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The third dimension of the actin cortex

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Abstract:

The actin cortex, commonly described as a layer of actin filaments beneath the plasma membrane, is beginning to be recognized as part of a more intricate and three-dimensional composite layer. In this review, we present an overview of recent research that unveils the three-dimensional architecture of the cortex. We emphasize key findings and address gaps in understanding its interaction with other structures, spanning from the outer plasma membrane to the cytoplasm. We also present an overview of the different techniques used to uncover the 3D architecture of the cortex. Furthermore, we draw attention to recent discoveries suggesting that actin-rich structures extending from the cell surface—outwards, such as filopodia, or inwards, such as stress fibers—can be considered as extensions of the cell cortex. This broadens our definition of the cortex, shifting from its simplified characterization as a thin, two-dimensional layer of actin filaments.

Introduction

The actin cortex, defined as a thin layer of actin filaments surrounding the cell, provides

structural integrity and regulates cell shape. Together with the plasma membrane – to which it is tightly linked through membrane-cortex linkers, it forms a composite material constituting the cell surface. The interaction between the cortex and various membrane-cortex linkers is vital for regulating cortical mechanical properties, crucial for successful division, migration, and development. Cortical dynamics and properties are extensively studied through newly developed approaches, including expansion microscopy, optical tweezers, AFM, cryo-ET and super-resolution microscopy (SIM, STORM, STED). This research has enabled the exploration of the three-dimensional architecture of the cell cortex, highlighting the relationship between structure, mechanics, and function. This review provides an overview of recent findings and the description of associated techniques used to visualize the cell cortex in three dimensions (Fig. 1), moving away from its traditional perception as a thin, two-dimensional layer.

Membrane-Cortex Composite Material

The plasma membrane is situated in close contact with the cell cortex and is tethered to it by membrane-to-cortex attachment (MCA) proteins, notably of the ERM family (ezrin, radixin, and moesin). The animal cell surface is therefore a composite, three-dimensional interconnected layered structure. ERM proteins play a vital role in organizing specialized membrane domains and locally altering the mechanical properties of the cortex [1,2] (Fig. 1A). However, the exact mechanisms through which they regulate cortex mechanics and structure remain unclear. A recent study highlighted that the membrane-cortex distance, regulated by the length of MCA-linkers, can impact cortex tension and thickness [3]. However, surprisingly, macrophages with all three ERM proteins knocked-out seem to show no changes in their migratory capacities or their cortical thickness and mechanics [4],

suggesting the presence of alternative MCA proteins in these cells.

Spectrin is another structural protein present in the membrane-cortex composite material; it forms a meshwork underlying the plasma membrane (Fig. 1A). The spectrin network in erythrocytes, devoid of an actin cortex, ensures mechanical integrity and adopts a triangular lattice arrangement [5]. Interest in the link between spectrin architecture and the actin cortex in other mammalian cells has only recently started to grow. Ghisleni *et al.* [6] first showed that spectrin and cortical actin in geometrically-confined fibroblasts accommodated spatially distinct plasma membrane territories – but cooperate to create a cohesive cortex in response to mechanical stimulation. They found that spectrin, in fibroblasts, is organized in erythroid-like lattices and periodic arrays. These arrangements can co-exist and a transition from one to the other can be mechanically induced [7].

In *C. elegans*, spectrin and the actin-bundling protein plastrin collaborate with each other to stabilize the cytokinetic ring – an extension of the cell cortex – during cell division [8]. The study emphasizes the collective effect of spectrin and plastrin, which differ in length (~80-200nm and ~9-12nm, respectively) as they connect the layers of the cortex-membrane composite material. Exploring the mechanism by which a variety of linkers interact within the cortex to alter its structure is an exciting area for further research.

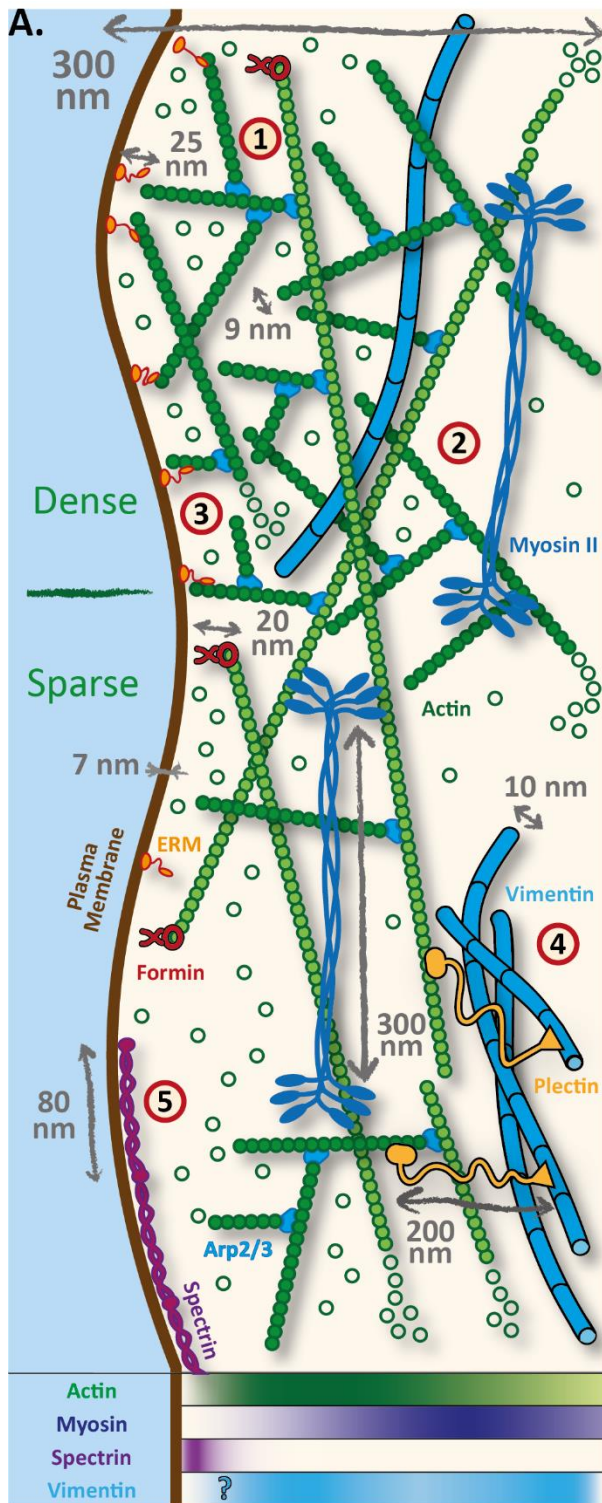
The 3D organization of actin and associated proteins inside the cortex

The cell cortex is primarily made up of an intricate meshwork of actin filaments, complemented by additional elements such as actin cross-linkers and myosin-II motors [Fig. 1A]. The various components are constantly undergoing turnover, occurring in a few seconds for actin crosslinkers [9,10] and hundreds of seconds for myosin minifilaments [11]. Cross-linker turnover is also influenced

by mechanical tension, contributing to the cortex's ability to sense mechanical forces [12]. Actin filaments undergo turnover in tens of seconds [13], as monomeric actin is polymerised at the plus ends of filaments and depolymerised at the minus ends. Joanny *et al.* [14] proposed a model that accounts for the polymerization at the membrane and depolymerization in the bulk to show, in the presence of internal stress, the existence of a cortex with a defined thickness. Arp2/3 and formins (e.g. mDia1) in concert with other regulators of actin polymerization kinetics, produce the bulk of the cortical actin, define its architecture, and also contribute to give to the cortex its considerable thickness [15,16]. Additionally, cortex properties are regulated by the RhoGTPases, which control the activity of Arp2/3, formins and other actin related players at the cortex as reviewed by Kelkar, Bohec and Charras [17]. The resulting dense and highly dynamic structure has been so far a challenge to image and resolve at the single filament level, preventing an 'in-depth' understanding of the cell cortex as a 3D object.

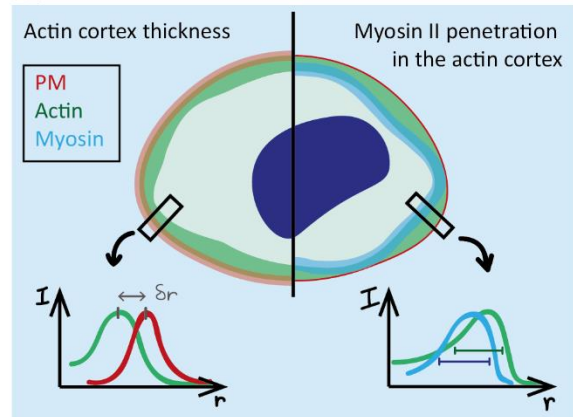
Techniques like super-resolution microscopy and cryo-EM have been widely employed to study the basal, adhered side of the cortical network of spread cells [18,19], which comprises a large variety of actin structures, such as stress fibers, in addition to the actin cortex. These methods allow measurements of 2D parameters like local orientation of actin filaments and mesh size, thanks to the availability of post-processing and segmentation pipelines for these imaging techniques [20,21] (Fig. 1D, E). 3D parameters like cortex thickness are more easily measured in rounded mitotic cells or cells plated on micro-patterns, which provide situations where cells have a clearly defined cortex spanning the whole cell surface.

The first precise measurements of cortex thickness were made using confocal imaging of fluorescently labeled actin and plasma membrane in live cells, followed by subresolution image analysis [22] (Fig. 1B). Cortex thickness is cell-type dependent but ranges around ~190nm in mitotic HeLa cells.

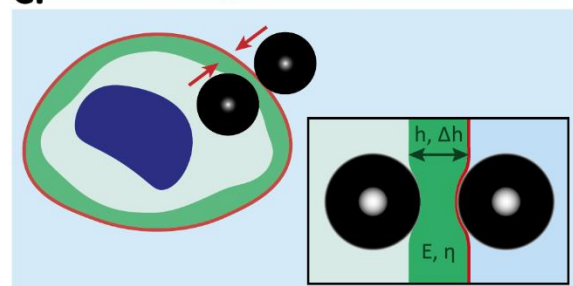


This study revealed that cortex thickness is important to regulate cell surface tension [23]. A similar imaging-based quantification of cortex thickness was also done by Clausen *et al.* [24]. Using dual-color super-resolution STED microscopy revealed that cortical actin density is asymmetric and measured the cortices of Jurkat T-cells to be ~ 230 nm.

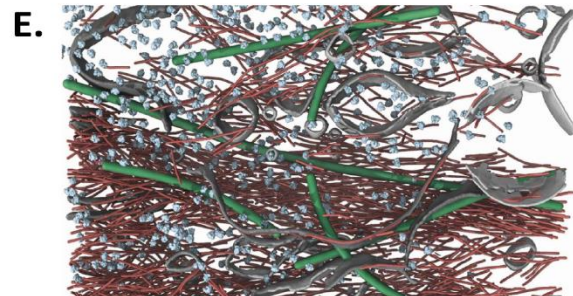
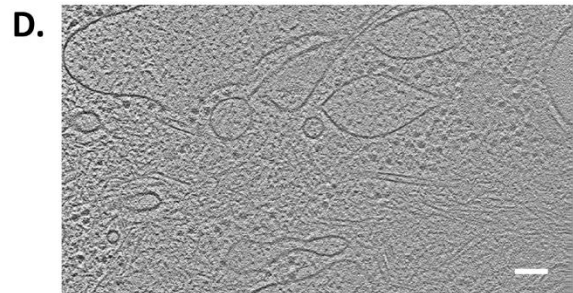
B. Fluorescence Microscopy



C. Magnetic Pincher



D. Cryo-Electron Tomography



An orthogonal approach to measure the cortex thickness is the magnetic pincher, a novel tool using superparamagnetic beads that 'pinch' the cell cortex [25] (Fig. 1C). It enables real-time measurement of cortex thickness in live cells with high precision. It revealed that the cortex is a very dynamic layer that undergoes myosin-dependent, large non-periodic fluctuations. The amplitude of

Figure 1 : Exploring the cortex 3D Architecture: Recent Results and Techniques. (A) Synthetic sketch of several cortical structures highlighted in this review. 1 – Two archetypes of actin filaments. Light green filaments: long, nucleated by formins (in red), slow turnover rate. Dark green filaments: short, nucleated by Arp2/3 (in light blue), higher turnover rate. 2 – Myosin II minifilaments (in dark blue) large size limits their penetration into the cortex, hence their contribution to cortical tension. 3 – The ERM proteins (in orange) tethering actin filaments to the plasma membrane are heterogeneously distributed and affect the width of the gap between the cortex and the membrane. 4 – Intermediate filaments such as vimentin (light blue) are an integral component of the cortex. In mitotic HeLa cells, a vimentin network forms on the inner side of the cortex, bound to actin filaments by plectin linkers (in yellow). 5 – A spectrin network (in purple) can coexist and co-organize with the actin cortex at the cell surface. Bottom part – tentative density distributions of four cortical proteins. (B) The plasma membrane, the actin cortex and myosin II minifilaments can be visualized as interpenetrating layers of fluorescent signal at the edge of the cell. Left side: the cortex thickness can be computed from the relative position of the fluorescence intensity peaks corresponding to actin and plasma membrane, using confocal microscopy in live cells. Right side: a similar technique to measure the distance over which the myosin II layer and the actin layer overlap, using dSTORM imaging on fixed cells. (C) Magnetic Pincher. A pair of superparamagnetic microbeads – one inside and one outside the cell – are magnetized thus attract each other, pinching the cell outer layer, mainly the cortex. The thickness h of the pinched layer and its temporal fluctuations Δh can be computed by tracking the bead on bright field images. At low force, this can be considered as a mechanical definition of the cortical thickness. The pinching force can be controlled to produce indentations of the cortex and further characterize its mechanical properties, such as its elastic modulus E and viscosity η . (D, E) Individual components of the cortex can be resolved using cryo-electron or super-resolution optical microscopy. D: Slice of a cryo-electron tomogram of the cortex. Scale bar 100 nm. E: Reconstructed 3D structure obtained from D, showing actin filaments in red, membranes and vesicles in gray, microtubules in green and ribosome in light blue. Reproduced from [21].

the fluctuations correlates with the cortex thickness, suggesting that thicker cortices are more “active”. These instabilities may favor thinning and rupture of the actin cortex, leading to bleb formation or cell protrusions.

Besides thickness, another important feature of the cortex that needs further exploration is the 3D local orientation of the constituting actin filaments. cryo-EM images of cells on micropatterns have shown that cortical actin filaments are oriented parallel to the plasma membrane [3]. This has also been observed in another study using a novel tool based on polarization microscopy to map the local orientation of actin filaments in a 3D cortex [26].

The spatial gradients of activity and concentration of myosin motors are known to shape cells during cell division, cell migration, and other morphogenetic processes within tissues. But, given the thickness of the cortical layer, there is not a lot of room for accommodating myosin minifilaments that are themselves ~ 300 nm long (Fig. 1A). Truong Quang et al. [27] explored the impact of the localization of myosin minifilaments in rounded mitotic cells, when surface tension increases. Their study reveals that during interphase, myosin minifilaments do not completely penetrate the cortex, resulting in a lower cortical tension (Fig. 1B). Conversely, in mitosis, there is a substantially larger overlap between cortical actin and myosin, leading to an increase in cortical tension. The restricted penetration of myosin II is hypothesized to be

a consequence of steric hindrance stemming from the small mesh size of the cortical network, which obstructs larger myosin minifilaments to penetrate into the bulk of the actin layer - the mesh size would change between interface and mitosis potentially due to a diminution in the density of actin branches, because of a reduced Arp2/3 activity in mitosis. This highlights how the 3D network structure of the cortex plays a role in regulating the internal stress and tension generated by myosin activity.

Interpenetration of other cytoskeletal components in the cortex

Microtubules are most often considered to occupy the cytoplasmic space [28] and only touch the cell cortex with their dynamic tips. During cell division, microtubules tips polarize the cortex, defining the division plane and aiding spindle elongation [29,30]. This picture is however too restrictive, and some cell types have abundant stable cortical microtubules. For instance, the cortices of differentiated epithelial cells in the epidermis are highly enriched with microtubules [31,32], suggesting a potential interplay between the two networks. However, the specifics regarding the localization of the cortical microtubule network in relation to the cortex, as well as descriptions of molecular or mechanical interactions, remain unclear. Future studies employing imaging techniques

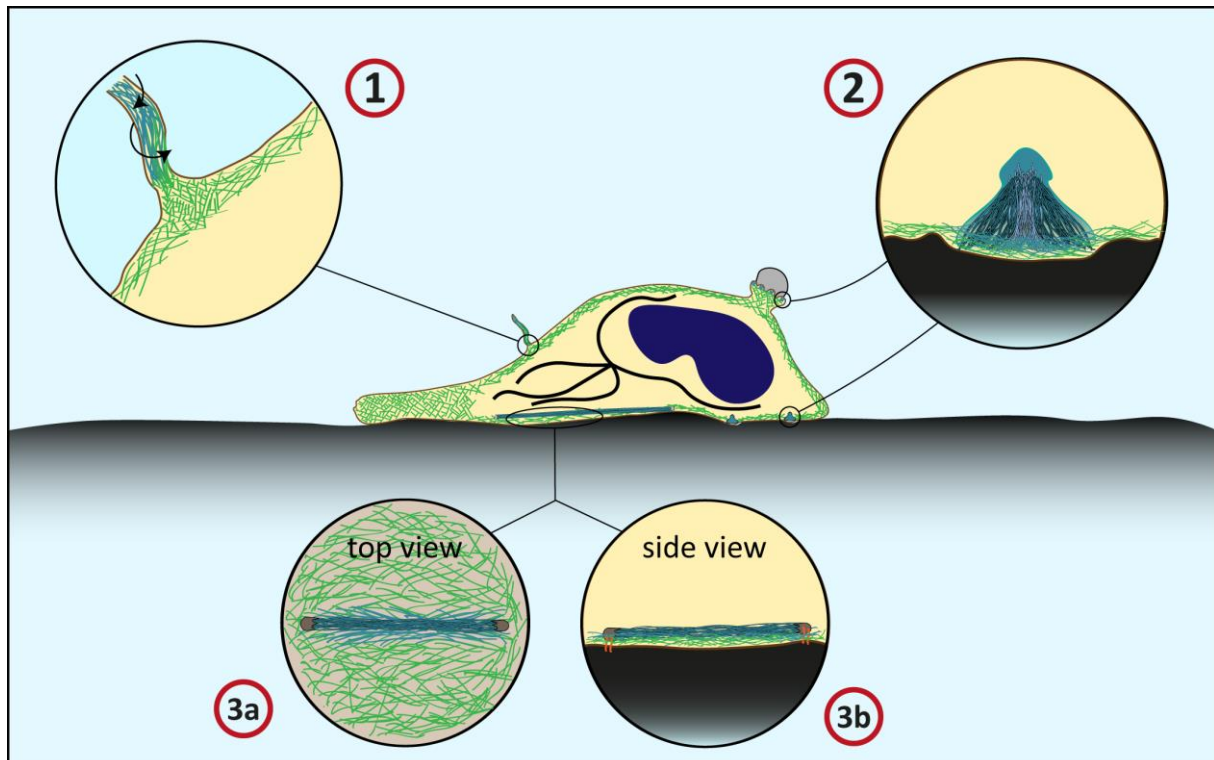


Figure 2: Actin-rich structures from the cell surface as extensions of the cortex in the third-dimension. 1 - Base of the filopodia emanating from the cortical network. While filopodial tips grow from actin polymerisation, its retraction is regulated by cortical flows experienced at the filopodial base. 2 – Lower actin core of adherent and phagocytic podosomes are embedded in the cortex as observed in Herron et al. (2022) [53]. Precise description of the actin organization at the junction between filipodia/podosomes and cortex still need to be defined. 3(a, b) - Stress fibers forming a mechanical continuum with the cortex as depicted in Vignaud et al. (2021) [38].

like super-resolution methods or expansion microscopy can shed more light on the presence and function of microtubules in the actin cortex.

Amongst intermediate filaments, the vimentin network plays a particular role in cell organization, being enriched in highly motile cells such as immune cells [33]. It has also been proposed to protect the nucleus [34]. Large quantities of VIFs have also been found to be present in the actin cortex of mouse embryonic fibroblasts, where they form an interpenetrating network together [35] (Fig. 1A). During mitosis, these filaments localize under the cell cortex, resulting in cortical thinning and increased cortical tension, contributing to mitotic rounding for successful division [36,37]. These results challenge the general view that the two networks are independent of each other in terms of structure and function.

However, there are still only a few studies asking how vimentin contributes to cortical mechanics. Guo et al. 2013 [38] utilized optical

magnetic twisting cytometry (OMTC), a technique applying torque to a magnetic bead attached to the cell surface to probe its mechanics. Their findings indicate that vimentin does not influence cortical mechanics, suggesting that either OMTC is not able to detect its contribution, or it is mechanically disconnected from the actin cortex. Alternatively, the actin cortex could dominate mechanics in the situation studied – the potential role of vimentin and other IFs in the cell cortex structure and mechanics remains an interesting question to investigate further.

Extensions of the cell cortex in the third-dimension

The precise definition of the 'actin cortex' remains a subject of ongoing debate, encompassing a wide range of interpretations and definitions. Positioned as the outermost

layer of the cell, the actin cortex plays a key role in sensing and responding to the surrounding environment. Its function exceeds merely providing structural integrity to the cell; it also contributes to forming structures that extend into the third dimension—outward into the cell's external environment or inward towards the cytoplasm. The extensive review on the actin cortex by Svitkina [39] also emphasizes the challenges in distinctly defining the boundaries of the cortex.

This section therefore aims to revisit various actin-rich structures connected to the cortex, with the goal of broadening our understanding and its definition to encompass its three-dimensional aspects.

Stress fibers were historically seen as separate from the cortex but connected to other stress fibers. It was recently observed that actomyosin bundles originating from the cortical network align with stress fibers, implying a transition from randomly oriented cortical filaments to increasingly aligned bundles near these fibers [40]. This study shows that the cortex and stress fibers actually form a mechanical continuum that helps in intracellular force-generation [Fig. 2]. Stress fibers can also spontaneously generate in the cortex triggered by stochastic myosin-II pulses [41]. These cortical stress fibers were observed beneath the nucleus of migrating mesenchymal cells. Their exact role is unclear, but they are hypothesized to offer protection to the nucleus during 3D migration.

Filopodia are actin-rich structures arising from the cell surface that are important for cell migration, sensing and invasion of matrices. They are around 100nm thick and contain around 10-30 actin bundles, cross-linked by fascin [42,43]. They have been observed to protrude not only from the lamellipodia, but also from other regions of the cell, including the cortex [44,45]. The extension-retraction dynamics of filopodia are regulated by the actin polymerization speed at the tip and a cortical retrograde flow at the base [46].

Leijnse et al. [47] demonstrate that the actin core within filopodia undergoes a spinning or twisting motion, concurrently with actin-polymerisation dependent growth at the tips. The two dynamics result in a complex set of behaviors including growth, shrinkage, helical buckling and traction force generation. This enables the filopodia to exert pulling forces of the order of tens of piconewtons [47] while actively exploring the three-dimensional extracellular matrix. The study models the twisting motion of filopodia as an active gel, which is also used to model the cell cortex [14]. In *Dictyostelium*, DdMyo7 is concentrated in dynamic regions within the cortex and along with VASP activity, initiates the formation of filopodia [48]. Delving deeper into the regulatory role of filopodia in local cortical organization could highlight the diverse range of mechanosensitive responses of the cortex [Fig. 2].

Podosomes are made of a core of slanted filaments surrounded by radial filaments. They have numerous functions, including adhesion and motility, and exist in various cell types, such as macrophages. A recent study used cryo-ET to obtain a 3D reconstruction of podosomes on the basal membrane of macrophages; the radius of the actin core was measured to be around 200nm with radial filaments of mean lengths 100nm [49]. The same study also showed that podosomes store elastic energy through dense actin filaments that are bent. As these filaments relax, the elastic energy is used to produce forces in the nanonewton range. Podosome-like condensates were also reported in the first actin cortices formed in fertilized *C. elegans* oocytes [50]. The formation of podosomes has been associated to the stabilisation of membrane-actin waves due to attachment of the cell surface to a substrate [51], a phenomenon demonstrated to reorganize a sparse cortex into denser networks in *Dictyostelium* [52]. These findings further emphasize the nature of podosomes as localized 3D extensions of the actin cortex.

Actin-rich puncta resembling podosomes were also recently discovered to appear on phagocytic cups [53,54]. It is well-known that the cortex contributes to phagocytic cup formation [55], but the actin architecture of the cup itself was unclear and was generally regarded as a homogenous network. These podosome-like structures are embedded in the cups, and in concert with myosin-II contractility, participate in producing normal forces on the particle during engulfment [54]. The 3D nanoscale architecture of F-actin in phagocytic podosomes was also uncovered using iPALM, TIRF-SIM and 3D-SIM on macrophages undergoing frustrated phagocytosis [56]. Interestingly, the study uncovered that phagocytic podosomes have an hourglass-like shape, measuring ~300nm in height, with two distinct actin cores enriched in cortactin positioned ~200nm apart. The lower actin core of phagocytic podosomes was embedded in a less organized, complex network of actin filaments, resembling the cortex [Fig. 2]. This reinforces the notion of podosomes as extensions of the cortex, making it that the cortex is a dynamic 3D structure, able to produce both lateral and normal forces, pulling and pushing, on the cell environment.

Conclusions and Future Perspectives

The recent studies of the cell cortex have revealed its 3D complexity and pivotal role in cellular mechanics and behavior. Moving beyond its traditional perception as a two-dimensional sheet, the cortex has emerged as an interconnected 3D structure. It interacts with the plasma membrane through ERM proteins and F-actin crosslinkers while engaging with cytoskeletal elements like microtubules and intermediate filaments. Consequently, the cortex forms part of a more intricate composite layer that mechanically and structurally adapts to sense and respond to its immediate environment. Studies

investigating the form and mechanics of diverse actin-rich structures extending from the cell surface, such as filopodia, phagocytic cups, podosomes, and stress fibers, provide substantial support for the hypothesis that these structures emerge from the cortex, form a mechanical continuum with it and can be considered as local specialized cortical extensions with a more organized structure and a specific set of functions.

Exploration into the three-dimensional organization of the cortex can be leveraged by advanced techniques like STORM, SIM, and STED, or expansion microscopy - offering high-resolution imaging for deeper insights on cortex architecture in 3D. Current efforts to combine live cell imaging and some of these superresolution approaches represent a promising way to additionally capture the dynamic nature of the cortex. An alternative way to investigate cortex dynamics can be found in novel biophysical tools like the Magnetic Pincher which recently revealed the fluctuating nature of the cortex thickness [23].

We summarize below the most important open questions we reviewed concerning the 3D nature of the cell cortex. The link between the actin cortex and other cortical components is a large area where more understanding is needed: the interplay with spectrin, intermediate filaments and microtubules for example is of prime importance to understand their contribution to the biochemical and mechanical regulation of the cell cortex. Steric exclusion of Myosin II from the actin cortex could be a new regulatory mechanism, in conjunction with the activity of small GTPases, for the formation of cortical contractile patterns and flows. Finally, understanding how filopodia, stress fiber, or podosome formation change the local cortical organization and mechanical properties would pave the way to redefine the role of the cortex in the integration of external mechanical cues.

CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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