscous elements as usually done in mechanical engineering [25, 47]. Actually, the viscoelastic complex modulus of the cell medium exhibits a weak power-law behavior over a wide frequency range. Using magnetic twisting cytometry (MTC) coupled to an optical detection of the motion of a bead coupled to membrane RGD receptor, Fabry et al. [20, 21] succeeded in probing the cell surface dynamics in the frequency range from 0.01 Hz to 1 kHz. During the bead displacement on the cell surface (forced by a twisting magnetic field), the cell responds with an opposing torque that reflects the cell mechanical strength. The ratio of the complex torque $T$ to the complex bead displacement $\tilde{d}$ in Fourier space is defined as the elastic modulus $\tilde{G}(f)$:

$$\tilde{G}(f) = f_s \frac{\tilde{T}(f)}{\tilde{d}(f)}$$

where the proportionality geometrical factor $f_s$ depends on the shape, thickness of the cell, and the degree of embedding of the bead in the cell cortex. $\tilde{G} = G' + iG''$, where $G'$ is the shear modulus and $G''$ the loss modulus. The range of stress and deformation used in this study was limited to the linear response regime for the cell. These authors found for five types of adherent cell models that both $G'$ and $G''$ increase with excitation frequency as a weak power-law over the whole frequency range. These power-law dependence of $G'$ and $G''$ on frequency was also
observed by other groups [22, 24, 48-50] and with other methods, such as atomic force microscopy [51, 52]. Except for a small additive viscous term that emerged only at high frequencies, mechanical responses collected from the cell surface did not appear to be tied to any specific frequency and in that respect was considered as (time) scale-invariant.

When a power-law behavior emerges in the rheological response of a cell, a wide range of frequencies is required to bring the experimental demonstration of the existence of scale invariance in the cell dynamical response to stress. Actually, a limited range of frequencies could still be parameterized by a combination of a small number of viscoelastic elements, as an exponential crossover between two regimes. The most impressive result of the above studies is the fact that all the curves captured from different cells of various types could be collapsed to single master curves typical of a soft glassy material (SGM) [25, 53], demonstrating the universality of this behavior [24, 54]. This universality law can be written as

\[
\tilde{G}(f) = G'(f) + iG''(f) = G_0 \left( i \frac{f}{f_0} \right)^{x-1} + 2\pi i \mu,
\]

where \(x\) is a unifying parameter, \(G_0\) and \(f_0\) are cell-type-dependent scaling factors for stiffness and frequency, \(\mu\) is an additive Newtonian viscosity term that is negligible for frequencies lower than 30 Hz. This equation tells us that (below 30 Hz) the phase angle \(\phi\) of \(\tilde{G}\),

\[
\phi = \tan^{-1}\left( \frac{G''}{G'} \right) = \frac{\pi}{2} (x-1),
\]

is independent of the forcing frequency. This unifying parameter \(x\) depends on the cell state; \(x\) decreases to 1 when the cell approaches an ideal elastic material (for instance, by increasing its contraction) whereas \(x\) increases toward 2 (limit of a Newtonian viscous fluid) when the cell prestress is diminished (e.g., by disrupting the actin CSK).

The common and generic features of SGMs are due to the fact that they are composed of numerous discrete elements which are interconnected in a random way via weak interactions. These materials are out-of-equilibrium metastable systems, very much like living cells. However, soft glassy dynamics as proposed by soft glass rheology theory [53] is not the only mechanism that can lead to scale-free mechanical behavior as expressed by power-law stress relaxation. Power-law behavior can also be produced by models containing a large number of viscoelastic compartments with a particular distribution of characteristic relaxation times \(P(\tau) \sim \tau^{-x}\) that must be related to intracellular processes.

With the same MTC device, it was also possible to track the spontaneous motions (without magnetic twisting) of small beads linked to cell membrane integrin receptors on adherent cells. Bursac et al. [48] observed that these motions were intermittent with periods of confinement (stalling) punctuated by directed movement (hopping). Plotting the mean-square displacement of the beads versus time revealed that they were subdiffusing (stalling) at short times
but superdiffusing at longer times (hoping) and that intermittent motions reflecting nanoscale CSK rearrangements depended on both the approach to kinetic arrest and energy release due to ATP hydrolysis. The percentage of hopping events in the bead motion was shown to be higher than in soft glassy systems where only thermal energy can push the system out of a microenergy well. Thanks to ATP-driven motors and polymerization/depolymerization cycles, the active properties of the cell CSK and cortex provide alternative ways to visit different microconfigurations and adjust to the ECM changes, with an effective local temperature which increases with the parameter $x$. Therefore, the exponent $x$ tells us the extent to which the cell behaves as a fluidic system ($x \sim 2$) or as an elastic solid ($x \sim 1$).

**Cytoplasm rheology** Microbeads engulfed inside living cells were used as tracers of the internal cellular activity [55-57]. Two different regimes of transport were observed: on the one hand, the passive fluctuations (local movements of the tracers) which characterize the local viscosity of the cytoplasm and, on the other hand, the active trajectories which are driven by molecular motors such as kinesins and dyneins along microtubules. The same type of experiments has also been performed more recently by nanoparticle tracing and manipulation inside A7 melanoma, MCF-10A and MCF-7 cells [58] with optical tweezers, leading again to the conclusion that the elastic modulus follows a power-law: $|G(f)| \sim f^\beta$, with $\beta = x - 1 = 0.15$, in agreement with previous measurements on the cell exterior [20, 21]. They also noted that the measured cytoplasmic modulus is approximately of a few Pa, much lower than previously estimated for its actin cortex [20]. Thus, these tweezers measurements confirmed the rubber-like elastic properties of the cytoplasm of these cells in two-dimensional (2D) adherent conditions.

**Fluctuation dissipation theorem and cell rheology** The equilibrium fluctuation-dissipation theorem (FDT) [19] assumes that the response of a system to a small perturbation is hampered by spontaneous fluctuations at equilibrium (damping term). Understanding the nature and the amplitude of nonequilibrium forces driving the dynamics of cells out of equilibrium is a very important challenge for statistical physicists and biologists. There are many evidences that FDT fails in living cells [59-62] as well as in active gel systems [63], and it is therefore important to estimate the critical timescale at which active-force-driven fluctuations are predominant over thermal fluctuations. Using micron-size silica beads attached to the wall of a living adherent myoblast cell, Bohec et al. [64] have recently identified a crossover time ($\tau \sim 1$ s) between thermally controlled fluctuations and active-force-driven fluctuations. This seems to corroborate that the short-time behavior has equilibrium-like properties, from which the subdiffusive nature of viscoelasticity emerges, while the long-time behavior is strongly governed by active nonequilibrium forces. Bohec et al. [64] also provided a quantitative estimation of the power dissipated by the active forces into the system, which turns out to be three orders of magnitude smaller than the chemical power injected into the underlying motors by the ATPase machinery.

**Long-range cell deformation capture with an atomic force microscope** As emerging in the late 1990s from scanning tunneling microscopy (STM) technologies, atomic force microscopy (AFM) was early recognized to provide a unique opportunity to investigate the structure, morphology, micromechanical properties, and biochemical signaling activity of cells under
physiological environment, and this with high temporal and spatial resolutions [65, 66]. The principle of AFM is to bring directly in soft (or hard) contact a sharp-tip cantilever probe over a cell surface and to capture with piconewton sensitivity the interaction force of the tip with the cell surface. AFM is a very powerful technique that has been used to detect single biomolecules (receptors, lipids) on single cell surface without the need for fixation or staining. AFM has such sensitivity that it can be used to measure interaction between and within single biomolecules [67-69]. Beyond its preliminary application for imaging the topography of biological objects [70-72], AFM has become a multitask scanning probe versatile tool (antigen recognition, molecular and membrane flexibility, single molecule, gel, cell and tissue elasticity, electric current, conductance, near-field electromagnetic field) [73, 74]. AFM force spectroscopy can be applied to probe the elastic properties of a cell, either adherent or confined in a narrow chamber [75-78]. Its unique ability to detect and to map the cellular elasticity of living cells with a few tens of nanometers’ resolution definitely outmatches the performance of other techniques such as magnetic or optical tweezers. However, it has as a main limitation that it cannot probe cell internal structure without crossing before the cell cortex. This difficulty has been partly overcome recently, thanks to a singular space-scale analysis of force-distance curves to disentangle the viscoelastic moduli of the cell cortex and of the underlying CSK [79]. Interestingly, 2D mapping of mean elastic modulus on a large variety of cells [75, 76, 80-83] was reconstructed, revealing for the first time intracellular interplay of mechanical forces in living cells.

The purpose of this paragraph is not to make a detailed review of AFM or to advertise its latest technological development which can be found in an increasing number of published reports [84-88], but rather to (i) pinpoint the few approaches which were focused on dynamical characteristics of living cells during large deformation and (ii) propose new research directions to perform real-time capture of the cell dynamics when the cell is not in a stationary phase. Unlike AFM-based micro rheology measurements [51, 52] discussed in the previous paragraph which were limited to very small deformations, we consider now much larger deformations (more than 1/10 of the cell size) and their temporal and/or frequency decomposition [89]. To perform large deformation cell study, a new experimental strategy has been recently proposed that consists in exciting the cantilever and recording the cell response over a band of frequencies rather than at a single frequency [90]. Note that broadband excitation of the cantilever can also be achieved by thermal excitation [91]. When the probed object is in a stationary regime, power spectral analysis of cantilever fluctuations, based on Fourier analysis, is the best way to understand how the interaction of the cantilever tip is changed when coming in contact with the sample surface. When the sampled surface is not stationary, it is no longer possible to perform a simple spectral analysis which only displays an averaged decomposition of the signal in frequency domain. The lower frequency part of the power spectrum is biased by the cell dynamical adaptation to the cantilever stress. To circumvent this difficulty, time-frequency analysis based on the wavelet transform has recently been proposed [92-95]. The continuous wavelet transform (WT) performs the spectral analysis of the signal on a compact window (given by the wavelet) and allows, therefore, to follow how the cell mechanics changes during its strain-to-stress response. Such a study has been recently performed on HOPG surfaces [96, 97] and on living myoblasts [98].
Intracellular stress measurements External forces that are transferred across integrins in FAs and channeled through the CSK can alter signaling activities deep inside the cell [99]. Another evidence for long-distance force transfer was provided by intracellular stress tomography measurements [89]. The long-distance force transmission mediated by intermediate filaments was observed in response to fluid flow-induced shear stress applied to the apical surface of endothelial cells [100]. Using the MTC technique, Laurent et al. [101] also found from alveolar epithelial cells, that the submembranous “cortical” CSK, which is mainly composed of actin, is less stiff and more responsive to external forces than the “deep” subcortical CSK, which also includes intermediate filaments and microtubules. Based on these findings, these authors concluded that “mechanical deformation is transmitted globally throughout the network, whereas the cell surface is able to ‘sense’ very local deformation forces.” To analyze the distribution and dynamics of traction stress within individual FAs, Plotnikov et al. [102] applied high-resolution traction force microscopy (TFM) [103, 104] to mouse embryonic fibroblasts expressing enhanced green fluorescent protein (eGFP)-paxillin as FA marker. These fibroblasts were plated on fibronectin-coupled elastic polyacrylamide supports (PAA) of known rigidity embedded with a mixture of red and far-red fluorescent beads. Cell-induced ECM deformation was visualized by spinning disk confocal microscopy, and traction fields were reconstructed at 0.7 μm resolution with Fourier transform traction cytometry [104]. FAs were found to exhibit tugging traction fluctuations on a wide range of ECM rigidities, but the choice of tugging versus stable traction states was shown to be regulated by both tension and a specific signaling pathway. These experiments suggest that strengthening the molecular clutch via the FAK/phosphopaxillin/vinculin pathway broadens the range of rigidities over which dynamic ECM rigidity sampling operates. The requirement for tugging focal adhesion traction in durotaxis suggests that tugging is a means of repeatedly sensing the local ECM rigidity landscape over time. Individual FAs within a single cell sense dynamically the sample rigidity by applying fluctuating pulling forces to the ECM and behave therefore as dynamical sensors to guide durotaxis.

3. Biochemical sensors based on fluorescence methods for capturing cell dynamics: from the nano to the microscale

3.1. Fluorescence-based nanomechanical sensors of intra- and intermolecular dynamics

In the late nineties, combined progress in the biology of fluorescent proteins, miniaturization of optical systems, and nanotechnologies have provided a tremendous asset throughout the investigation of the kinetic properties of macromolecules in living cells [105]. The chemical interaction between two molecular complexes of a metabolic pathway is conditioned by their ability to come in contact, which is often assisted by ATP driven molecular motors. Transport of protein actors in a randomly crowded space such as the cellular cytoplasm differs markedly from a batch reactor. A common form of biochemical regulation is allostery, where an effector molecule binds to a regulatory site and favors a global conformational change that alters further down the structure and function of the active site. Mechanical forces regulate receptor-
ligand binding conformation through control of allosteric conformational changes [106]. This general idea of mechanical regulation of active site functions through allosteric-like regulation of a distal site is termed mechanochemistry and is well accepted for motor proteins [107, 108]. We focus here on cytoskeletal proteins, since they are directly involved in mechanosensing pathways; however, the approaches discussed below could be generalized to a wide variety of biochemical interactions.

To dissect how mechanical stress impacts the structure of cytoskeletal proteins, molecular labels have been designed by physicists to provide a fluorescence signal that could report on the molecular strain. Whereas many proteins have been shown in vitro or predicted by numerical simulations to undergo conformational changes in response to external mechanical stress, we had to wait until the 2000s to get the demonstration of these changes in vivo. Most of these molecular sensors use the Forster resonance energy transfer (FRET). FRET is a technique that can measure the proximity or spatial distance between a donor and an acceptor molecule. It has been widely used to detect protein conformational changes, thanks to molecular constructions with various fluorescent proteins [110]. The principle of stress FRET sensors was elaborated by combining two mutants of a fluorescent protein [111] with either a stable α-helix linker [112, 113], a spectrin linker [114], or a spider silk domain as linker [115]. These force sensors share a common mechanism for interpreting force: tension in the host induces strain in the linker, leading to increased distance between the donor and acceptor. The dynamic range of these sensors is limited by the nearly linear relationship between FRET efficiency and strain [113].

Recently, using the high flexibility of the vinculin linker domain, a sensor based on force transmission through FAs was developed [115, 116]. When the head integrin domain Vh binds to talin, it recruits vinculin to FA, whereas on the other side the tail integrin domain Vt binds to F-actin and paxillin. This intermediate flexible vinculin linker plays an important role in the transmission of adhesion strength from the FAs to the actin CSK. This calibrated biosensor has piconewton (pN) sensitivity, and the tension across vinculin in stable FAs was estimated to 2.5 pN. It was also demonstrated that higher tension across vinculin favors adhesion assembly and enlargement, and conversely that low tension vinculin favors disassembly or sliding of FAs at the trailing edge of migrating cells. Finally, this study [115] revealed that FA stabilization under force requires both vinculin recruitment and force transmission, and surprisingly, that these processes can be controlled independently.

Another type of strain sensors was elaborated from proximity imaging microscopy (PRIM) combined with GFP dimers [117] and further called PRI-based strain sensor module (PriSSM). If two GFP molecules are brought into physical contact, changes in the ratio of fluorescence emitted when excited with 395 nm and 475 nm light occur. Proximity imaging exploits these changes to reveal homotypic protein-protein interactions in vivo. Unlike FRET, PRIM involves only 2 types of fluorescent excitation spectra corresponding to monomeric and dimeric GFP, so that an estimated excitation ratio should simply reflect a mixing ratio of the monomer and the dimer. By combining the GFP-based PRIM technique and myosin-actin as the model system, Iwai et al. [117] used this genetically encoded fluorescent sensor to visualize the interaction between myosin II and F-actin in Dictyostelium cells. Both spectroscopic and
microscopic studies suggested that the fraction of PriSSM-myosin bound to F-actin is low in normal cells.

We have just given few examples of application of FRET to probe cellular internal molecular structures and their transformation under mechanical stress and association with molecular partners. If one can use this method to identify the mechanical organizing centers in a mechanotransduction pathway, the range of forces estimated \textit{in vivo} can be biased by several limitations. FRET is sensitive to changes in distance between 0.5 and 2 times the Forster radius (\textit{i.e.}, between 2 and 10 nm) and to the fluorescence lifetimes of the donor (free or engaged in FRET). Indeed, the dynamic range accessible to this technique is rather low because the interactions which are probed by FRET are typically in the range of a few ns (the fluorescence lifetime of the donor) and difficult to discriminate from background thermal or shot noise. The donor fluorescence lifetime decreases due to energy transfer in the excited state. In adhesion, molecules interact with their closer neighbors but also with other partners on much longer distances which can reach several tens of nm, and thus may be missed by FRET. Conversely, molecules that do not actually participate to a mechanotransduction complex can nevertheless be in juxtaposition and show FRET. FRET experiments suffer from additional artifacts: the method is aimed at probing the interaction of two partners but it is quite impossible to separate the fluorescence responses coming from multiple donor and acceptor interactions even if extensive controls for every FRET pair studied have been performed \textit{a priori}. Finally, none of these methods can determine the number of molecules of a certain component and stoichiometry within an adhesion. A partial correction of these limitations has recently been proposed by coupling FRET biosensors with fluorescence lifetime imaging microscopy [118-120]. Note that this method also provides a millisecond temporal resolution.

3.2. Spanning short- to long-range interactions and transport with fluorescence correlation spectroscopy

Analyzing the fluorescence fluctuation signals offered a simple, high-resolution, quantitative method to probe the intracellular dynamics that other fluorescence imaging techniques could not afford. From a single fluctuation temporal signal it is possible to get several informations over a wide range of frequencies, such as molecular densities, interaction rate and stoichiometry, intra- and extracellular transport (diffusion, advection, etc.). These fluctuation signals should therefore be a very good candidate to capture the multiscale properties of cells in space and in time. Fluorescence correlation spectroscopy (FCS) was originally developed [121] to measure diffusion coefficients and chemical rate constants of biomolecules in solution. It has also been applied successfully to characterize the nature of transport processes of colloidal particles in complex flows [122]. This method uses a focused laser beam to define a very small focal volume (\sim 1 femtoliter) from which the fluctuations of fluorescence intensity are recorded. These fluctuations are analyzed in the nanosecond-to-hour temporal range, and can therefore give information about many different processes including transport, exchange and binding interactions, fluorescence bleaching or blinking. The characteristic times of these different processes are uncovered by computing autocorrelation functions (ACF). Modeling of these ACFs allows the estimation of diffusion, transport, and reaction rates, but it can also
be generalized to cross-correlation analysis to quantify molecular interactions if two fluorophores are used simultaneously in the confocal volume [123-125].

In experiments where the fluorescence signal is too weak or lacks contrast, thereby preventing separation of features from background signals, the spatial and temporal fluorescence cross-correlation functions allow to recover enough contrast thanks to their temporal fluctuations. This method was recently applied by Chiu et al. [126] to capture actin flow as well as F-actin dynamics and location of F-actin bundles in human breast adenocarcinoma cells (MDA-MB-231) grown in three-dimensional collagen gels. By recording simultaneously the collagen signal using confocal reflection microscopy, they also showed that collagen fibers move in concert with the actin-bundle flow. This experimental study is an impressive demonstration of the impact of fluctuations on internal cell dynamics, and of the power of fluorescence cross-correlation methods to discriminate different molecular entities inside living cells to reach a quantitative model of the intracellular architecture that resembles a random obstacle network for diffusion proteins [125]. Correlation of fluorescence amplitude fluctuations with two colors was also applied to detect the presence of molecular complexes in FAs [127]. In addition to their participation in the structural linking of the ECM to actin filaments, these complexes also serve as signaling “hubs” that regulate many cellular processes, including their own assembly and turnover, migration, gene expression, apoptosis, and proliferation. Capturing the dynamics of assembly and disassembly of these complexes is therefore of major importance to understand how a cell senses its environment. Indeed, there is not a single irreversible event of adhesion but rather a fully orchestrated sequence of adhesion events, which may take from seconds to many minutes to be established. This is typically a multifrequency behavior and applying FCS was a definite step toward deciphering the intricate mechanisms of adhesion. Adhesion complexes and interacting protein actors comprise more than 100 different molecules; some are stably associated and others only transiently [128]. When staining FAK, paxillin (Pax) and vinculin (Vn), with enhanced green fluorescent protein (EGFP) and mCherry, respectively, Digman et al. [127] evidenced a heterogeneity in the dynamics and aggregation state of paxillin in different regions across the cell. Taking diffusion as the main mode of transport for adhesion molecules through the cytoplasm, they showed that exchange (binding-unbinding) kinetics with a broad range of rates (0.1-10 s) dominate in the vicinity of adhesion zones. They observed large clusters and complexes exchanging rather slowly in the vicinity of the disassembling adhesion regions, whereas small aggregates (largely monomers) were observed exchanging rapidly in assembling adhesion zones.

In a very recent paper, Baum et al. [125] used FCS to link protein mobility and cellular structure in single cells at high resolution. They mapped the mobility of inert monomers, trimers, and pentamers of the GFP domain on multiple length and timescales in the cytoplasm and nucleus via parallelized FCS measurements. From the perspective of these proteins that cover the range of size of most enzymes, they showed that the cellular interior appears as a porous medium made up by randomly distributed obstacles that reorganize in response to intra- and extracellular cues for small molecules and acts as a viscous medium on large polymeric molecules.
3.3. Confining fluorescence measurements with near field optical probes to improve sensitivity

Reflection interference contrast microscopy (RICM) [129, 130] has been used since the seventies for imaging the internal structure of cells adhering on solid surfaces. Due to a lack of quantitative interpretation of these images, this method was early abandoned. This technique relies on reflections from an incident beam passing through materials of different refractive indices. The interference of these reflected beams is either constructive or destructive, depending on the thickness and index of the layer of both the liquid medium and the cell in contact with the glass coverslip. More recently, thanks to fast progress in data acquisition and storage and improved modeling of the reflection signals, RICM was applied to a variety of biological situations, such as adhesion of vesicles and cells [131]. It has the practical advantage of not requiring any staining or labeling of the sample, and can be implemented with relative ease and very little investment on a standard inverted microscope. It can also be combined with several other microscopy techniques such as fluorescence or other scanning probe microscopies (AFM, optical or magnetic tweezers [130, 132]). Reflected light imaging has also been coupled to fluorescence excitation in total internal reflection fluorescence (TIRF) microscopy [133-135] to capture the cellular structures involved in FA complexes. As compared to transmission microscopy, this planar confinement (evanescent field) of light not only provides a higher signal-to-noise ratio but also minimizes photodamage to the cellular material [136]. Interestingly, the fact that RICM can be performed without staining the cellular sample was exploited to capture the spontaneous fluctuations (called Fluctuation Contrast RICM or Dynamical RICM) of a soft interface to identify the organization of specific ligand-receptor bonds in cellular adhesion [137, 138].

More recently, surface plasmon microscopy has been proposed for imaging internal structures of cells without staining [139-144]. This microscopy offers also the possibility to recover both the amplitude and the phase of the reflected field and in some situations to retrieve the index of the layer in contact with gold without needing to know its thickness [145-148]. This microscopy combines total internal reflection of light with surface plasmon resonance excitation to achieve high contrast and high resolution images. Lately, surface plasmon resonance imaging ellipsometry (SPRIE) has been applied to capture cell-matrix adhesion dynamics and strength [149].

3.4. Beyond fluorescence methods: quantitative phase microscopies for living cell data capture

In the fifties, phase contrast (PC) and differential interference contrast (DIC) microscopies [150] have revolutionized the biologist view of living systems, by inferring their morphometric features without the need for exogenous contrast agents [151]. However, both PC and DIC remain qualitative in terms of optical path-length measurement, since the relationship between the incoming light power and the optical phase of the image field is generally nonlinear. Quantifying the optical phase shifts associated with biological structures was expected to give access to important information about morphology and dynamics at the nanometer scale [152-154]. However, imaging large field of view samples required time-consuming raster
scanning. Full-filed phase measurement techniques were also developed [155, 156], providing simultaneous information from a large number of points on the sample. Fourier phase microscopy (FPM) [157], digital holographic microscopy (DHM) [158] and quantitative phase microscopy (QPM) [159-164] have recently been implemented to provide quantitative phase images of biological samples with remarkable sensitivity and stability over extended periods of time. Thanks to its sub-nanometer path-length stability over long periods and efficient algorithms to retrieve the phase maps from fringe patterns [161, 165], QPM is well suited for studying a wide range of temporal scales. This technique has been applied to capture red blood cell fluctuations (spontaneous flickering), which manifest as submicron motions characterized by membrane displacements in the millisecond (or less) timescale. Amin et al. [166] showed that the frequency behavior of the complex modulus $G(f)$ of healthy red blood cells is similar to that obtained in SGMs. Over the frequency range 5 - 50 Hz, the storage and dissipation moduli approach power-law behavior, $G' \propto f^{0.5\pm0.02}$ and $G'' \propto f^{0.7\pm0.05}$, where the errors indicate cell-to-cell variations ($N=13$). As already discussed for MTC, the intermediate exponent of 0.7 tells us that normal (discoid) red blood cell membranes behave neither as purely elastic nor as purely viscous media, but as viscoelastic gels. For red blood cells switching to echinocyte and spherocyte shapes, the $G''$ exponent decreases consistently, indicating stronger confinement of the membrane viscous motions. Finally, above 35 Hz, the viscous modulus becomes dominant, i.e., the cell transits toward a dissipation-dominated regime, which has been ascribed by the authors to the culture medium viscosity.

4. From fluctuations to deterministic behavior

4.1. Emergence of coherent dynamics in cellular systems

So far, most cellular models have been established at specific scales, those which focus on molecular mechanisms are not suited to pave macroscopic scales and inversely. Establishing a connection between the discrete stochastic microscopic and the continuous deterministic macroscopic descriptions of the same biological phenomenon is likely to give new clues toward the understanding of mechanotransduction and mechanosensing processes. The scale invariance properties of the cell rheology revealed by MTC [24, 54] suggest that for very small mechanical deformations, no characteristic timescale emerges. Even if fluctuations have been shown to play an essential role in many biological systems, e.g., Brownian ratchets [167-169], does that mean that the molecular motors dynamics is not cooperative or synchronized? Progressive molecular motors such as myosin, kinesin and dynein, RNA and DNA polymerases, and chaperonins are macromolecules which hydrolyze ATP while moving unidirectionally along a linear macromolecular track. These progressive transport processes cannot operate without the presence of thermal fluctuations, their directionality resulting from the rectification (asymmetrization) of the Brownian motion [170].

A typical mechanism that crawling cells use to probe their environments is called protrusion, which is a thin (sharp or flat) actin gel extension that the cells generate to move and invade their environment. These protrusions result from many dynamical multicale processes namely...
polymerization/depolymerization of cytoskeleton filaments (actin, microtubules, and interfilaments), progressive molecular motors, and FA complexes recruitment [171]. These outer cellular extensions are called filopodia and lamellipodia depending on the shape and dynamics of the protrusion; they also vary with the presence of intra- and extracellular factors [172]. Protrusions grow and shrink in a random manner around the cell on a few minutes’ timescales over micrometers. When protrusions are temporarily stabilized, adhesion mechanisms are triggered and the cell can develop traction forces on its ECM. If the cell is polarized, an imbalance between the protrusions at the cell ends may lead to a directional motion. Filopodia stochastic dynamics was shown to play a key role in turning the nerve growth cone to face the chemical signal of a specific partner cell [173-175]. In a recent experimental work, Caballero et al. [176] have illustrated the key role of fluctuating protrusions on ratchet-like structures in driving NIH3T3 cell migration. They have shown that stochasticity affects the short- and long-term cell trajectories. They confirmed with a theoretical model that an asymmetry in the protrusion fluctuations is sufficient for predicting the long-term motion, which can be described as a biased persistent random walk. Depending on the type of cells and their environment (ECM stiffness, culture medium), fairly nondeterministic ruffling- and bubbling-like shape dynamics [80, 177] or low-dimensional “periodic” and coherent dynamics [178] can be observed. When placed in conditions for adhesion and spreading on fibronectin-coated glass plates, mouse embryonic fibroblasts (MEFs) show two modes of spreading [178, 179]: on the one hand, anisotropic spreading extensions supported by randomly emerging filopodia [180] and, on the other hand, deterministic spreading extensions that otherwise are rather smooth and continuous. In the latter case, these smooth extensions were shown to be periodically interrupted with a period of about 24 s [178] and to depend on the rigidity of the ECM, integrin binding and myosin light chain kinase (MLCK) activation. Giannone et al. [178] suggested a local cytoskeletal signal transport via the actin cytoskeleton from the tips of the lamellipodia to the back where contraction can be activated to start a new cycle. However, in that situation the oscillating signal remained local and did not synchronize over the whole cell cortex. In different situations, global cortical oscillations in spreading cells were observed and attributed to a cyclic depolymerization of microtubules [181], to Arp2/3 complex [182], or to calcium oscillations [183, 184]. These studies question the nature of the transition from stochastic and dissipative [185, 186] local protrusions or membrane pearling [187, 188] processes to global periodic morphological protrusions. This accumulation of experimental evidences of the impact of fluctuations on cell dynamics together with our improved ability to quantify and to model them are pushing the whole cellular biology community to revisit our traditional models of cell shape and dynamics.

4.2. From mechanotransduction to mechanogenetics: is there a genomic signature of the cell dynamics?

We have seen above that the cell mechanosensing mechanisms involve many length and temporal scales; they are definitely out-of-equilibrium processes which can manifest as stochastic in some situations or low-dimensional periodic dynamics in other situations. Cellular systems have a unique property that no physical/chemical system can reproduce. Depending on the external perturbation, they have the ability to evolve as they synthesize...
some cytoskeletal elements and/or biochemical activators, which may drastically change gene expression and their mechanical phenotype. Cancer stem cells are vivid examples of very drastic transformations [189]. The mechanical environment of a cell has a direct impact on its genetic expression and reciprocally the interplay between the cell mechanics and its geometrical constraints is conditioned by the gene expression level of all the cytoskeletal and adhesion proteins. Large-scale cellular mechanosensing leads to an adaptive response of cell migration to stiffness gradients [11, 190]. This two-way communication initially termed as mechanotransduction could also be called mechanogenetics of a cell to enlighten the interplay of genomic and mechanical functions. Recent advances in cellular biology have put forward mechanical forces as major actuators in cell signaling in addition to biochemical pathways [191]. Within the cell, the cytoskeleton provides a physical continuity from the ECM down to the interior of the nucleus, enabling direct mechanical links between the cellular microenvironment and chromosome organization. Sensed mechanical signals influence information processing through complex cellular signaling and transcriptional networks that may or may not be specifically force dependent [192]. In many cases, these responses feedback to remodel the cytoskeleton and/or nuclear architecture and consequently modify also the mechanosensitive structures that were initially involved in the response. It has been shown that both integrin-mediated and cadherin-mediated adhesion foci enlarge and strengthen in response to tension in the range of a few tens of seconds [193]. On longer timescales, signaling pathways are activated over minutes (e.g. the small GTPase RhoA), which stimulates the formation of actin stress fibers [32], whereas gene expression pathways that operate over hours or days (e.g. the induction of vinculin through serum response factor [194]) change the composition and structure of FAs and of the CSK. Although it seems reasonable to assume that cell mechanics and motility require coordinated protein biosynthesis, nowadays the links between cytoskeletal actin dynamics and correlated gene activities are still poorly understood. Olson et al. [194] recently filled this gap by discovering that globular G-actin polymerization can modulate myocardin-related transcription factor (MRTF) cofactors, thereby inducing the nuclear transcription serum response factor (SRF) and subsequently impacting the expression of genes encoding structural and regulatory effectors of actin dynamics. In cancer, the genome architecture is often impacted directly by mutations and/or translocations or chromatin rearrangements, but the influence of the cellular microenvironment may also change the spatiotemporal program of replication and gene expression [195-199]. Recent large-scale sequencing efforts have also helped scientists to delineate the enormous complexity of cancer and the degree to which signaling, drug resistance and genomic alterations vary from patient to patient and even within one patient [200].

**Abbreviations**

ACF: autocorrelation functions  
AFM: atomic force microscopy  
ATP: adenosine triphosphate
CSK: cytoskeleton
DHM: digital holographic microscopy
DIC: differential interference contrast
DNA: deoxyribonucleic acid
ECM: extracellular matrix
EGFP: enhanced green fluorescent protein
FA: focal adhesion
FAK: focal adhesion kinase
FCS: fluorescence correlation spectroscopy
FDT: fluctuation dissipation theorem
FPM: Fourier phase microscopy
FRET: Forster resonance energy transfer
GFP: green fluorescent protein
GTPase: guanosine triphosphatase
HOPG: highly ordered pyrolytic graphite
MCF7 (-10): Michigan Cancer Foundation-7 (-10), a model of breast cancer cell
MEF: mouse embryonic fibroblast
MLCK: myosin light chain kinase
MRTF: myocardin-related transcription factor
MTC: magnetic twisting cytometry
PAA: polyacrylamide
PC: phase contrast
PRIM: proximity imaging microscopy
PriSSM: PRI-based strain sensor modulus
QPM: quantitative phase microscopy
RICM: reflection interference contrast microscopy
RNA: ribonucleic acid
SGM: soft glassy material
SPRIE: surface plasmon resonance imaging ellipsometry
SRF: serum response factor
STM: scanning tunneling microscopy
TFM: traction force microscopy
TIRF: total internal reflection fluorescence
WT: wavelet transform

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