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Cracking the membrane lipid code

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Abstract

Why has nature acquired such a huge lipid *repertoire*? Although it would be theoretically possible to make a lipid bilayer fulfilling barrier functions with only one glycerophospholipid, there are diverse and numerous different lipid species. Lipids are heterogeneously distributed across the evolutionary tree with lipidomes evolving in parallel to organismal complexity. Moreover, lipids are different between organs and tissues and even within the same cell, different organelles have characteristic lipid signatures. At the molecular level, membranes are asymmetric and laterally heterogeneous. This lipid asymmetry at different scales indicates that these molecules may play very specific molecular functions in biology. Some of these roles have been recently uncovered: lipids have been shown to be essential in processes such as hypoxia and ferroptosis or in protein sorting and trafficking but many of them remain still unknown. In this review we will discuss the importance of understanding lipid diversity in biology across scales and we will share a toolbox with some of the emerging technologies that are helping us to uncover new lipid molecular functions in cell biology and, step by step, crack the membrane lipid code.

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Lipid composition matters

Membrane structure and function is dictated by the chemical diversity and the stoichiometry of their building blocks, *i. e.* their lipid composition. Even though it has been known for decades that lipids are extremely diverse in their structure and physicochemical properties, scientific models depicted in textbooks, reviews or articles fail generally to reflect this lipid diversity but rather seem to present membranes as passive scaffolds that support processes independently of their lipid composition. Several data contradict this notion. First, Nature has kept thousands of different lipids (some of them with very elaborate synthetic pathways) that are not randomly distributed across species, tissues or organelles [1–5]. Furthermore, changing the stoichiometry of specific membrane lipids has a great impact on membrane properties and, as a consequence, on membrane-dependent processes for example, on intracellular trafficking [6]. Second, lipids have been shown to interact in a highly specific manner with the transmembrane domains of proteins [7]. Moreover, membrane lipids and transmembrane proteins seem to have undergone convergent evolution to meet the needs of each organism [8]. Third, mutations in enzymes working on lipid metabolic pathways give rise to very severe genetic diseases and lipid dysregulation underlies many metabolic diseases [9–11]. Therefore, the cell requires a strict control of its lipid composition and of the lipid intracellular distribution in order to support membrane function and, ultimately, life [5]. The difficulty of this task comes from the fact that lipid composition is highly plastic and sensitive to external stimuli (temperature, medium or diet) and is also dependent on circadian rhythm, age and sex [12–15]. This implies that cells exquisitely detect or sense changes in membrane properties and consequently adapt and fine-tune the lipid composition accordingly [16]. However, the mechanisms by which cells regulate the abundance of different lipids to maintain an adaptive and functional membrane remain largely unknown and represent a major challenge in membrane research. Recent data in bacteria indicate that headgroup-specific acyl chain remodeling underlies adaptation to allow growth at different temperatures [17,18]. Moreover, hydrophobic mismatch has been established as the sensing mechanism for the regulation of membrane lipid

composition [19]. Research in yeast has identified the protein Mga2 as a novel eukaryotic sensor for lipid saturation using a rotation-based mechanism [20]. These studies represent, however, the tip of the iceberg and many basic facts about lipid molecules remain still obscure such as their enrichment in specific organelles or their interaction with specific proteins through which they also exert their effects.

In summary, classic biochemistry has been useful to advance our understanding of the complexity of membrane lipid composition but multidisciplinary approaches based on advanced mass spectrometry and chemical biology among others are now uncovering new lipid functions in cells.

I am a cell biologist wishing to study lipids: where do I start?

While membranes are universally recognized as integral constituents of cellular architecture, our understanding of their physiology lags behind most other aspects of cell biology, due to serious technical limitations. First, lipid species are not directly encoded in the genome. Consequently, manipulating individual lipids has been challenging, as the only way so far has been to knock down/out or overexpress the corresponding metabolic enzyme, thus modifying the amounts of dozens of lipids at a time. Recent advances in chemical biology are now allowing us to manipulate lipids *in cellula* with a high spatio-temporal resolution. Second, the various lipid metabolic pathways are often interconnected. This must be considered when studying the effect of the lack of a specific lipid, as the cellular responses could be related to secondary up- or down-regulation of other lipids following the primary effect. These networks of co-regulated lipids can be detected using lipidomics, based essentially on mass spectrometry of lipids [6,21,22]. A further degree of complexity arises from trying to understand the contribution of individual lipid species to membrane fitness *in vivo*. The difficulty to track lipid molecules using microscopy and the limitation of biophysical techniques for *in vivo* studies have opened a gap between the fields of membrane biophysics and the physiology community. Luckily, novel technologies that combine high-throughput genetics with mass spectrometry and chemical biology with advanced microscopy allow us now to uncover previously unthinkable aspects of lipid biology. In the next sections we propose a pipeline with the most recent technical developments that might help overcome the cell biologist's frustration when coming across a lipid-related phenotype.

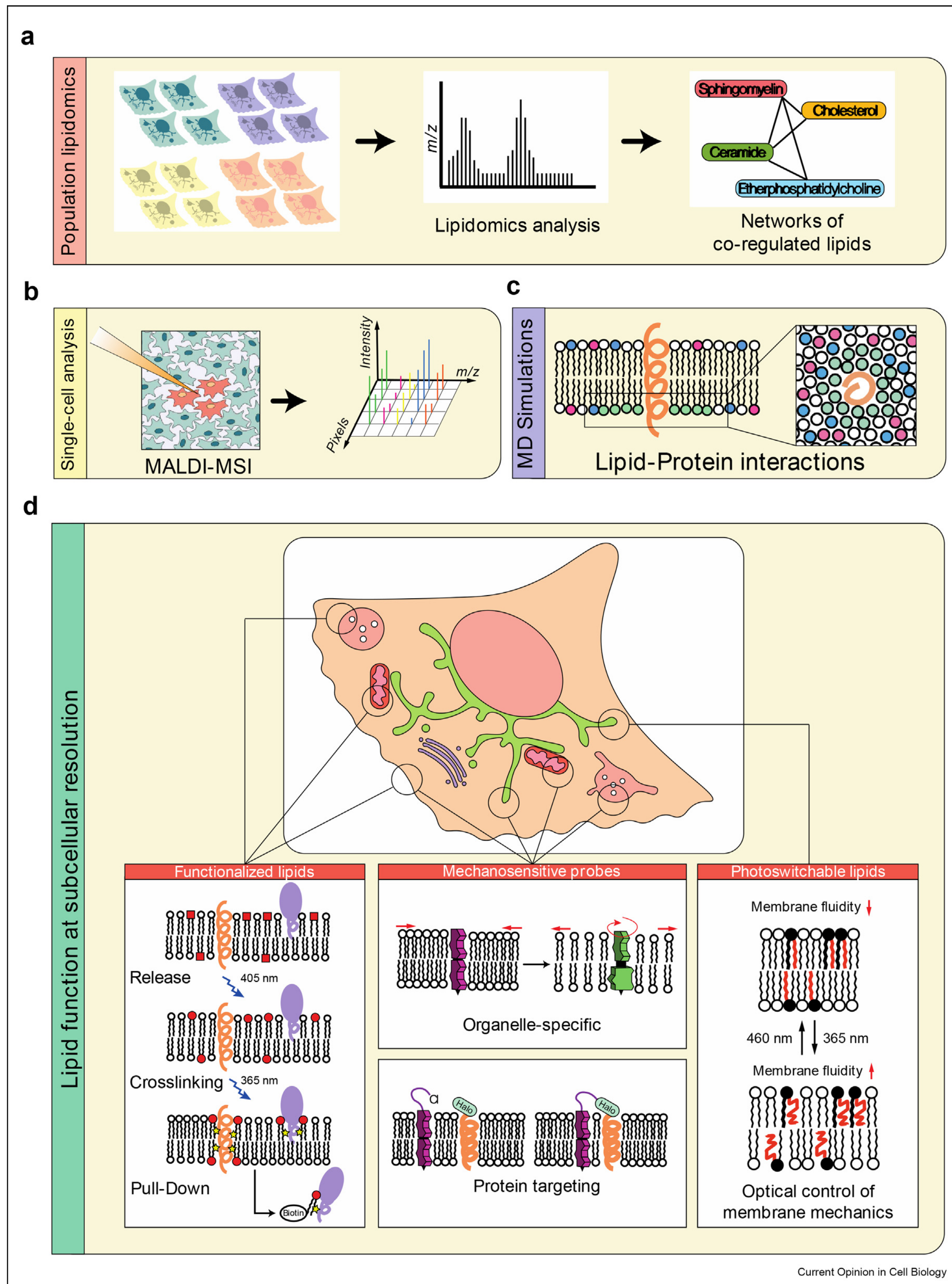
Exploring the lipidome in the quest for novel lipid functions

Since lipids are of central importance in biology it is very common to find lipid metabolism affected under a wide range of conditions when applying untargeted research approaches or discovery research. Along these lines, lipid related pathways have appeared as hits in several genetic screens that aimed to study processes in principle non-related to lipid biology. Some of these studies have succeed in uncovering novel lipid functions. Jain et al. used a CRISPR screen of cells under high and low oxygen conditions and accumulation of peroxisome-derived ether lipids was found to be a characteristic feature of hypoxia [23]. More recently and using a very similar approach, Zou et al. found that peroxisomes and ether lipids drive the susceptibility to ferroptosis using an untargeted approach [24]. Part of the success of these discoveries relies on the use of lipidomics to detect variations in the cell lipidome under those conditions. Mass spectrometry of lipids has also been useful to uncover networks of co-regulated lipids that may share common functions in cells (Figure 1a), and, combined with other methodologies it has served, for example, to propose novel roles for ether lipids in the early secretory pathway [6]. Thus, many of the biological roles of ether lipids have become apparent in the last years by using high-throughput genetics in combination with lipidomics analysis. Lipidomics analysis in cell biology should be the preferred technique to detect lipidome rearrangements, but it must be combined with other methodologies to expand the static picture that it provides as well as the lack of information on spatial distribution. The most promising strategy to overcome this limitation is the use of imaging mass spectrometry (IMS), in particular, matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) a technique sensitive enough to perform single-cell lipidomics (Figure 1b) [25]. Secondary ion mass spectrometry (SIMS) in combination with metabolic labeling has also allowed the high-resolution mapping of lipid distribution in cellular membranes, showing promise to improve accuracy when detecting lipids in cells [26–28].

Approaching lipid biology with subcellular resolution

General mass spectrometric technologies lack subcellular resolution. This aspect is of particular importance because, depending on its localization, a lipid may play different molecular roles and have a distinct metabolic fate. The classical methods for visualizing lipids, that include lipid modifications using bulky fluorophores, have major drawbacks as they affect the physical-chemical properties of the molecule. Alternatively, approaches that combine affinity-purification of organelles with lipidomics can help to overcome those limitations offering quantitative information about the spatial distribution of lipids in cells [29,30]. Furthermore, recent advances in chemical biology are opening novel avenues in lipid cell biology, offering an unprecedented spatio-temporal resolution. Functionalized lipids are allowing us to pre-localize a particular lipid in a subcellular

Figure 1



Toolbox to approach the complexity of lipid cell biology. (**a**) Analyzing the lipid composition of different cellular populations can unravel networks of co-regulated lipids which may help uncovering new lipid functions. (**b**) Single-cell lipid analysis reveals cell-to-cell variabilities that have been shown to drive different cellular states. (**c**) *In silico* analysis of lipid–protein interactions can be used to understand lipid function at the molecular level. (**d**) Methods to study lipid function at the subcellular level: functionalized lipids can be used to pre-localize a particular lipid within the cell (caging) and to follow their

compartment and to follow its metabolic fate and interactome only with the use of light (Figure 1d) [31,32]. These lipids can combine a photocleavable group or "cage" that protects the lipid from being metabolized, a photo-sensitive diazirine group that allows crosslinking of the lipid to neighboring proteins, and an alkyne or azide group for detection and isolation. Using this type of methods, scientists have shown that sphingosine has a different metabolic fate depending on the organelle where it is released [33]. This proves that lipids do not freely diffuse and showcases the importance of a proper functioning of lipid trafficking machineries to maintain cellular compartmentalization. A recent study using lysosome-targeted photoactivable sphingosine has uncovered a potential role for two known cholesterol transporters in sphingosine export from the lysosomes which could lead in the future to strategies to treat lysosomal storage diseases [34]. Direct detection of lipids within a bilayer using high spatial resolution techniques such as cryo-electron microscopy (cryo-EM) is typically not possible due to the poor electron-scattering by lipid molecules. In a recent study, however, Moss et al. have used brominated lipid probes as contrast enhancing agents for cryo-EM, enabling the direct detection of lipid species [35].

Finally, computational methods such as molecular dynamics (MD) simulations permit the prediction of high-resolution lipid–protein interactions (Figure 1c). A good example is the case of the p24 complex, the cargo receptor needed for the export of glycosylphosphatidylinositol-anchored proteins (GPI-AP) that cycles between the ER and the Golgi apparatus. p24 has been shown to interact in a highly specific manner with a particular sphingomyelin species (SM18) [7]. This interaction seems to be important for protein oligomerization and function. Recent work has shown that the yeast p24 transmembrane domain (TMD) is enriched in ceramide molecules on its luminal side, probably driving membrane asymmetry at endoplasmic reticulum (ER) exit sites, while the mammalian TMD is enriched in ether lipids [6,36]. Interestingly, GPI-AP in yeast have a ceramide anchor and in animals they are ether lipid-based [37,38], thus, these data support the conserved roles of those two lipid classes, sphingolipids and ether lipids.

Studying the function of membrane lipids in their native environment

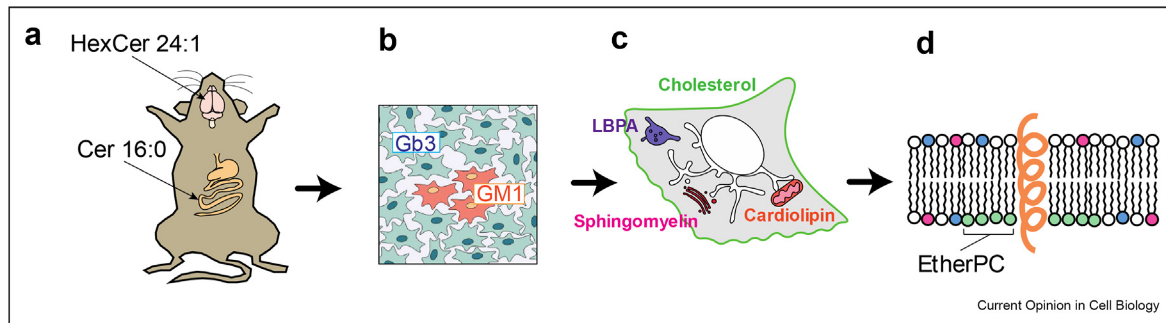
Most lipids do not behave as individual entities, they rather collaborate and work together forming cellular membranes. Particular lipid species are directly required for membrane protein function through specific lipid–protein interactions, but they are also indirectly involved through their effects on membrane biophysical properties such as tension, bending rigidity, and viscosity [1]. For decades we have been able to analyze the properties of lipids in reconstituted membrane systems, and this has greatly advanced our knowledge of e.g. lipid–lipid interactions, melting temperatures of different lipid molecules and their effect on membrane packing. However, as an example of the limitations of these studies, how melting temperature and packing can be correlated, and how those results can be extrapolated to an *in vivo* situation has not been fully established yet. It was clear for a long time that novel technologies were needed to tackle membrane biophysics in living cells. In recent years, fluorescent mechanosensitive probes known as flippers, have been developed to measure biophysical forces within membranes of living cells [39,40]. Flippers are sensitive to changes in membrane tension or to compressive forces within the bilayer. Such forces in turn modify the excitation wavelength of the molecule, enabling imaging of membrane tension using fluorescence lifetime imaging microscopy (FLIM) by monitoring changes in flipper lifetime. Modifying the flipper molecule to incorporate chemical organelle-targeting motifs ensures the accurate delivery to mitochondria, ER or lysosomes, among others, without loss of function of the probe [41]. Targeting through genetic engineering is possible if expressing a HaloTag fusion protein in the membrane of interest, thus enabling a HaloFlipper to bind covalently [42] (Figure 1d).

Solvatochromic probes based on Nile red represent another group of environment-sensitive fluorophores, suitable to detect changes in viscosity or lipid order, thus reporting on the physical state of membranes [43]. Functionalization of Nile red with organelle-targeting groups now allows the super-resolved quantification of membrane biophysical parameters with high temporal and spatial resolution [44,45]. Moreover, the probe can be covalently linked to a membrane protein to explore its local membrane environment [46]. Altogether, these techniques, are now allowing cell biologists to map the biophysical parameters of cellular membranes with an unprecedented resolution.

Finally, recently developed photo-pharmacological tools are allowing us to not only measure membrane properties like viscosity or fluidity, but also to control them. Photo-switchable lipids based on molecular photoswitches such as azobenzenes, have emerged as powerful tools to control protein-membrane interactions and membrane mechanics in model membranes and, more recently, *in cellula* (Figure 1d) [47–50].

export, metabolism and interactome with high-spatiotemporal resolution upon controlled release, crosslinking and pull down experiments; organelle and protein-targeted membrane probes that report on biophysical parameters can be used to study the effect of changes in lipid stoichiometry on cellular membrane homeostasis. Finally, photoswitchable lipids can be used to control membrane mechanics and understand the role of membrane fluidity in cell biology.

Figure 2



Lipid asymmetry across scales. (**a**) Tissue specific reference lipidomes are helping us to understand lipid diversity and its impact on disease. The amount of HexCer 24:1 is significantly higher in all brain regions while Cer 16:0 is the most abundant species in the intestine [3]; HexCer, hexosylceramide; Cer, ceramide. (**b**) At a different scale, cell-to-cell lipid variability within a tissue as detected by MALDI-MSI can drive cellular differentiation [25]; Gb3, globotriaosylceramide; GM1, monosialotetrahexosylganglioside. (**c**) At the subcellular level, lipid trafficking routes ensure a proper lipid distribution between organelles and thus, cellular homeostasis [53]; LBPA, lysobisphosphatidic acid. (**d**) Finally, membranes are asymmetric and laterally heterogeneous in their lipid composition [54,6]; EtherPC, Etherphosphatidylcholine.

Final considerations: lipid asymmetry across scales

Why do cells spend so much energy to synthesize so many different lipid species? At the organismal level, lipids are different from organ to organ and between tissues (Figure 2a). Adipose tissue samples from subcutaneous and visceral tissue depots differ in their lipid composition, and this has been shown to be a key element delineating the obese phenotype [22]. At the cellular level, the lipotype hypothesis proposes that distinct cellular lipidomes are both a consequence and a component of differentiation programs which, in the end, dictate cellular identity (Figure 2b) [51]. Currently, specific lipid signatures have been shown to underly cell differentiation in skin [25]. At the subcellular level lipids are heterogeneously distributed between organelles a consequence of both vesicular trafficking and lipid trafficking through membrane contact sites (MCS) (Figure 2c) [52,53]. Moreover, the same lipid has been shown to have a different metabolic fate depending on its subcellular localization, a phenomenon termed as “metabolic bias” [33]. Finally, membranes are asymmetric in their protein and lipid composition [26,54–57]. Heterogeneous distribution of lipids within the membrane can happen in two axes, vertical and lateral and both appear to be driven by lipid–protein interactions of various “strengths” (Figure 2d). Additionally, lipid translocases and transfer proteins enable the establishment and maintenance of transbilayer lipid asymmetry, an important feature that is crucial for cell viability [58,59]. Most of this knowledge has been acquired using multidisciplinary approaches that include state-of-the-art mass spectrometry techniques and chemical biology. Further developments to increase sensitivity and spatiotemporal resolution in those techniques will help relieve most of the deficiencies of genetics and classic biochemistry when navigating the complex (and exciting) world of lipid biology.

Author contributions

Alejandro Melero: Conceptualization, Writing, Review & Editing, Visualization. **Noemi Jiménez Rojo**: Conceptualization, Writing – Original draft, Writing – Review & Editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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