



Intermediate filament mechanics *in vitro* and in the cell: from coiled coils to filaments, fibers and networks

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Intermediate filament proteins form filaments, fibers and networks both in the cytoplasm and the nucleus of metazoan cells. Their general structural building plan accommodates highly varying amino acid sequences to yield extended dimeric α -helical coiled coils of highly conserved design. These 'rod' particles are the basic building blocks of intrinsically flexible, filamentous structures that are able to resist high mechanical stresses, that is, bending and stretching to a considerable degree, both *in vitro* and in the cell. Biophysical and computer modeling studies are beginning to unfold detailed structural and mechanical insights into these major supramolecular assemblies of cell architecture, not only in the 'test tube' but also in the cellular and tissue context.

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'Nanofilaments': fibrous protein assemblies that comprise a major cytoskeletal moiety and the nuclear lamina

The fibrous intermediate filament (IF) proteins constitute the nuclear lamina network as well as a 10-nm-diameter filament system in the cytoplasm of metazoan cells [1]. Supposedly, they all originate from a common ancestor, most probably a kind of a 'primordial nuclear lamin' [2]. All IF proteins follow a common structural principle including a central α -helical 'rod' of conserved

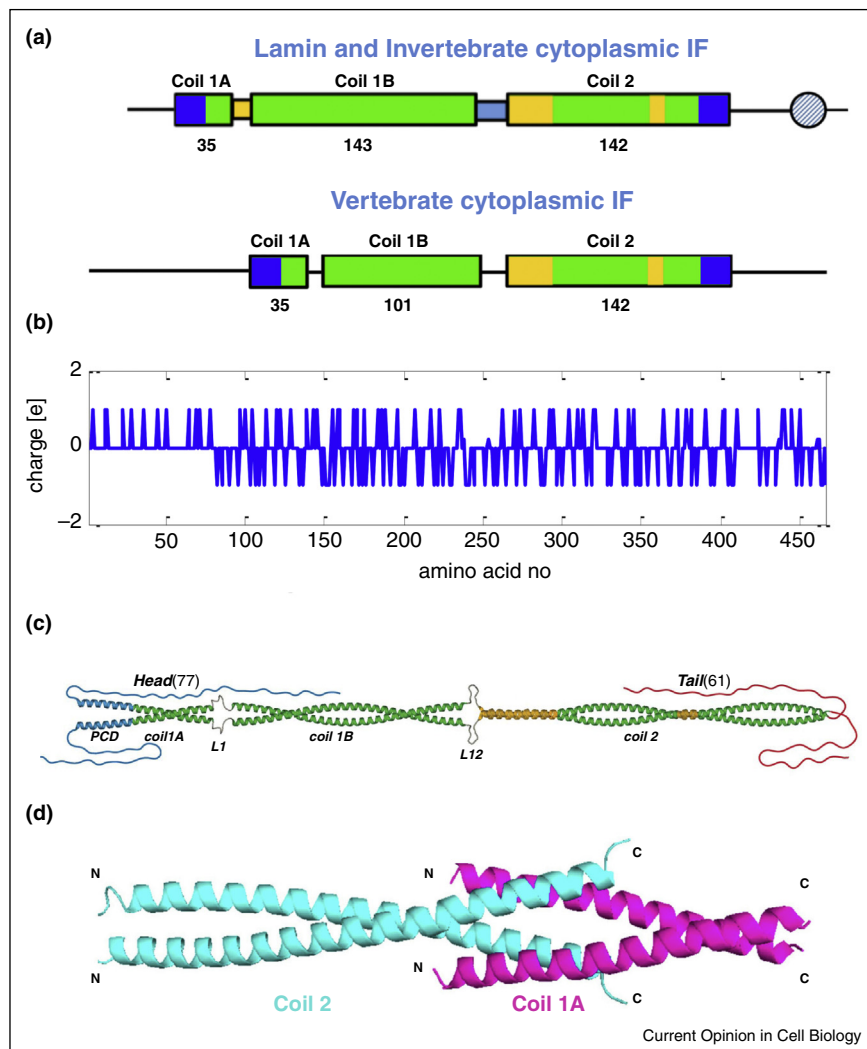
size that is flanked by non- α -helical N-terminal ('head') and C-terminal ('tail') end domains both of highly variable size [3]. The central α -helical rod domain is comprised of three segments separated by two linkers: coil 1A; linker L1; coil 1B; linker L12; and coil 2 (Figure 1a). All three segments exhibit a distinct pattern of charged amino acid clusters (Figure 1b) that are important for a given IF protein to assemble into higher order structures. In addition, a heptad repeat pattern of hydrophobic amino acids yields a 'hydrophobic seam' along the α -helical segments that mediates the formation of an unstaggered parallel coiled-coil dimer. This rod dimer is the basic building block of all IF-protein assemblies with an approximate length of 46 nm for the vertebrate cytoplasmic IF proteins and 52 nm for the nuclear lamins and the invertebrate cytoplasmic IF proteins (Figure 1c) [3].

IF proteins form filaments, fibers and networks

The dynamic nature of IF proteins reflected in the assembly process is accompanied by extreme stability; IF filaments are notoriously insoluble under physiological conditions and therefore have to be solubilized with chaotropic agents (e.g., 8 M urea or 6 M guanidine-HCl) to employ them for *in vitro* assembly [4]. In cells, IF structures retain this remarkable resilience, and contribute considerably to mechanical stability. Generally, individual IF proteins can be renatured without the help of chaperones into soluble complexes (e.g., dimers, tetramers, octamers) by dialysis into low ionic strength buffers. In fact, assembly already starts during reconstitution of the urea-denatured molecules in the course of lowering the urea concentration. For example, monomeric vimentin denatured in 8 M urea forms a coiled-coil dimer in 6 M urea, and a tetramer in 5 M urea. Further dialysis into low ionic strength buffers preserves the tetrameric state [5]. In these tetramers, two dimers associate laterally by their coil 1 domains in an anti-parallel orientation, thereby yielding apolar, approximately 65-nm long rod-shaped particles with tapered ends. These so-called A_{11} tetramers have been clearly visualized by electron microscopy of rotary metal shadowed specimens [5], and more recently by modeling the three-dimensional structure of a tetramer using the atomic structure of the vimentin coiled-coil dimer [6].

In a subsequent assembly step, lateral association of tetramers leads to unit-length filaments (ULFs), or

Figure 1



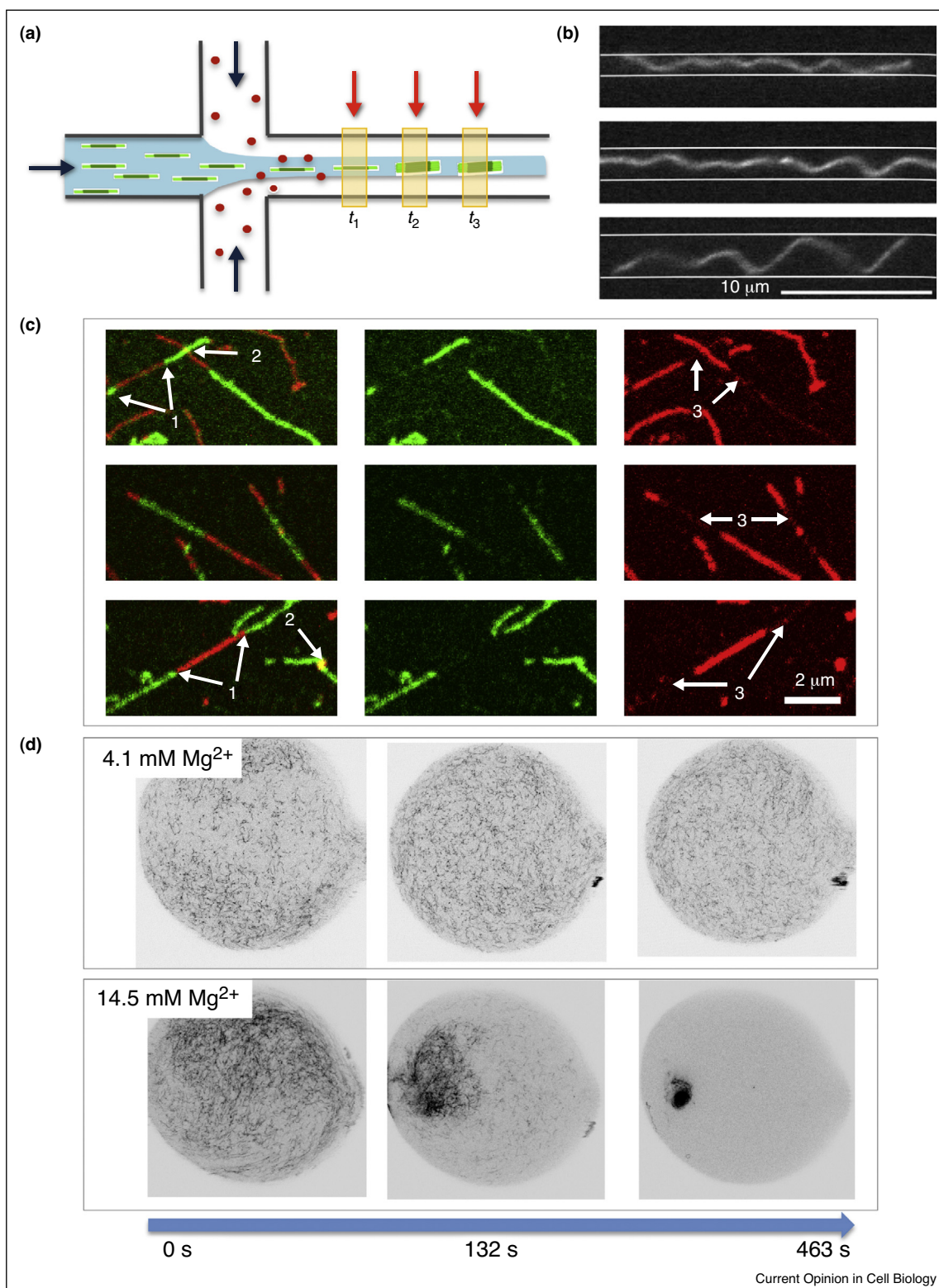
IF protein organization. **(a)** Domain organization of lamin A and vimentin as representatives for nuclear and cytoplasmic IF proteins: A central α -helical 'rod' is flanked by non- α -helical 'head' and 'tail' domains. Boxes represent amino acid sequence segments engaged in coiled-coil (green) or 'paired bundle' (yellow) formation; the IF-consensus motifs are indicated in blue. The 'linker' segments between coil 1A and coil 1B as well as those between coil 1B and coil 2 may be α -helical in the case of lamins, but are probably of unique fold in cytoplasmic IF proteins. The circle in the lamin tail represents an Ig fold. **(b)** Charge distribution of vimentin demonstrating the very basic nature of the head (12 arginines, no acidic residue) and the rather dense pattern of alternating basic and acidic charges along the rod; however, the total charge of the rod is very acidic (70 acidic versus 46 basic residues). The charge of the tail is slightly acidic (8 acidic versus 6 basic residues). **(c)** Model of a vimentin coiled-coil dimer. Vimentin-like IF proteins exhibit pre-coil domains probably adopting an α -helical fold (PCD) not seen in lamins and keratins (redrawn from Ref. [6]). Paired bundles are in orange, the 'linker' domains are designated L1 and L12. The numbers in brackets indicate the number of amino acids in the 'heads' and 'tails', respectively. **(d)** Potential hetero-coiled-coil formation exhibited by two coiled-coil dimers in the ~3 nm head-to-tail overlap region directly observed for lamins (for details see Ref. [13]).

'mini-filaments', of approximately 65 nm length [5]. These ULFs then further engage in an elongation reaction by longitudinal annealing of ULFs with one another and with already elongated filaments. In the focus of the molecular mechanism is the 'head-to-tail' association of the end domains of individual coiled coils (Figure 1d).

According to mass determination of individual ULFs and mature IFs by scanning transmission electron microscopy

(STEM), IFs can be highly 'polymorphic' with their mass-per-length (MPL) ranging between 20 and 60 kDa/nm along one and the same filament [5]. Indeed, this heterogeneity could potentially be of importance for the cell by providing a means to *locally* adjust the mechanical properties. This potential MPL heterogeneity of the ULFs has to be kept in mind when performing biophysical measurements, in particular when assembly is performed in a 'kick-start' mode rather than by a 'slow'

Figure 2



Vimentin *in vitro* assembly. **(a)** Vimentin assembly in a diffusive mixing device. Assembly buffer injected from the two side channels are mixed diffusively with vimentin tetramers (green bars) injected from the main channel. As a result of the increased ionic strength, higher order complexes and eventually unit-length filaments form (green blocks at t_2 and t_3). The red arrows indicate examples for measurement positions, by for example, X-ray scattering or fluorescence spectroscopy, which correspond to different time points in the assembly process (adapted from Ref. [7]).

process such as dialysis that generally yields more uniform filaments [5]. In addition to electron microscopy (EM) and atomic force microscopy (AFM), more recently the very rapid association of tetramers to ULFs has also been monitored '*in situ*' using microfluidic methods, that is, by employing a 'protein-jet' [7]. Here, one takes advantage of the fact that on a small length scale diffusive mixing is fast and the continuous flow maps the temporal progression of the assembly reaction on a spatial axis. Such an experimental setup is depicted in Figure 2a with the red arrows denoting different positions for measurements of the resulting protein complexes by X-ray scattering or fluorescence spectroscopy. After extended assembly times, long IFs are obtained that are about one order of magnitude more flexible than actin filaments and much more flexible than microtubules (Figure 2b).

By contrast to the cytoplasmic IF proteins, much less is known about the hierarchy of assembly steps that occur during the formation of nuclear lamin filaments, fibers and networks both *in vitro* and *in vivo*. For lamins, dimeric and not tetrameric complexes are obtained after renaturation both in low salt [8] and high salt [9,10] buffers. Starting from these two rather extreme *in vitro* assembly conditions, different structures are formed depending on the further assembly regime. In one scenario, several dimers first associate head-to-tail to a dimeric fiber of variable length [8]; in the next step, two such head-to-tail units associate laterally in an approximately half-staggered, antiparallel manner into apolar tetrameric protofilaments. Subsequently, they further laterally associate into IF-like structures of generally heterogeneous diameter. Under most conditions lateral association does not come to a halt at this stage but continues. At the endpoint of assembly, large needles exhibiting regular banding patterns with an axial repeat of 48–49 nm and also referred to as paracrystalline fibers, are formed [8–10]. Lamin paracrystals were originally considered to be artificial structures, because relatively high concentrations of divalent cations were used to generate them in a very regular organizational state suitable for high-resolution structural analyses [10]. However, paracrystals are the major assembly products when lamin A dimers are dialyzed from high salt conditions directly into physiological buffers [11]. These results correlate well with *in vivo* experiments employing the overexpression of A-type lamins in cultured cell systems, where at high cellular lamin expression large paracrystalline arrays were observed by electron microscopy both in the cytoplasm and the nucleus, probably because of a lack of cellular chaperones and other organizing factors [12]. Hence, for

lamins the formation of single filaments and the further association of filamentous precursors to paracrystalline fiber arrays are intimately coupled processes. In a somehow analogous manner, keratin IFs also further laterally associate into extensive fiber bundles under certain ionic conditions (see below).

Dissecting the molecular mechanism of the 'head-to-tail' interaction

According to the assembly scenarios presented above, for both cytoplasmic and nuclear IFs the principal interaction for elongation is the head-to-tail association of dimers as shown by the use of 'half-minilamins' [13]. These represent N-terminal and C-terminal fragments of lamins with a truncated central α -helical rod domain. Accordingly, the IF consensus motifs residing at either end of the α -helical rod domain (see Figure 1a in blue), including conserved sequence motifs of the flanking non- α -helical end domains, mediate the basic elongation reaction of two coiled-coil dimers. As illustrated in Figure 1d, this interaction may engage the *de novo* formation of parallel coiled coils between the C-terminal end segments of one coiled coil (in cyan) with the N-terminal end segments of the connecting coiled coil (in magenta). Alternatively, a lateral type of interaction of these two structural modules may occur. In both cases, adjacent short segments of the head and tail domain are important, as in the case of lamins both of them harbor phosphorylation sites for the protein kinase CDK1. Phosphorylation of these sites at the onset of mitosis leads to a disassembly of the lamina to the level of dimers. Since exactly the same amino acid rod end segments are conserved in all cytoplasmic IF proteins (see Figure 1a), the same type of longitudinal association of dimeric coiled coils might mediate the head-to-tail interaction of the cytoplasmic IF as well (see also Ref. [1]). It appears indeed to be this head-to-tail interaction that specifies what makes a structural protein an IF protein, and the occurrence of these two highly conserved motifs in newly found coiled-coil proteins, as revealed by genome sequencing of new species, may qualify the respective candidate as a potential IF protein [14]. Indeed, this criterion may serve as a suitable 'test' for inclusion of proteins into the IF family, especially with respect to organisms outside the vertebrates.

Excitingly, it has recently been shown using single-molecule force spectroscopy by optical tweezers that the C-terminal end of a parallel dimer made from two coil 2 segments considerably resists pulling forces. This observation argues that this highly conserved part of all IF proteins provides a stable interaction module with a

(Figure 2 Legend continued) (b) Individual vimentin IFs confined in microchannels. The width of the channels was 1.2 μm , 1.6 μm and 2.7 μm , top to bottom (adapted from Ref. [18]). (c) Subunit exchange along mature filaments. The overlay of the red and the green channel shows different modes of interaction between differently labeled subunits: 1 – end-to-end annealing; 2 – overlapping of filaments; 3 – exchanged subunits (adapted from Ref. [22]). (d) Impact of divalent ions on vimentin filament networks. Pre-assembled vimentin IFs were challenged with different concentrations of magnesium in microfluidic drops: above $\sim 10 \text{ mM Mg}^{2+}$ the networks aggregate strongly (adapted from Ref. [32]).

rather distinct structural design to allow proper longitudinal assembly by interacting with the amino-terminal end of coil 1A of a second dimer [15]. In stark contrast, the amino-terminal end domains of the coil 1A dimer have been demonstrated to be rather flexible indicating that their structural dynamics contribute to the ‘locking-in’ reaction of the head-to-tail interaction [16].

Mechanical properties and molecular architecture of single IFs

The persistence length of IF is in the range of a few hundred nm to a few μm [17,18] classifying them — in a physical sense — as semiflexible biopolymers. Thus, eukaryotes are equipped with three filaments systems spanning nm to mm persistence lengths. Confinement in microchannels has been used to measure the equilibrium persistence length of freely fluctuating vimentin IFs in solution, that is, unadsorbed (Figure 2b). Whereas the resulting bending rigidity is only one order of magnitude smaller than for actin filaments, the specific construction of IFs from laterally oriented fibrous molecules that strongly interact with each other results in the extreme extensibility of IFs, that is, they are highly stretchable, though possibly not in a reversible manner, which is not found for the other cytoskeletal filaments. Hence, experimentally, single IFs can be stretched by about 240% before they rupture [19].

Molecular dynamics simulations have shed some light on the mechanism enabling this extensibility, some of which has its origin in an α -helix-to- β -sheet transition [20] accounting for about 100% extension. Hence, another and even larger contribution must come from ‘subunit gliding’, that is, relative longitudinal movements of dimeric or tetrameric subunits against each other along the filament axis. As the lateral association of tetramers provides a multitude of single molecular interactions, both ionic and hydrophobic, the resulting complex system of binding activities enables IFs to withstand strong forces, and, upon being stretched along the filament axis, to re-associate laterally when pulled apart because of the periodic type of longitudinal structural design of IF. As demonstrated by an AFM-based nanomechanical approach, laterally bent desmin IFs exhibit a complex force–displacement curve showing a robust, strain stiffening-type behavior when extended above 50% [21]. Eventually, the ‘head-to-tail’ associations of the individual continuous dimeric coiled-coil strands within an IF are physically broken at increasing force and the filaments are irreversibly torn apart.

Despite the notorious insolubility of IF subunits, IFs do exchange subunits over time, although at a rather slow rate, *in vitro* amounting to about 1 tetramer per hour along a 1- μm -long filament stretch corresponding to 1 in approximately 200 tetramers [22]. Interestingly, the polymorphism of IF diameters influences subunit exchange:

evidently, polymorphic filaments are prone to a higher subunit exchange rate than filaments with a uniform diameter (Figure 2c). A possible reason for this difference is the fact that filaments with a non-uniform diameter harbor not just two but ‘many ends’ (that is, binding sites for subunits), thereby allowing subunit exchange all along the filament. However, the exchange scenario of IF *in vivo* differs significantly from the one encountered *in vitro*: in a cell disassembly is accelerated by phosphorylation reactions such that an entire IF network may disappear within minutes [23].

In vitro IF ‘superstructures’

IFs form bundles and networks

Like other filamentous biopolymers, IFs exhibit polymeric and polyelectrolyte properties [24^{••},25^{••}]. The interplay between their intrinsic mechanical bending rigidity and interactions between the filaments leads to the formation of superstructures. Distinct bundling and cross-bridging proteins such as plectin link IFs to one-another and to actin filaments and microtubules [26,27]. Hence, within a cell the three principal cytoskeletal filament systems — actin filaments, intermediate filaments and microtubules — do not exist in ‘splendid isolation’. Rather, they form cell type-specific ‘composite filament networks’ thereby combining different persistence lengths, extensibilities and surface charge densities. Hence, these composite filament networks are a major determinant of the mechanical properties of a cell and even more so of cell layers and tissues. Last but not least, as a consequence of the distinct surface charge patterns exhibited by IF protein monomers and polymers, even small cations such as Ca^{2+} , Mg^{2+} , Mn^{2+} , Gd^{3+} , mediate filament–filament interactions thereby causing the formation of IF bundles and networks.

Structure of IF networks

Despite their sequence homologies and similar monomer domain organization, different IF types yield distinct network structures. For example, vimentin and desmin IFs form networks of small mesh size and high connectivity, with the deformation of these networks being affine. By contrast, keratin IFs form bundles or fibers, and eventually these bundles produce sparsely connected networks [28^{••},29^{••}]. Rather than forming filament bundles, nuclear lamins assemble into filaments and paracrystalline fibers [8–10]. Moreover, IF proteins from these three groups do not form ‘hybrid’ assemblies, that is, vimentin-lamin, vimentin-keratin or lamin-keratin IFs, and hence have been divided into three separate assembly groups [30].

Direct imaging of filaments and higher-order structures is facilitated by fixing them in place. However, surface properties such as their charge may significantly impact the state of the filaments upon chemical fixation. Hence, an alternative is, for example, encapsulation of the networks in microfluidic water-in-oil drops thereby taking

advantage of the 3D-confinement, similar to the 3D confinement encountered in a cell. Furthermore, such a picoliter-sized environment can be tuned individually in terms of buffer, salt concentration and pH [31,32], parameters that play a vital role in the cell. In Figure 2d examples of vimentin networks in the presence of different Mg^{2+} concentrations are shown: accordingly, high divalent salt concentrations lead to fast condensation of the filaments into dense aggregates.

Physical properties of the networks

Rheology experiments have yielded much valuable insight into the mechanical properties of IF networks, in particular concerning the response of vimentin IFs to increasing mechanical strain: they are less rigid at low strain but ‘harden’ at high strain, a property called ‘strain-hardening’ (or strain-stiffening [33]). Following up these pioneering experiments of Janmey and colleagues, divalent cations like Mg^{2+} and Ca^{2+} have been studied in particular in combination with vimentin [32,34–36] keratin [28^{••},29^{••},37,38^{••}], as well as neurofilaments [39]. Under these conditions the IFs become cross-linked thereby yielding filament networks that display rheological properties similar to those found for actin filaments cross-linked by actin binding proteins (ABPs). A recent study on keratin IFs demonstrated that interactions via charge patterns on their rod domains, which are even stronger than those mediated by hydrophobic patches on their tail domains, give rise to distinct filament networks [38^{••}]. Notably, the addition of low concentrations of non-ionic detergents to assembling keratin K8 and K18 impaired strain-stiffening indicating that hydrophobic interactions of the tail domains, which are supposed to reach out of the filament body, mediate strain-stiffening of the networks at low shear. Accordingly, tail-less variants of IF proteins such as desmin do not exhibit strain-stiffening neither in the presence nor in the absence of detergent [40]. The importance of the tail domains in filament cross-linking has also been shown for vimentin very impressively with variants of progressive deletion into the tail domain [34]. Moreover, strain-stiffening of vimentin IFs depends strongly on the ionic strength as at medium ionic strength (50 mM NaCl) no strain-stiffening is observed whereas at higher ionic strength (160 mM NaCl) a robust response is recorded, even though under both assembly conditions the same storage modulus is reached. This behavior is in sharp contrast to that of desmin IFs, which exhibit a nearly identical strain-stiffening response under ionic strength both conditions (see Figure 6 in Ref. [17]).

Cell mechanics

Whole cell experiments

In the cell, the situation is much more complex than in the test tube due to a largely unknown environment, which also makes it much more difficult to unambiguously dissect whole cell scenarios. Despite these challenges, it has been found important to approach the

problem ‘bottom-up’, as described above, and ‘top-down’ looking at cell experiments, and to eventually combine both complementary approaches.

To test the contribution of individual proteins to the deformability of cell components such as the nucleus or of whole cells, various methods have been employed: magnetic beads bound to surface receptors to pull on the cytoplasm, AFM cantilevers for indentation experiments, microaspiration with glass capillaries for nuclei, ‘cell stretching’ of adherent cells on silicone membranes as well as of suspended cells by lasers, and microfluidic channels to ‘squeeze’ whole cells [41,42^{••},43^{••},44,45].

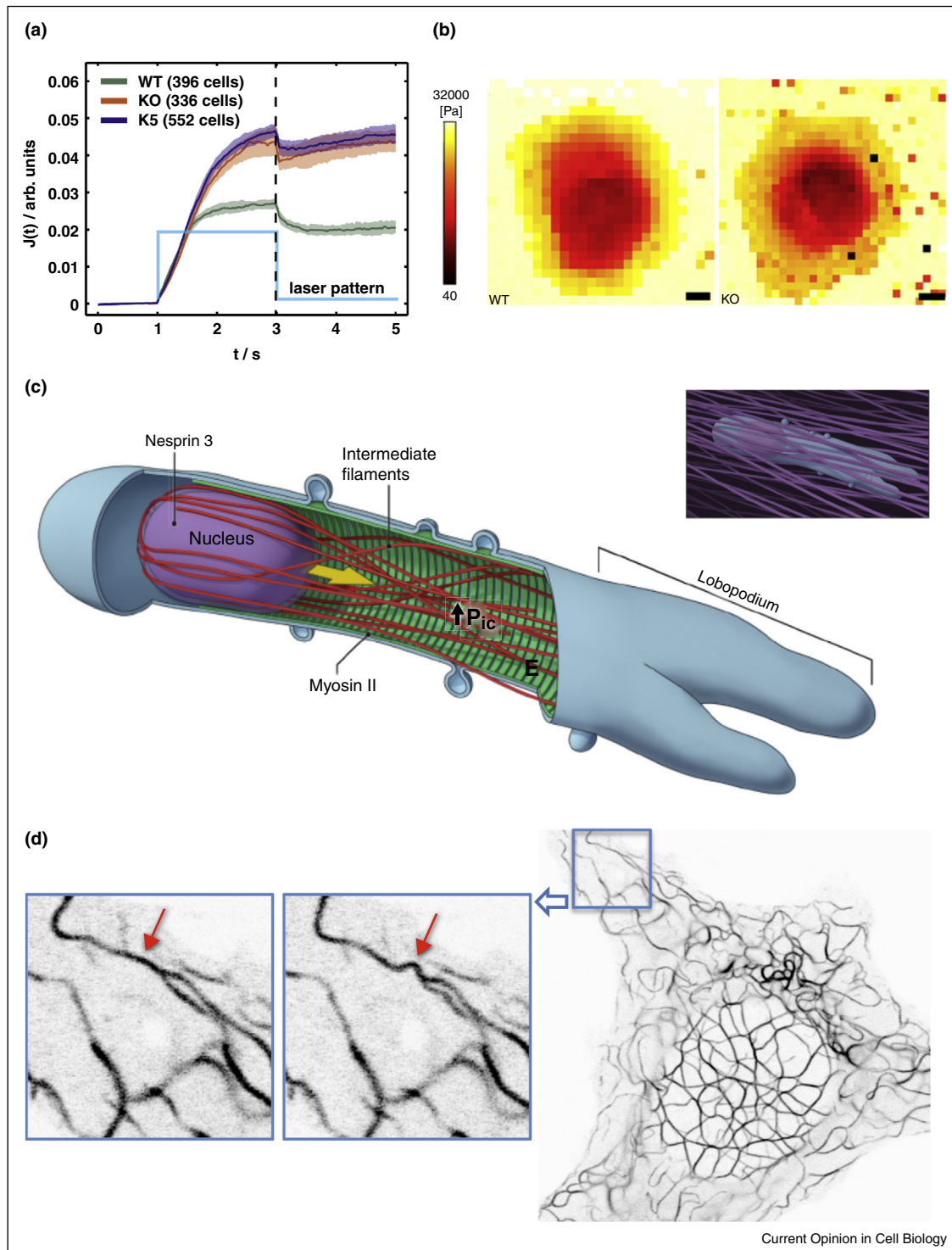
Keratinocyte mutants with the keratin network removed show a much higher deformability than the corresponding wild type cells, as consistently shown using optical stretchers [46^{••}] (Figure 3a) and AFM [47^{••}] (Figure 3b). Vimentin, as well, contributes the deformability of cells as shown by microrheology [48^{••}].

Cells on the move

Cultured fibroblasts are a classical object of cell biology to study cell locomotion. They constantly alter their shapes as they form both leading edge lamellipodia and trailing edge ‘tails’ during locomotion on flat substrates. These shape transitions reflect changes in the viscoelastic and mechanical properties of the cells. Recent studies of resting versus moving fibroblasts have shown that upon stimulation of the cells by serum, vimentin filament arrays are decomposed in regions where lamellipodia form, yielding large numbers of IF ‘particles’ as defined by fluorescence microscopy, that is, probably both short IFs and ULFs. These latter structures are evident within the active ruffling membranes that are the hallmark of the advancing leading edge powered by the actin cytoskeleton [49]. The organizational changes in vimentin IFs appear to be caused by site-specific phosphorylation of vimentin. Furthermore, the microinjection of a vimentin coil 2 peptide, shown to depolymerize mature IFs into non-IF structures *in vitro* [50], also induces the rapid disassembly of vimentin IFs in live cells and the formation of lamellipodia. Hence, vimentin IFs and cell locomotion are coupled in an interdependent regulatory network. Accordingly, vimentin IFs play an important role during the epithelial to mesenchymal transition (EMT). Epithelial cells typically exhibit complex IF networks of keratins but no vimentin. The expression of vimentin in epithelial cells results in their rapid transition to a mesenchymal shape combined with a significant increase in cell motility and focal adhesion dynamics [51].

In this context, a new twist in the concept of how cells move confined in three-dimensional matrices has revealed that vimentin filaments cooperate with the actomyosin-system to establish a pressure gradient within the moving cell. In this process, the IF-stabilized nucleus

Figure 3



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In vivo IF mechanics. (a) Data from optical stretcher experiments show that wild type keratinocytes are less deformable than keratin knockout cells ($J(t)$ shows creep deformation; from Ref. [46**]). (b) Atomic force measurements; shown are stiffness maps of a live wild type keratinocyte (left) and a keratin knock-out cell (right); scale bar $10\ \mu\text{m}$ (from Ref. [47]). (c) Pressurization of lobopodia by the 'nuclear piston'. (Inset) Migration of primary human fibroblasts in a 3D extracellular matrix. (Main figure) Nesprin 3 connects the nucleus via plectin to intermediate filaments and actomyosin contractility. These connections help pull the nucleus forward to pressurize the forward cytoplasmic compartment and sustain high-pressure lobopodia-based 3D motility; from Ref. [52**]. (d) Buckling event in a network of keratin bundles in a SW13 cell stably transfected with CFP-K8 and YFP-K18. (Left) two time frames, 2 s apart, showing the buckling event. (Right) marked ROI (region of interest) as shown on the left hand side [55].

serves as a piston to push cells through the matrix via lobopodium formation (Figure 3c). Here, already single IFs and not their networks may indeed serve as mechanical stress-absorbers [52**].

Networks and bundles within cells

Interestingly, the absence of vimentin in fibroblasts does not influence the generation of intracellular forces as determined by force spectrum microscopy [53**]. These novel results suggest that vimentin IFs have little influence on intracellular force generation, despite their — at the molecular level still only vaguely defined — role in cell mechanics. Different from vimentin, keratins form thick bundles both *in vitro* and in cells. These bundles display considerable dynamics, which are myosin motor dependent, confirming the strong linkage to the actin cytoskeleton. When the cells are exposed to external shear forces, the dynamics decrease, hinting at a ‘protection mechanism’ of the cells against potentially harmful forces [54]. Looking more carefully at the bundles, which are on a higher hierarchical level organized in a network of bundles, they show frequent short wavelength buckling events and much stronger bending than the high persistence length of the bundles would predict [55] (Figure 3d). One interpretation of this phenomenon is a cellular response to compression, another possibility is that the actomyosin network pulls locally on keratin bundles via plectin linkages.

Conclusions

In most metazoan cells, the three cytoskeletal filament systems form networks that are linked to one another by so-called cytolinker proteins, thereby allowing ‘cross-talk’. This cross-talk, in turn, provides the link that is necessary to couple cell mechanics, in particular cell stiffness, deformation and stability, to intracellular transport and cell locomotion. Thus, we would like to stress the nanocomposite nature of the cytoskeleton as a whole. This sophisticated architecture allows the cell to fine tune its mechanical properties in a dynamic and local way to support movement, cell division, organelle placement and cellular integration into tissues. Since IFs are apolar structures, they are probably not directly involved in intracellular transport and cell locomotion. However, for both cells and tissues they are a primary determinant of stiffness and deformation, and these properties together with cell motility are very important for basic processes such as cell invasion and hence metastasis. Moreover, the IF network provides intracellular stabilization connecting cell adhesion structures with organelles such as mitochondria and the nucleus. IFs can withstand rather large strains, thereby helping cells to respond locally to mechanical insults that otherwise would lead to significant deformations. Last but not least, their contribution to manage mechanical stress at the tissue and organ level is obvious from the deleterious effects of human IF disease mutations, resulting in one of the many so-called

IF-pathies [56]. For example, a point mutation in the muscle-specific IF-protein desmin destroys the sarcomere arrangement in myofibers as a result of the continuous mechanical load exerted on the cells by the beating muscle. As a consequence, this disturbance ‘translates’ over time into the fatal reorganization of a whole organ, that is, the heart, as has recently been directly demonstrated by a knock-in approach of the mutated gene in mice [57].

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