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REVIEW ARTICLE

# Membrane Homeostasis: The Role of Actin Cytoskeleton

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**Abstract** |

Introduction: The plasma membrane of a cell undergoes continuous deformations and turning-over of its constituents required for a diverse set of functions. And yet, the membrane retains its steady-state surface tension—without which these functions are adversely affected.

Objectives: In this review, we discuss how the establishment and maintenance of the homeostatic state of the membrane has a major contribution from the actin cytoskeleton. The regulation of tension at the global (or cellular) scale is extensively studied for the past few decades, leading to our understanding of how actin polymerization forces, myosin-II based contractile forces, and ezrin-mediated attachment to the membrane—each have a separate and sometimes multiple possible effects on membrane tension.

Conclusions: Drawing examples from cell motility and blebbing cells, we highlight how the dynamics of the cytoskeleton decides if the steady-state tension has a uniform profile, front-rear gradients, or temporally varying tension profiles in single cells. Non-invasive studies open up new avenues especially allowing the investigations focusing on local regulation of tension—at the plasma membrane and inside cells.

**keywords:** *Actomyosin cortex, Membrane tension, Homeostasis*

## 1 Introduction

Homeostasis, in general, refers to the state of a dynamic equilibrium. The plasma membrane is proposed to be at a dynamic mechanical equilibrium in live cells which ensures that its tension is actively maintained at a particular level[1]. Membrane trafficking, osmotic pressure differences, and flattening/unflattening of membrane folds are some of the key processes contributing actively to the membrane tension regulation. While each of these cellular processes is affected majorly by the cytoskeleton, the actomyosin cortex also directly contributes to the generation of surface tension. Herein, we first briefly discuss the historical evolution of the understanding of the structure and dynamics of the membrane and cortex, followed by summarizing the definitions, techniques, key insights, and future challenges.

Several naturalists from the seventeenth to nineteenth centuries helped in the construction of the concept of cells[2–4]. However, it took ~ 100 years after the cell theory was proposed in 1839[5], for the concept of membranes in every cell to be proved, appreciated, and believed.

Early studies on water–lipid interfaces can be dated to Benjamin Franklin's experiments with one teaspoon of oil on water/table in 1772[6]. While Pockels in 1891 developed a way to measure the lipid monolayer covered surface at the air–water interface[7], Langmuir in 1917 explained the concept of molecular hydrophily. He showed the flexibility of the hydrocarbon chains and talked about amphipathic molecules, even in protein monolayers[8]. Though, the first 'membranologist' Overton in 1899 showed that cell membranes were in lipid structure[9]; it took many years to calculate the thickness of cell membranes. Fricke was the first to report values of ~ 3.3–4 nm-thick membranes in erythrocyte[10] and yeast cells[11]. However, a major breakthrough in the study of

**Membrane tension:** Describes how taut the membrane is. Tensed membranes retard processes requiring local membrane shape changes.

**Actomyosin cortex:** A crosslinked filamentous network of actin, found as a thin layer under the membrane.

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membrane structures was the report by Gorter and Grendel in 1925, who extracted lipids from blood cells and showed that the ratio of lipid covered surface to the total cell surface was 2:1, indicating the presence of membranes as lipid bilayers[12]. In 1935, Danielli and Davson proposed a Pauci-molecular concept of cell membranes where they suggested that the lipid bilayer was sandwiched between two monolayers of proteins, giving rise to a tri-laminar structure of biological membranes[13]. Though contradicted in the upcoming years, Robertson's EM studies identified the constituents of the unit membrane[14]; one cannot disregard that the Danielli–Davson–Robertson model was the first to talk about asymmetry in the biological membranes[15].

Thus, extensive studies in the 1900s[15–17] led to the proposal and acceptance of first modern theory of the membrane—the Fluid Mosaic Model by Singer and Nicholson in 1972[18]. While the "mosaic" was believed to be due to the varied composition of the membrane, comprising of phospholipids, proteins, and carbohydrates; the "fluid" aspect was added due to the mobility of these molecules. This model has remained fairly unchanged over the years, with certain modifications arising due to studies conducted in the late 1990s[19]. The presence of detergent-resistant microdomains in the cell membrane has helped the field of membrane biology evolve further.

With the advancement of microscopy, our knowledge about the structure and the composition of both the membrane and the cytoskeleton has grown immensely. Interestingly, a decade before the 1972 model of membranes came into being, the cell cortex was discovered as the first element of the cytoskeleton with the help of electron microscopy of free-living amoeba cells in 1964[20], and was identified as a dense layer of actin filaments. Several years later, studies on patients with Duchenne muscular dystrophy (DMD) showed that proteins could attach crosslinked actin networks to glycoproteins on the cell membrane in muscle cells[21], indicating the presence of membrane–cortex linkages. The ERM (ezrin[22]–radixin[23]–moesin[24]) family of proteins which are hugely credited for being membrane–cytoskeleton linkers was discovered later between 1983 and 1991.

Discovering the dynamic nature of the membrane and the cytoskeleton has brought a paradigm shift in the field by revealing the constant intermingling of various components together maintaining homeostasis. While lipid diffusion was studied in the late 1950s[25], protein diffusion on the membrane was discovered in 1970 when lateral diffusion of proteins was observed by immunofluorescent antibodies in heterokaryons[26]. In 1976, FRAP (fluorescence recovery after photobleaching) experiments clearly demonstrated the concept of membrane fluidity[27]. The dynamics in the cytoskeleton[28] has been studied over many years (1970s–2010s) and spans several properties. These range from actin treadmilling[29], branching[30], severing[31], capping[31], and self-organizing into higher order actin structures[32] in eukaryotic systems. However, experiments leading to the discovery of myosin-II, the first molecular motor to be found out, can be traced back to 1864[33]. While the occurrence of endocytic pits and vesicles can be dated back to studies in 1964[34], the presence of membrane trafficking events was seen in 1979[35], who showed receptor-mediated endocytosis.

As studies probed the ubiquitous role of membrane and cortical mechanics in controlling membrane homeostasis, the ability of light to impart forces[36] on objects started gaining importance. With this knowledge, techniques using light - optical trapping (OT)[37], micropipette aspiration (MA)[38,39], atomic force microscopy (AFM)[40], and interference-based methods[41] began to be used extensively to measure membrane tension in cells between 1990s and 2000s. These techniques are used till date and often give a major insight into believing the concept of membrane tension homeostasis[42] and surface area regulation[1] (in late 1990s). Fluctuation-based experiments which measure mechanics in erythrocyte membranes often probe the dynamics of the RBC membrane (RBC flickering[43]) was discovered back in 1951. Recent work in 2018 with a fluorescent molecular probe (FliptR) which quantifies changes in membrane tension in living cells has been identified[44]. In addition to direct observations and measurements in cells, reduced systems—supported bilayers, unilamellar vesicles, and vesicles with reconstituted cytoskeleton in them—have had a great impact on our understanding of the basic physical mechanisms involved in membrane homeostasis.

## 2 Membrane, Cytoskeleton, and Their Linkages

Ranging in thickness between ~ 5 and 8 nm due to the presence of proteins associated with thebilayers[45], cell membranes outline the boundaries of the cells and are usually described by the fluid mosaic model[46]. These membranes are lipidbilayers where their hydrophobic regions in each monolayer see the interior of the bilayer and their hydrophilic head groups interact with the aqueous phases on each side. Depending on the type of interactions they have with the

**Treadmilling:** New actin monomers are added to filaments facing the membrane at the front facing the membrane. Any old monomer appears to be moving backwards till it falls off due to enhanced depolymerization at the back.

**Myosin-II**: A motor protein (uses ATP hydrolysis to perform work) continuously tries to contract the actin network.

**Fluid mosaic model:** Describes the membrane as a 2D fluid composed of lipids and protein laterally distributed as a mosaic pattern and mobile.

membrane,proteins can be classified into integral, peripheral, or lipid-anchored proteins[47]. The distribution of lipids in themembrane can differ in the two leaflets, with cell types and due to continuous flow of membranes from one compartment to another[45, 48]. Reports show that the cholesterol content in the plasma membrane is larger than ~ 20 weight% in most eukaryotes[49]. We know from studies that while the plasma membrane of myelin sheath in humans contains 30% phospholipids, 30% proteins, and 19% sterol; those in mouse hepatocytes contain 27% phospholipids, 45% proteins, and 25% sterol[45]. Several membrane proteins (like membrane pumps, membrane channels, curvature sensing proteins, etc.) are believed to be implicated in membrane homeostasis[50–58]. Lipids can also dynamically partition into nanometric platforms because of cholesterol altering the lipid packing around it[59,60] and it is aided by short actin filaments[61,62].

Though lipids in biological membranes usually exist in a liquid-ordered state below 37 °C, increase in temperature can lead to a liquid-disordered phase in bilayers[63]. Lipids have appreciable lateral mobility[64] and can free diffuse laterally in a bilayer, about $10^7$ times a second[65]. The lateral diffusion coefficient of phospholipids/cholesterol mixtures has been estimated to be around 1–10 $\mu m^2/s$[66–68]. The lateral diffusion of membrane proteins can vary[45] and are much slower than lipids[27,69,70], both due to their size and due to their interactions with the cytoskeleton.

One of the major components of the cytoskeleton is the crosslinked tightly woven meshwork of contractile actomyosin, called cell cortex or cortical actin (Fig. 1), which is found underlying the plasma membrane. Actin treadmilling, the binding and turning-over of motor proteins and crosslinkers—sall together determines the structure of the actomyosin network[71]. Actin filaments of different lengths are created by the differences in rates of actin polymerization (Fig. 1b) and depolymerization at the two ends, the presence of optimum monomeric actin concentration along with ATP and other regulatory factors[72]. These filaments (~ 100 nm to microns in length in non-muscle cells[73,74]) interact with myosin proteins (Fig. 1c), which can themselves form mini-filaments, about ~ 300 nm in length[75]. The contractility in the network is brought about by the myosin motors walking on multiple actin filaments which induces compaction to the crosslinked (by proteins like α-actinin and filamin) network[76]. Depending on the method of measurement, degree of cell spreading, stage of cell cycle, and the type of cell, the average thickness of the cortex can vary ~ 50–400 nm[74,77,78]. The cortex is studied to be to thicker in low spread-out cells[77], and the thickness is more in de-adhered cells than in mitotic cells[77–79]. The mesh size of the cortex can also vary from ~ 20 nm in mitotic HeLa cells to ~ 300 nm in some interphase cells[71].

All actin-binding proteins undergo cycles of attachment and detachment from the meshwork at varying rates and adds to the dynamic nature of the cytoskeleton. With the help of several accessory protein, the actomyosin network can form a variety of structures, showing varied filamentous actin organization, in the cell[80]. The cortex is attached to the membrane with the help of certain cytoplasmic proteins. The ERM (ezrin, radixin, moesin) class of proteins[81,82] are known to attach to the PIP2 domains of the membrane on one hand and to actin filaments on the other hand (Fig. 1d). These proteins contain three conserved domains, an N-terminus FERM (Band 4.1, ezrin, radixin, moesin) domain, a central helical coiled-coil domain, and a CTD (C-terminal domain)[83]. Activation by PIP2 mediated uncoupling of the N- and C-terminal domains allows the inactive cytosolic ERM proteins to bind with the plasma membrane either directly or indirectly through membrane-associated proteins[84,85]. The myosin-1 family of proteins also mediate this membrane–cortex linkages by binding to actin filaments by their calmodulin-dependent motor domains and to acidic phospholipids on the membrane[86]. Studies report that based on the angle of polymerization, the membrane can extend by ~ 2.75 nm (Fig. 1b) with each polymerization event[87].

In the next section, we define membrane tension and discuss the different techniques used to measure it, to understand the role of membrane and its underlying cytoskeleton on membrane tension homeostasis.

## 3 Membrane Tension and Its Measurement

Over the years, it has been seen that several biological processes have been reported to be affected by membrane tension, namely membrane trafficking[88] (Fig. 1e), phagocytosis[89], cell motility[50,90], cell division, and cellular morphological changes[91], in cells. Even in vitro reports suggest that changes in membrane tension can affect clathrin binding[92] and polymerization of coat proteins[93]. But what is membrane tension?

**Lipid packing:** Lipids at the membrane are packed laterally depending on various factors like lipid–lipid interaction and temperature. If the membrane is under tension, packing can change.

**Polymerization:** Process of adding monomers to form polymer (filament). When enough monomers are present, and ATP is present, polymerization is favored.

**Contractility:** The ability to contract. For actin network, depends on myosin-to-actin ratio and needs the network to be crosslinked.

**Membrane trafficking:** The process of membrane transport to and fro the plasma membrane and membranous organelles in the cell.

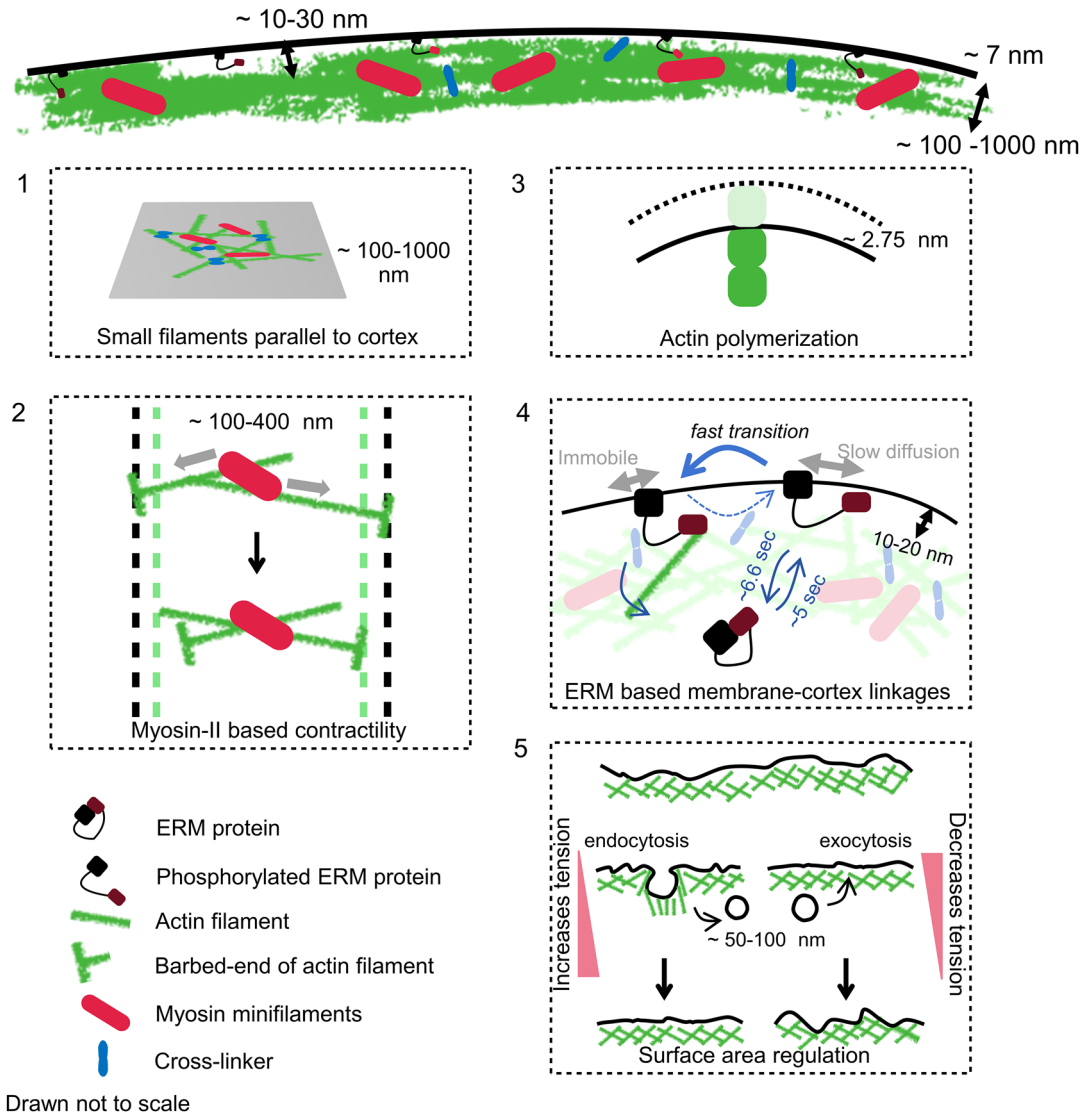


***Figure 1:*** Schematic representation of the organization of submembranous actin network under the membrane. Membrane thickness (~ 7 nm), typical cortex thickness (~ 100–1000 nm), and expected gap between cortex and membrane (~ 10–20 nm) are indicated. Box 1 shows how filaments are arranged parallel to the plane of the membrane at the cortex with size of small filaments expected ~ 100–1000 nm. Box 2 shows how a myosin-II minifilament (~ 100–400 nm long) leads to compaction (initial black dashed to green dashed) of the actin network by binding simultaneous to two different actin filaments using its two ends and walking towards barbed ends of both the filaments. Box 3 shows a zoomed-in schematic of extension of the membrane brought in by a new monomer (shown in light green, ~ 2.75 nm) being added on to an existing polymer of actin—a special case where the filament is normal to the plane of the membrane, like at the lamellipodia. Box 4 highlights a typical ERM family protein (Ezrin, here) adding on to the membrane in its phosphorylated form (and diffusing slowly). It quickly transits to an actin-bound state which makes it almost immobile. Box 5 highlights how the cortex may restructure during endocytosis (process that is believed to enhance membrane tension) and allows exocytosis (process that is believed to reduce membrane tension) due to its mesh like structure with typical sizes of vesicles being ~50–100 nm.

The pure lipid bilayer membrane at the molecular scale is a 2D fluid. It is believed to not-experience mechanical stress and be 'tensionless'[94].However, as they form closed structures like vesicles (or cells), they start experiencing tension—arising, for instance,due to the osmotic pressure differences between the outside and inside of thevesicle[95]. Increasing the pressure differenceresults in membrane deformations and increases tension. Pascal's law measurement of

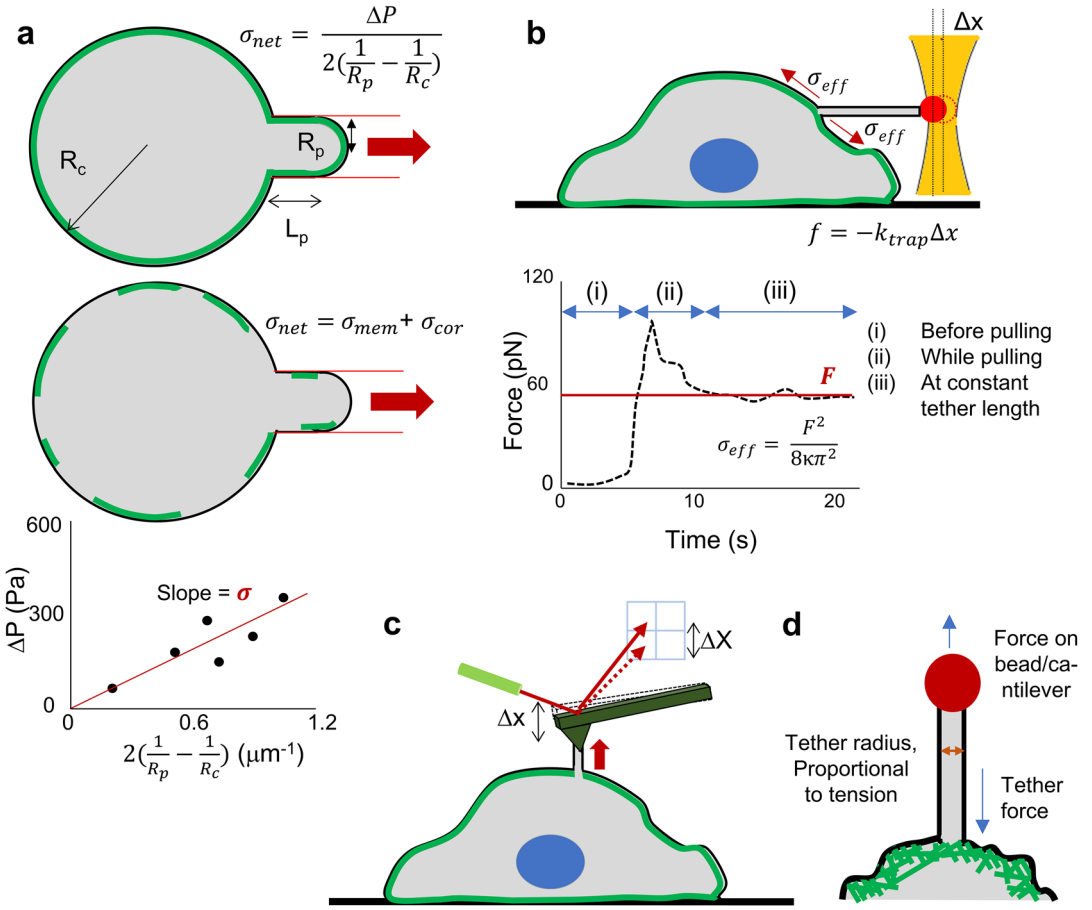


***Figure 2:*** Schematic representation of the invasive techniques used to measure membrane tension. **a** Diagram of micropipette aspiration where the membrane of a suspended cell is aspirated into the micropipette where pressure (P1) is applied, the pressure in the sample being, say P2. The pressure difference created ($\Delta P$=P2–P1), and the varying radii of the pipette and the object are used to calculate net tension. It is the summation of membrane tension and cortical tension. Membrane tension can only be calculated using this technique in the presence of a cytoskeletal drug which removes the underlying cytoskeleton and nullifies cortical tension. **b** Diagram of optical trapping where a latex microsphere (red) is used by a laser to manipulate the membrane of cells and extract tethers. The force calibrated from the trap stiffness ($k_{trap}$) and bead displacement ($\Delta x$) is used over time to measure the force at the plateau phase of tether pulling. This is used to calculate the apparent membrane tension, using a known value of bending modulus. The apparent tension is a summation of membrane tension and the contribution from membrane–cytoskeleton attachments. **c** Diagram of atomic force microscopy where a cantilever is used to extract tethers from cells. The force in this technique is calibrated by the cantilever stiffness and its displacement ($\Delta x$), using the reflected laser's displacement ($\Delta X$). Apparent tension is calculated as mentioned above. **d** A zoomed-in view of a tether being pulled by a bead in optical traps or by a cantilever in AFM where the membrane linkages are broken and membrane, devoid of cytoskeleton, is pulled into the tube.

deformations allow micropipette aspiration-based measurement of tension (Fig. 2a) in lipid vesicles[39]. Admittedly, cells are more complex structures due to the presence of the cytoskeleton, and measurements of the 'net' tension by micropipette aspiration have contributions both from the cytoskeleton and the membrane[96]. Such measurements are usually complemented with conditions under which the cytoskeleton is disintegrated and can no longer contribute to the net tension[97]. Typical net tension in the presence of the cortex in fibroblast cells yields values ~ 0.4 pN/nm, while it drops to 0.04 pN/nm in the absence of a contractile cortex[97]. Similarly, techniques utilizing nano-tether extraction using optical traps (Fig. 2b) or AFM (Fig. 2b) are based on Helfrich's elastic model of the membrane. They measure the force required to maintain a nano-tether (Fig. 2d) extracted out of vesicles or cells expected to depend on the membrane

**Tether:** A membranous tube (hollow cylinder surrounded by lipid bilayer), devoid of cytoskeleton, formed when beads or cantilevers pull out the membrane in vesicles or cells.

tension and bending rigidity. When performed on cells, due to the additional cost involved of breaking membrane–cytoskeleton attachments, tension derived from the force is termed as the "apparent membrane tension"[91,96,98].

In both micropipette aspiration and nanotether extraction, the tension can be thought as the lateral mechanical tension. To understand it, one can imagine a hypothetical boundary around a local patch of membrane[99] and define tension as the "force applied to the unit length of this imaginary boundary by the surrounding membrane in the direction tangential to the membrane plane". While tension extracted from MA experiments (based on Pascal's law) is consistent with this description, Helfrich's model includes tension as the energy cost per unit area of increasing local surface area. Note that Helfrich's model[100] has been extended to predict membrane height fluctuations and forms the basis of measurements utilizing fluctuation spectra to estimate tension[101–104] (Fig. 3a–c). Intuitively, enhanced tension should suppress transverse fluctuations[105]. It can be distinguished from other factors that also contribute to fluctuations like bending rigidity, cytoplasmic viscosity, and temperature due to the different frequency dependence of their effect on the amplitude of fluctuations[106]. In addition to lateral tension and fluctuations tension, the recent breakthrough of fluorescence-based measurement (Fig. 3d) of tension reflects the internal tension[44]. The fluorescent reporters bind to the hydrophobic region of bilayers and undergo changes in their fluorescence lifetime depending on the immediate lipid packing[44]. With such different possible routes of measuring tension, it is important to understand if these descriptions of tension are comparable with each other.

Studies address this by measuring the internal tension (dependent on lipid–lipid distance, thus related to real microscopic area occupied by a fixed number of lipids), lateral frame tension (manifested by changes in projected area for a fixed number of lipids), and fluctuation tension (derived from the fluctuation spectra) of the same simulated sheet of a fluctuating membrane[107]. The study predicts that internal tension deviates from lateral frame tension and fluctuation tension for very low tensions at which fluctuations are significantly high or the real microscopic area is $> \sim 3\%$ of the projected area. The lateral frame and fluctuation tension, however, match for a large (5 decades) window of tension[95,107,108].

Unlike lipid vesicles, cells have additional factors influencing measurements—which need to be properly accounted for the correct interpretation of measured tension. The cytoskeleton, per se, affects the membrane in many ways: it confines the membrane, the actin gel, and/or pinning of transmembrane proteins with the membrane increases the effective viscosity—and it can also impart direct forces[109]. Fluctuation-based techniques directly follow the movement of the membrane—and can hence be utilized to study the different effects of the cytoskeleton on membrane mechanics[102,103,109]. While direct measurement of membrane tension from tether-based measurement is difficult in cells, fluorescent reporters still are expected to reflect the internal tension. Tensional inhomogeneities are best measured by fluorescent reporters[110] or fluctuation-based techniques[103], although a recent demonstration of slow flow of membrane tension utilized two-point measurement of tension using optical trap-based tether extraction[111].

The next sections compare evidence accumulated from different techniques to summarize how different aspects of the actin cytoskeleton affect membrane tension regulation.

## 4 Effect of Actin Polymerization

Actin polymerization can impact membrane tension depending on the network organization. At the lamellipodia of keratocytes; for example (Fig. 4a), actin polymerization occurs almost normally to the membrane and thus maximizes the displacement of the membrane[87]. OT as well as FliptR measurements show tension to be enhanced at the leading edge in motile cells[110,112], showing the direct contribution of actin polymerization forces on membrane tension. In non-motile cells, most of the membrane is free of lamellipodia like protrusions and have an intact layered cortex[80,113]. In the presence of this intact cortex, actin polymerization occurs almost tangential to the membrane and does not contribute majorly to membrane displacement. Proper polymerization rates, however, ensure longer filaments, resulting in a stronger, well-crosslinked, intact cortex.

The timescales of polymerization/depolymerization events, however, are much smaller (these are faster events) than the timescales over which the effect builds up and manifests as functional states. Reports using speckle microscopy have shown that the polymerization velocities in cells

**Pascals Law:** Says pressure build-up in a closed compartment is uniformly felt at all patches in the boundary and pushes the boundaries creating tension that can counteract the push and bring the system of mechanical equilibrium. Tension (T) build-up at any part is dependent on the local curvature such that $T/R = \Delta P$.

**Optical Trap:** Focused beam of (usually) infra-red laser that strongly attracts di-electric particles (like micron-sized polystyrene spheres) to its center. Once trapped, particles can be moved around in the sample (of cells) by moving the beam around keeping sample fixed or moving sample around keeping beam fixed.

**Fluctuations:** Membranes incessantly vibrate due to being hit by surrounding water molecules which themselves incessantly move around because of thermal energy. Fluctuations can be shape fluctuations of membrane-to-coverslip height fluctuations for the basal membrane of adherent cells.

**Lamellipodia:** A very actin-rich projection at the front edge of a moving cell especially having branched actin network.

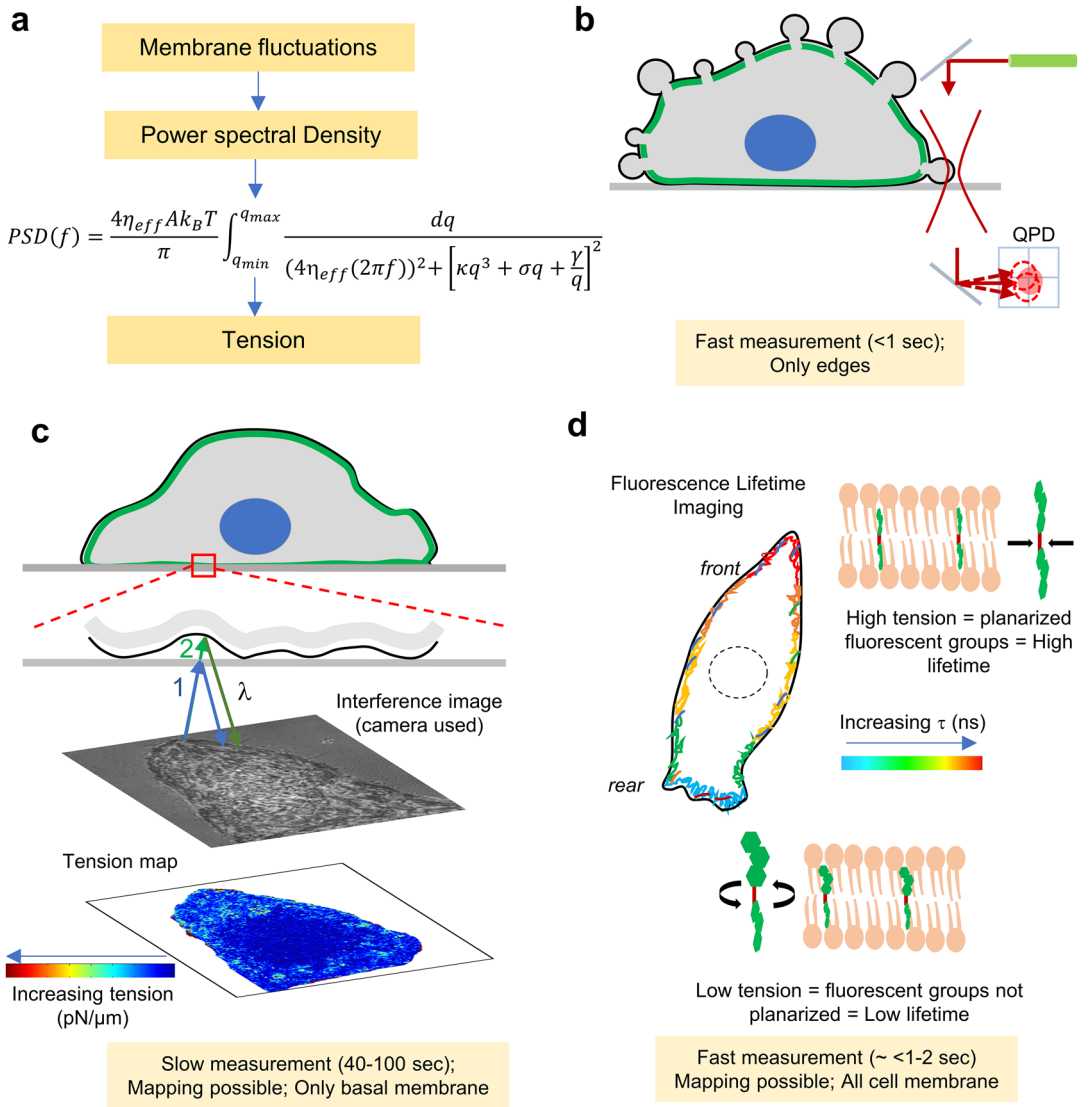


***Figure 3:*** Schematic representation of some noninvasive techniques used to measure membrane tension. **a** Flowchart to show that membrane fluctuations (used in techniques discussed in **b**, **c**) can be used to calculate membrane tension using power spectral density as suggested in the equation $PSD(f) = \frac{4\eta_{eff} A k_B T}{\pi} \int_{q_{min}}^{q_{max}} \frac{dq}{(4\eta_{eff}(2\pi f))^2 + \left[\kappa q^3 + \sigma q + \frac{\gamma}{q}\right]^2}$. **b** Schematic representation of an experimental design that uses a laser and a QPD to detect fluctuations at cell edges. The measurement is fast and has been reported on cellular blebs. **c** Schematic of interference reflection microscopy (IRM) where the basal membrane of adherent cells can be imaged due to interference of reflected rays. Light from a lamp source suffers reflection due to a difference in refractive indices. A camera is used for capturing interference images over time which helps to measure fluctuations and tension. IRM can create maps of tension and requires measurement over 40–100 s. **d** A cartoon of a migrating cell with front and rear end showing a gradient of membrane tension due to incorporation of FliptR, a molecular probe. The changes can be captured by fluorescence lifetime imaging (FLIM). The probe can detect tension changes because of the fluorescence lifetimes alterations created due to tension planarizing the fluorescent groups. Detecting tension differences by FliptR is a fast process which can used be used all over the cell membrane.

can vary from 7 nm/s in fibroblasts[114] to 170 nm/s in keratocytes[115], which amounts to actin monomer addition rate (perpendicular to the membrane) to be ~ 3–63 /s, since the displacement caused by each monomer addition[87] is ~ 2.75 nm. The rate of actin turnover, measured by FRAP (fluorescence recovery after photobleaching) studies, ranges from ~ 20 s in keratocytes[116]

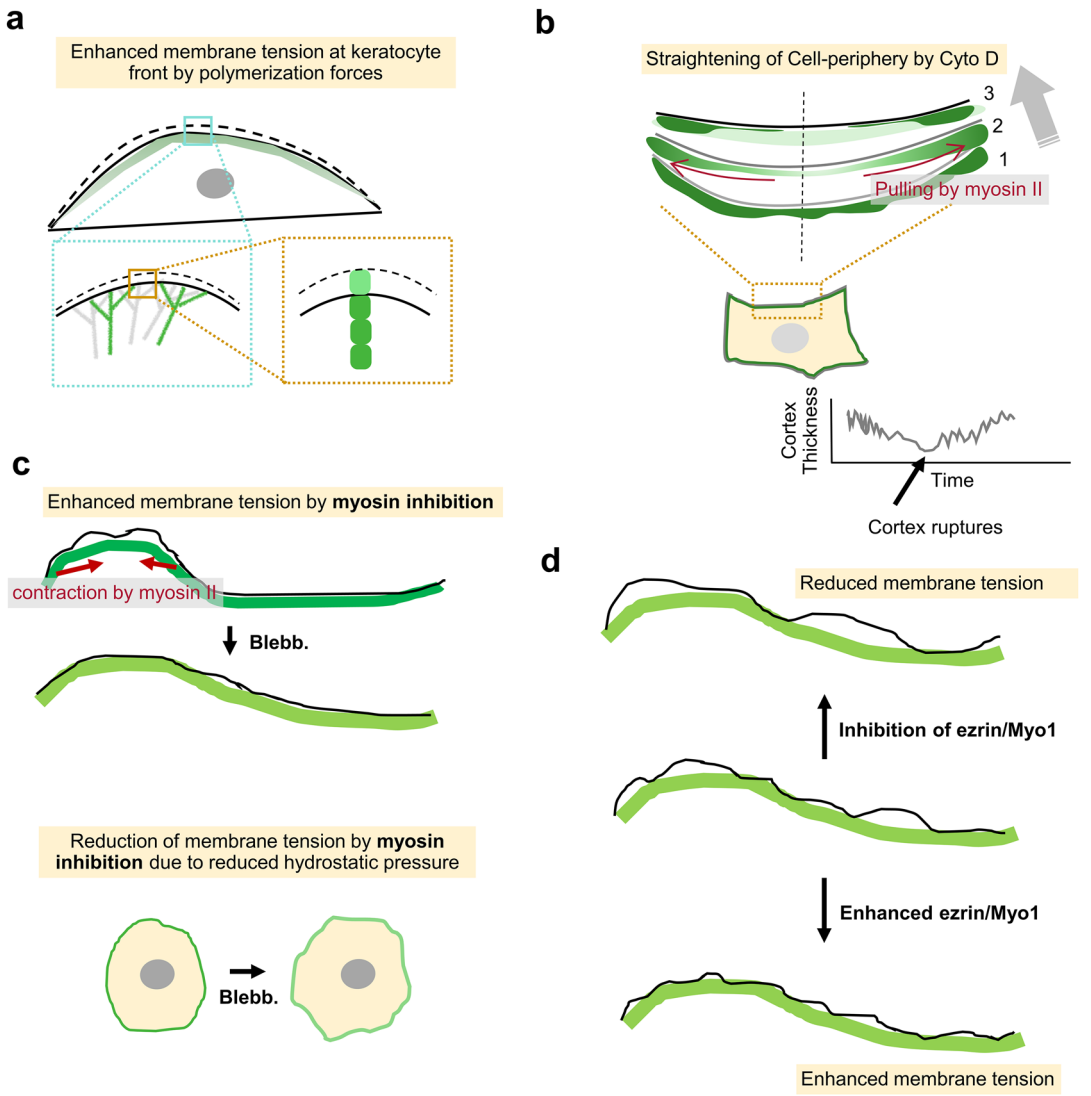


***Figure 4:*** Schematic representation to show how the cortex can affect membrane tension. **a** Effect of actin polymerization forces in motile cells: actin polymerization of the cortex at the lamellipodia in motile cells, like keratocytes, occurs normal to the membrane, thereby extending and 'taut' ing the membrane. This directly contributes to the enhanced membrane tension at the leading edges of migrating cells. **b** Effect of actin polymerization forces in non-motile cells: cytochalasin D treatment not only stops actin polymerization, but it also ruptures and clears the cortex due to myosin-II's contractile forces. Cartoon depicts a case where cell edge has been seen to straighten (and cortex thickness decreased) till the cortex ruptures. After rupturing the cortex is not sharp at the rupture point (dotted line) and thickness increases. **c** Effect of contractility: depiction of how myosin-II inhibition may cause an increase in apparent membrane tension, by preventing local curvature-based contractions, which clearly demonstrates the role of contractility to reduce tension during actin flow. However, inhibiting myosin-II can decrease hydrostatic pressure and reduce the apparent membrane tension. **d** Effect of membrane–cortex attachments: Cartoon depicts how reduced linkages may lead to a decrease in tether force, and increased attachments enhance apparent membrane tension.

to ~ 500 s in certain endothelial cells[117]. Reports suggest that cofilin enhances actin depolymerization at the pointed end by a factor of ~ 30[118]. Since turnover rates are a combination of uncapping rates and depolymerization rates, studies have calculated depolymerization rate to be ~ 3 s for 100 subunits long filament[119]. At the site of polymerization, the force is instantaneously felt. However, the time for the force to alter tension of the whole front (of a motile cell, for example) is currently debated. While instantaneous equilibration has been long believed[120], recent studies

report much slower flow of tension[111] (with an effective diffusion constant ~ 0.024 $\mu m^2/s$). Faster measurements of tension in live cells are therefore required to understand how tension is affected by actin dynamics.

To understand its importance in maintaining membrane homeostasis, we next discuss experiments that measure the new membrane tension inhibiting actin polymerization in non-motile cells by drugs like Cytochalasin D (Cyto D) or Latrunculin A/B (Lat A/B). It needs to be noted that often such treatments not only prevent polymerization of actin but also result in a myosin-II-based clearing of the cortex[103,121], leaving the membrane bare in certain regions while accumulating actin in others. Cyto D has been shown to cause a straightening of the cell periphery (cortex + membrane) together with thinning of cortex[77] at the central section till it ruptures (Fig. 4b). The heterogenous nature of cortex is amplified on drug treatment, such that some regions are weakened more and pulled by myosin-II-based contractile forces. The progress of this process may be different in different cell types.

The apparent tension or net tension has been reported to decrease in drug-treated cells[89,97,120]. Results of net tension are expected, since the dissolution of the cortex results in a significant reduction of the massive contribution of membrane–cytoskeleton attachments which tether extraction forces sense. However, whether membrane tension also changes is unclear from these studies.

Fluctuation-based force spectroscopy experiments are extremely sensitive to membrane movements. However, even in these studies, conflicting results can be found probably due to the use of three different systems—blebbing M2 cells[102], neurites[104], and HeLa cells[103], which have different effects of these drugs. Although the most consistent reports are those of reduction of tension on lowering polymerization rates, it is important to note that the report of enhanced tension (by Cyto D[103]) reports fluctuation tension, and pools data from local patches—and is therefore distinct from the other studies. A corresponding increase in amplitude of fluctuations (on Cyto D) found in the same study. Although the rise in mean tension accompanied by rise in mean fluctuations is non-intuitive, the matter is clear when instead of comparing two different systems, one compares regions in Cyto D-treated cells. Once the parameters of same regions are compared, the dependence of fluctuations on tension remains inverse. The study further corroborates this by experiments that use a pre-treatment ofBlebbistatin (Blebb.) to stop cortex clearance. On comparisons between Blebb. and Blebb. + Cyto D-treated cells, membrane tension is seen to significantly increase in the latter case. We believe that this is because the pressure load, although reduced by cortex dissolution, is now completely balanced by membrane tension—hence resulting in its enhancement. We hope that future studies implementing other non-invasive and local measurements like using FlipTr will resolve the mechanism.

MA-based measurements report membrane tension only when the cortex is disintegrated, and hence, do not contribute to the debate about the effect of the cytoskeleton's polymerization. Interestingly, ATP depletion studies also show similar discrepancies between OT-based measurements and fluctuation-based tension measurements. Note that ATP depletion is expected to drastically reduce actin polymerization as well as contractility of the cortex and does not clear the cortex[103]. While OT-based tension measurements show a huge drop in apparent tension[122], IRM on HeLa[103], and similar fluctuation-based study in RBCs[101,123] show an increase in tension. These studies emphasize that at the homeostatic state, the plasma membrane is actively maintained at a lowered tension state and increases when specific processes such as mitosis occurs[124]. To thoroughly understand the role of the cytoskeleton, mapping of tension must be further explored. Also, a combination of OT-based methods with fluctuation-based methods is needed to address the differences observed, for example, when ATP is depleted.

It is important to note that even controlled experiments with video microscopy of GUVs (giant unilamellar vesicles) have shown tension to be reduced on activating membrane pumps[125] and that micropipette studies on fixing tension have long reported ATP to enhance fluctuations and excess area[126]. OT-based tension measurements in such systems would resolve the origin of the conflict, because these systems lack membrane–cytoskeleton linkages which hugely offset tether extraction-based tension measurements. Although fluctuation-based studies report an increased tension (on ATP depletion) even after considering the enhanced fluctuations due to activity (factor A, Fig. 3a), whether the enhanced tension in cells is a misinterpretation due to the lack of incorporations of the actual effect of activity (like allowing for frequency dependence of A) should be definitely addressed.

**Cytochalasin D:** A widely used drug which perturbs the cytoskeleton by inhibiting actin polymerization.

**IRM:** Interference reflection microscopy sense distance of the basal membrane from the coverslip. It can be used as a technique to study adhesion patterns of objects and to measure membrane fluctuations/tension in adherent cells.

**Blebbing:** The process in which spherical protrusions, called blebs, are formed due to uncoupling the membrane and the cytoskeleton and the action of hydrostatic pressure.

## 5 Effect of Myosin-II Based Contractile Forces

**Hydrostatic pressure:** Outward pressure felt at the membrane when the compression of the cortex cannot compress the fluid in the cell. Fluid pushes back the cell periphery: cortex + membrane.

The contractile nature of the actomyosin network acting on the closed geometry of a cell results in creating hydrostatic pressure that is balanced together by cortical tension and membrane tension[99]. This pressure leads to the formation of natural blebs in cells when the membrane has appreciably detached from the cortex like in M2 cells[102,127], newt blastomeres[128], walker carcinoma cells[129], *Dictyostelium*[130], and zebrafish germ cells[130]. Fluctuation-based measurement in M2 cells have revealed that growing blebs have low tension initially, which increases during expansion[127]. Authors highlight that the effect is probably connected to the newly formed cortex. However, the experiment shows how contractility of the rest of the cell enhances tension of a patch of membrane that has lost its attachment from the cortex. Here, we point out that these high-speed measurements were possible due to the use of fast measurement of fluctuations—either QPD-based[102] or imaging-based[131].

**Spectrin:** A cytoskeletal protein which lies underneath the plasma membrane and is of utmost importance in erythrocytes, providing them with structure and membrane integrity.

Studies with RBCs are important to be noted here, where linkages of the membrane with the spectrin network have been reported to provide inward forces[132] due to the biconcave morphology and "soften" the membrane[101]. However, experiments on adherent nucleated cells reveal at first glance conflicting results. In line with results from RBCs, are measurements on migrating keratocytes. Myosin-II's ability to contract (and create inward forces on an imaginary frame) when inhibited by the drug Blebbistatin increases the tension at rear ends of these cells[120]—supporting the role of myosin-II in lowering membrane tension (Fig. 4c). However, similar OT-based tether extraction from other non-motile cells have revealed both an increase[50] as well as decrease[133] in tether forces (proportional to tension) on myosin-II inhibition. The decrease may be explained by the fact that on inhibiting myosin-II decreases the hydrostatic pressure[134,135] (without compromising cortex contractility completely) and results in a decrease in both membrane tension as well as cortical tension (Fig. 4c). The increase, on the other hand, may be expected in systems where curvature results in contractility causing local inward forces reducing tension. Inhibiting myosin-II in these cases would increase tension. We think that in cases where the hydrostatic pressure reduction is still shared by the still partly contractile cortex, myosin-II inhibition by Blebbistatin reduces tension. However, when the cortex and its load-sharing capacity is compromised, an increased membrane tension is observed.

**Blebbistatin:** A widely used drug which is known to inhibit myosin-II contractility.

Therefore, although conflicting, the kind of cell, the initial state of the cell (state of adhesion and hydrostatic pressure), and the level of inhibition together are expected decide how myosin-II inhibition affects tension.

The cortex can affect the membrane only, because it lies under the membrane. It stays there because of membrane–cortex attachments. We next discuss their role in membrane homeostasis.

## 6 Effect of Membrane–Cortex Linkages

Membrane–cortex attachments have been believed to be important for membrane tension, especially since they have been suggested to provide resistance to membrane flow into tubes during tether extractions[90]. In zebrafish progenitor cells, it has been shown that reduction of attachments by Ezrin or myosin 1b depletion results in reduced static tether force[136]. Studies have also reported that increasing membrane–cortex attachment by ezrin phosphorylation in lymphoblasts[137], adding PIP2 in epithelial cells[138], or by incorporating synthetic linkers in stem cells[139] lead to an increase in the tether force. Recent studies on HEK cells suggest that the reduced tether forces in epithelial cells in comparison to embryonic cells are due to naturally high expression of membrane–cortex attachments in embryonic cells[140].

The effects of perturbing the Myosin 1 family of proteins on membrane tension have also been explored. In line with ezrin studies, reducing attachments by myosin 1g in lymphocytes also reduces tether force[141]. Overexpression of each Myo1a, Myo1b, Myo1c, Myo1d, or Myo1e individually increases the tether force in NIH 3t3 fibroblast cells; however, overexpression of dominant-negative construct Myo1c decreases it[142]. Enhanced or reduced membrane-cytoskeleton attachments are expected to alter the tether force (and apparent membrane tension) as reported, since tether extraction in cells includes the cost of detaching these membrane–cortex linkages (Fig. 4d).

It remains to be understood if membrane tension is altered in a similar way. Fluctuation-based experiments/fluorescent tension probes may prove to be useful in this context.

## 7 Challenges and Future Prospects

Besides global changes, cellular processes may locally alter membrane tension—as expected during endo/exocytosis. Mechanochemical feedbacks are reported to be triggered by global

change in tension[143]. Are local perturbations to tension sensed and corrected locally? Tension controls many processes, ranging from cell–cell fusion, membrane–membrane fusion, motility, membrane trafficking, etc. Future studies need to evaluate the correlation of the local tension profile with spatial heterogeneities of actin polymerization, contractility, and membrane pinning, in addition to curvature sensing proteins. This will provide a platform to explore the role of tension in other phenomena like sorting of endocytic pathways, membrane fusion, etc. We believe that interference and fluorescence-based techniques are best suited toward these goals by providing the required spatial resolution in tension measurement and by allowing specific targeting of fluorescent tension probes to different intracellular organelles[144].

The challenges faced by fluctuation-based study is mainly to properly account for active forces. Although in certain systems (HeLa, CHO, and C2C12), a window of frequency has been found (0.01–1 Hz) in which activity alters fluctuations similarly across the band[103], it still needs to be characterized before interpreting fluctuations using standard Helfrich-based model to extract tension. Recent studies in RBC, for example, show frequency dependence at the above-mentioned band but a frequency-independent effect at frequencies >10 Hz[132]. The challenge of utilizing fluorescent probes of tension, however, is to account properly for the altered levels of lipid "order", and not tension in changing the fluorescence lifetime of the probes.

Despite the challenges, a holistic understanding of the role played by the cytoskeleton in membrane homeostasis will require such new directions of investigations—covering regulation of tension at a local level, in intracellular structures as well as in cells embedded in deep tissues.

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## Compliance with Ethical Standards

## Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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**Membrane pinnings:** A combination of intracellular membrane–cortex linkages and extracellular membrane–substrate adhesions, together giving rise to undulations on the membrane.

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**Fluorescence lifetime:** The time which a fluorophore takes in an excited state before it emits a photon and returns to the ground state. It's a measure used extensively in fluorescence lifetime imaging microscopy (FLIM).

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