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The beauty of simplicity in membrane biology

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For the past 40 years, minimal reconstituted systems have helped cell biologists to understand the mechanisms that underlie membrane traffic. Having progressed from minimal synthetic and cell-derived ensembles to direct comparison with living systems, reconstitution is poised for ever more precise and informative understanding of membrane biology.

For cellular membranes, change is the only constant. Contorting to form protrusions and vesicles at one moment, only to break apart or fuse together at the next — the restlessness of these dynamic surfaces is fundamental to all cells. A myriad of functions, from membrane trafficking to cellular motility, require the continuous flux and remodelling of membranes. The biological and medical importance of membrane dynamics, coupled with its alluring beauty, first observed by George Palade and colleagues in the 1960s and 1970s[1], has long motivated scientists to search for the underlying molecular mechanisms. Much has been learned by observing cells, but the complexity and resilience of a living system make it difficult to pinpoint the components required to achieve individual functions. This challenge has inspired reconstituted approaches, which have shaped the past and present of membrane biology. In particular, minimal systems have driven major discoveries in membrane traffic, which is the focus of this Comment. Although we lack the space to explore them here, similar narratives have played out in parallel in many other areas of membrane biology.

In the late 1970s, shortly after the first visualizations of cellular membranes by electron microscopy, molecular reconstitution emerged as a powerful tool to decipher the physiology of membrane traffic[2]. This approach was motivated by earlier fermentation experiments by Eduard Buchner in the late eighteenth century and the enzymology experiments of Arthur Kornberg in the 1960s and 1970s. Molecular reconstitution seeks to build functional biological systems from a minimal or reduced set of biochemical components. It has distinct advantages that have often enabled researchers to cut through the overwhelming complexity of living membranes. In particular, the ability to control the components, concentrations and physio-chemical conditions within an experiment provides a unique opportunity to pose precise mechanistic questions and differentiate between competing mechanistic models. Reconstituted assays often provide the type of quantitative data that can constrain and validate simulations. Perhaps more importantly, the ability to construct a functional system from its component parts provides a unique level of insight. As Richard Feynman said, "what I cannot create, I do not understand".

Since their inception, reconstituted experiments have grown increasingly sophisticated and precise, gradually transitioning from ensemble to single-molecule, real-time investigations of cell-derived or purified components. An example of this progression is the discovery of the mechanisms of membrane fusion, which began in the late 1970s with pioneering studies on viral fusion proteins[3] and continues today. James Rothman and Erik Fries sought to understand how transport vesicles move proteins from one organelle to another[2]. The first step was to determine whether fusion of transport vesicles within the Golgi apparatus was driven by the physical proximity of these vesicles to the organelle or was encoded biochemically. To differentiate between these hypotheses, the researchers isolated and mixed Golgi-containing fractions from two types of hamster cell. The first group of cells expressed a radiolabelled version of the surface glycoprotein of vesicular stomatitis virus (VSV-G), but lacked the crucial Golgi-resident enzyme, GlcNac, to complete its glycosylation. The second cell type did not express VSV-G, but contained Golgi equipped with the complete machinery for glycosylation. Full glycosylation of VSV-G required fractions from both cell types, which suggested that transport vesicles arising from the first cell type had fused with Golgi from the second cell type, where glycosylation was completed. Because physical proximity between transport vesicles and the Golgi is lost during fractionation, this key experiment, in combination with complementary findings from Randy Schekman's laboratory in yeast cells[4], indicated that the specificity of membrane fusion was encoded biochemically. Several laboratories rapidly built on these findings using diverse systems, including in vitro fusion of yeast vacuoles by Bill Wickner and colleagues[5], such that by the mid-to-late 1990s, the major components of the vesicle fusion machinery, which we know today as the SNARE complex, had emerged.

Purification of the full set of SNARE components followed, and membrane fusion was reconstituted in a minimal system with purified lipids and proteins in the late 1990s[6]. Here, and in subsequent studies, fusion was measured using ensemble methods such as fluorescence dequenching after fusion-mediated dilution of labelled lipids. These data, coupled with structural studies[7], elucidated the zippering mechanism of membrane fusion by SNAREs. Using this platform, the impact of a rapidly emerging group of SNARE effectors was characterized, refining our understanding of the fusion mechanism, and gradually explaining the exquisite speed of calcium-triggered synaptic vesicle fusion[8]. As fluorescence microscopy methods grew in sophistication, it became possible to observe individual vesicles fusing with a target membrane[9]. Collectively, these studies revealed the stochasticity of fusion and its dependence on the stoichiometric ratio of lipids and proteins.

In this narrative, like many others that have occurred in parallel, reconstitution experiments have progressed steadily from ex vivo complementation assays, typically performed on bulk ensembles of membranes and proteins isolated from cells, to systems of lipids and proteins of defined composition, and more recently to experiments on single molecules and vesicles (Fig. 1a). Each of these broad classes of experiments has distinct advantages and have uncovered elegant mechanisms in membrane traffic.

Ex vivo 'complementation'

The biology of membrane traffic is staggeringly complex. Depending on the question and the tools available, a complete in vitro reconstitution

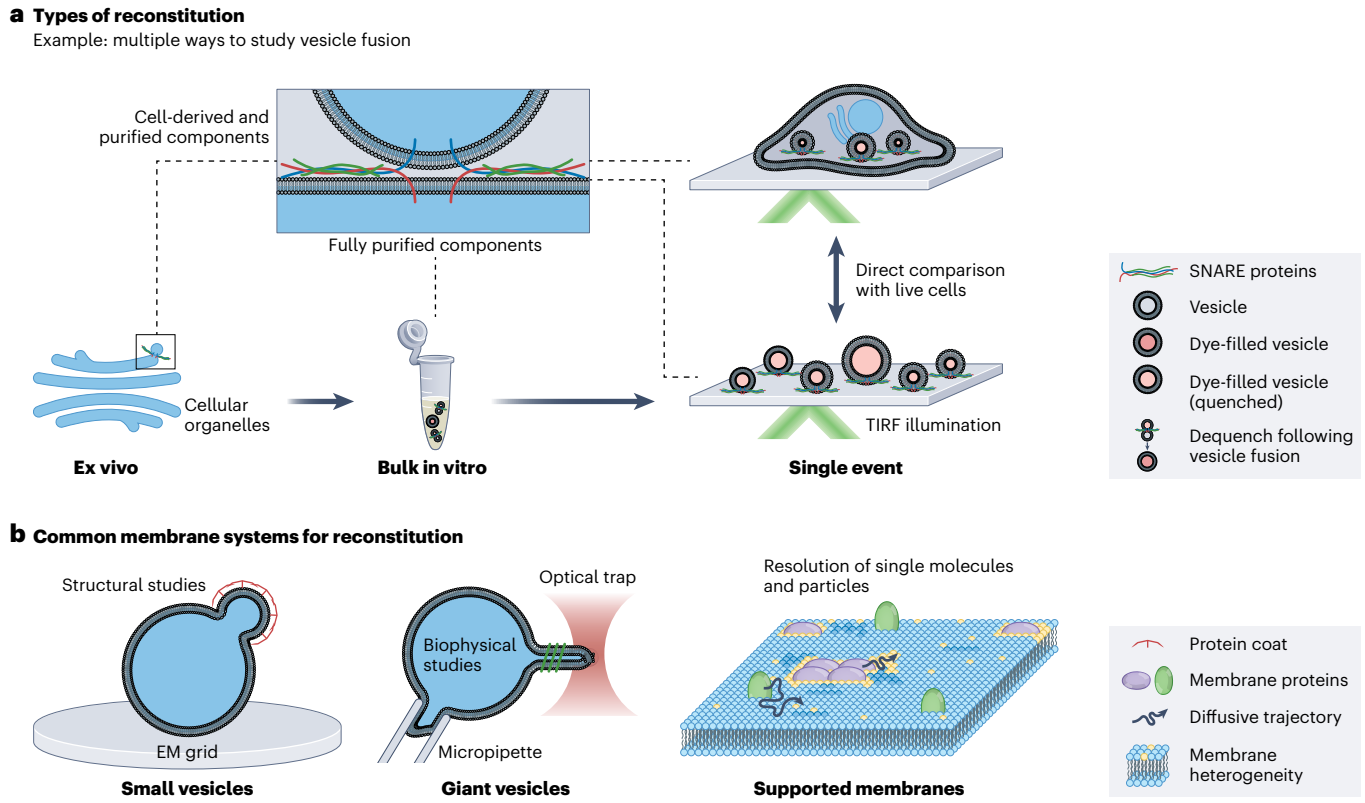

Fig. 1 | Methods and classes of reconstitution in membrane biology and traffic. a, SNARE-mediated fusion of a vesicle to a target membrane is used as an example to illustrate the three major types of reconstitution experiment. Here, the major components of the SNARE complex are illustrated as coloured lines: v-SNARE (blue), t-SNARE (red, green). The three types of reconstitution are: (1) Ex vivo complementation assays, which use cell-derived membranes to examine key steps in membrane traffic, such as membrane fusion, fission, coating and bending. In some cases, cell-derived membranes from two or more different types of cell are mixed. In other cases, purified protein or lipid components, or cell-derived extracts are added to cell-derived membranes to determine the dependency of these processes on exogenous components. (2) Bulk in vitro experiments use fully purified components, rather than complex, cell-derived membranes and extracts. Here, they are typically assayed using methods that examine large ensembles, rather than individual events. These assays include various forms of spectroscopy, light-scattering, fractionation and blotting. (3) Experiments on single events in membrane traffic can be visualized with single-molecule resolution using modern microscopy techniques such as total internal reflection fluorescence (TIRF) illumination or lattice light sheet imaging. Single events can consist of individual vesicles or virions fusing to a synthetic membrane, individual membrane budding events, or individual proteins and lipid diffusing and assembling within the surface of a supported lipid bilayer. This approach can increasingly be used in both synthetic systems and live cells and organisms, allowing more accurate comparisons between minimal and living systems. **b**, Common membrane substrates for reconstitution include: (1) small vesicles with diameters ranging from tens to hundreds of nanometres. These vesicles have been frequently used as substrates to observe the effects of proteins, such as coats, on membrane shape under electron microscopy. They are also frequently used in assays of protein-lipid binding. (2) Giant vesicles have diameters in the micrometre range, making them suitable for fluorescence microscopy. They are frequently used to visualize in real time the assembly and function of proteins involved in membrane deformation, fission and fusion. Pipette aspiration, as shown in the figure, can be used to control membrane tension, whereas optical trapping can be used to pull out membrane tethers of controlled diameter. (3) Supported membranes typically consist of a lipid bilayer on a flat substrate such as a coverslip, grid or mica surface. They can be combined with high-resolution microscopy to observe the diffusion and assembly of individual protein and lipid molecules within the membrane. They are also useful for examining interactions between viral particles or live cells with synthetic membranes.

of the underlying machinery from purified components can be impractical, if not impossible. These limitations were even more pressing in the 1970s and 1980s, during the early decades of the field, when cloning, sequencing and modern purification methods were not available. Working within these constraints, many remarkable insights came from assays in which membranes taken from cells were 'complemented' with purified or other cell-derived components. This 'top-down' approach combines the sophistication of a functional biological membrane with the opportunity to test the impact of specific components added to the system. A prominent example is the discovery of the classic protein coats. Relatively early in their work to understand vesicular traffic, the Rothman and Schekman laboratories used purified COPI and COPII protein coat components to drive vesicle budding from cell-derived membranes[2,4]. In another example, Min Wu and Pietro De Camilli used cell-derived membrane sheets as platforms from which to observe, by fluorescence microscopy, the successive steps of protein recruitment during clathrin-mediated membrane budding[10]. Furthermore, by combining live T cells with reconstituted membranes and protein

components, the labs of Michael Dustin and Jay Groves reconstituted the assembly of the immunological synapse, using the ability to control the reconstituted target membrane as a tool for determining the underlying biophysical mechanisms[11]. Despite these notable successes, the complexity of cell-derived components can obscure precise molecular mechanisms, driving researchers towards reconstitutions with purified components.

Reconstitution with purified components

When a biological mechanism relies on a tractable set of components that can be purified, an in vitro reconstitution becomes possible. Protein and lipid components may be isolated from tissues, purified from host cells, or synthesized. This approach limits the complexity of the process under study. In exchange, it presents the unique opportunity to determine the set of components that is sufficient to achieve a particular function. In these experiments, interactions between purified proteins and lipid membrane substrates can be observed using innumerable analytical tools based on structural biology, biochemistry and quantitative imaging. Lipid substrates can range from small vesicles with diameters of a few hundred nanometres or less, to giant unilamellar vesicles, which are a micrometre or larger in diameter (Fig. 1b). While small vesicles mimic the curvature of trafficking vesicles and organelles, electron microscopy is required to resolve their structure. By contrast, giant vesicles are easily resolvable under the fluorescence microscope, enabling real-time visualization of membrane morphology, albeit at diffraction-limited resolution. Beginning in the late 1990s, in vitro reconstitution experiments, coupled with traditional cell biology, determined the proteins and mechanisms responsible for membrane budding and fission. Insights from structural biology and biophysics were combined to determine the function of the BAR family of protein scaffolds, along with membrane bending and fission by diverse proteins[12], including dynamin, endophilin and ESCRT scaffolds[13,14]. Similarly, the precise mechanisms of coat assembly, the first structures of purified vesicular coats[15–17], and the process of membrane budding by clathrin and its adaptors[18] have each proceeded from experiments with minimal systems of purified lipids and proteins. In many cases, the use of mutated proteins, often designed with the assistance of high-resolution structural data, has proven essential. These collective successes established in vitro reconstitution as a crucial part of the modern toolkit for studying membrane traffic.

Single particles and events

With the advent of super-resolution approaches in the early 2000s, and the steady improvement of fluorescence imaging in the decades since, it has become increasingly feasible to examine the dynamics of single membrane vesicles, proteins and assemblies during reconstitution experiments. This approach, which can be applied to experiments involving either fully purified proteins and lipids or cell-derived components (such as membranes, lysates and vesicles), has brought about the most recent, and perhaps most exciting phase of reconstitution in membrane biology. An early example of this approach in 2008 involved tracking individual enveloped virion particles as they fused with synthetic target membranes[19], revealing the dynamics of discrete and separable lipid and content mixing phases of the fusion process. Other in vitro experiments were used to demonstrate the ability of proteins to sense the curvature of individual membrane vesicles[20] or to monitor the disassembly of individual clathrin coats[21]. In both cases, individual particles (vesicles and coats) were tethered to the surfaces of coverslips so that they could be visualized individually with high spatiotemporal resolution, ultimately revealing the stochastic nature of the underlying processes while providing new mechanistic and functional understanding.

Current limitations and future opportunities

The fundamental challenge of reconstitution is often one of interpretation. However beautiful our simplified systems may be, how do we know that the mechanisms they suggest are true to what is going on in the cell? After all, a reconstitution experiment merely shows what 'can' happen when a limited set of biological components come together under controlled conditions, often far removed from those found in the natural cellular milieu. How do we narrow down this list of possibilities to identify a bona fide biological mechanism? In the words of Arthur Kornberg, "half of what we know is wrong, the purpose of science is to determine which half".

Admittedly, this task is not an easy one, and data from reconstitution experiments have sometimes led to detours along the path to understanding. One such example is the over-interpretation of data derived from protein assemblies on purified membrane substrates. In particular, the observation that dynamin proteins involved in membrane scission can form extended cylindrical scaffolds in vitro led, for a time, to the expectation that fission in cells should depend on such structures. However, live-cell measurements ultimately showed that the assembly of these structures is much more limited and transient[22]. Similarly, in the effort to identify mechanisms of membrane remodelling, if the ratio of proteins to lipids is too high, membrane bending and vesiculation will eventually result from non-specific physical effects such as steric pressure and electrostatic repulsion[23] – the physiological relevance of which depends on the stoichiometry in vivo.

Encouragingly, innovations in quantitative imaging such as those offered by lattice light sheet microscopy[24] and cryo-electron microscopy tomography[25] are increasingly providing live cell data on the stoichiometry, morphology and dynamics of biomolecular structures, which is needed to complement reconstitution experiments. Looking towards the future, we envision being able to compare the same process side by side in reconstituted systems and living cells, or even entire organisms. Although reconstituted systems can provide key insights on their own, a direct comparison with living systems will reveal how complex, regulated functions emerge from a set of biochemical components. This emerging toolset will make it increasingly possible to understand how cellular membranes organize themselves against a backdrop of staggering biochemical complexity. In particular, we are poised to probe the mechanisms that proteins and lipids use to assemble together, often on sub-second timescales, to provide robust, yet flexible, biological function. This is an enduring question that touches many areas of membrane biology from host cell–pathogen interactions and organelle contact sites, to the emerging roles of liquid-like protein condensates.

And perhaps one day, reconstitution will also enable abiotic systems and materials that are increasingly cell-like in their sophistication, yet simple enough to control. Cell biologists are traditionally motivated by fundamental questions. However, their growing skill in creating functional systems from minimal sets of purified proteins and lipids could be repurposed to provide the next generation of drug carriers, wound-healing agents, and immunomodulatory materials. Such an achievement would truly illustrate the beauty of simplicity.

We regret that we have only been able to cite a very small fraction of the many inspiring papers that have used reconstitution, in its many forms, to advance understanding of membrane trafficking.

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Competing interests

The authors declare no competing interests.