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# Reconstituting the dynamic steady states of actin networks in vitro

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Despite the constant renewal of their components, cellular actin networks maintain their overall appearance, through a subtle balance of filament assembly and disassembly. This balance is key to the remodelling of cellular architecture. We discuss the significance of in vitro reconstitutions in deciphering the complexity of actin regulation.

Reconstitution approaches can provide valuable insights into cellular complexity. In this Comment, we discuss how these methods have been used to help to determine the molecular processes involved in regulating the dynamic steady states of actin networks.

## Dynamic steady states

**Actin network dynamic steady state.** All living systems, at all scales, from whole organisms to intracellular organelles, are driven by a constant flow of matter. Unlike inert matter, which is maintained by the strength of the bonds between its components, living systems are made up of weak bonds, and owe their lasting appearance to the active breaking of these bonds and the constant renewal of their components. They are therefore in a so-called dynamic steady state (DSS), as their appearances remain identical, but they are supported by the constant turnover of their building blocks.

In cells, the actin network is also in a DSS. Actin filaments assemble along the plasma membrane and form different structures in specific subcellular regions (Fig. 1a). All of these structures are constantly renewed. In each structure, the rates of assembly and disassembly result in a turnover of actin subunits, whose dwell times range from seconds for lamellipodia or filopodia to minutes for stress fibres and hours for microvilli[1]. Notably, for the network to be in steady state, the average rate of assembly of actin filaments in all subcellular structures needs to match precisely the average rate of filament disassembly[2]. A small mismatch would eventually lead either to cell extension and fractionation in the case of excessive assembly, or to cell retraction and collapse in the case of excessive disassembly. There might be a transitory imbalance, but this phase must be quickly compensated so that the network returns to its basal state.

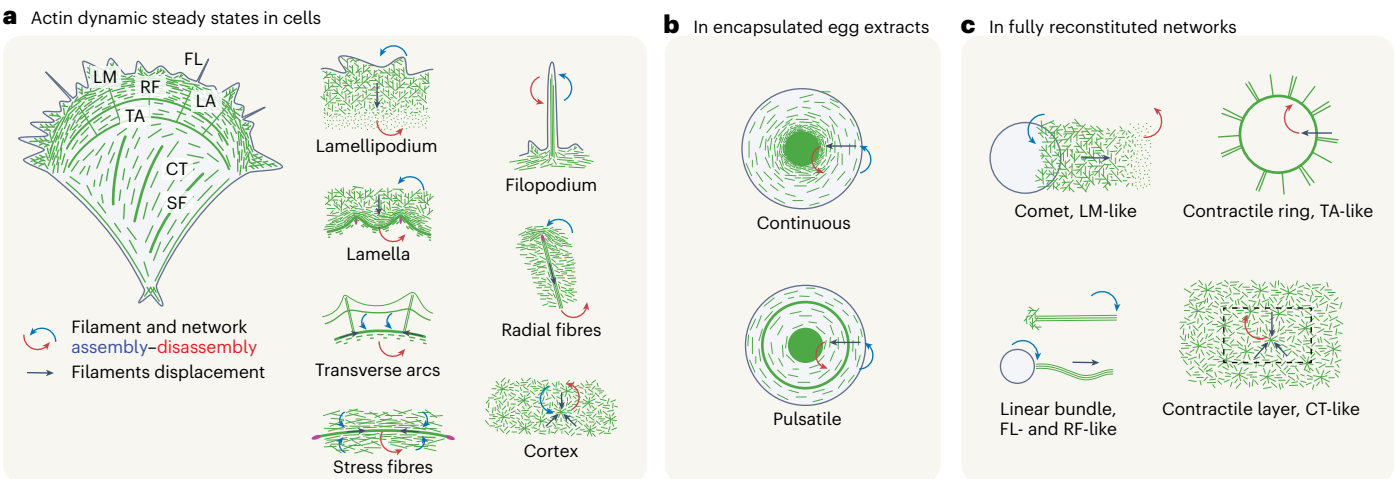


**Fig. 1 | Dynamic steady states of actin networks.** a, Schematic representations of subcellular actin modules in cells. The actin network can be divided into the lamellipodium (LM), lamella (LA), filopodium (FL), transverse arc (TA), radial fibre (RFs), stress fibre (SFs) and cortex (CT). Curved arrows show sites of filament assembly or incorporation (blue) and disassembly (red). Straight arrows show the flow of filaments in the module (black). b, Schematic representations of actin network DSSs in encapsulated egg extracts. Filaments assemble at the periphery and disassemble throughout the network. Myosin-based network contraction can generate either a continuous (top) or a pulsatile (bottom) inward flow of filaments when assembly and disassembly do not match perfectly. c, Schematic representations of attempts to reconstitute a module DSS. Comets, which are LM-like networks, are not robust and need to be tuned to display a genuine DSS. Linear bundles assembled from formin-coated beads or by formin-based elongation of filaments from branched networks, aimed at resembling FLs or RFs, only elongate, and do not display a DSS. Contractile rings (TA-like networks) are initially assembled on micropatterned networks of branched filaments, and disassemble as they undergo actomyosin-mediated contraction. Contractile layers formed of stabilized filaments – which resemble the random, thin layer of a CT-like network – can be forced to contract in a specific region (dashed frame). So far, contractile rings and layers can undergo a single contraction step only, and do not display a DSS.

The mechanisms that balance the rates of assembly and disassembly must be complex, as the two processes are regulated by distinct molecular machineries[1]. It is likely that there is feedback between assembly and disassembly that ensures their balance, but the underlying mechanisms are largely unknown. Cells would have difficulty surviving conditions that alter the maintenance of actin DSS, highlighting the importance — but also the challenge — of studying this mechanism. Moreover, it is likely that compensatory mechanisms would kick in if the system were to deviate from steady state, making this difficult to study in living cells.

**Dynamic steady states of cellular modules.** The balancing mechanism is likely to be robust, as it occurs in all cell types, despite their very different architectures. Interestingly, these different architectures are based on various 'modules'. A 'module' encompasses all structures of a given type — for instance, all filopodia, lamellipodial protrusions, or stress fibres (Fig. 1a). Although each of these individual structures might grow or shrink, a module itself remains in a DSS, as its elementary structures are preserved. The overall growth of some structures within a module compensates for the shrinkage of others, so that the module permanently integrates new monomers and releases as many. This implies that the DSS of cellular networks is made up of the multiple DSSs of its modules. A balance of assembly and disassembly rates must thus be ensured at the levels of both cells and modules. However, the molecular regulators of filament assembly or disassembly act in multiple modules, precluding the identification of their precise contribution to a given process by genetic removal or biochemical inactivation.

In addition, it should be noted that the different modules can independently exchange monomers with the cytoplasmic pool, but they can also be coupled, as filaments can transit from one module to the other. Direct connections between modules through the exchange of filaments, and indirect connections through the sharing of a common pool of monomers, make it difficult, if not impossible, to dissect their individual and overall balances in cells. Reconstituting actin DSSs in vitro, under simpler conditions than in cells, may thus help to shed light on these important but complex processes.

## Reconstituting actin DSSs in cell extracts

Cell extracts are obtained by centrifuging cell lysates to remove membranes and organelles. They are thus more homogeneous and structurally simpler than cells. Although they exhibit the assembly of branched and contractile actin meshworks, they do not allow the formation of contractile bundles or filopodia. Owing to the removal of plasma membranes, cell extracts can be placed in microengineered compartments to impose controlled geometric constraints. This can reveal key properties of cytoplasmic self-organization that are obscured by the complexity of living cells.

*Xenopus* egg extracts clearly show the emergence of a DSS in the form of a propagating actin wave[3], which is, in essence, driven by the coordinated assembly, condensation, contraction and disassembly of filaments. However, unlike in whole eggs, this wave stops in vitro after a few minutes. The limited lifetime of these dynamic states remains unexplained.

Interestingly, encapsulation of egg extracts in water-in-oil droplets has resulted in a contractile DSS lasting up to several hours. These contractile networks show a continuous inward flow of actin filaments, while the spatial profile of the concentration of filaments remains constant[4] (Fig. 1b). The filament flow slows down linearly from the periphery to the centre, with a concurrent, progressive increase in filament density. Interestingly, the decrease in flow towards the centre — that is, the contraction rate — is constant and does not appear to depend on the filament network density. In addition, filament disassembly appears to be proportional to network density. Together, these properties enable the rate of contraction to match the rate of network turnover, ensuring a constant flow of filaments, even in the face of variations in network density. This property is robust and appears to arise from inherent properties of the contractile system, rather than from fine-tuning of assembly and disassembly rates[4,5]. However, the molecular and physical mechanisms underpinning these key relationships remain to be elucidated.

In cell extracts, the continuous inward flow may become less regular and pulsatile, in the form of concentric waves (Fig. 1b). Such a transition is a sign of perturbation of the DSS, as it results from a transient imbalance between network growth at the periphery and coalescence towards the centre. Indeed, it can be induced in vitro by an increase in contraction, or a decrease in polymerization[6]. An interesting potential explanation for this observation is that, in systems that are driven by rapid turnover, network connectivity depends on local assembly and disassembly rates, which are dynamic and variable. Therefore, in large networks, heterogeneities will inevitably force the network to fragment into more or less regular subnetworks. The mechanism that ensures both the cohesion required for continuous flow and the disassembly required for network turnover is key to the contractile DSS, but requires further molecular characterization and filament-scale exploration to be uncovered.

However, cell extracts retain the molecular complexity of a cell. They therefore cannot be used to isolate the minimal molecular processes and reveal the elementary and fundamental mechanisms that govern complex network dynamics and rearrangements.

## DSS in reconstituted systems

In reconstituted systems that are made up of purified proteins mixed at defined concentrations in a given environment, all components and structural parameters are controlled, which allows the identification of the key parameters that influence network formation. In particular, such systems have been used to reconstitute the assembly of lamellipodia-like or filopodia-like networks, as well as contractile networks that mimic the cell cortex or stress fibres (Fig. 1c). However, so far, most of these assays have only been able to recapitulate the assembly and elongation of the network or its disassembly and contraction, but not the coexistence and balance of these two behaviours.

**DSS based on assembly and disassembly.** A proper balance between assembly and disassembly becomes apparent in vitro through the formation of dense meshworks, or 'comets', of constant length, generated by continuous filament renewal at the surface of beads[7] or on micropatterned surfaces[8]. However, these DSSs are not robust, and small variations in assembly or disassembly rates lead to network growth or shrinkage[7,8]. Recently, the confinement of biochemical mixtures forming comets within closed microwells — coupled with the addition of cyclase-associated proteins — has enabled the efficient recycling of subunits from disassembled filaments, allowing for their reassembly at the front of the comet[9]. Indeed, monomers could be reused several times to assemble the comet. These conditions thus lead to a true DSS, in which assembly is coupled to disassembly, while the length and appearance of the network remain constant for several hours[9].

**DSS based on assembly, contraction and disassembly.** Contractile DSSs, in which the appearance of contractile structures is maintained

by filament flow and renewal over time, have not yet been fully reconstructed in vitro. However, a first step in this direction was achieved by associating filament nucleators at the surface of a lipid bilayer with myosin proteins. The resulting network showed successive steps of local contraction, randomly forming pulses, and some degree of filament renewal[10]. Nevertheless, finding the optimum balance between contractility, connectivity and disassembly in a reconstituted system that fully recapitulates the situation in a cell remains a challenge.

More generally, the central question of DSSs is still open: what enables the assembly rate to match the disassembly rate, and how is the contraction rate tuned to the turnover rate? Different mechanisms, regulated by distinct molecular components, cannot adopt exactly the same rate just by chance. This fine-tuning requires feedback between assembly and disassembly, so that one process speeds up or slows down in accordance with the other. The nature of this feedback is still unknown, but its elucidation is central to a true understanding of the DSS.

**Mechanical coupling.** One possible feedback mechanism is that mechanical forces produced in a growing network affect its rate of assembly. Such mechanisms have been described for formin-based[11] and Arp2/3-complex-based[12] assembly. Several experiments have suggested that applying pulling forces to filaments connected to formins speeds up their assembly[11]. But the potential underlying mechanism remains unclear and controversial. Although exerting tension on a torsionally unstrained filament accelerates linear filament assembly by formins, when the filament can be stretched, actomyosin tension tends to block formins. Furthermore, it has been reported that frictional tension along hydrodynamic flow in a microfluidic chamber promotes the detachment of formins from actin filaments[13]. In other studies, the pressure exerted by network densification on nucleation sites tends to promote the polymerization of branched networks through the Arp2/3 complex, rather than inhibiting such networks[12]. In addition, and independently of the mechanosensitive properties of filament nucleators, it should be considered that mechanical coupling could also be mediated by signalling enzymes such as Rho GTPases, as they regulate both actin assembly and myosin-based contraction and disassembly.

**Chemical coupling.** A possible, more straightforward, feedback mechanism is the direct modulation of monomer concentration by the assembly and disassembly processes. If the monomer pool is limited, a slow disassembly will decrease the concentration of free monomers and thus reduce network assembly, whereas rapid disassembly will have the opposite effect. Such a coupling through the monomer pool will constrain the two rates to match[9]. In addition, a scarcity of free monomers can force competition and thus mutually influence several structures that share the same monomer pool[14]. Whether the pool of actin monomers is limited in cells is still unclear, as their concentration is quite high[1]. However, proteins that interact with free monomers, such as thymosin, might limit the actual availability of monomers for polymerization and so restrict the size of the 'usable' pool, de facto limiting it[15].

**Lifetime of DSSs.** Finally, another important aspect that can be addressed with reconstituted assays is the parameters that limit the lifetime of actin DSSs. In cells, monomers are actively degraded and replaced by constant synthesis, but in vitro assays pointed at a crucial ageing process, as magnesium replaces calcium and ignites the polymerization capacities of the monomers[9]. Other parameters might also contribute to the loss of conformation and activity of actin monomers, and further studies will be necessary to reveal the intrinsic limitations of the lifetime of modules that are exposed to heat, oxidation or mechanical stress. Such studies would inform us about cellular needs in term of monomer synthesis and degradation in order to compensate for these inescapable ageing processes. In addition, the energetic consumption of the DSS remains unclear, although it is a major limiting factor in the DSS lifetime[9]. It thus appears crucial to study the metabolic pathways that supply energy in the form of ATP, and their relative impact on network assembly and disassembly rates in specific modules. Such studies could clarify whether limited energy resources could distort module turnover rates and allow certain modules to outcompete others.

## Multiple modules at DSS

In cells, the rates of assembly and disassembly might temporarily match imperfectly, and this could induce the expansion or contraction of specific modules. The control of these transient phases could therefore be used as a mechanism for reorganizing the respective sizes of the various modules and reconfiguring network organization, for example as cells differentiate. Indeed, each cell type is characterized by a specific size and architecture, the regulation of which could be elucidated by identifying the mechanisms that regulate the size and shape of actin modules in DSS.

Furthermore, it would be interesting to study the coupling and balance between multiple DSSs, as this could reveal how they adapt their turnover rates and exchange filaments. For instance, monomers could be exchanged with the pool to give rise to two independent DSSs; existing DSSs could exchange monomers and become coupled, or exchange filaments and form a single DSS. Achieving such complex reconstitutions could allow us to study the processes that results in cell coherence – that is, a cell's ability to remain a single entity or to divide into two, or even to fragment into several pieces. Furthermore, studying the coupling between multiple DSSs into a global DSS is necessary to explain the remarkable robustness of the actin network DSS, which, although it can take multiple forms, is present in all living cells.

Finally, advancements in technology, enhancing our ability to modulate compartment shapes or protein mixtures in real time, might allow us to design more sensitive and adaptable networks. Such progress promises a deeper understanding not only of the structural adaptation of cells to their environment, but also of the complex integration of modules that is required for cells to move. In particular, this could shed light on the synchronization between expansion and retraction phases, and on the spatial segregation of these behaviours. The identification of such mechanisms will be necessary to gain further insight into the computing and coordination that underlie key cellular functions such as chemotaxis and haptotaxis.

Notably, as is always the case, reconstitution approaches can clarify basic mechanisms, but do not take into account the contributions that arise from cellular complexity. The cellular mechanisms that support the amazing robustness of the actin DSS are likely to involve several signalling pathways, as well as regulation at the transcriptional level. Detailed investigation of such complex regulations will require further studies in living cells, building on the insights obtained with reconstitution approaches.

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## Competing interests

The authors declare no competing interests.

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