Visualizing Biological Membrane Organization and Dynamics☆

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Abstract

Biological membranes are fascinating. Santiago Ramón y Cajal, who received the Nobel prize in 1906 together with Camillo Golgi for their work on the nervous system, wrote "[…]in the study of this membrane[…] I felt more profoundly than in any other subject of study the shuddering sensation of the unfathomable mystery of life"[†]. The visualization and conceptualization of these biological objects have profoundly shaped many aspects of modern biology, drawing inspiration from experiments, computer simulations, and the imagination of scientists and artists. The aim of this review is to provide a fresh look on current ideas of biological membrane organization and dynamics by discussing selected examples across fields.

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Introduction

The field of biological membranes

Membranes are and have always been essential for biology, starting from their catalyzing role in the origins of life, be it by harnessing energy as ion gradients across them or by forming compartments for chemical reactions to take place, later on shaping organelles and transportation vessels. In their role as barriers, they maintain concentrations, form (electrochemical) gradients used for energy conversion, ward off access of unwanted substances, enable a fine control over influx and efflux into a given compartment, and thereby complexify the delivery of drugs. Furthermore, they are essential as matrices to support other (macro)molecules, in particular membrane proteins. They are able to store energy, and they are intrinsically linked to signaling processes. Biological membranes have shaped important parts of biophysics, structural and molecular biology, and molecular modeling. A compelling image of several biological membrane structures in the neuron is depicted in Fig. 1. The visualization of these objects is at the core of this review. By visualization, a term that may depend very much on the question being addressed is meant the understanding that is gained about these objects and their properties by making them visible to our eyes and minds through abstractions, images, pictures, and conceptualization. This interpretation closely follows the definition given by Ben Shneiderman: "The purpose of visualization is insight, not pictures" [1].

Early days of biological membrane investigation

A brief (and by no means complete) account on how membrane-related questions have accompanied the scientific progress over the last 50 years may start with the early visualization of bilayers and proteins in bilayers by freeze-fracture electron microscopy (EM) [2] as well as the observation of lateral diffusion of proteins in live cells [3,4]. X-ray diffraction measurements of Engelman [5] and calorimetric studies by Reinert and Steim [6] were critical to the acceptance of the lipid bilayer as the fundamental structural element of the membrane.

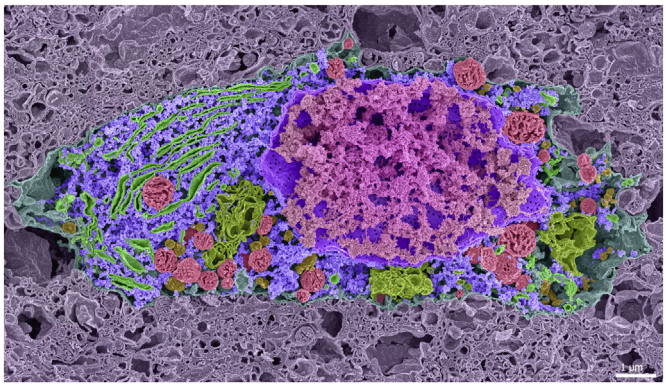

Fig. 1. Colorized scanning electron micrograph showing the internal structure of a single neuron. The membranes of the endoplasmic reticulum are bright green. The ER and other parts of the cell are covered in membrane bound cytoplasmic proteins and ribosomes, which are blue. The Golgi complexes are a dull green, and their products, lysosomes and vesicles, are yellow. The mitochondria are red. The outer membrane of the cell is turquoise. Microscopy and image processing: James Tyrwhitt-Drake, UVic AMF. Sample preparation: Dr. Patrick Nahirney, UVic Medical Sciences. See also: http://gigapan.com/gigapans/147187.

The fluid mosaic model of the structure of cell membranes introduced by Singer and Nicolson [7] provided a molecular vision that has deeply influenced many scientists. At about the same time, it had become clear that the association of protein complexes with membranes had functional consequences. Mitchell's [8] work has significantly contributed to this realization, for instance, by recognizing the importance of the electrochemical proton gradient for energy transformation. In these early days, Henderson and Unwin's [9] pioneering investigation paved the way for profound and consequential effects on the field of membrane structure through the determination of the low-resolution EM structure of bacteriorhodopsin. Early computational attempts look at the order–disorder transition in model bilayers [10].

Between 1980 and 1995

Between 1980 and 1995, the first molecular simulations of membrane systems appeared, investigating first mono- [11] and then bi-layers [12,13] on a 10- to 100-ps timescale, and in particular their phase behavior and the diffusion of solutes [14]. This progress made in molecular modeling enabled a straightforward atomic-level visualization of membrane systems filling an important gap with other existing techniques. During this period, in 1985, the Nobel prize in physiology or medicine was awarded jointly to Brown and Goldstein for their discoveries concerning the regulation of cholesterol metabolism [15,16]. Another Nobel prize rewarded Deisenhofer, Huber, and Michel for determining the three-dimensional (3D) structure of the photosynthetic reaction center [17]. This seminal work proved that the structure of membrane proteins could actually be determined, thereby providing detailed structural views of such objects which represents an important turning point in membrane biophysics. Pioneering structural studies use two-dimensional (2D) and 3D electron and x-ray crystallography. At this time, the two-stage model of membrane protein folding and oligomerization was introduced [18].

From 1995 to 1999

From 1995 to 1999, molecular simulations gained an order of magnitude in timescale, from 100 ps to 1 ns, examining peptides and small membrane proteins inserted in lipid bilayers [19]. Nevertheless, it became obvious that such calculations are extremely expensive in terms of computational resources and specific methods as well as simplified models were developed to go further. For instance, in coarse-grained models [20], the number of particles is typically reduced by about an order of magnitude. It became obvious that the structure determination of membrane proteins is lengthy and complicated; hence, many avenues were pursued to improve this situation. In this context, lipidic cubic phases have been studied extensively [21,22].

From 2000 to 2007

From 2000 to 2007, the 1- to 100-ns timescale becomes accessible and more complex phenomena are investigated, such as, for instance, electroporation [23] and the behavior of double bilayers. The scope of systems accessible through computational approaches has been strongly extended by the exponential development of coarse-grained simulation methods and force fields, leading to depictions of large membrane machinery and complex membrane mixtures. Membrane protein folding and insertion receive continued attention [24,25]. High-resolution microscopy techniques and cryo-electron tomography enable to go further in understanding membrane systems at the cell level [26,27]. More Nobel prizes concern protein structure determination by NMR, a technique later on applied to membrane proteins in a micellar environment [28,29], and the structural elucidation of important classes of membrane proteins such as aquaporins [30] and ion channels [31,32]. Around this time, the vision of membrane architecture based on the classical fluid mosaic model was refined to take into account the patchiness of the membrane [33], crowded by proteins, leading to segregated

domains and variability in properties such as thickness and lipid composition.

For 10 years now

For 10 years now, simulation times are within the 100-ns to 10-μs regime, and the complexity of systems under consideration has gone up steeply to reach multiple molecular assemblies such as membrane proteins in a trafficking organelle [34]. The attention received by these biomembrane investigations is due to several aspects. First, concerning membrane proteins, but also the ability to overcome membrane barriers, the pharmaceutical industry has major interests in these objects [35]. Membrane proteins, and ion channels in particular, are linked to many conditions such as Alzheimer, Parkinson, general anesthesia, tobacco addiction, drug resistance, bioenergetics, and vision. Antimicrobial peptides [36,37] bear importance as an alternative to antibiotics, to complement immune defenses and to fight bugs. Fundamental processes related to membranes and membrane proteins such as electroporation and the world of lipid phases are equally of interest. Some concepts continue to spawn animated discussions among scientists, such as lipid rafts [38,39]. Note that in this manuscript, the term raft will be used in a broad sense to denominate nanodomains in biological membranes, the existence of which seems beyond dispute. Lipids themselves are intrinsically linked to our health, be it through cholesterol levels or conditions such as atherosclerosis, angina, and infarct. Over recent years, the steep rise of the emerging field of lipidomics has led to renewed interest in biological membranes and an increasingly finer view on the role of individual lipid compositions [40–42].

Scope of this review

In this review, I will discuss how visualization contributes to shaping our mental image of membranes, membrane proteins, and related processes. It is by no means an exhaustive review, but rather a personal view based on my own experience as well as exchanges with colleagues active in the broad field of biological membranes. The aspects of visualization—in the broad sense that was introduced above—and conceptualization are central for the viewpoint adopted in this review. The discussion will address three complementary aspects: depictions obtained from experiments, visualizations from computational methods, and illustrations representing educated guesses to fill in missing data. The goal is to capture how the visualization (again, in a rather broad sense) of biological membranes supports and in some cases drives our understanding in the molecular biology of these objects.

A first focus concerns the organization of biological membranes in terms of a molecular view of these objects; hence, spatial arrangements, geometry, and deformations will represent important aspects. The focus may be on the lipids themselves, on protein within the bilayer or on reporter molecules. Adding dynamics (and hence to some extent noise) to initially static views is essential, as lipids are extremely dynamic objects with many different characteristic timescales, forming the second focus of the review. Dynamic views do not always make things clearer or easier to understand. On the other hand, many membrane properties can be described collectively, without considering the detailed dynamics of each individual lipid, and hence, such an approximative averaged picture may be sufficient and convenient to gain insight into certain questions. A general difficulty is that much of the information obtained provides an indirect view only, for instance, by following a given species in the membrane environment to better understand a biological process or mechanism. Hence, the required interpretation and extrapolation to all pieces of the membrane puzzle is in itself a major challenge. The review attempts to highlight important ideas and advances on how visual cues are shaping scientists' understanding of biomembrane landscapes and molecular interactions. It reflects my personal view of what may be a critical overview of the biomembrane field. The perspectives focus on current challenges and the bottlenecks to future major advances. A few thoughts on areas that may deserve more attention from the scientific community are provided as well.

Experiments

Visualizing membrane structures

Experiment is a primary source of information

Experiment is a primary source of information for membrane objects, and over the years, the great variety of experimental approaches and their improvements extended the accessible window of observation to many levels of size, resolution, and timescales. The lipid bilayer represents a fundamental object of interest. Different views contribute to our understanding, such as a physico-chemical picture of lipids based on model membranes, phase diagrams, diffraction of bilayer stacks, and so on, complemented by structural biology of isolated systems through x-ray diffraction and cryo-EM of membrane proteins. Then, atomic force microscopy (AFM) provides another level of detail, followed by structural biology approaches on more or less intact systems through cryo-tomography and superresolution optical microscopy. At the entire cell level, the repertoire includes depictions through fluorescence and optical microscopy. In all these approaches,

the resolution or underlying number of observables is a critical parameter. Static images are complemented by dynamics (or disorder) by a variety of approaches, for instance, NMR. Let us start our journey with detailed information on bilayer structure, down to an atom level description, which can be gained in particular from crystallographic and AFM studies as illustrated in Fig. 2.

Gaining detailed information on bilayer structure from experiment

The size scale spanned is about 4 orders of magnitude, in the nanometer range, between 0.2 and 800 nm. Crystallographic and neutron diffraction provided important details about lipid bilayers, going back to ground-laying neutron diffraction studies by Büldt *et al*. [48] and subsequent joint x-ray and neutron diffraction studies of fluid bilayer structure by Wiener and White [43,49]. The archetypical visualization derived from such studies is a scattering density profile (Fig. 2a) that allows to measure the bilayer thickness in a direct way [43]. These early x-ray and neutron diffraction studies of bilayers were crucial in the development and testing of MD simulations, as they provide an experimental metric to compare to. Actually, such lipid bilayer profiles played a critical role in the acceptance of the bilayer hypothesis by showing the transbilayer distribution and dynamics of the lipid component groups such as phosphates and carbonyls along the bilayer. A different visualization using an electron density map can nicely depict the bigger picture of bilayer orientation in specific phases (Fig. 2b) [44]. This picture can be further refined through AFM at submolecular resolution depicting the membrane surface as shown in Fig. 2c [45]. More complex processes can be observed, such as membrane remodeling by peptides (Fig. 2d, [46]) or the formation of raft-like structures (Fig. 2e, [47]). The question how these rafts are built in terms of their thermodynamic equilibrium, their induction by raft fabric-binding proteins such as bacterial toxins and other lectins, and so on, is precisely at the heart of ongoing investigations. Some influential work was performed by Kai Simons trying to understand the dynamic segregation of proteins and lipids into subdomains in the plasma membrane [50,51]. Important contributions to the raft topic are reviewed elsewhere [52,53]. AFM is a tool of choice for

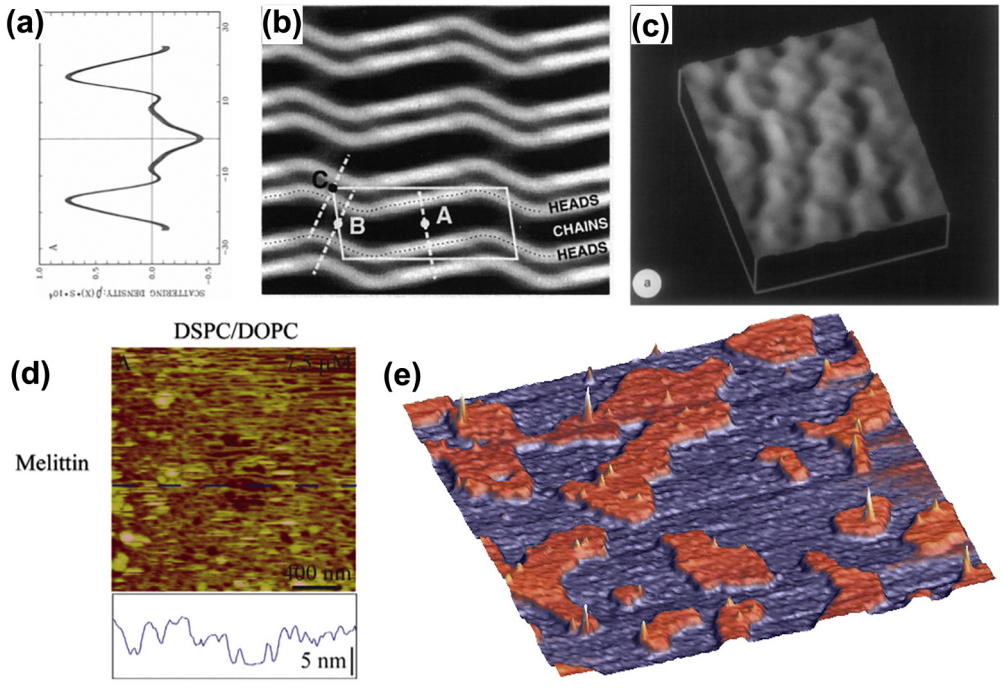

Fig. 2. Visualization of lipid bilayer structural properties. (a) Neutron scattering density profile of a fluid dioleoylphosphatidylcholine bilayer determined by joint refinement of x-ray and neutron diffraction data [43]. (b) electron density map of the ripple phase in lecithin bilayers [44]. Image copyright 1996 National Academy of Sciences. (c) AFM image of the surface of a DMPG membrane; the image is 2.4 × 3.0 nm^2, its height about 0.05 nm [45]. (d) Membrane remodeling by the melittin peptide showing a gross disruption at high peptide concentration [46]. (e) Sphyingomyelin rafts protruding from a DOPC background with an almost exclusively raft-associated glycosylphosphatidylinositol-anchored protein shown as yellow peaks [47].

imaging the surface topography of membrane domains [54]. At the other end of the length scale, membrane objects in a cellular context have been known and visualized for a long time with stunning details.

Toward larger-scale direct observations of membrane structure

We typically jump ×1000 from the nanometer to the micrometer scale, in the 1/100 to 10-micron range, to observe cellular objects constituted by membranes. Much information on the conformation of membranes can be obtained by optical light microscopy on a 10-micron scale [55]. The phenomena that have been observed include flexibility and undulations of membranes and lipid vesicle shape transformations. On a 10× smaller scale, Fig. 3a illustrates how electron-opaque stained lipids provide detailed transmission electron microscopy pictures of various tissues, here highlighting the Golgi apparatus and the distribution of very low density lipoprotein particles. Such staining techniques enabled early estimates of the width of lipid bilayers [62]. A particular interest has been devoted to the membrane curvature [63], and Fig. 3b to d depicts chosen examples from the review by McMahon and Gallop [57] emphasizing its key role in growth, division and movement. Earlier images obtained by freeze etching provided some of the first views on such objects, albeit with artifacts due to the sample preparation (Fig. 3e, [58]). Among others, visualizations of liposomes, lipid phases, and lipid rods could be obtained by this technique, at scales down to a tenth of a micron [64]. In combination with thin section EM [59], the rapid-freeze deep-etch images provide stunning details as shown in Fig. 3f

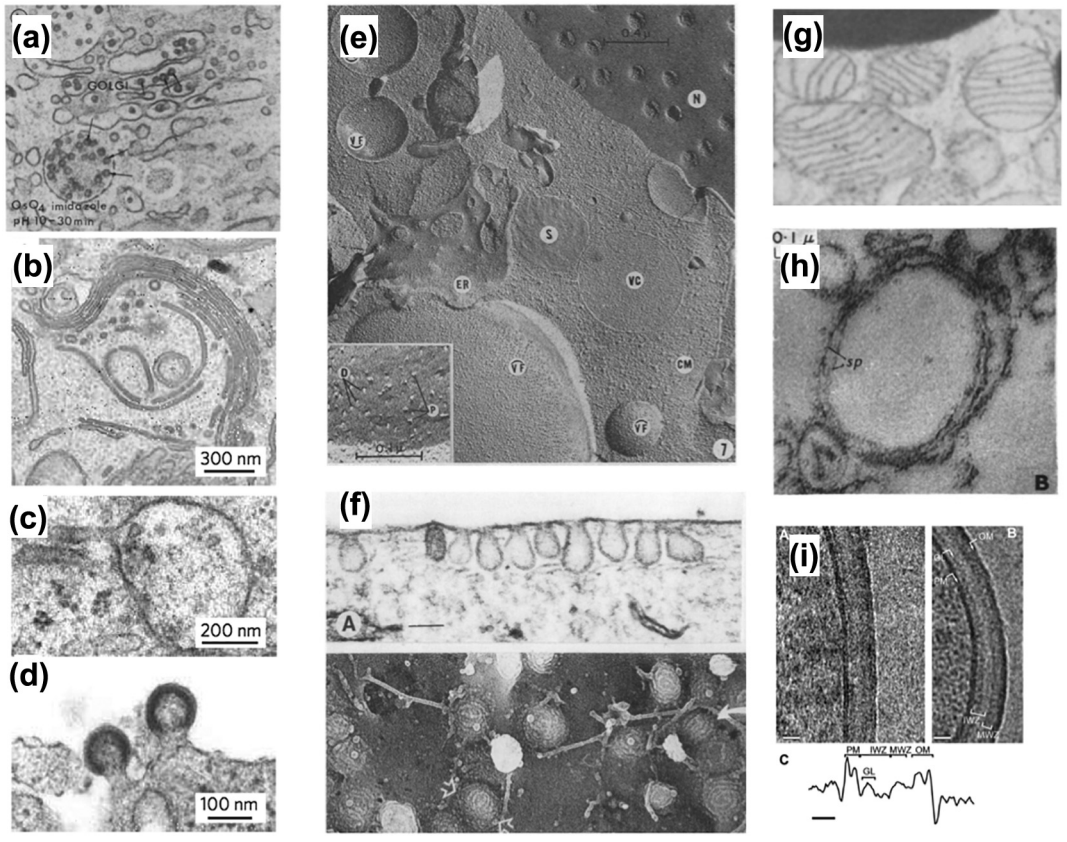

Fig. 3. Cell-integral biological objects formed by membranes. (a) Transmission electron microscopy of stained lipid bilayers depicting the Golgi apparatus [56]. (b) Fenestrations in the Golgi (from C. Hopkins and J. Burden, Imperial College London) [57]. (c) Tubule on endosomes (from P. Luzio and N. Bright, University of Utah) [57]. (d) HIV-1 viral budding (from W. Sundquist and U. von Schwedler, University of Utah) [57]. (e) An onion root tip shown with etching and featuring the surfaces of the cytoplasmic matrix (CM), vacuolar contents (VC), vacuolar membrane faces (VF), spherosomes (S), nuclear membrane (N), and endoplasmic reticulum (ER) [58]. (f) Caveolae in the plasma membrane of a fibroblast from ref. [59], Fig. 1. (g) TEM image highlighting mitochondrial membranes [56]. (H) Section of an isolated mitochondrial membrane with a three-layered structure and small particles attached [60]. (i) Cryo-EM of vitreous sections (CEMOVIS) of the cell envelope of *Mycobacteria smegmatis* featuring plasma membrane (PM), inner wall zone (IWZ), medial wall zone (MWZ), granular layer (GL), and outer membrane (OM) [61].

for a plasma membrane with a high density of caveolae, revealing their specific coat texture. Complementary depictions of objects such as mitochondria can be obtained, for instance, globally highlighting their membranes (Fig. 3g, [56]) or focusing on small particles attached to the surfaces of isolated mitochondrial membranes (Fig. 3h, [60]). With techniques such as cryo-EM of vitreous sections (CEMOVIS), one can zoom in to the very details of membrane structure in cell envelopes, for instance, revealing the fact that bacteria have an outer membrane (Fig. 3i, [61]). Another helpful approach is to focus on the membrane plane using supported phospholipid bilayers and epifluorescence microscopy to depict liposomes, tubular structures, and membrane defects [65].

An obvious question arises about how we can fill the space between those two extreme scales at the atomic level and the organelle one, as well as enrich the scope of objects and processes that can be addressed. Several solutions exist. Several classes of proteins may have intrinsic links with membranes and may hence act as reporters for the underlying membrane structures as illustrated in Fig. 4.

Using reporter molecules to bridge scales of observation

The red blood cell has been studied for a long time, representing an emblematic membrane-based biological object. Its membrane is supported by a spectrin-actin lattice termed the membrane skeleton, which can be visualized, for instance, by negative staining EM (Fig. 4a, [66]). Hemagglutinin membrane distributions in relation to its possible ability to cluster in lipid rafts have been investigated by combining different approaches, for instance, fluorescence photoactivation localization microscopy, reaching subdiffraction resolution [67] with EM

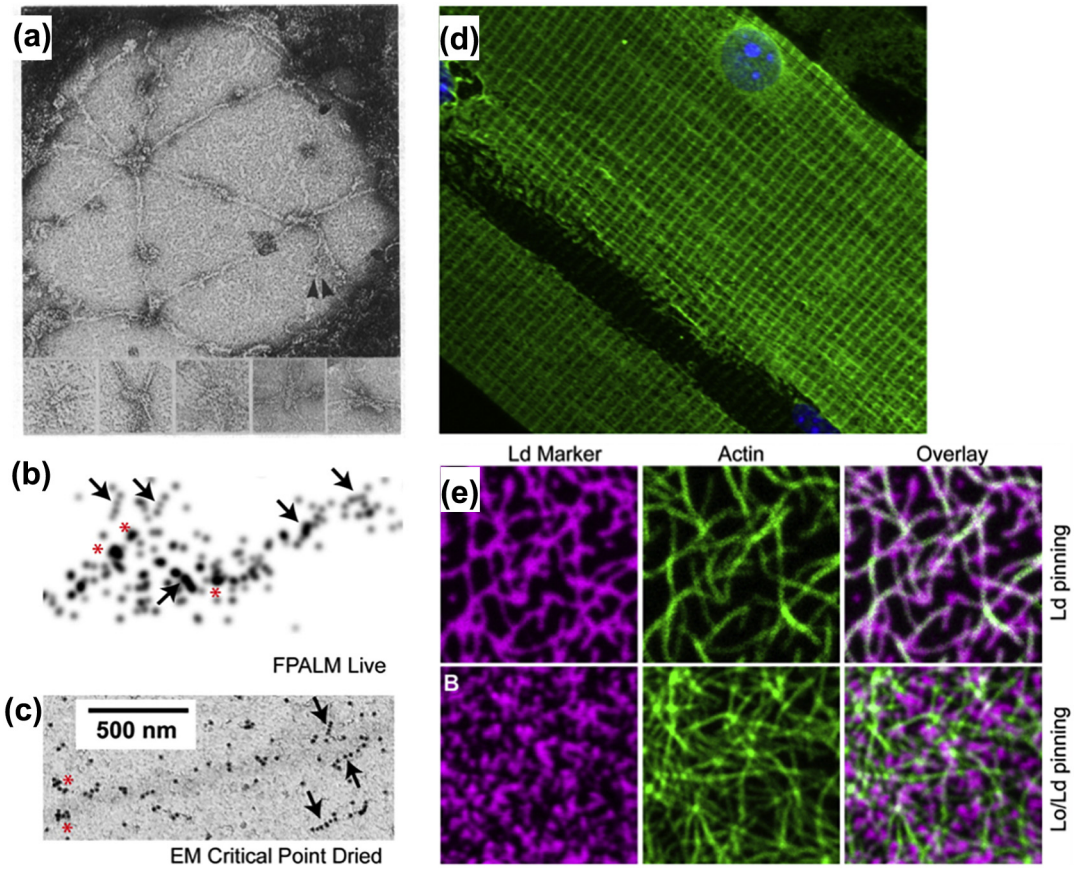

Fig. 4. Insight on biological membranes through characteristic protein structures. (a) Protein associations in the erythrocyte membrane skeleton, in particular junctional complexes involving spectrin [66]. (b) Hemagglutinin protein clusters in live cells obtained by fluorescence photoactivation localization microscopy [67]. Copyright 2007 National Academy of Sciences. (c) TEM image of hemagglutinin clusters in fibroblast membrane sheets [67]. Copyright 2007 National Academy of Sciences. (d) Dystrophin organization on the sarcolemma [68]. (e) Actin network influence on lipid phases in model membranes [69].

(Fig. 4b, c). The question of recruitment to lipid rafts has been investigated using single-molecule near-field optical microscopy, localizing reporter molecules to approximately 3-nm accuracy in 250-nm small domains [70]. The sarcolemma is the cell membrane of a striated muscle fiber cell and represents another source of insight into biological membranes. Figure 4d shows sarcolemmal features revealed by staining the dystrophin network attached to it [71]. The eukaryotic cell membrane as well features characteristic protein connections to dense actin networks. Fluorescence correlation spectroscopy and stimulated emission depletion experiments imaged by confocal microscopy have been used to illustrate the influence of this network on lipid phase separation [69].

A zoom on EM, x-ray, and AFM approaches

As mentioned above, EM and AFM techniques have from their beginning been key approaches, sometimes in close link to crystallography, shaping our mental images of membrane objects. Through subsequent refinement, and also by choosing specifically suitable objects, very in-depth studies and visualizations could be achieved as depicted in Fig. 5.

The purple membrane is one such particularly suited object studied extensively in the literature. Early insights from EM revealed some of its features with great detail, down to about 0.7 nm [9] as shown in Fig. 5a and b. Crystallographic studies provided additional insight, also resolving the lipids surrounding the membrane protein and essential water molecules for proton translocation (Fig. 5c, [72]). AFM images refined the view by exposing the overall arrangement in the membrane to a high precision [73] as in Fig. 5d and recording conformational changes, for example, of surface-exposed loops [74] as in Fig. 5e. These loops could be further refined by a combined view from several techniques [76]. To what extent x-ray crystallography provides an accurate picture of membrane lipids [77,78] has led to discussions and a critique in the scientific literature [79]. An alternative for structural characterization of membrane proteins themselves is

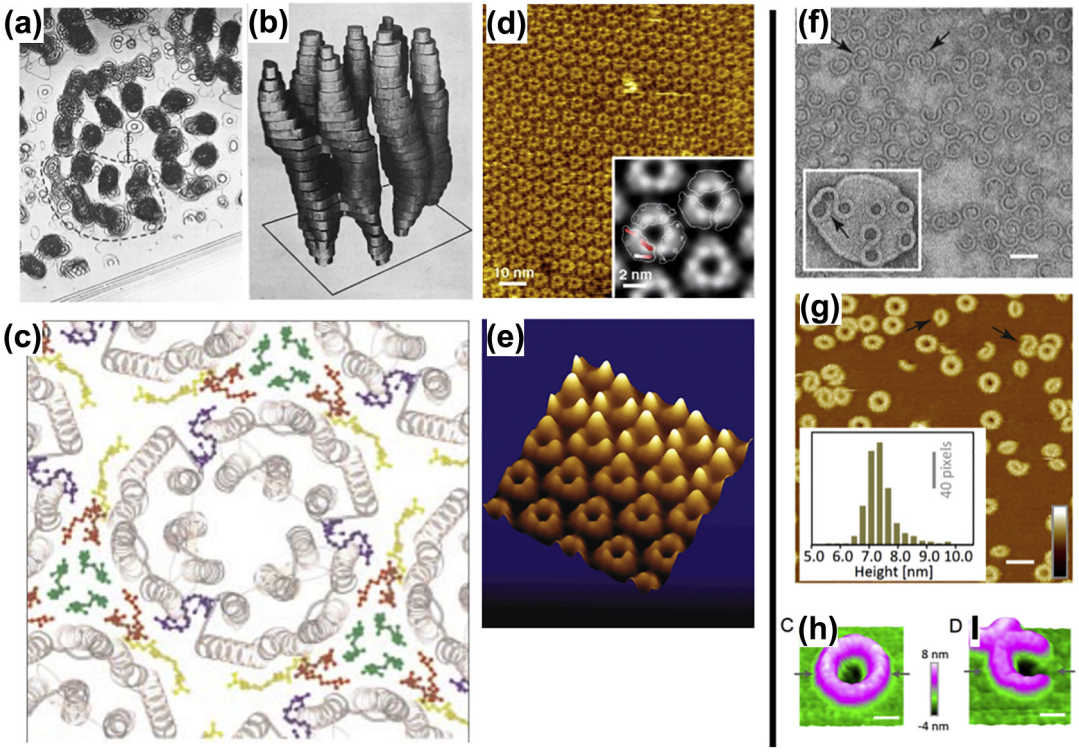

Fig. 5. Near atomic scale insights on membrane objects. (a) Three-dimensional potential map spanning the purple membrane obtained by EM [9]. (b) A model of a single bacteriorhodopsin molecule constituting the purple membrane [9]. (c) Crystal structure highlighting protein, lipid, and water organization in the purple membrane [72]. (d) Cytoplasmic surface of the purple membrane showing individual bacteriorhodopsin molecules at subnanometer resolution [73]. (e) Force-induced conformational change of bacteriorhodopsin surface loops [74]. (f) Negatively stained EM of arc- and ring-shaped suilysin oligomer assemblies that perforate a liposome membrane [75]. (g) AFM topography of suilysin on a supported lipid bilayer [75]. (h) Detailed AFM topography of a complete suilysin ring [75]. (i) A suilysin arc only partially enclosing the dark hole in the membrane [75].

provided by NMR spectroscopy [29] and can be extended to lipid–protein interactions [80]. The purple membrane is not the only object suitable for in-depth characterization. Vesicle-bilayer complexes have been visualized [81,82] among the many important features on the membrane surface. Figure 5f to i provides one other example on the stepwise visualization of membrane pore formation by a bacterial cytolysin that forms either ring- or arc-shaped oligomers [75]. The time evolution of such processes can be followed.

Selected examples highlighting recent evolutions

Our insight into such membrane structures has recently significantly been heightened by the most advanced EM approaches. In particular, single-particle cryo-EM reconstructions and tomographic studies have significantly enriched the portfolio of available techniques to probe complex and large-scale membrane structures as depicted in Fig. 6.

Membrane structures are ubiquitous and have diverse shapes, as, for instance, illustrated by the SEM image of vesicles at a nerve ending shown in Fig. 6a or the various membrane structures evidenced in the colored micrograph of Fig. 1. Using more recent cryo-EM approaches, through the so-called resolution revolution [85], even more detailed depictions can be obtained. Specific interactions may be highlighted, such as between liposomes and amyloid fibrils [83] in Fig. 6b, the end binding of actin filaments to the plasma membrane [26] in Fig. 6c, or between SNARE proteins and lipid bilayer nanodiscs [86]. Intermediate stages of important biological processes such as mitochondrial fusion can also be witnessed as shown in Fig. 6d where two mitochondrial outer membranes are connected by a mitofusin docking ring, leading to the formation of a pore [84]. Tomography now enables the 3D reconstructions of whole cells, as illustrated in Fig. 6e, that can be co-refined by a combination with computational approaches [27]. Many

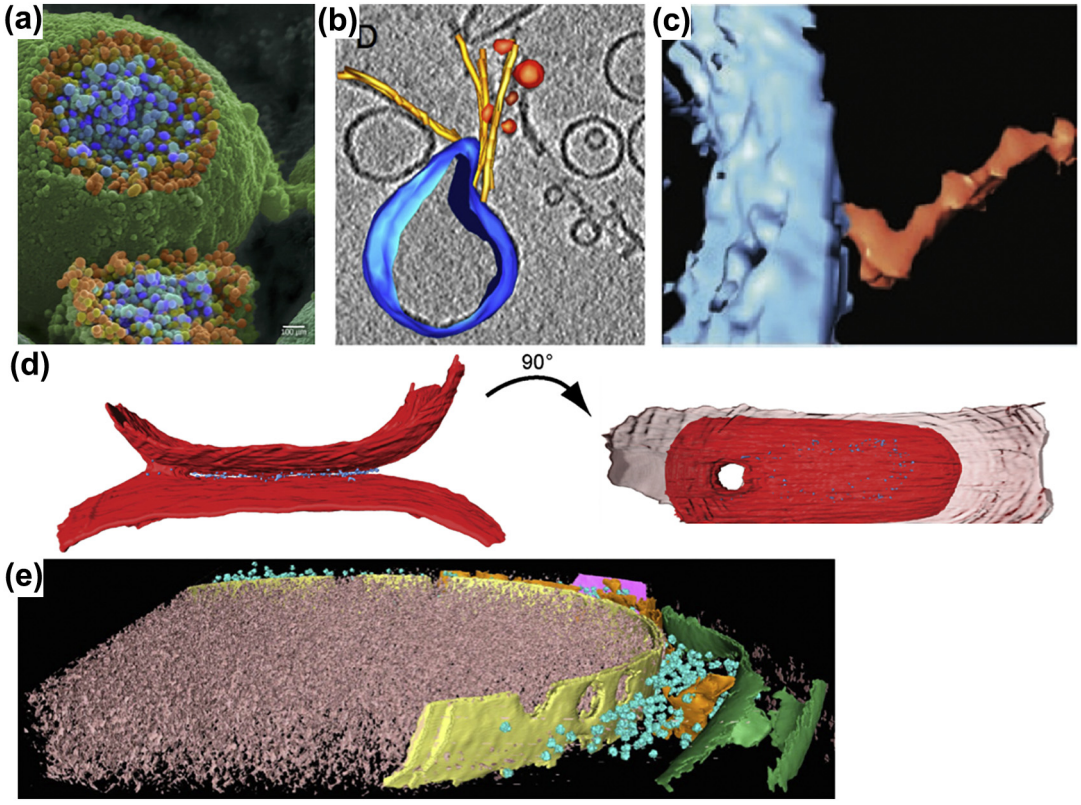

Fig. 6. Toward membrane machinery at a larger scale and in three dimensions. (A) Scanning electron microscope image by Tina Carvalho, University of Hawaii at Manoa, depicting a nerve ending. It reveals vesicles shown in orange and blue color. Image under a CC-BY-NC-SA license. (B) A rendered 3D model of a distorted liposome interacting with fibrils and surrounded by small vesicles [83]. (C) The actin cytoskeleton of a eukaryotic cell visualized by cryoelectron tomography, here zooming in on the actin–membrane interaction [26]. (D) Two docked mitochondria (red) at an intermediate stage of outer membrane fusion with an apparent toroidal pore of 40-nm diameter [84]. The blue volumes at the interface are attributed to a docking ring, possibly formed by mitofusin protein assemblies. (E) Tomographic reconstruction of a *S. cerevisiae* cell with a portion of the cell wall in magenta, mitochondrion in green, and ER in orange. Reprinted with permission from Ref. [27]. Copyright 2017 American Chemical Society.

membrane structures are thereby revealed. In specific cases such as viruses, this technique even permits to resolve secondary structures of (membrane) proteins within their membrane environment [87]. An interesting extension to these approaches is to combine EM with other techniques. For example, *in vivo* fluorescence video microscopy has been demonstrated as a suitable technique together with EM for following the morpho-functional organization of the intracellular membrane trafficking pathways and monitoring structures such as transport carriers [88].

Adding dynamics to the picture

Membranes are intrinsically fluid and dynamic objects as already mentioned in passing in the previous section. Specific experimental techniques have been developed to be able to visualize the temporal dynamics of membrane phenomena. A very widespread approach is to track the movement of domains or reporter molecules in membranes.

Tracking the movement in the membrane

Using single dye tracing, Schutz *et al.*[89] imaged the lateral motion of single fluorescence labeled lipid molecules in native cell membranes on a millisecond time scale, achieving a positional accuracy of ca. 50 nm (Fig. 7a). Thereby, they followed lipid-specific membrane microdomains. Such a spatial resolution is necessary to distinguish different cases of lipid diffusion as induced by barriers such as rafts, which is illustrated in Fig. 7b,c [90]. Such tracking experiments can also be combined with high resolution imaging, for instance, enabling insight into how the actin meshwork may condition the diffusion of membrane proteins [91] as depicted in Fig. 7d and e. This combination provides a view on the compartmentalization and barriers inducing anomalous diffusion in a membrane environment. High-speed single-particle tracking techniques and their influence on our understanding of the plasma membrane dynamics have been reviewed in light of different underlying concepts [92] and extend the accessible timescales down to about 1-μs temporal resolution, by achieving 2-nm spatial precision [93]. Such techniques have enabled to observe single lipids moving about rafts [94]. However, maybe more important than the precise "image" produced by such approaches, their statistical treatment and analysis provide further insight [95]. The tracking of single molecules is not the only approach to gain temporal insights. With improvements in the AFM technique, it has been possible to track the surface of membranes in incredible detail and with continuously improving time-resolution as depicted in Fig. 8.

AFM-based membrane surface tracking

Early studies investigated phenomena such as drug–membrane interactions, here the interaction of

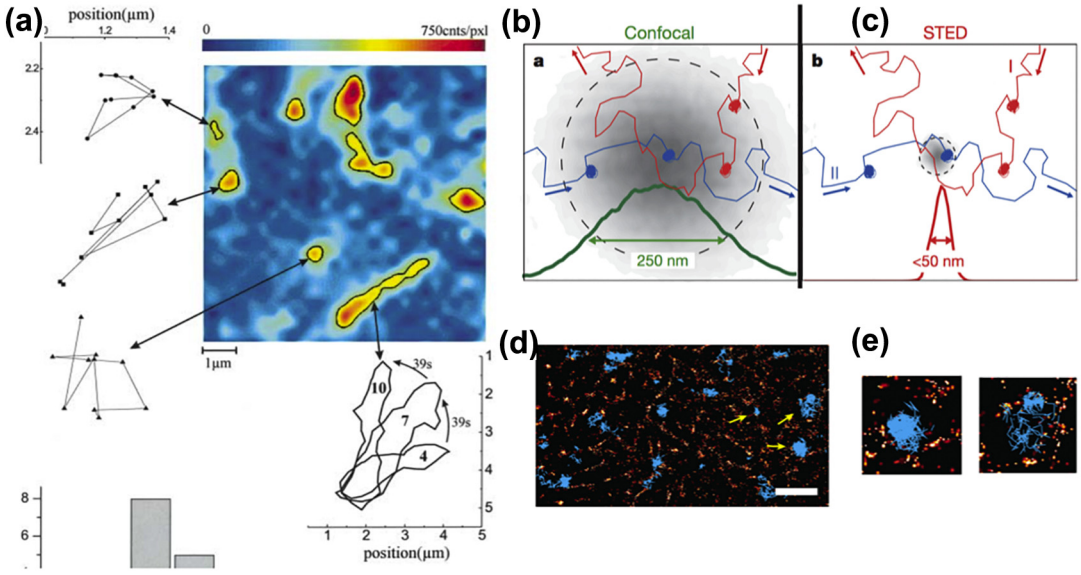

Fig. 7. Reporting on membrane dynamics by tracking individual molecules or domains. (a) Single-molecule microscopic visualization of lipid microdomains in a muscle cell membrane [89]. (b) Following molecules in a confocal microscope is limited in detail due to the large detection area [90]. (c) stimulated emission depletion is able to discriminate between freely diffusing and hindered lipids due to its small subdiffraction spot size [90]. (d) The image depicts the trajectories of individual potassium channels shown in cyan overlaid on an actin photoactivated localization microscopy image shown in red [91], scale bar is 2 μm. (e) Enlargement of two areas of panel d illustrating confinement in a large compartment (left) and hopping between two compartments (right) [91], scale is ×4 compared to panel d.

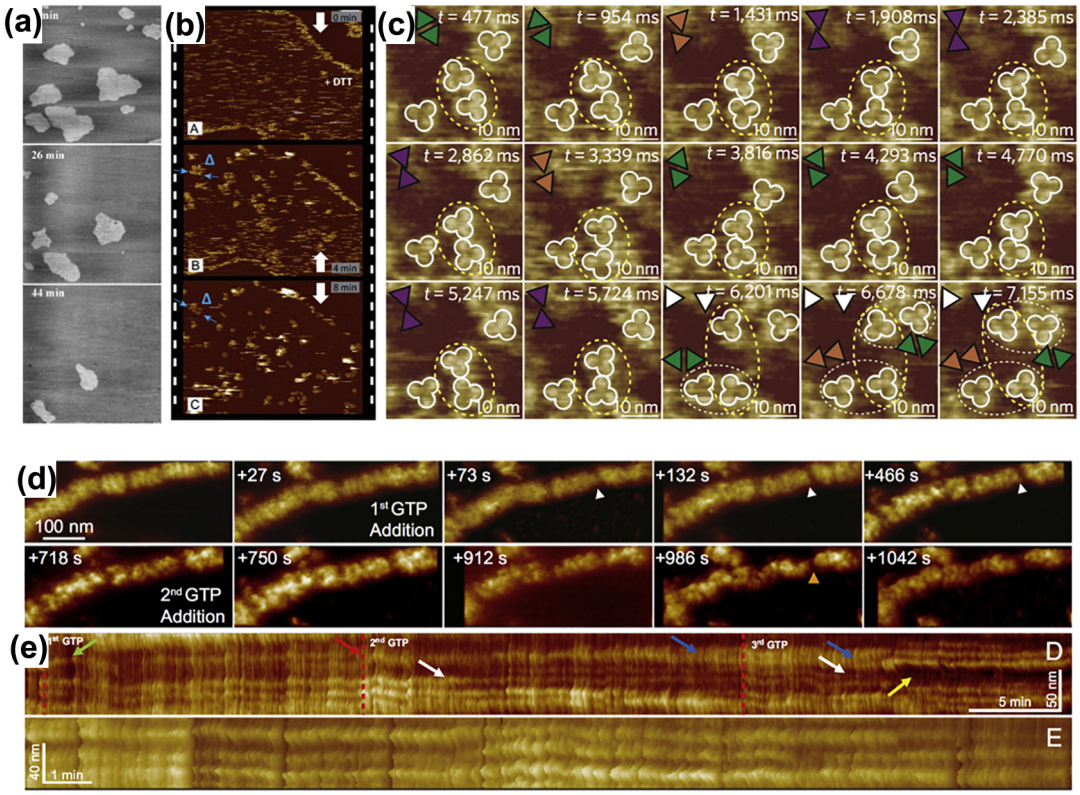

Fig. 8. (High-speed) AFM tracking of membrane processes. (a) Real-time imaging of drug–membrane interactions [96]. (b) Real-time imaging of the prepore-to-pore transition by suilysin with prepore intermediates appearing as diffuse streaks in the top panel, becoming more clearly defined in the center panel, leading to the pore state shown on the bottom [75]. (c) High-speed AFM of the outer-membrane protein F extends timescale resolution to hundreds of microseconds [97]. (d) Membrane tubule constriction through the dynamin helix related to membrane fission [98]. (e) Kymograph illustrating morphological changes of the dynamin helix along the tubule axis depending on GTP treatment or not [98].

the antibiotic azithromycin with lipid domains in model biomembranes (Fig. 8a, [96]). By using time-lapse images over 60 min, a progressive erosion and disappearance of the bilayer gel domains were observed with a certain type of bilayer, providing evidence that the perturbation of lipid domains strongly depends on the lipid nature. Figure 8b revisits the suilysin example discussed previously, now explicitly adding the temporal dimension completing the process on a ~20-min scale. Much higher time resolution can now be achieved using high-speed approaches [97]. Figure 8c features the sequence of separation of two OmpF proteins implying molecular shear and rotational movements, visiting a range of interaction states fully compatible with a coarse-grained simulation model. As previously discussed, our information is not always direct in terms of observing the membrane or membrane proteins themselves, but can also be guided by reporter molecules. Such is the case in the study of membrane fission-related dynamin helix changes depicted in Fig. 8d and e [98]. The dynamin helix acts upon a membrane tubule. Another interesting feature of AFM is to probe force-induced conformational changes as has been done by monitoring the unfolding pathways of individual bacteriorhodopsins from the purple membrane [99]. Other sources of information on dynamics exist, often applied to model membranes from supported lipid bilayers, giant unilamellar vesicles, and giant plasma membrane vesicles [100,101]. By combining several approaches, additional insight may be gained and complementary pictures emerge as in the study of the formation of liquid ordered domains combining confocal microscopy with fluorescence microscopy and AFM [102]. In some cases, the dynamics of objects can be tracked in three dimensions [103,104].

The dynamic information that is obtained by these various experimental approaches, either a series of fixed images at certain time intervals or more recently videos, depicts the sequence of events but cannot properly account for the stochastic and random aspects of processes that are typically ensemble phenomena. Therefore, one must keep in mind that our (partial) observations are driven by a given kinetics and only represent certain aspects of the true biological process.

Characterizing membrane dynamics naturally leads to imaging approaches, some of which have already been touched upon.

Imaging, just imagine

Much progress has been made in imaging, and many techniques and variants thereof are available. Lyman *et al*. [105] point out the general progress made in visualizing dynamics and membrane organization through new experimental approaches, some of which are directly related to imaging. At the core, we find fluorescence-based approaches, able to reach submicron molecular dynamics (MD) measurements [106]. This may require specific developments, such as, for instance, nano-antennas capable of probing single-molecule dynamics in the plasma membrane of living cells [107,108]. Improved spatial (super)resolution of the imaging has been achieved for electron and near-field microscopy, as well as fluorescence-based approaches [109–111], which had a clear impact on the study of membrane rafts [112,113] and caveolae [114]. The most recent advances deliver volumetric 3D image series of subcellular processes, including endocytosis and membrane dynamics [115].

One of the many fluorescence-based techniques that provide useful insight into membrane systems is fluorescence lifetime imaging microscopy (flimscopy), which can, for instance, be applied to liposomes [116] and living cells [117]. Time-resolved fluorescence images may provide detailed insight into phenomena such as cell membrane permeabilization and DNA/membrane interaction by electric pulses at subsecond resolution [118] or in dynamin-catalyzed membrane fission and vesicle release [119].

Limiting the perturbation induced by labels and further progress

A general concern with many approaches is that tags might interfere with *in vivo* function. In comparison to single-particle tracking of membrane components [120], where relatively large gold particles or antibodies are used for tracking, smaller molecular labels can be used for imaging membrane regions, for instance, to monitor plasma-membrane proteins [121]. The smaller labels reduce the impact such particles may have on altering the very dynamics under study. Label-free approaches such as interferometric scattering microscopy [122] and coherent brightfield microscopy [123] can completely elude the issue of probe perturbation [124]. Single dyes can be detected as well in fluorescence images, enabling the imaging of single molecule diffusion [125]. The combination of photoactivated localization microscopy with live-cell single-particle tracking leads to spatially resolved maps of single-molecule membrane protein motions [126].

Some existing techniques that lacked imaging capabilities have been extended, as is the case for Fluorescence correlation spectroscopy. By imaging total internal reflection fluorescence cross-correlation spectroscopy data, diffusion phenomena in lipid membranes could be probed with good temporal (millisecond) and spatial (microns) resolution [127].

Another area of progress concerns the increasing repertoire of (membrane) probes, some of which are capable of sensing properties of their environment such as membrane polarity [128], orientation [129], or tension [130]. Ratiometric biosensors have been developed as well [131,132]. Alternatively, very detailed insight into physico-chemical membrane properties may be obtained by combining techniques. For example, by coupling laser confocal scanning microscopy to microelectrochemistry, a pH profile for membrane permeation processes can be imaged [133]. Similarly, electrical measurements co-recorded with second harmonic generation micrographs lead to images describing structural and dynamic variations within a single bilayer [134]. Second harmonic generation is a powerful technique, able to probe even fine details such as water chirality and environment in a membrane environment [135].

Many of the experimental techniques described so far rely on reporter molecules, which can typically be among the lipids or among the membrane proteins. Hence, the acquired data are indirect and an important aspect is what is actually "seen" by a given technique compared to what remains hidden. The probes may induce a preference for specific lipid phases, hence introducing some bias in the measurements.

From average properties to simulations

Many ideas and concepts, less so comprehensive pictures, can be derived from the measurement of average properties [136]. The bilayer density shown in Fig. 2a is a typical example; lipid order parameters as measured by NMR are another. Information on the lipid–protein interface was obtained early on by ESR and NMR [137–139]. NMR spectra thereby enable insight into bilayer properties, polar lipid headgroup features, their angle with respect to the bilayer, and so on, and are sensitive to the environment, for instance, the ionic force. To provide a single emblematic example of the unique role, such information may play to shape our conceptual images, lipid bicelles should be mentioned, for which indirect techniques such as NMR and neutron diffraction provide measurements [140,141], which are, however, compatible with a range of models. They develop their full scope when combined with computational approaches [142–145] that will be discussed in the next section.

Simulations and Computational Approaches

A distinctive feature of molecular simulations is that all ingredients of a simulation system are seen

with high fidelity, and no reporter molecules or probes are necessary to observe biological membranes in atomistic detail. Hence, molecular simulations, and in particular the MD technique, were crucially important as the primary tool for direct membrane visualization. Their accuracy comes with the limitation of comparatively small spatial scales, typically within the tenths of a nanometer to 1000-nm range. This scale is appropriate to compare to above-mentioned diffraction experiments, which were crucial for validating the transbilayer distribution and dynamics in the bilayer in the early days of MD simulations; see, for instance, the studies described in Refs. [12,146,147]. Timescales are short as well, from picoseconds to tens of microseconds, although this is somewhat depending on the underlying model of representation. Both time and spatial resolutions are high, routinely on the order of tenths of nanometers and picoseconds, respectively. An intrinsic limitation is that one can only observe the molecular species that were built into the model to be simulated. Hence, building accurate starting models for molecular membrane simulations including all relevant compounds is essential.

Building biological membrane models

A famous quote by Richard Feynman states, "What I cannot create, I do not understand." Many tools for putting together membrane-related molecular constructions exist and a few of them are discussed here as they represent an important cornerstone for our molecular understanding of these systems. Unless the computational power behind simulations

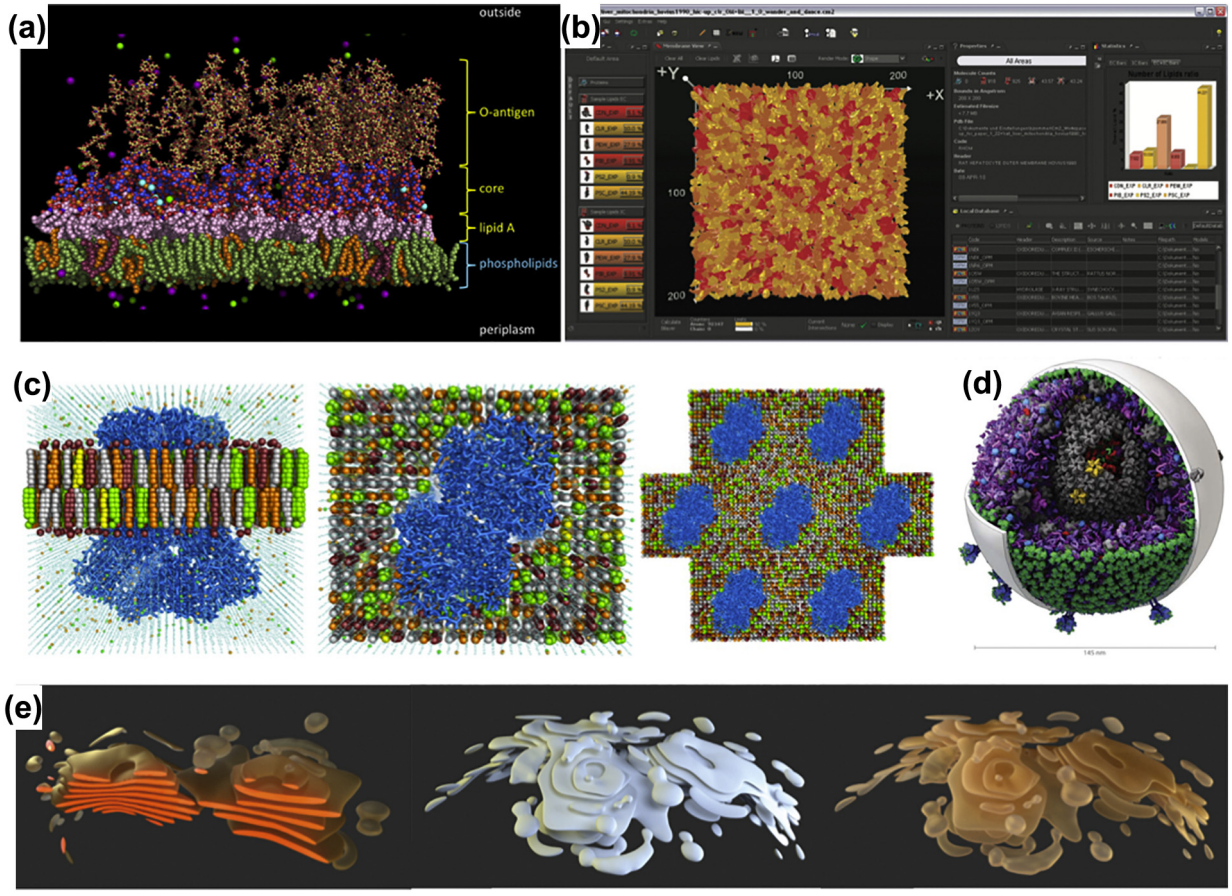

Fig. 9. Membrane building tools. (a) Molecular system representing a typical gram-negative bacterial outer membrane with lipopolysaccharide [155]. (b) Building an outer mitochondrial membrane model with the CELLmicrocosmos MembraneEditor. Reprinted with permission from Ref. [156]. Copyright 2011 American Chemical Society. (c) Setting up a cytochrome bc1 complex in a hexagonal prism within a five-component bilayer. Reprinted with permission from Ref. [157]. Copyright 2015 American Chemical Society. Views are from the side, from the bottom and overviewing the lattice. (d) Result of applying a cellPACK recipe for stochastic packing of a whole HIV-1 virion model in its envelope [158]—published by The Royal Society of Chemistry. (e) Modeling the Golgi apparatus in three dimensions from freeze-fracture SEM data [159]. On the left, a cut model highlighting cleaved surfaces as in an SEM image. In the middle, the 3D model; on the left, rendering with final material.

is sufficient for a model to fully equilibrate, it is essential to build the best possible initial systems for the computations to produce reliable results [148,149].

Some of the available tools for building membrane systems [150] are directly integrated into the molecular modeling workflow, for instance, as extensions to the popular Visual Molecular Dynamics software [151] or as part of widely used web services such as CHARMM-GUI [152–154]. Figure 9 illustrates a few examples of membrane building tools. A central issue is the packing of the lipid bilayer [160], which has been addressed for example through shape-based approaches [156], 2D bin packing [161], or by using coarse-grain lipid templates [157]. Another trend is to take into account the full complexity of lipid compositions [162–166]. Modern tools attempt to integrate both aspects of packing and lipid composition as illustrated in Fig. 9a–c. For setting up membrane proteins in their environment, automated pipelines such as the MemProtMD (http://memprotmd.bioch.ox.ac.uk) resource exist [167,168]. The curvature of membrane objects offers another challenge for building models, for instance, in the case of vesicles. If the strain induced by such curvature is not compensated for, a model may be in a metastable tense state by construction, leading to biases that could, for instance, substantially facilitate vesicle fusion. A link between curvature and packing defects exists [169,170]. Nowadays, the ambition is to build and visualize cell-scale models with astonishing accuracy [155,158,159,171–173]; see Fig. 9d and e, for examples, an undertaking that can be greatly helped by efficiently exploiting GPUs [174]. An emblematic example where such advanced model building yielded extremely valuable and scientifically sound insights is the study of a trafficking synaptic vesicle [34] (Fig. 10). The model provides a detailed picture in terms of protein and lipid composition, vesicle size, and copy number of its major constituents by integrating quantitative data and structural models.

Quite naturally, such models require a rich repertoire of complementary simulation approaches to bridge the different timescales and length scales.

Simulating biological membrane dynamics at multiple scales

The vast majority of simulation and modeling approaches described hereafter integrate motion and provide a dynamic view of lipid bilayer-related

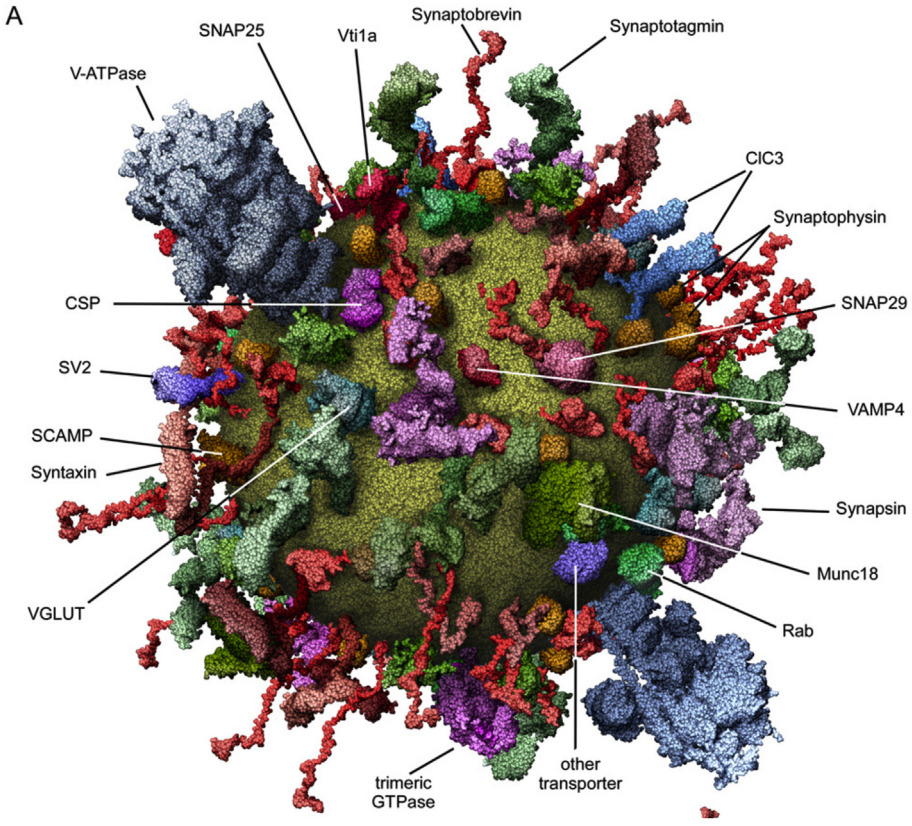

Fig. 10. Molecular model of an average synaptic vesicle. An outside view of the vesicle based on space-filling models of all macromolecules at near atomic resolution [34].

structure and function. An average image can be extracted as well, for instance, in relation to aspects such as ionic conductance, osmotic permeability, electrostatic potential, pressure profile, and so on [175,176]. Most simulations discussed here rely on a force field to represent the lipid bilayer [177–181]. An overwhelming number of exciting studies exist in this field, yet only a tiny fraction will be alluded to in the present review. For a more complete account, the reader is referred to comprehensive reviews such as Refs. [182–189]. The fundamental role that MD simulations played and still play in our understanding of membrane structure has to be stressed, as it cannot be discussed in detail within the scope of the present review.

Challenges and limitations

Concerning the description of the various modeling scales used to represent membrane systems, we will follow the nomenclature from Ref. [190], mostly discussing representations at the bead- and elastic scales. Every detail of a chosen model system can be followed using MD; hence, the choice of the extent of the model and of the aspects that are to be analyzed is crucial. Therefore, dealing with the complexity of membrane systems in terms of their represented size and the multiplicity of lipid components is essential. Examples include attempts to capture plasma [165], thylakoid [191], and bacterial [163,192] membranes. An accurate representation of the diverse glycolipids poses some modeling challenges; the asymmetry in membrane leaflet composition adds to the complexity. Similar issues exist for the (membrane) protein representations. For example, the latter often neglect post-translational modifications that play crucial biological roles as has been shown for the epidermal growth factor receptor [193]. In the spirit of the famous quote “all science is either physics or stamp collecting” by Lord Rutherford, one may point out that observing a single (or even a handful) of molecular trajectories is more akin to stamp collecting than to capturing the true MD (“physics”) of a biological system, which is often overlooked when MD results are interpreted. Much more extended sampling—in our experience, typically tens of replicas of relevant duration (which depends on system size, but as an order of magnitude say on the order of several microseconds at least)—is needed to obtain statistically reliable and significant data.

Atomistic MD simulations

Atomistic MD simulations were among the first approaches to generate insight into membrane dynamics [10,12–14,194–196], for instance, with respect to long-range undulatory and peristaltic modes [147,197–199]. At the same time, the case of such membrane undulations illustrates the intrinsic timescale and length scale limits such simulations have to come up against. Figure 11 attempts to retrace a small selection of historic landmarks in the field. Figure 11a to c depicts a progression in the simulations in terms of membrane complexity. Figure 11a is among the first depictions of a simulated pure lipid bilayer. Inclusion of peptides and proteins into the membrane simulations paved the way to study large biological machinery, starting from model systems such as the gramicidin A channel [19,202] shown in Fig. 11b. The next step is to encompass a more complete membrane environment, for instance, including the cell wall structure as depicted in Fig. 11c [200]. Many membrane processes have been investigated, and in addition to complexifying the membrane itself, more intricate membrane phenomena have been simulated and visualized even for pure membranes. Among these, electroporation is particularly spectacular [23,203] (Fig. 11d). More recently, cavitation bubble collapse has been investigated for drug delivery [204,205]. Figure 11e shows another level moving toward the simulation of large functional membrane patches: a landmark study investigates a multicomplex photosynthetic membrane as it most likely appears in the cell [201] (Fig. 11e). It consolidates several experimental results in an MD simulation of a large multiprotein–membrane system.

Coarse graining of membrane systems

Coarse graining of membrane systems provided a significant step forward in terms of improving sampling, spatial and temporal scales [206–210] involving many simulation groups such as those of Orsi and Essex [211], Klein *et al*. [20,212], Marrink *et al*. [213], Sansom *et al*. [214,215], and Izvekov and Voth [216]. Observing the lipid phase behavior as illustrated in Fig. 12a and b is a typical application [217] and bears, for example, some relevance in relation to the previously mentioned hemagglutinin distribution of Fig. 4c [218]. Membrane fusion is another fascinating topic of foremost importance where our ideas have been shaped at least in part by molecular simulations [222–225]. Membrane remodeling in general can be investigated, for instance, zooming in on tethers [219] (Fig. 12c). Complex membrane protein environments can be treated with fewer limitations than at the all-atom level. For instance, the *Escherichia coli* cell envelope incorporating two membranes and various native membrane proteins has been simulated, revealing that both membranes curve in a manner dependent on the size of the embedded proteins [220] (Fig. 12d). Similar approaches enabled the simulation of enveloped viruses whose shells encompass complex compositions [226–228]. A current challenge to further optimize coarse-grained membrane models

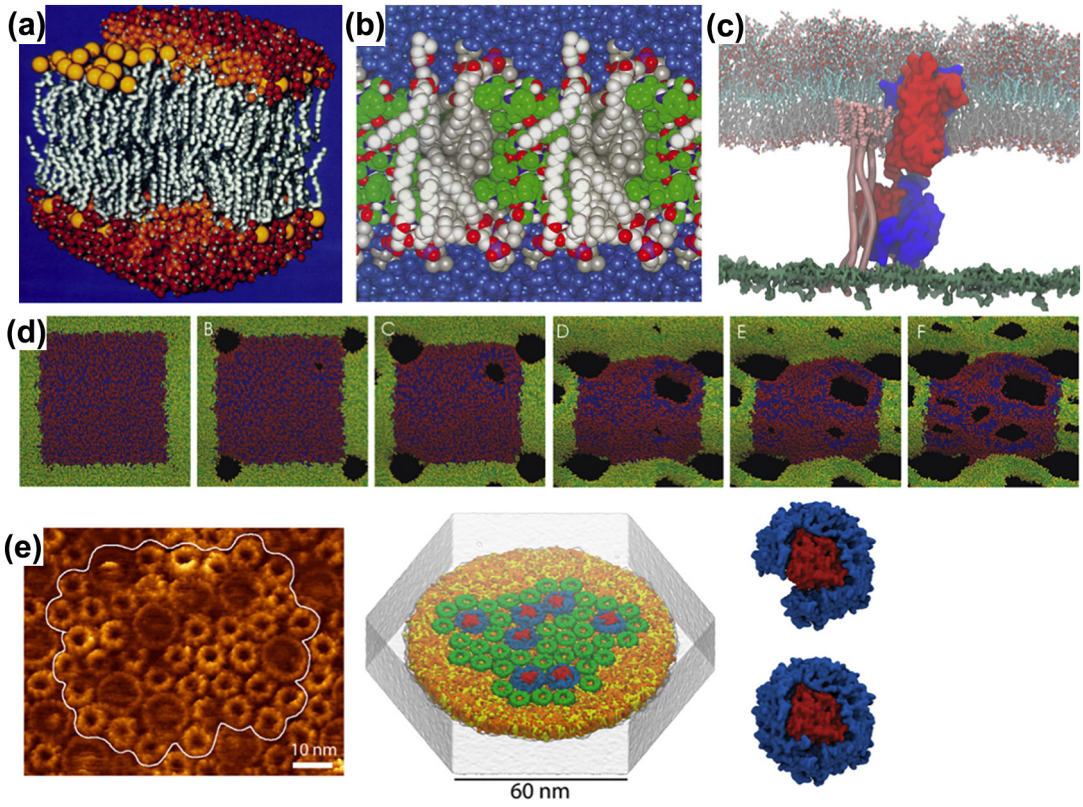

Fig. 11. Atomistic molecular simulation examples. (a) Simulation of the gel phase of the POPC bilayer. Reprinted with permission from Ref. [12]. Copyright 1993 American Chemical Society. (b) Gramicidin A channel in a 1:8 ratio simulation in DMPC [19]. (c) Membrane protein simulation snapshot from the end of a 200-ns atomistic simulation with a cell wall model [200]. OmpA dimer is red and blue for each protomer, Braun's lipoprotein is pink, the membrane is cyan (LPS) and gray (other phospholipids), and cell wall is green. (d) Top view of the lipid bilayer of initially ca. 25 × 29 nm at six different times during an electroporation simulation spanning ca. 4 ns [23]. © Tieleman; licensee BioMed Central Ltd. 2004. (e) Modeling of a photosynthetic membrane patch, showing a corresponding AFM topograph, the model of the chromatophore patch containing 39 LH2 and 7 LH1-RC complexes, with a close-up on an open and closed LH1 monomer [201].

is to remove the explicit solvent [229–232], which calls for some treatment of hydrodynamics [233]. We have recently set up an implicit water membrane model capable of describing, for instance, hydrodynamics within a lipid vesicle nanoreactor by extending previous work with the MUPHY software in line with the concepts described in Ref. [234]. An example of a vesicle nano-reactor in its flowfield is illustrated in Fig. 12f. This effort is part of a general trend to bridge coarse-grained scales with the mesoscopic scale. Further examples involve understanding the formation of large membrane protein clusters, which has been investigated by training a mesoscale model incorporating thousands of outer-membrane proteins [235] on coarse-grained MD simulations of large protein assemblies [236], as previously performed on GPCRs [221,237] (Fig. 12e). A typical target of such mesoscale simulations is to recapitulate the protein diffusion characteristics. Other groups have investigated lateral diffusion in membranes [238,239].

Such mesoscale and ad hoc modeling approaches enable another step toward bigger biological objects. The previously described coarse-grained bead models can be further approximated through coarser beads. A series of very coarse coarse-grained models, some qualified as shape-based, others as ultra-coarse-grained [240], have been derived in several cases, for instance, for transmembrane proteins [241] and as illustrated in Fig. 13b for membrane sculpting BAR domains [243,245]. Using dissipative particle dynamics, very large membrane sections and their remodeling can be investigated, providing a step forward on topics such as the previously mentioned fusion processes [242,246] (Fig. 13a). An alternative approach, already alluded to above, is to use implicit representations, which has been described for the membrane [247].

For specific questions, ad hoc tuned coarse-grained representations can be designed, as, for instance, in relation to the dynamics in the photosynthetic thylakoid membranes [207]. The phase

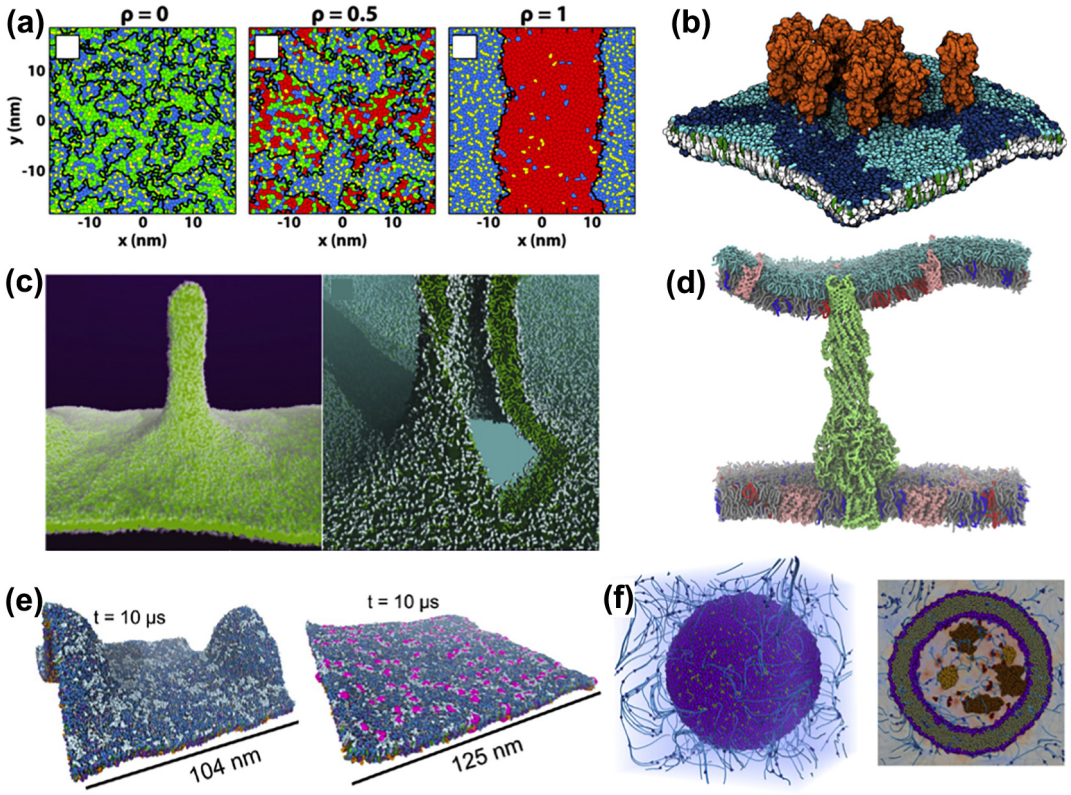

Fig. 12. Examples of coarse-grained simulations. (a) Determination of Lo and Ld lipid phase patches demarcated by a thick black line in a coarse-grained simulation of a four-component DPPC (blue), PUPC (green) DUPC (red), and cholesterol (yellow) lipid mixture. DUPC ratio increases from left to right. Reprinted with permission from Ref. [217]. Copyright 2015 American Chemical Society. (b) CG Model of a hemagglutinin A cluster in domain-forming membranes of DPPC (headgroups in light blue), DLiPC (headgroups in dark blue), and cholesterol in green [218]. The protein is shown in orange, and phospholipid tails in gray and white. (c) A membrane tether pulled by external force, shown in side and cross-sectional views [219]. (d) Coarse-grained model encompassing the two membranes of *E. coli* with several membrane proteins [220]. The AcrABZ-TolC efflux pump is lime green, inner membrane protein AqpZ and outer-membrane protein OmpA are pink, and the lipids are cyan (LPS), gray (POPE), blue (POPG), and red (cardiolipin). This is a snapshot from the end of a 10-μs CG simulation. (e) Mesoscale fluctuations of a plasma membrane model with increasing protein content [221] revealing organization and dynamics of receptor proteins. On the left, without any proteins present; middle; on the right, with GPCRs of the S1P1 receptor (in pink). (f) Illustration of a vesicle nano-reactor simulation using a dry Martini-like lipid representation coupled with Lattice–Boltzmann hydrodynamics. On the left, the exterior water flow around the vesicle is depicted; on the right, a cross-sectional cut reveals the overall fluid dynamics in the system. Unpublished results courtesy of A. Brandner, S. Timr, and F. Sterpone.

behavior of proteins in such photosynthetic membranes is recapitulated by such a model [248]. Phase separation in mixed lipid bilayers has also been investigated by bridging CG MD simulations to a mesoscopic model based on the phenomenological Landau–Ginzberg free-energy functional [249]. The red blood cell membrane was mentioned and depicted previously (Fig. 4a). It can be simulated using elastic modeling [250,251] as depicted in Fig. 13d. Such elastic representations can be used to study protein mobility [252] and can be compared to coarse-grained models [253], showing that discrepancies arise for large distances. Another approach applied to the red blood cell, in particular to reproduce its static and dynamic light scattering features, has been to model it as triangulated closed surfaces [244] (Fig. 13c), an approach that can also be applied to fluctuating membranes [254]. Similar strategies have been used to understand lipid membrane-mediated attraction between curvature inducing objects [103,104], in close link with experiment. Such a link is needed, and adapted modeling techniques may fill an important gap in complementing the most recent experimental data on membranes, as convincingly argued in [105].

Making sense: visualization and analysis

Visualization is key to understand and analyze the complex processes in molecular membrane simulations [255]. For instance, taking the preponderant MD technique as an example, all information,

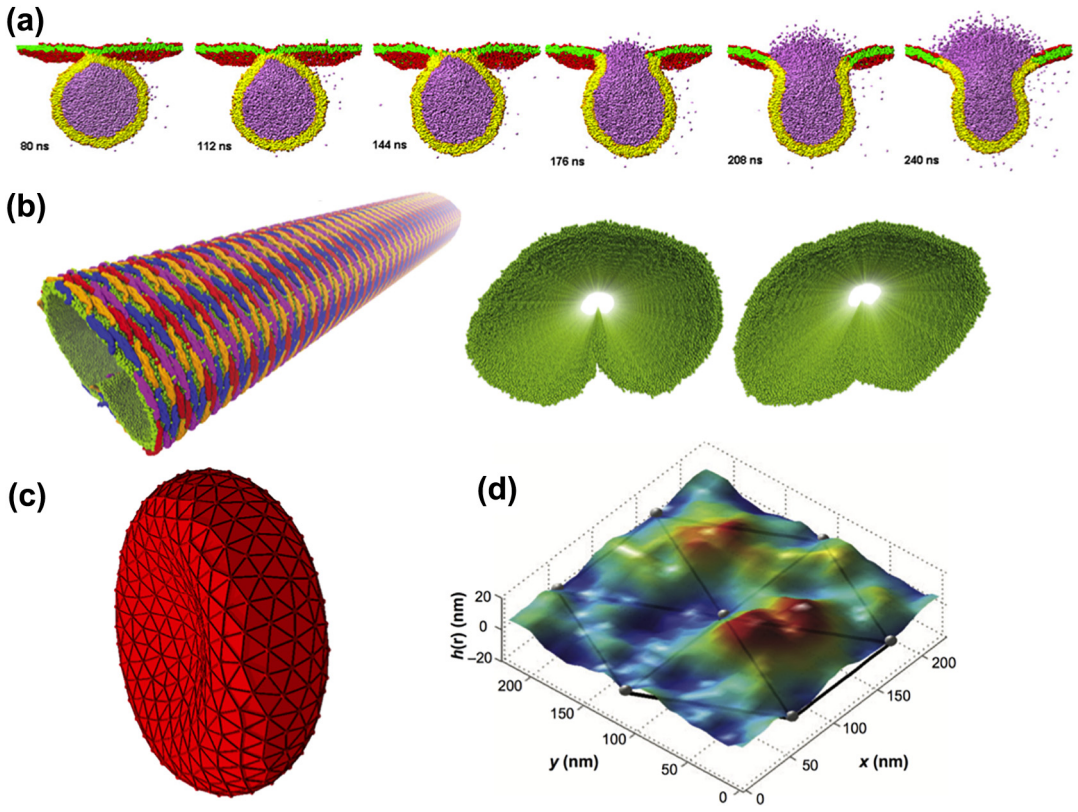

Fig. 13. Examples of mesoscopic membrane simulations. (a) Tense vesicle fusion with a tense square membrane patch observed by dissipative particle dynamics [242]. (b) Membrane tubules induced by an F-BAR domain lattice, starting from an edge-to-edge fusion forming a T-junction [243]. On the left, the membrane tubule is shown with F-BAR domains from the side. In the middle and on the right, the tubule lipids are shown from top at 0 and 30 μs, respectively. (c) Triangulated model of the red blood cell used for studying its light scattering features [244]. (d) An elastic representation of the red blood cell membrane with anchoring to the spectrin network highlighting specific pinning sites [232].

visualization, and analysis are derived from the time series of positions and velocities of the atoms (or particles for coarse-grained simulations) of the simulation system. The repertoire of visual representations is mostly the typical one for atomistic systems, applied to the membrane constituting molecular species, with a few additional ones that will be discussed hereafter. To highlight the lipid bilayer in a non-intrusive and simplified way, one may fit a deformable sheet through selected reference atoms or positions [256] (Fig. 14a), or connect reference atoms such as the phosphorus from lipid headgroups as illustrated in Fig. 14b. This representation is particularly useful with large systems, eventually containing several lipid bilayers [257]. To recapitulate the dynamics of lipid flow within the membrane, a combination of path line, vector field, and streamline techniques can be used [258] as illustrated in Fig. 14c. Many membrane simulations are carried out at coarse-grained resolution; hence, an effortless way of displaying such systems facilitates the visualization task. Molecular visualization software such as UnityMol [263] starts to incorporate native import filters for coarse-grained topologies. This visualization capacity was used to visualize carbon nanotube perturbations of a lipid bilayer [259] (Fig. 14d). Zooming into the fine details of glycolipids, specific—possibly abstracted—representations for the sugar moieties [260] (Fig. 14e) are yet to be generalized, which applies to, for example, glycans as well. It seems likely that volume rendering approaches, as already used for some experimental membrane data representations in three dimensions [115], should bear promise for visualizing simulations of biomembranes. Concerning the representation of membrane proteins and their properties, the particular example of the depiction of hydrophobicity is compelling. Typically, it is implemented through color variations. A confusingly large number of different color scales can be found in the scientific literature, without a clear consensus. An original method combines a black-to-white gradient with animated field lines [261] and has been applied to membrane proteins [262] (Fig. 14f). Another aspect

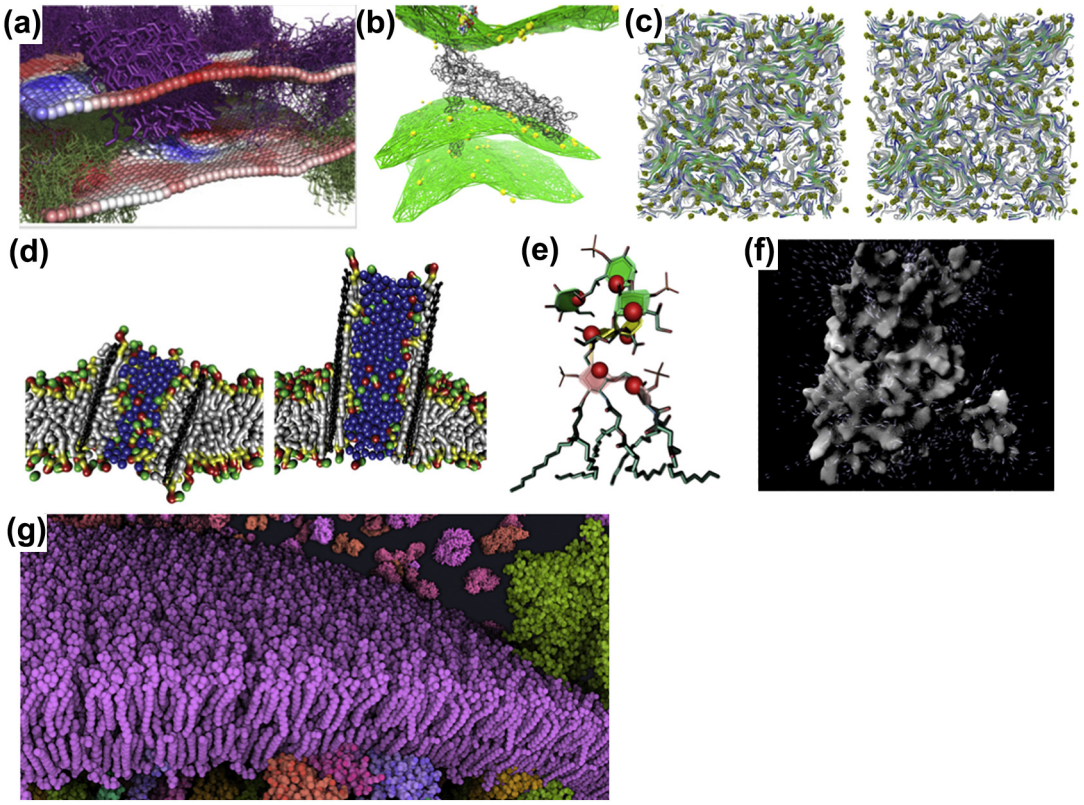

Fig. 14. Specific visualization methods applicable to membranes and membrane proteins. (a) Sheet representing the membrane center colored by local order parameter with cholesterol molecules represented on top [256]. (b) SNARE complex in a double-membrane system from [257], with each headgroup represented by a green network connecting phosphor atoms. (c) Correlated dynamics for upper and lower leaflet of a planar membrane depicted by streamlines [258]. (d) Native coarse-grained representation of a membrane-inserted carbon nanotube filled with water [259]. (e) Representation of an LPS lipid combining SugarRibbons and HyperBalls-licorice representations in SweetUnityMol [260]. (f) Hydrophobicity representation of a GPCR following [261,262] highlighting electric field lines through animation and hydrophilicity through darkening. See http://www.baaden.ibpc.fr/pub/blt2/jbn11.html for an animated version on-line. (g) Cross-sectional view of a lipid-bilayer membrane populated by lipid tiles [174].

concerns our ability to display large membrane systems, in line with building cell-scale models as discussed above. Hence, performance and visualization efficiency are important aspects to be considered. The Wang tiling concept has been extended to membranes for precisely this purpose [174] as shown in Fig. 14g.

Visualization for analysis

Such visualizations are typically closely connected to the analysis of simulation trajectories, a vast domain. Often the 2D nature of the bilayer is exploited in these analyses, which requires defining membrane leaflets in case of deformed membranes. Voronoi diagrams and Delaunay triangulation can be used for such analysis [264] (Fig. 15a), as well as grid-based approaches [270]. Some solutions are extensions to visualization software such as VMD [271]; others are intended for command-line use, such as FATSLiM [265]. The latter is particularly apt to handle curved membranes, as it relies on a local calculation of membrane normals illustrated in Fig. 15b. MDAnalysis is another feature-rich command-line analysis tool that has been applied to many large membrane systems featuring a LeafletFinder algorithm [272]. A particular tool was developed to focus on local rather than averaged membrane properties, including area per lipid, order parameters, curvature, and bilayer thickness [256]. Lipid bilayer packing defects are shown in Fig. 15c, representing intrinsically local properties that can be analyzed with a geometrical approach [170,266]. Image-processing techniques (Fig. 15d) are another source of specific membrane analysis tools, as applied to detection of lipid phases during phase separation [267]. Concerning membrane proteins, the analysis of helix geometry and its visual abstraction (Fig. 15e) was proposed [268] as well as the characterization of their particular topologies

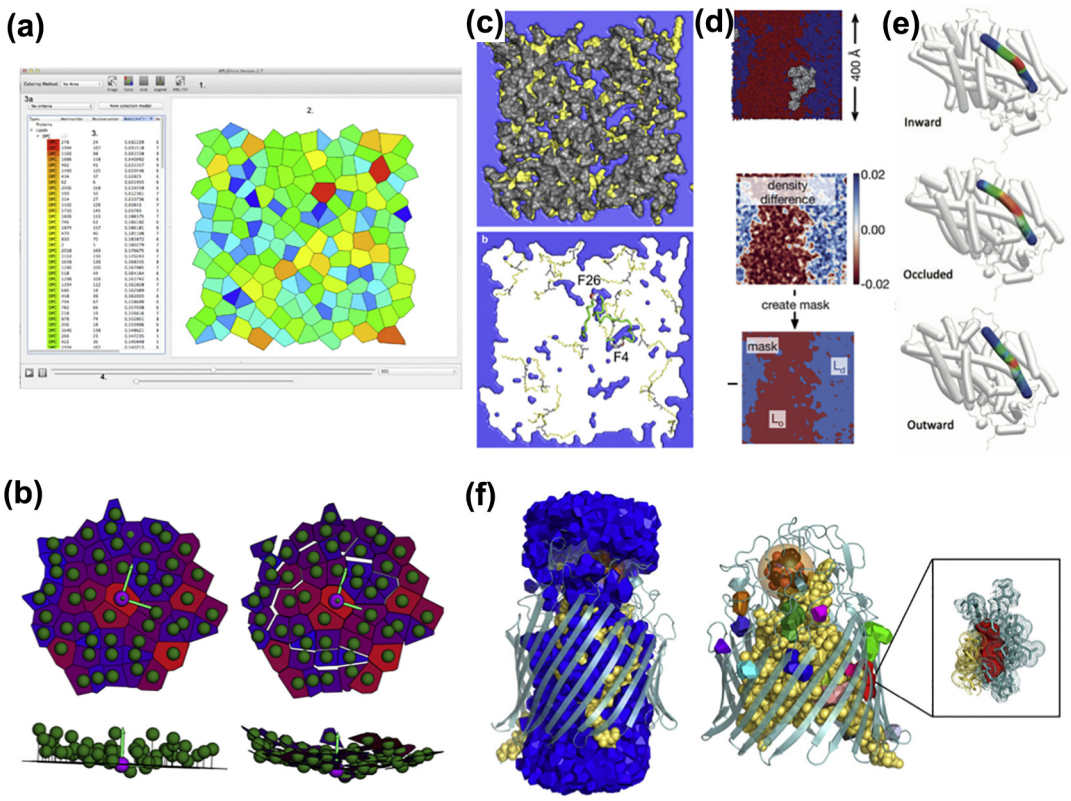

Fig. 15. Analysis examples producing visual representations. (a) APL@Voro user interface and typical color-coded analysis result [264]. (b) Difference between area per lipid analysis for a curved membrane patch. On the left, analysis in a plane; on the right, FATSLiM approach using local Voronoi cells [265]. (c) Packing defects determined by a geometrical method shown in blue, acyl chains in yellow, and polar heads in gray. A peptide inserting into the membrane is colocated [170,266]. (d) Image processing to determine lipid domain boundaries from Ref. [267]—published by The Royal Society of Chemistry. (e) Bendix membrane protein helix representations colored by an angle-indicative heatmap [268]. (f) Laguerre tesselation analysis of the water network through the FepA membrane protein showing a water path through the protein bridging the media on both sides of the membrane (left) and isolated water inclusions (center) with an inset zooming in on a selected inclusion [269].

(Fig. 15f), notably in relation to water networks prefiguring solute transport, based on Laguerre tesselation [269].

Conceptual illustrations, animations, and artistic depictions of membrane systems

In order to conceptualize ideas about bilayer structure and fundamental principles of biological membranes, researchers have from the early days on recognized the crucial importance of visual representations thereof. Historically, illustrations as in Fig. 16a and b were abundant in early papers on membranes in order to fill the gaps between the "raw" experimental and simulation data and their interpretation, often pre-dating the realization of the first experimental approaches providing actual visualizations. Such illustrations typically represent schematic views of lipid bilayers [7] or pseudo-3D representations of their phases [273]. These images were triggered by research results requiring conceptualization to convey the underlying new ideas to the scientific community, be it about membrane protein insertion [18,274] (Fig. 16c to e), hydrophobic mismatch [276] (Fig. 16f), the fluid mosaic model [7,277] (Fig. 16g), diffusion within the bilayer [278] (Fig. 16h), or many other ideas. Hence, this figure not only is of historical importance but also illustrates the way in which the scientific community crystallizes ideas and concepts in this field, which is intrinsically visual. A broad variety of membrane depictions can be found in the literature, yet many among them are based on more (or sometimes less) educated guesses. The sheer scales at which membranes operate require some mental analogies for us to fully apprehend the characteristic size and length spectrum. To aid our imagination of these dimensions, a "perceptive scale" [279] has been proposed to facilitate the interpretation of cellular scales.

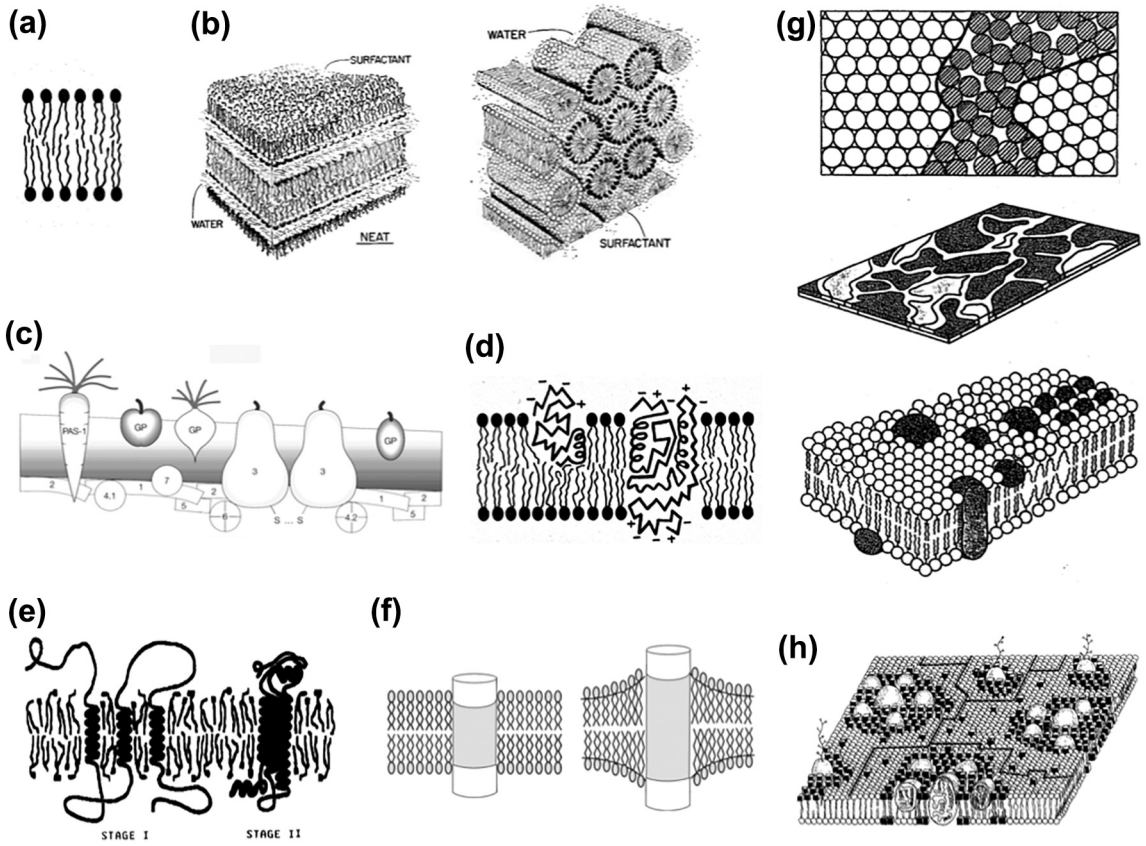

Fig. 16. Selected historic illustrations from the literature. (a) Schematic cross-sectional view of a phospholipid bilayer with filled circles representing headgroups and wavy lines depicting the fatty acid chain [7]. (b) Alternating layer structure of a neat surfactant phase (left) and middle phase structure of surfactant/water systems (right) [273]. (c) Hypothetical membrane protein insertion for the major erythrocyte membrane [274,275]. Carbohydrates exposed to the extracellular space are drawn attached to the proteins. (d) Cross-sectional view illustrating the lipid–globular protein mosaic model of membrane structure [7]. (e) Two-stage model of membrane protein insertion illustrated for an alpha-helical integral membrane protein. Reprinted with permission from Ref. [18]. Copyright 1990 American Chemical Society. First trans-bilayer alpha-helices form, then they assemble into their final tertiary architecture. (f) Hydrophobic thickness illustrated. On the left, a perfect match between protein and bilayer; on the right, a hydrophobic mismatch leading to a lipid bilayer distortion around a membrane protein [276]. (g) A typical biomembrane in various degrees of schematization. The top panel illustrates organized and disorganized lipids forming discrete regions. The center panel schematizes ordered and disordered regions as plates. The bottom panel depicts a molecular view on the long range organization and compound distribution [277]. (H) A possible pathway for a molecule diffusing randomly in a biological membrane [278].

Toward communication approaches

Subsequently, the motivation for illustrations expanded as recognition of the beauty and complexity of nanoscopic molecular assemblies grew, a move from data analysis, interpretation, and (research) hypothesis generation to broader education, communication, and learning operated [280–282]. This evolution opened the available toolset toward software from animation, illustration, and cinema [283,284], with specific extensions for biomolecules and applications to membranes [285] as depicted in Fig. 18a. Some new tools were designed to tackle the challenges of illustrating biological complexity [288]. Such illustrations have been marked by the touch of their creators with a certain artistic license [289], with a range of famous contributors such as David Goodsell [290–294] (Fig. 17b), Janet Iwasa [282] (Fig. 17a), Graham Johnson [297], Gaël McGill, and many more. Some general guidelines have been suggested for illustrating biomolecular structural data [286]. The same content may be depicted differently according to the targeted audience as shown in Fig. 18b and c. Illustrations may also serve to raise awareness and stimulate scientific discussion. Such is the case for the pneumococcal life cycle, visually reviewed through watercolor paintings (Fig. 17d) with a consistent scale recapitulating currently available experimental data [296]. Hence, quite naturally, such depictions

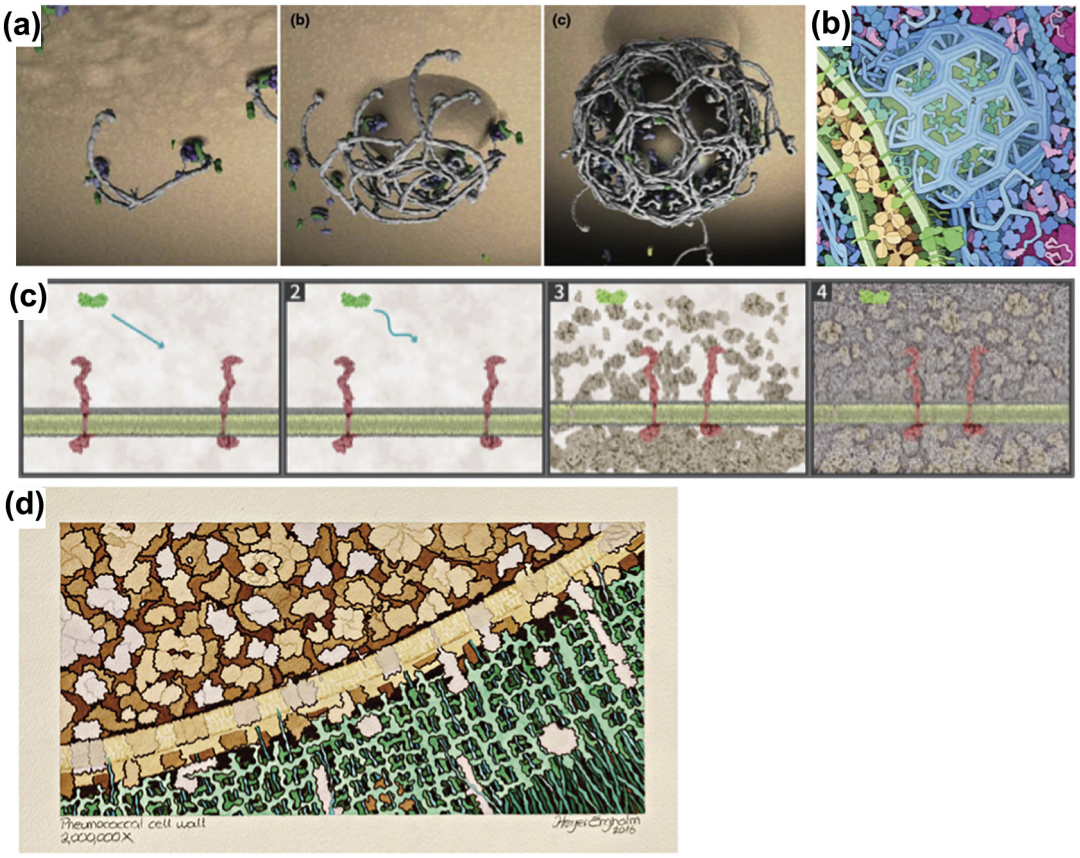

Fig. 17. Contributions from medical illustrators and artists. (a) Three still frames from an animation depicting clathrin-mediated endocytosis by Janet Iwasa in collaboration with Tomas Kirchhausen [282]. (b) Transport from the Golgi featuring a clathrin cage illustrated by David Goodsell [293]. (c) Opening frames from four animations representing the same receptor–ligand binding event with varying degree of detail and crowding by Jodie Jenkinson and Gaël McGill [295]. (d) Watercolor of pneumococcal cell wall synthesis with apparent membrane, membrane proteins, cell wall structure, and peptidoglycan layers drawn by Høyer Engholm [296].

evolve over time, when new knowledge becomes available. A good example, discussed in more detail in [298], may be the animation "The Inner Life of the Cell" from 2006 by Harvard BioVisions and Xvivo (https://www.youtube.com/watch?v=wJyUtbn0O5Y). Since then, our awareness of the crowdedness of cellular environments has risen significantly and is not conveyed in the initial movie. Motions as well are unlike the very agitated perpetual molecular jiggling that we are now aware of. An updated version of the animation, "Inner Life of a Cell | Protein Packing" has since been released (https://www.youtube.com/watch?v=uHeTQLNFTgU).

The efficiency of illustrations and animations for learning concepts such as structure and composition of the cell membrane, chemical properties of the relevant molecules, membrane barrier function, and transport mechanisms has been investigated [299]. The visual language used needs to be adapted and has to remain sufficiently accurate and complex to convey, for instance, the dynamic nature of binding events in a membrane [295] as illustrated in Fig. 17c. Tangible models can be used, for instance, to convey the solubility properties of membrane proteins [300]. Serious games [301] may offer new opportunities for learning and some already address membrane systems, such as DocMolecules (Fig. 18d, https://youtu.be/gZyneEqaWcQ), targeting to dock a drug to the right membrane receptor or Eukaryo (Fig. 18e) [287] simulating a eukaryotic cell.

Perspectives and Conclusion

The perspectives on visualizing biological membrane organization and dynamics are both extremely encouraging and full of challenges. The first challenge, already mentioned, concerns the complexities in composition, in scales and in diversity that are starting to be addressed by experiments, simulations,

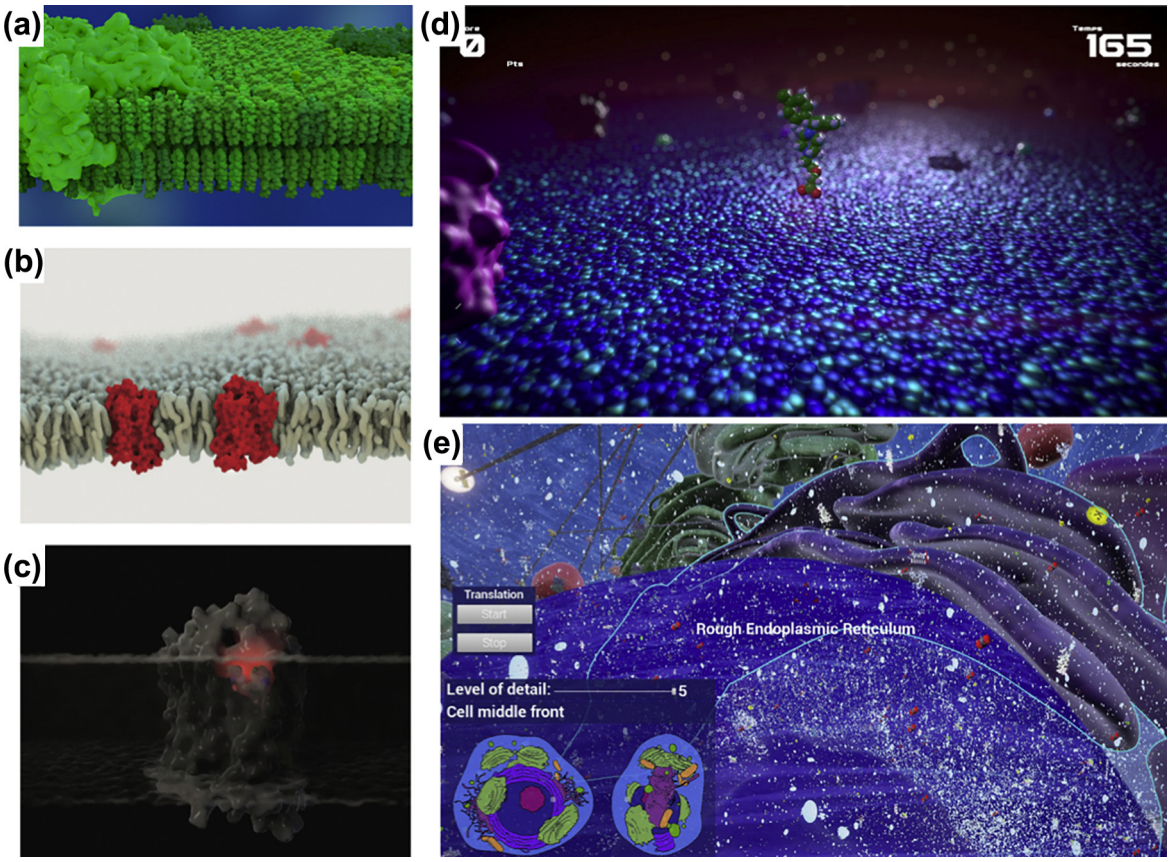

Fig. 18. From 3D modeling to communication and serious games. (a) Lipid bilayer perspective on a 3D thylakoid membrane model rendered with Blender [159,285]. (b) Choice of molecular representations according to a given audience. Here, a GPCR represented in a detailed lipid bilayer to make an engaging web banner image [286]. (c) The same GPCR as in panel B rendered for a game prototype to engage teenagers [286]. (d) Screenshot of the DocMolecules serious game prototype showing a drug molecule floating over a membrane in search for its receptor. (e) Eukaryo simulation screenshot depicting the eukaryotic cellular space with control elements to adjust the level of detail and a minimap for location reference [287].

and illustrations. On the experimental side, many key players remain largely hidden from scrutiny, such as, for instance, the ubiquitous cholesterol. Finding an efficient yet non-perturbing fluorescent cholesterol analog would allow to attend to our "blindness," and work is under way in that direction. On the simulation and visualization front, our tools are not yet as mature as for proteins, DNA, or even sugars, where many representations with various degrees of abstraction exist, tuned to capture essential parts of the biology of these molecules. As has been discussed above, our repertoire for pictorial lipid representations is still rather limited. Another tendency, that is to be largely welcomed, is the convergence of the various methods, be it experimental, computational or illustrative ones, to combine and confront related information. This is fully in line with what Lyman *et al.* [105] described in their "modeling manifesto" for the pair of experiments and simulations.

It should, however, be noted that we are still at the level where we attempt to accurately describe structure and dynamics of membranes, and only to some limited extent their (mostly) equilibrium energetics. In the future, such a molecular view may need to embrace thermodynamics more fully, to characterize where and in what form energy is distributed in a given configuration. In addition to the MD, it is essential to capture the dynamics of energy flow. This requires to move beyond the equilibrium picture, as life in its essence is a non-equilibrium state. Our conceptual images need to evolve from a currently rather vague picture of enthalpy associated with biological membranes to a comprehensive mental representation of energy fluctuations and flow. This major challenge may become tractable by using modern tools to render the multidimensional massive data that can nowadays be acquired intuitively explorable [302]. Virtual reality approaches, visual analytics [303], and in general advanced data display and mining technologies [304] will assist this transformation [305,306].

To conclude, our picture of biological membranes is already very rich and detailed, with meso- to nanoscale information from a variety of complementary sources, enabling us to relate molecular structures to the biological phenomena, as well as to characterize the pertaining biophysical properties. Many of the underlying techniques have seen significant progress enabling us to probe these membrane systems in depth. These improvements enable a convergence between real (experiments), virtual (simulations), and imaginary (illustrations) views. However, we may still miss new comprehensive representations for these systems, sometimes necessary to develop new ideas. Therefore, we may still be overlooking some fundamental concepts. For other biological objects of studies, such breakthroughs were made in close relation to our pictorial or mental conceptualization. Three emblematic examples include unraveling the mystery of the alpha helix, to which Pauling contributed decisively, depicting the secondary structure of proteins by cartoon representations pioneered by Richardson, or understanding the DNA double helix based on the works by Watson, Crick, and Franklin. Entire branches of biology rely on these discoveries.

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Note added in proof: During typesetting of this article, a related review on the simulation of biological membranes became available and is cited for completeness: Multiscale Simulations of Biological Membranes: The Challenge To Understand Biological Phenomena in a Living Substance by Giray Enkavi, Matti Javanainen, Waldemar Kulig, Tomasz Róg, and Ilpo Vattulainen Chemical Reviews Article ASAP https://doi.org/10.1021/acs.chemrev.8b00538.

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†The full quotation is "I must not conceal the fact that in the study of this membrane I for the first time felt my faith in Darwinism (hypothesis of natural selection) weakened, being amazed and confounded by the supreme constructive ingenuity revealed not only in the retina and in the dioptric apparatus of the vertebrates but even in the meanest insect eye. There, in fine, I felt more profoundly than in any other subject of study the shuddering sensation of the unfathomable mystery of life." from the autobiography Recollections of My Life.

Abbreviations used:
EM, electron microscopy; AFM, atomic force microscopy; MD, molecular dynamics; ESR, Electron spin resonance; NMR, Nuclear magnetic resonance.

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