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REVIEW

SPECIAL ISSUE: RECONSTITUTING CELL BIOLOGY

More from less – bottom-up reconstitution of cell biology

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ABSTRACT

The ultimate goal of bottom-up synthetic biology is recreating life in its simplest form. However, in its quest to find the minimal functional units of life, this field contributes more than its main aim by also offering a range of tools for asking, and experimentally approaching, biological questions. This Review focusses on how bottom-up reconstitution has furthered our understanding of cell biology. Studying cell biological processes *in vitro* has a long tradition, but only recent technological advances have enabled researchers to reconstitute increasingly complex biomolecular systems by controlling their multi-component composition and their spatiotemporal arrangements. We illustrate this progress using the example of cytoskeletal processes. Our understanding of these has been greatly enhanced by reconstitution experiments, from the first *in vitro* experiments 70 years ago to recent work on minimal cytoskeleton systems (including this Special Issue of Journal of Cell Science). Importantly, reconstitution approaches are not limited to the cytoskeleton field. Thus, we also discuss progress in other areas, such as the shaping of biomembranes and cellular signalling, and prompt the reader to add their subfield of cell biology to this list in the future.

KEY WORDS: Bottom-up synthetic biology, Giant unilamellar vesicles, *In vitro* reconstitution, Model membranes, Synthetic cell

Introduction

Cell biology by its very nature faces the challenge of handling the complexity already contained within a single cell: how do its underlying molecules and modules work together to create the fascinating properties of living matter?

To elucidate which molecules are constitutive for cellular functions, we usually monitor a change in the cell's behaviour upon controlled perturbation of either its environment or of its molecular and genetic composition. For example, the necessary set of molecular components for cell motility can be determined by monitoring the speed of cell movement during treatment with a chemoattractant or upon deletion of specific genes. After many years of performing these classic cell biology experiments, a detailed picture of cells on the molecular level has been painted, from metabolic and signalling pathways down to the molecular origin of their mechanical properties. Genome and proteome research suggests that this manifold of cellular processes is mediated by complex networks of molecular interactions, showing considerable cross-talk, feedback loops, as well as redundancies. This is perhaps unsurprising, given that redundancy confers robustness and therefore resilience to cells and organisms. However, while being clearly evolutionarily advantageous, the high degree of both interconnectivity and redundancy makes it hard to define something like the minimal cellular machinery required for any cellular process when using a top-down approach.

The field of synthetic biology has gained considerable traction over the last years (Shapira et al., 2017). Recent reviews have been devoted to laying out the main goal of bottom-up synthetic biology: recreating life in its simplest form (Göpfrich et al., 2018; Schwille et al., 2018). But apart from addressing questions about the origin of life and conceptualising 'designer cells', what, if any, is the virtue of such an engineering-based approach for studying complex cellular phenomena? In fact, bottom-up synthetic biology approaches have also shed light on many cell biological phenomena. By reconstituting cellular processes from isolated, well-characterised components, experimentalists can witness and quantify the emergence of more complex cell-like behaviour, such as membrane remodelling and spatial patterning (Liu and Fletcher, 2009; Vahey and Fletcher, 2014). At this point, it is worthwhile noting that the marriage of reconstitution experiments and biology is not new. From the first days of biochemistry, reconstituting cell biology *in vitro* has been going hand-in-hand with traditional biology experiments. Consequently, one could see Eduard Buchner's discovery of cell-free fermentation in 1897 not only as the birth of biochemistry, but also of reconstitution experiments (Buchner, 1897). What can we learn from reconstitution experiments more than a century later? And what can we learn beyond using our increasingly sophisticated techniques to interrogate living cells, ranging from genetic to pharmacological manipulation? First, reconstitution experiments offer a route to distinguish between 'necessary' and 'sufficient', both regarding the role of individual molecular players and entire pathways, which is hard to do with top-down methods. Second, testing the predictions of our mechanistic models generated from, for example, *in vivo* data is greatly facilitated by the control over all parameters that reconstitution offers. Third, this synthetic biology data can itself produce input for quantitative modelling, and thus generate new predictions as well as insight into the physical principles governing life. Fourth, reconstitution experiments can reveal emergent properties, i.e. the tendency of a system to show a behaviour that cannot be deduced from the properties of the isolated, constituent components, so is more than the sum of its parts.

In this Review, we want to make a case for including mimics of cellular membranes in reconstitution experiments, and for considering the spatial and mechanical aspects of the cellular context in which the reconstituted processes occur. We illustrate this point by showing how our understanding of cytoskeletal processes has been greatly enhanced by minimal cytoskeleton systems. We discuss how recent technological advances have enabled researchers to address questions of unprecedented complexity. It has become possible to reconstitute systems of defined multi-component biomolecular composition, including physical variables such as position and geometry, in addition to chemical ones such as concentration. Given the enormous nature of this undertaking, we present merely a few important examples of cell biology areas in which reconstitution approaches have led to new insights (Fig. 1).

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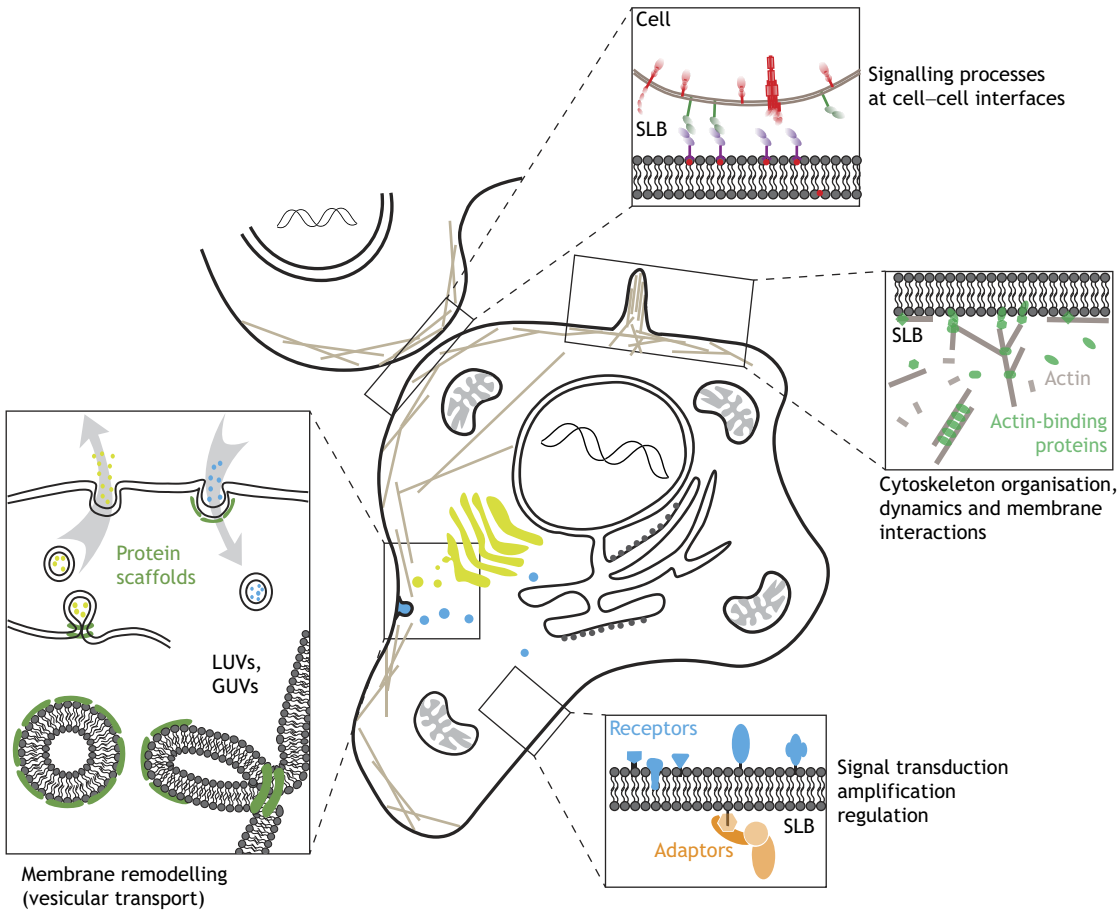

Fig. 1. Illustration of questions that can be addressed by interfacing bottom-up synthetic biology and cell biology as discussed in this Review. Different artificial membrane systems, such as supported lipid bilayers (SLBs) and large or giant unilamellar vesicles (LUVs, GUVs), can be used to mimic cellular membranes and membrane–membrane contacts. In combination with purified proteins or cell extracts, minimal machineries for different cell biological processes can be reconstituted and studied in these model membranes. Examples of phenomena discussed in this Review include the remodelling of the membranes themselves, membrane-associated cytoskeletal rearrangements and signalling processes.

Beyond reconstitution approaches, synthetic biology tools such as opto-genetics are also increasingly attractive to cell biologists as a means to modulate key components in a given cellular pathway both rapidly and specifically, without off-target effects. However, we will not cover this aspect and instead refer the interested reader to a recent review (Kim et al., 2016). We close with a perspective on how the approach of synthetic biology, combined with the knowledge of the cell biology and membrane biophysics communities, could help us gain a holistic understanding of the living organism, and to connect the results of ever more complex *in vitro* studies of cellular function seamlessly to those obtained from studying cells.

A case study: reconstituting the actin cytoskeleton – from identifying the minimal contractile unit to the recreation of functional actin networks

The beginnings – seeing them contract for the first time

The cytoskeleton is involved in a vast number of cellular processes, from shaping cells to their propagation (cell division) and locomotion. This filamentous scaffold of proteins, comprising the actin cortex that supports and shapes cell plasma membranes, is also a prime example of how reconstitution experiments have been indispensable for dissecting its molecular make-up and function. In the 1940s, Szent-Györgyi observed that the tension that built up in *in vitro* muscle preparations upon addition of magnesium and ATP was comparable to that in living muscles (Szent-Györgyi, 1949). The demonstration that contraction can be reproduced *in vitro* by only two proteins, actin and myosin, showed the power of a reductionist approach to deciphering the molecular origins of cellular behaviour, and paved the way for the modern understanding of muscle and cytoskeleton function.

Discovering actin cytoskeleton architecture, dynamics and regulation using *in vitro* reconstitution experiments

Beyond muscle contraction, the actin cytoskeleton in all cells is fundamental to their function because numerous cellular processes are driven by actin reorganisation. Actin dynamics themselves are regulated by various mechanisms, mediated by hundreds of actin-binding proteins. These control elongation, shortening and architectural organisation of actin filaments in response to a variety of environmental and physiological cues (Blanchoin et al., 2014; see also Plastino and Blanchoin, 2019 in this Special Issue). Beginning in the 1960s, Oosawa and co-workers pioneered *in vitro* reconstitution assays to study actin filaments (Kasai et al., 1965; Oosawa and Asakura, 1975). From these studies, we learnt important physical and mechanistic properties of actin filaments, e.g. that ATP and bivalent cations are dispensable for, but accelerate, actin polymerisation (Fig. 2A). The use of fluorescently labelled phalloidin to both label and stabilise actin filaments for imaging was already proposed by Yanagida et al. (1984), but it was when Blanchoin and Pollard pushed this microscopy approach to new spatiotemporal resolution by using

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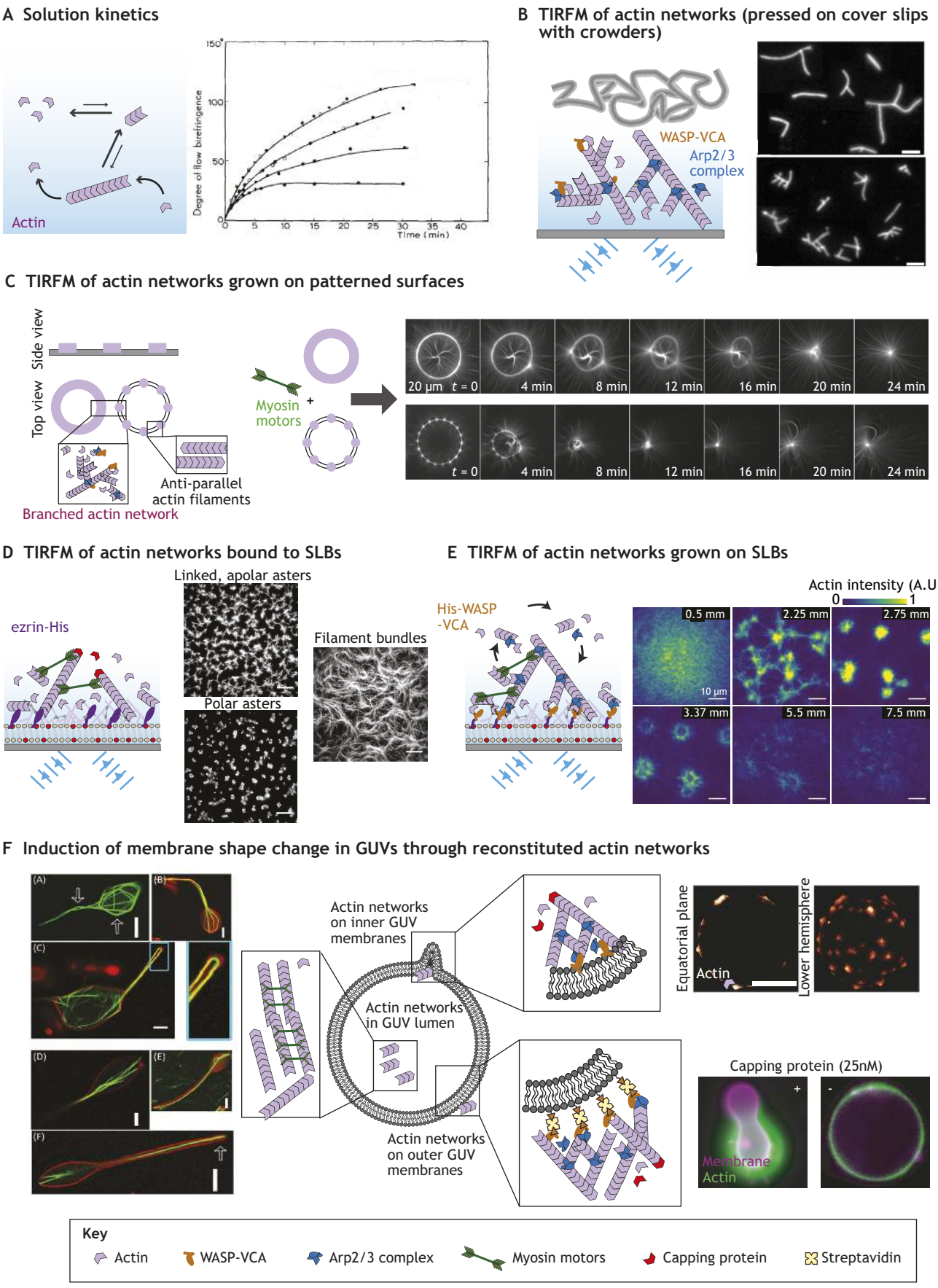

Fig. 2. See next page for legend.

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Fig. 2. The growing complexity of reconstitution experiments used to study the cytoskeleton. (A) Reconstitution of actin polymerisation in solution shows the dependency of polymerisation rates on mono- and divalent ions and nucleotides (data sets depicted) using flow birefringence (*y*-axis) as a readout for actin polymerisation. Graph adapted from Kasai et al. (1965), with permission from Elsevier. (B) Seminal work imaging WASP-VCA and Arp2/3 complex-dependent actin branching in solution with TIRFM. Blue bars/arrows depict evanescent field from TIRF illumination. Images (right) from Blanchoin et al. (2000a), reprinted with permission from Springer Nature. (C) Myosin was shown to selectively contract and disassemble antiparallel actin structures (patterned rings) over branched actin network (full rings) at ring-shaped micropatterns. Depicted here is a time series of myosin-induced contraction of actin networks nucleated from full (top) and dotted (bottom) rings. Time series images from Reymann et al. (2012), reprinted with permission from AAAS. (D) Reconstitution of actomyosin networks bound to SLBs via the actin-binding domain of ezrin shows that myosin induces three distinct network configurations with different actin filament connectivity: polar asters, apolar asters or bundles. Red circles depict Ni-NTA lipids used to anchor ezrin to the SLB. Network configuration images from Köster et al. (2016) reprinted with permission. (E) Reconstitution of a minimal dynamic actin cortex nucleated at SLBs by the N-terminal WASP-VCA fragment and the Arp2/3 complex. This shows that myosin addition reorganises the actin network and induces a new steady-state actin network structure (image at 7.5 min); this new distribution persists for hours. Actin reorganisation time series images from Sonal et al. (2019), reprinted with permission. (F) Reconstitution of actin networks inside and outside of GUVs shows that actin polymerisation in combination with very few actin-binding proteins, such as crosslinkers (left) (confocal images from Tsai and Koenderink, 2015, reprinted with permission) and membrane-bound nucleators (top right) (confocal images from Dürre et al., 2018, where figure was published under CC-BY 4.0 licence), can induce membrane protrusions that are reminiscent of biological phenomena, such as filopodia, and different overall membrane shapes if a branched actin network is formed at GUV membranes in the presence of capping protein (bottom right) (confocal images from Simon et al., 2018, where figure was published under CC-BY 3.0 licence).

total internal reflection fluorescence microscopy (TIRFM) that for the first time, polymerisation dynamics and filament topology could be investigated simultaneously (Amann and Pollard, 2001; Blanchoin et al., 2000a,b; Kuhn and Pollard, 2005). This new method led to a flurry of *in vitro* reconstitution studies on actin and actin-binding proteins (Breitsprecher et al., 2009). With the advent of single-molecule fluorescence microscopy, the mechanisms could now be resolved at the molecular level of single monomers and actin-binding protein complexes (Fujiwara et al., 2010; Smith et al., 2013a,b), reviewed in Smith et al. (2014). These techniques enabled the precise measurement of the kinetics and parameters underlying actin assembly.

Since TIRFM is a surface-based technique, pre-assembled filaments were originally confined to the surface of glass cover slips by molecular crowding agents such as methylcellulose (Blanchoin et al., 2000a) (Fig. 2B). Alternatively, filaments can also be directly immobilised by the use of either surface-bound streptavidin and biotinylated actin (Smith et al., 2013b), or surface-adsorbed actin nucleation factors (Bieling et al., 2016; Reymann et al., 2010, 2012). Beyond facilitating data collection, the need to bring filaments close to a surface can also be exploited to generate defined boundary conditions for network growth using microfabrication or micro-patterning approaches (Box 1). Studies that controlled the spatial distribution of nucleation sites by micro-patterning techniques have been able to probe how actin network architecture affects network remodelling by motor proteins (Reymann et al., 2012) (Fig. 2C). In another study, micro-patterned actin network patches were used to test their mechanical properties by atomic force microscopy, while simultaneously monitoring network structure by TIRFM (Bieling et al., 2016). It was found that applying force to self-assembling branched actin networks, somewhat counterintuitively, increases their density, strength and the efficiency of force generation by actin

Box 1. Current methods and techniques used for the bottom-up reconstitution of cell biology

Model membrane systems: Vesicles whose membranes are made from lipids are called lipid vesicles or liposomes. Depending on their size, they are grouped into small (r<100 nm), large (r<1 µm) or giant (r=1–100 µm) unilamellar vesicles (SUVs, LUVs or GUVs) (Walde et al., 2010). Because of their cell-like size, GUVs are well-suited for reconstituting cellular processes with cell-like geometric and volumetric boundary conditions. They are straightforward to image with optical microscopy and can be micro-manipulated, e.g. to generate membrane tubules or measure membrane tension. Supported lipid bilayers (SLBs) are another versatile model system, formed by initiating the rupture and fusion of SUVs on substrates. Interactions with the support can be limited by functionalising lipids or surfaces with polymers (Sackmann and Tanaka, 2000).

Protein purification: Protein biochemistry can still be a bottleneck in reconstitution experiments. However, in addition to purification from bacterial, insect and mammalian cells, cell-free protein expression is becoming increasingly accessible, and promises to be particularly advantageous for the synthesis of difficult proteins including toxins and membrane proteins (Bernhard and Tozawa, 2013; Kai et al., 2012).

Micro-patterning and microfabrication tools: *In vitro* reconstitution experiments can now make use of microfabrication and micro-patterning tools to test the effect of spatial constraints on cellular processes, e.g. using micro-contact printing or direct photo-patterning (Thery, 2010). In addition to using GUVs, these are also useful tools for recreating the spatial constraints present in cells in *in vitro* experiments (Laan et al., 2012).

Microfluidic techniques for sample preparation and handling: After an initial investment in terms of a more sophisticated experimental setup, microfluidic technologies offer a high level of control over experimental parameters and the benefit of easy automation (Damiati et al., 2018; Scheler et al., 2019). They can be used to control reaction conditions on SLBs, or as a route to form cell-sized compartments such as droplets and lipid vesicles (Elani, 2016), thereby offering control over droplet or GUV size, content and membrane (a)symmetry (Richmond et al., 2011). Downstream of sample production, vesicles or droplets can be manipulated and analysed on-chip, for example in microfluidic traps (Robinson et al., 2013) or by pico-injection modules (Weiss et al., 2018).

polymerisation. Importantly, controlling local network architecture by micro-patterning is not only a useful handle to dissect mechanistic questions, it also allows researchers to better mimic the tight spatial control of actin organisation in cells in reconstitution experiments.

Reconstituting the interplay between membrane and cytoskeleton – minimal models of the cell's actin cortex

Cells are shaped by interaction of the dynamic actin cytoskeleton with the plasma membrane. Actin networks on model membranes (minimal actin cortices), and in particular synthetic supported lipid bilayers (SLBs), have long been used to study processes in membranes, and they are an ideal experimental system for TIRFM (Box 1). Therefore, membrane–cytoskeleton interactions have recently been reconstituted on SLBs, offering a more physiological mimic of the membrane-bound cytoskeleton. For example, actin filaments can be anchored to SLBs using magnesium ions (Mg^{2+}), which bind to both phospholipids and the negatively charged actin filaments (Häckl et al., 1998), or using biotinylated actin filaments attached to biotinylated lipids via streptavidin (Heinemann et al., 2013; Vogel et al., 2013). However, cellular conditions can be mimicked even more closely by reconstituting proteins that naturally mediate interactions between actin filaments and the plasma membrane, such as N-WASP (also known as WASL) in combination with L-a-phosphatidylinositol-4,5-bisphosphate (PIP2) lipids (Lee et al., 2010; Liu et al., 2008), fimbrin (Fim1 in yeast, also

known as plastin in mammals) (Murrell and Gardel, 2012), ezrin (Bosk et al., 2011; Köster et al., 2016) or ponticulin (Barfoot et al., 2008). For example, Köster et al. have studied the reorganisation of actin bound to the His-tagged actin-binding domain of ezrin attached to Ni-NTA SLBs, and found that the system shows multiple modes of actin organisation depending on factors such as actin filament length and actin:myosin ratio (Köster et al., 2016) (Fig. 2D). In phase-separated lipid bilayers and monolayers, the actin–myosin reorganisation also induced changes in the shape and dynamics of the membrane domains (Köster et al., 2016; Vogel et al., 2017), indicating how the cytoskeleton may impact lipid membrane organisation. This Special Issue of Journal of Cell Science includes our work on using a membrane-attached form of the VCA domain of N-WASP to generate a dynamic, membrane-proximal actin network, in which actin polymerisation is balanced with myosin-II driven network disassembly, supporting a role for myosin in cellular actin turnover (Sonal et al., 2019) (Fig. 2E). Importantly, if nucleation-promoting factors are membrane-anchored, they can diffuse and rearrange in response to network growth, reminiscent of the situation in a cell. These bilayer-based minimal actin cortices offer the potential for reconstituting multi-component actin networks, and will be an important asset to study the dynamics, organisation and emergent behaviour of membrane-associated cytoskeletal components.

Toward recreating the full complexity and functionality of the actin cytoskeleton *in vitro*

While processes at SLBs are readily observable using high-resolution microscopy, SLBs are two-dimensional, in contrast with the three-dimensional structures of cellular membranes that can be deformed and remodelled by forces exerted by the cytoskeleton. This membrane shaping is not only crucial for the remodelling of overall cellular shape, but also the generation of the structures required for cell motility, such as filopodia, or for the controlled generation of membrane vesicles in endo-, exo- and phagocytosis. Giant unilamellar vesicles (GUVs) are an ideal model system for mimicking both deformability and dimensions of cell membranes (Box 1). Actin networks have been reconstituted on GUVs with the same anchoring strategies used on SLBs outlined above. The ‘inside-out’ configuration, in which actin is polymerised at the GUV surface, is inverse to the situation in cells, but more experimentally accessible. Reconstituting minimal actin cortices on GUVs has offered many insights into the interplay of the membrane and the actin cytoskeleton. Work from the Fletcher laboratory showed that bundled filaments that initiate membrane protrusions can emerge from a membrane-attached branched actin network in the absence of bundling proteins (Liu et al., 2008), reminiscent of filopodia. Studies of GUV-bound actin networks, either grown from a membrane-bound actin nucleator, or built by adsorption of pre-assembled actin filaments, have revealed that network tension can be induced by both active actin polymerization or by myosin II, and that this depends on filament branching and length (Caorsi et al., 2016; Carvalho et al., 2013; Simon et al., 2018). This induced network tension resulted in the actin distribution undergoing symmetry breaking on the GUV membranes (Carvalho et al., 2013), which *in vivo* is essential during the polarisation processes that lead to cell shape modifications and cell division.

To recapitulate the actin–membrane interactions that control cell shape more closely, they need to be reproduced in a geometry akin to that of a cell. Since the size of GUVs is similar to that of cells, they generate boundary conditions of the relevant dimensions if cytoskeletal components are encapsulated within them. The first studies in which actin was encapsulated in GUVs date back around 20 years (Häckl et al., 1998; Limozin and Sackmann, 2002); however, efficient encapsulation of multiple cytoskeletal components has been difficult. Recently, actin network assembly on inner GUV membranes has been facilitated by several methodological advances (see Box 1) (Abkarian et al., 2011; Li et al., 2009; Pautot et al., 2003; Pontani et al., 2009; Stachowiak et al., 2008; Tsai et al., 2011; Weinberger et al., 2013), although all these methods are still low-throughput and suffer from either complex implementation protocols, or low reproducibility (Tsai et al., 2011; Walde et al., 2010). Nevertheless, even when only a limited set of actin cytoskeleton components and motors was reconstituted in GUVs, basic membrane deformation modes could be recreated, such as membrane blebbing, as well as inward and outward membrane protrusions (Dürre et al., 2018; Loiseau et al., 2016; Tsai and Koenderink, 2015) (Fig. 2F). Actin networks have also been successfully reconstituted in droplet-stabilised GUVs (Weiss et al., 2018) or water-in-oil droplets (Claessens et al., 2006), including the reconstitution of a model actin cortex from droplet-encapsulated *Xenopus* cytosol that exhibited spontaneous symmetry breaking (Abu Shah and Keren, 2014).

The polymerisation of actin does not only produce forces that drive membrane protrusions. Cell-invading pathogenic bacteria such as *Listeria* monocytogenes can move rapidly through the cell’s cytoplasm by initiating actin polymerisation at their membranes via ActA, resulting in an actin ‘comet tail’ that propels the bacterium forward. Using solely purified and reconstituted actin cytoskeletal components (actin and three regulatory proteins), the motility of (dead) bacteria or ActA-coated microspheres could be recreated *in vitro* (Cameron et al., 1999; Loisel et al., 1999), demonstrating that actin and only five additional components are sufficient for comet tail formation and conferring motility. These experiments not only demonstrated the power of *in vitro* reconstitution experiments, but they also inspired many subsequent reconstitution studies, as evidenced by the fact the original publication (Loisel et al., 1999) has been cited ∼1000 times at the time of writing.

While all the proteins used in the reconstitution studies discussed above had previously been identified by experiments in cells, it would be very challenging to dissect these processes quantitatively in the same setting. *In vitro* reconstitution suggests that it is the delicate balance between various network-modulating factors and the actin assembly kinetics that determines the final outcome and functionality of the interactions of the cytoskeleton with membranes. Although this Review does not cover other cytoskeleton components, such as microtubules, our knowledge of these polymers has also been extended by reconstitution experiments (Dogterom and Surrey, 2013). In the future, ever more complex *in vitro* studies of cytoskeletal filaments and their interactions with motors and other binding proteins, as well as of the cross-talk between the different cytoskeleton substructures (Dogterom and Koenderink, 2018), will continue to illustrate how cells assemble these components and substructures to generate the dynamic micrometre-scale architecture characteristic of cells. While cell experiments will always be needed to place these findings in a physiological context, *in vitro* reconstitution studies are particularly useful to disentangle the contributions of physical interactions, boundary conditions and signalling mechanisms, as we will continue to lay out in the next sections.

Reconstituting cellular processes beyond actin cytoskeleton

Membrane (re)organisation and transformation in intracellular trafficking

In the previous section, we discussed how the actin cytoskeleton can cause membrane deformations *in vitro*. The fact that cellular

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membranes are constantly remodelled accounts for their diverse biological functions, including vesicular transmembrane transport, for example endo- and exocytosis, modulation of membrane composition, changing cell surface area and shapes, as well as cell fusion and fission. In addition to the actin cytoskeleton, many other proteins have evolved to facilitate these fundamental processes. Classic examples are coat proteins, such as the clathrin protein family, and the COP-I and COP-II complexes, which are required for the budding of vesicles shuttling cargo between the plasma membrane and intracellular compartments (Béthune and Wieland, 2018; Haucke and Kozlov, 2018).

Much of what we know about the basic biophysical requirements of these processes stems from reconstitution experiments (Bassereau et al., 2018). Reconstitution experiments by Takei et al. were the first to conclusively show that the ATP-independent assembly of a clathrin coat on artificial liposomes was fully sufficient to drive membrane vesiculation (Takei et al., 1998). More recently, *in vitro* clathrin assembly on GUVs has shown that clathrin can only deform membranes below a certain tension, suggesting a potential regulatory mechanism (Saleem et al., 2015). *In vivo*, scission of clathrin-coated cell membrane vesicles requires the assembly of dynamin on the membrane neck (Damke et al., 1994), and consistent with this model, dynamin formed a coat on lipid tubules (Takei et al., 1998). The addition of GTP to these coated tubules led to their twisting, and also scission if tubules were under longitudinal tension (Roux et al., 2006, 2010). Similar experiments studying the mitochondrial dynamin homologue Drp1 (also known as DNM1L) showed, however, that this pulling-force-induced fission is not a universal feature of all dynamin-like proteins (see Ugarte-Uribe et al., 2019 in this Special Issue). COP-I- and COP-II-coated vesicles are required for cargo transport between the endoplasmic reticulum and the Golgi apparatus. *In vitro* reconstitution of COP-I on GUVs showed its self-assembly into coats on membranes, with the extent of membrane deformation being tension-dependent, analogous to clathrin (Manneville et al., 2008). When a COP-II coat was assembled on GUVs, their membranes were deformed into beads-on-a-string-like constricted tubules, similar to those observed in cells (Bacia et al., 2011).

Recent work suggests that in addition to these classic ‘coatamers’, other components of the vesicle coats can also contribute to membrane bending. *In vitro* experiments have shown that proteins with Bin/amphiphysin/Rvs (BAR) domains that have intrinsically

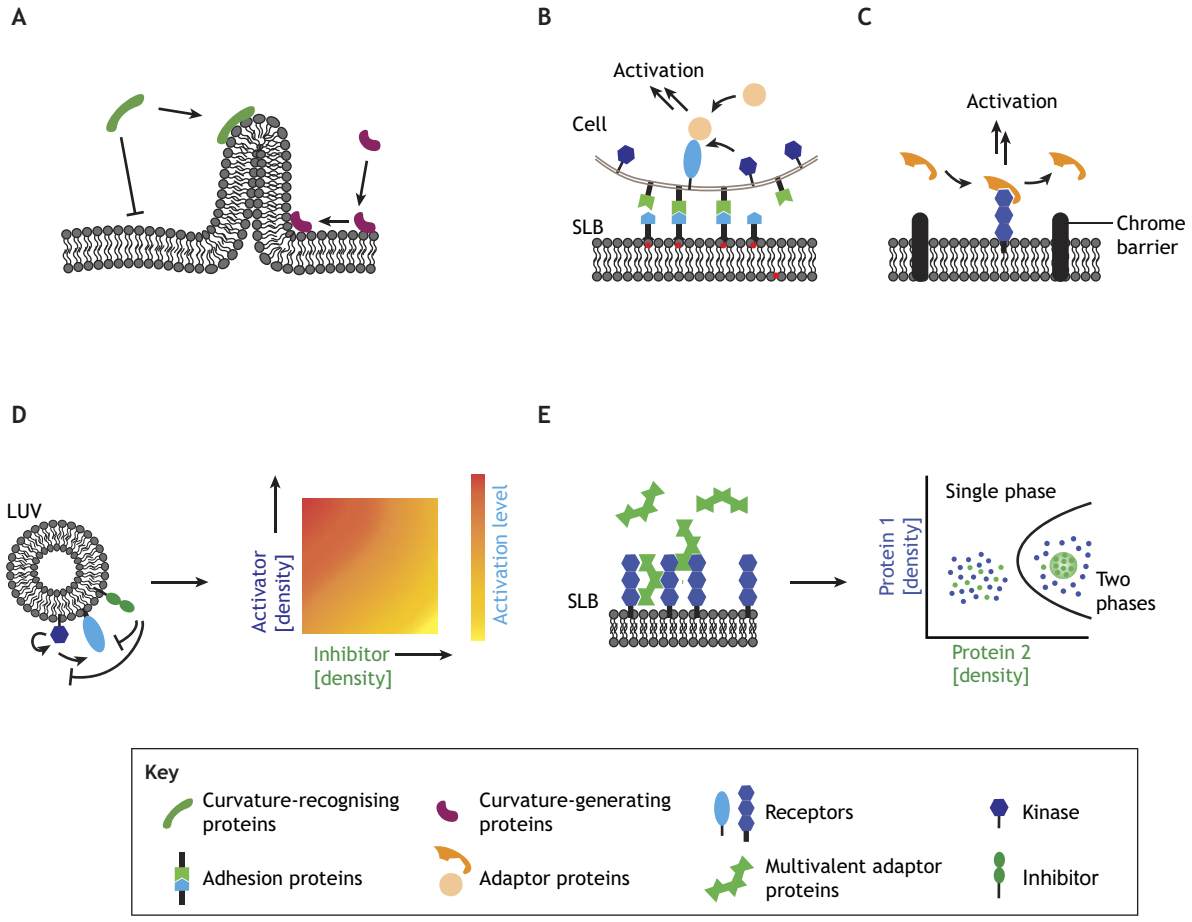

Fig. 3. Information that can be obtained from applying reconstitution experiments to cell biological questions. (A) Reconstitution of membrane scaffolding protein recruitment to model membranes can answer whether these proteins induce or detect membrane curvature, or both. (B) Cell–cell interactions are reconstituted in hybrid interfaces between cells and SLBs to study, for example, the recruitment of adaptor proteins to cell-surface receptors. See Dustin and Groves (2012) for a review on how this system is used to study T-cell activation. (C) SLBs functionalised with metal (chrome) barriers to limit protein mobility can be used to study the activation of single proteins in these membrane corrals. See, for example, Lee et al. (2017). (D) Reconstitution of minimal signalling networks bound to LUVs can be used to map the phase diagram of signal output (e.g. receptor phosphorylation) under different input conditions (e.g. densities of activators and inhibitors). See Hui and Vale (2014) for a study on the proximal T-cell receptor signalling network. (E) In addition to following the kinetics of single molecules reconstituted in SLBs (cf. panel C), reconstitution of minimal signalling networks on SLBs can be used to reveal higher-order organisation of proteins, such as protein–protein phase separation induced by multivalent interactions. See, for example, Li et al. (2012).

curved shapes are capable of sensing and inducing local membrane deformation (Prévost et al., 2015; Renard et al., 2014; Sorre et al., 2012) (Fig. 3A), thereby aiding the function of other membrane-remodelling proteins, e.g. dynamin (Yoshida et al., 2004).

Another protein machinery that drives membrane remodelling by polymerisation is the endosomal sorting complex required for transport (ESCRT) system, which is essential for multivesicular body biogenesis and cytokinesis (Schöneberg et al., 2017). Early reconstitution experiments have defined the minimal set of proteins required for the function of the ESCRT-III complex (Wollert et al., 2009); however, it was recently found that an additional component is required for membrane scission (Schöneberg et al., 2018). The molecular mechanism by which ESCRT-III polymerisation curves membranes is still debated, but experiments on SLBs coupled with atomic force microscopy have now suggested that a main component of the ESCRT-III complex self-organises into spiral 'springs', which store the energy required for membrane deformation upon triggering the spring's release (Chiaruttini et al., 2015). In an article published in this Special Issue, De Franceschi and colleagues exploited a novel method based on laser-induced fusion of GUVs to reconstitute ESCRT-III proteins inside GUVs, allowing the study of their localisation on membranes in the physiological membrane topology (De Franceschi et al., 2019).

In addition to dedicated membrane scaffolding, basic physical principles, such as protein clustering and crowding, have recently emerged as more general mechanisms for inducing membrane invagination. For example, Shiga toxin undergoes clustering by inducing membrane nanodomains enriched in globotriaosyl-ceramide (Gb3), whose negative spontaneous curvature leads to membrane invagination (Pezeshkian et al., 2017; Römer et al., 2007). In the case of Shiga toxin, the spontaneous curvature generated dominates over the opposite effect exerted by the steric pressure from protein crowding, which by itself is sufficient to drive membrane tubulation (Stachowiak et al., 2010, 2012) and fission (Snead et al., 2017). These mechanisms, unveiled by reconstitution experiments, probably contribute to all physiological membrane-bending mechanisms, and will have to be considered when studying the curvature-generating capability of coat proteins or other scaffolding proteins.

Reconstituting signalling and signal transduction pathways

All processes discussed so far are tightly regulated in response to internal and external stimuli, such that cells can constantly adapt and respond to their environment. Membranes in particular serve as a signal transduction hub where numerous plasma membrane receptors decode and relay signals to the cell's interior by binding to their respective ligands. In most cases, receptor activation is not directly coupled to the cell's response to the ligand. Instead, the binding events are translated into the activation of secondary messengers that turn on effector proteins, e.g. transcription factors. These complex signal transduction pathways also confer sensitivity and robustness by including signal amplification mechanisms and signal modulation mechanisms such as feedback loops. A whole field of synthetic biology is devoted to studying commonly occurring regulatory network 'modules' in isolation from larger networks by genetically engineering pathways such that they are orthogonal to those present in bacterial or mammalian cells. Recent reviews discuss how this experimental approach, combined with mathematical modelling, has produced invaluable insights on regulatory protein networks and gene circuit architecture (Lim et al., 2013; Wang et al., 2016), and how this knowledge is beginning to be exploited for personalised medicine (Xie and Fussenegger, 2018). *In vitro* reconstitution is useful to identify the core components in a pathway that are necessary to elicit particular cellular behaviours, as well as shed light on their molecular mechanisms. For example, it is now consensus that receptor tyrosine kinase activation occurs by the intermolecular interaction of its kinase domains, which are brought into proximity by formation of the receptor dimer. This model was proposed for the epidermal growth factor receptor (EGFR) when it was found that its kinase domain can be activated by increasing its local concentration on liposomes (Zhang et al., 2006). The same *in vitro* reconstitution method was later used to confirm this triggering mechanism for all other members of the EGFR family (Monsey et al., 2010).

In recent years, efforts have been made to reconstitute not solely individual components of signalling pathways, but to recreate entire signalling cascades *in vitro* while preserving the natural arrangement of the signalling proteins. A paradigm for the interplay of spatial arrangement of signalling molecules and signal transduction is T-cell receptor (TCR) signalling, which mediates T-cell activation when T-cells form contacts with antigen-presenting cells of cognate antigen. Being pivotal for immune cell function, TCR proximal signalling events are one of the best-studied juxtacrine signalling processes that we will briefly highlight here, because *in vitro* reconstitution experiments have greatly contributed to our current understanding of them. The requirement of a cell–cell interface, termed the immunological synapse, to transduce signals between these cells makes it immediately obvious that this spatial arrangement may influence signalling mechanisms. Here, semi-reconstitution approaches have proven highly successful, in which contacts between cells and cell-emulating SLBs are studied instead of cell–cell contacts (Fig. 3B). In a landmark paper, Grakoui et al. observed the formation of the protein patterns characteristic of the immunological synapses in contacts between T-cells and SLBs containing the adhesion protein ICAM-1 and the TCR ligand, which is normally presented by the antigen-presentation cell (Grakoui et al., 1999). This model system has since been extensively used to study early T-cell activation, because it allows researchers to probe T-cell responses to different 'cell' surface conditions (Groves and Dustin, 2003; Manz and Groves, 2010). These studies led to the discovery that upon activation, the TCR and other signalling molecules organise into clusters that sustain signalling by recruiting adaptor proteins, connect to the cytoskeleton and show directed motion towards the centre of the cell–SLB contact (Varma et al., 2006; Yokosuka et al., 2005). When the functionalised SLBs were combined with nanometer-scale structures (chrome barriers) to impose geometric constraints (Box 1, Fig. 3C), the positioning of these clusters within the contact determined their function (Mossman et al., 2005). These semi-synthetic experimental systems allowed direct control of the type and density of adhesion proteins and ligands, key to testing the molecular mechanism of TCR triggering. For example, titration of ligands in SLBs revealed that the minimal triggering unit is four ligands in a single cluster for this system (Manz et al., 2011), highlighting the exceptional sensitivity of TCRs. Using similarly functionalised SLBs, single-molecule microscopy experiments suggested that a TCR is capable of triggering ligand-independently and that signal amplification must occur downstream of TCR triggering (Chang et al., 2016; O'Donoghue et al., 2013). These are just a few examples of how synthetic SLB–cell contacts have provided an easily-modifiable platform to study T-cell activation. Nevertheless, they illustrate that spatial reorganisation of receptors – which do not only play an important role in T cell signalling – can be studied using a semi-reconstitution approach, including other immune cell interfaces (Liu et al., 2009), neuronal synapses (Pautot et al., 2005) and EphA2–integrin signalling cross-talk in cell–cell adhesion (Chen et al., 2018).

Of course, this semi-synthetic approach is not only limited to SLBs, but contacts between cells and cell-emulating GUVs can also be studied (see Jenkins et al., 2018 in this Special Issue).

In the case of the semi-reconstitution approach used to study signalling at interfaces, the synthetic side is used to control the input of a given signalling pathway, e.g. the ligand or its density, and the spatial constraints under which it is delivered, e.g. by controlling the contact area. The output generated by the receptor and signalling pathway, however, is analysed in the live cell. In recent years, efforts have also been made to reconstitute the cytosolic protein interaction network for a given signalling process. In T-cell receptor signalling, *in vitro* reconstitution of intracellular TCR domains, together with the TCR-kinase Lck, the TCR-phosphatase CD45 (also known as PTPRC), and Lck-inhibitory kinase Csk on liposomes has allowed Hui and Vale to probe the phase diagram of this network (Hui and Vale, 2014) (Fig. 3D). Importantly, by measuring the enzyme kinetics and network behaviour on membranes, thus recapitulating the geometry of the molecules' natural environment, the derived parameters have the correct units (surface densities rather than concentration) and can thus serve as a basis for the mathematical modelling of the network (Fernandes et al., 2017; Mukhopadhyay et al., 2016; Rohrs et al., 2018a,b). Furthermore, reconstitution of signalling molecules on SLBs rather than liposomes makes them accessible to single-molecule studies, yielding insights into how the kinetics and dwell time distributions of the receptor-mediated assembly of adaptor proteins could give rise to noise reduction in signalling pathways (Fig. 3C). For example, Huang et al. studied LAT-mediated SOS activation by Grb2 in a reconstituted system (Huang et al., 2016). Increasing the complexity of the reconstituted network even further, Su et al. have now assembled a Herculean total of 12 components of the proximal TCR signalling network on model membranes (Su et al., 2016). Analysis of the behaviour of this reconstituted signalling network revealed that upon TCR phosphorylation, downstream signalling proteins spontaneously phase-separate into liquid-like compartments, similar to what was observed in live cells and in experiments with the reconstituted adaptors LAT and NCK (also known as NCK1) (Banjade and Rosen, 2014) (Fig. 3E). In these clusters, the kinase-to-phosphatase ratio was shifted in favour of TCR phosphorylation, and initiated localised actin filament assembly that affected the shape of the phase-separated domains.

All these studies suggest that *in vitro* reconstitution experiments can be helpful to recapitulate the assembly of signalling hubs under controlled conditions, revealing how spatial organization impacts biochemical reactions inside a cell, and their emergent physical properties, in the case of multivalent interactions such as the formation of fluid clusters. Additionally, these experiments can provide access to the thermodynamic properties of complex assemblies, which are almost impossible to obtain from live cell measurements, but essential for computational modelling. Generating predictive models will continue to improve our understanding of the crucial functions of signalling pathways, such as signal discrimination, propagation and modulation, in particular by providing sometimes non-intuitive hypotheses that can then be experimentally validated.

Conclusions and outlook

Cell biologists are being equipped with an ever-growing repertoire of techniques and approaches to unravel the inner life of the cell. In the age of systems biology and big data, reconstitution experiments that are, by their very nature, reductionist, may thus seem somewhat antiquated, if not irrelevant. Certainly, there is a caveat to all reconstitution experiments aimed at dissecting cellular phenomena: how can we know whether a reconstituted system is behaving 'physiologically'? Results obtained with reconstituted systems, therefore, should be first regarded as a means to build new models, rephrase questions if required and then devise new experiments – in cells – to test their predictions. Conversely, we should also use *in vitro* experiments to rigorously challenge the hypotheses generated by systems biology, e.g. from high-throughput RNA-seq and mass spectrometry data. These top-down approaches can inform us how processes in specific cells are coupled, and which components are required for them. This information can, and should, be integrated into the design of our increasingly complex, but still minimalistic, reconstitution experiments.

With the recent advances in bottom-up synthetic biology, reconstitution of multi-component systems including protein–protein and protein–membrane interactions, has become feasible. Where technical constraints such as protein purification (Box 1) still hamper experimental progress, 'hybrid' reconstitution experiments, which, for instance, substitute cell extracts for highly purified proteins, can provide a complementary approach to full *in vitro* reconstitution and live-cell studies. For example, filopodia-like structures have been recreated on SLBs using *Xenopus* extracts, showing that their tip structure is not templated, but generated through self-organisation – something that would have been hard, if not impossible, to discover in a classical cell biology experiment (Lee et al., 2010). In addition, cell extracts have been used to study the behaviour of proteins in reconstitution experiments that are difficult to purify, such as full-length SOS family proteins (Lee et al., 2017). This semi-reconstitution approach can also be applied to membranes, where so-called giant plasma membrane vesicles (GPMVs), cytoskeleton-free vesicles generated from cell membranes, have been exploited as models of cell membranes. For example, studies on GPMVs have provided conclusive evidence that the actin cortex plays a crucial role in maintaining constraint diffusion of membrane molecules (Schneider et al., 2017), and that even proteins binding weakly at cell–cell interfaces can accumulate there as a result of cooperative effects induced by geometry and membrane bending (see Steinkühler et al., 2019 in this Special Issue). Even one step up in complexity, keratocyte lamellipodial fragments have been recently used to study local actin network turnover in lamellipodia-like structures in the absence of a cell body with a cortical actin network (Raz-Ben Aroush et al., 2017).

In this Review, we have emphasised how reconstitution experiments have always been part of cell biological discoveries, although in contrast with classic biochemistry, modern reconstitution experiments are more holistic and focus on isolating a functional unit, rather than an individual molecule. Thus, already very complex cellular processes have been recreated *in vitro*, from cytoskeleton-driven membrane deformations and 'endocytic' vesicle creation to multi-component signalling pathways. These bottom-up experiments have mostly been a multidisciplinary effort requiring the expertise from biochemistry, biophysics and engineering. Today, with many inspiring experimental designs and methods in hand, cell biologists are always well advised to ask whether full – or partial – reconstitution is feasible for the cellular function they study. This would not only add a new perspective on their research objectives but, more importantly, make the phenomenon accessible to rigorous quantitative modelling down to the molecular level.

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