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Review

High throughput quantitative molecular lipidomics ☆

Hye R. Jung [1], Tuulia Sylvänne [1], Kaisa M. Koistinen, Kirill Tarasov, Dimple Kauhanen, Kim Ekroos *

Zora Biosciences, Biologinkuja 1, Espoo, FI-02150, Finland

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ABSTRACT

Applications in biomedical research increasingly demand detailed lipid molecule information acquired at high throughput. Although the recent advances in lipidomics offer to delineate the lipidomes in detail, the challenge remains in performing such analyses at the requested quality and to maintain the quality also in a high throughput setting. In this review we describe a high throughput molecular lipidomic solution based on robotic assisted sample preparation and lipid extraction and multiple lipidomic platforms integrated with a sophisticated bioinformatics system. As demonstrated, the virtue of this lipidomic toolkit lies in its high throughput delivery of comprehensive quantitative lipidomic outputs at the molecular lipid level, its ease of scalability and its capability to serve in a regulatory setting. We anticipate that this toolkit will contribute to basic research, nutritional research and promote the discovery of new disease biomarkers, disease related mechanisms of actions and drug targets. This article is part of a Special Issue entitled Lipodomics and Imaging Mass Spectrometry.

1. Introduction

Eukaryotic cells comprise hundreds to thousands molecular lipid species that serve as cellular building blocks such as cell and organelle membranes, store metabolic energy and function as bioactive molecules [1,2]. As each lipid structure is an essential determinant of the biological system, a defect in the underlying lipid regulation can lead to deleterious effects on the organism and assist in the pathophysiology of diseases such as obesity, atherosclerosis, diabetes and cancer. To elucidate lipid-related dysfunctions, the biological role of each individual molecular lipid species needs to be identified. However, to delineate the physiological functions of molecular lipids in a biological system or in a sub-fraction of that system is a daunting challenge. Traditional lipid analysis techniques such as thin-layer chromatography are not sensitive enough and therefore inadequate for such work. Another layer of molecular detail or better yet, several layers deeper into the lipidome have to be achieved. Here, lipidomic techniques show the most significant promise in delivering the biological understanding of each individual molecular lipid [3,4].

Lipidomics can be defined as a systems-level analysis of lipid species, their abundance, biological activities, subcellular localization and tissue distribution [5]. The evolution of lipidomics has been strongly influenced by the recent advances in mass spectrometry (MS). The present MS technology driven lipid analysis makes it possible to resolve complex lipidomes by identifying and quantifying hundreds of molecular lipid species in a number of lipid classes that make up the organism's lipidome [6–11]. As a result of the high sensitivity and selectivity of the methods, a lipidome-wide analysis of minute sample amounts has become feasible. Latest advances even permit to perform such analyses at high throughput without endangering the analysis quality [12].

Common lipidomic approaches are capable of elucidating lipids with different sum compositions, e.g. phosphatidylethanolamine (PE) 36:4 [13]. Already in a full mass spectrometry analysis such type of information can be obtained. Since no selective analysis modes are usually required, a profile of the sum lipid composition can be very rapidly acquired, either in conjunction with liquid chromatography (LC) or direct infusion approaches. For instance, in the latter, by taking advantage of the high mass resolving power of instruments, such as an orbitrap or a fourier transform ion cyclotron resonance mass spectrometer, a broad profile of "brutto" lipids can be readily identified and quantified in only minutes from unresolved samples. Here, the high mass accuracy is used to separate the actual lipid peaks from the chemical noise. These types of global lipidomics approaches, including unselective LC-MS on high resolution instruments, have become very attractive due to their simplicity, reliability and the speed assisted by the lipid software advancements. Such setups are therefore appealing for high throughput lipidomic screenings. However, it must be noted that currently these approaches primarily produce lipidomic outputs based on sum lipid composition only.

Most lipids are located in cell membranes and their bioactive outputs are determined by the individual molecular lipids present. Furthermore,

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* Corresponding author. Tel.: +358 407448997.
E-mail address: kim.ekroos@zora.fi (K. Ekroos).

[1] Both authors contributed equally to this work.

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physiological response will be dependent on the structure of and membrane distribution of these molecular lipid species. Shinzawa-Itoh and colleagues showed by sophisticated experiments that the oxygen transfer mechanism in cytochrome c oxidase requires a specific phosphatidylglycerol molecular lipid with palmitate and vaccenate at the *sn*-1 and *sn*-2 positions respectively on the glycerol backbone [14]. In addition, Menuz and colleagues showed that C24- to C26-carbon ceramides mediate the death of a *C. elegans* mutant that fails to resist asphyxia whereas ceramides with shorter chains have the opposite effect [15]. These examples underscore the importance and the specificity of the detailed molecular lipid structures determining the biofunctionality. Therefore, it is essential to identify and quantify lipids at the molecular lipid species level, e.g. PE 16:0/20:4, where the information of the type of fatty acids and their positions attached to the glycerol backbone making up the particular PE molecule are retrieved. Analytically this becomes much more challenging and time consuming. However, molecular lipidomics is a prerequisite for increasing the biological knowledge of molecular lipids and their mechanism of action, supporting both basic research and medicine and promoting the discovery of disease biomarkers and new drug targets in coming years.

Studies of dysfunctional lipid metabolism can be done using a variety of cell culture models, animal models, tissues and biofluid samples from humans. Such studies have provided valuable insight into the metabolic consequences of atherosclerosis [16], obesity [17], diabetes [18] and insulin resistance [19]. In particular, mouse models have been applied to assess the molecular mechanisms of lipid disorders, albeit the mechanisms of action in humans might be different. While animal studies typically consist of a low sample number the situation dramatically changes when the focus turns to clinical trials in man with hundreds to thousands of samples. In case of plasma or serum samples, the challenge is to characterize in-depth and quantify the lipidomes similar to the approach recently published by Quehenberger and colleagues [20]. However, to routinely and rapidly quantify over 500 molecular lipids at high quality in sample sets exceeding several hundreds of samples becomes an extremely challenging task. This suggests it is necessary to perform high throughput molecular lipidomics for the discovery of prognostic and diagnostic marker as well as biomarker that will serve as a read-out of experimental or approved therapies while the appreciation for the bioactivity of lipids has the potential of identifying novel drug targets. Undoubtedly, high throughput molecular lipidomics is demanded, which suits good laboratory practice, regulatory environment and is cost beneficial.

In this review, we describe a high throughput molecular lipidomics solution based on 96-well robot-assisted sample preparation and lipid extraction, multiple lipidomic analysis platforms and an integrated bioinformatic system (Fig. 1). We discuss the validation and key features of the workflow, and emphasize practical issues associated with this workflow. High-quality comprehensive and quantitative molecular lipidomic read-outs can be achieved instantly. We highlight the efficacy of this workflow by discussing its application for high throughput molecular lipidomic studies.

2. Automated sample preparation and lipid extraction

It is known that manual lipid extraction is labor-intensive and prone to errors thereby prohibiting the large scale studies containing hundreds or thousands of samples. Automation of the sample preparation and extraction process is therefore required for large scale lipidomics studies. Automation has the additional benefit of significantly improving the study cost-effectiveness. The process should be accomplished such that no artifacts will be generated while maintaining the endogenous lipids intact. Appropriate selection of solvents, reagents, sample amounts, lipid standards, hardware, and protocols should be carefully considered since all components of the process will impact the quality of the final lipidomic dataset. Over the past years we have in our laboratory continued to advance the robot-assisted sample preparation and lipid extraction procedure based on the 96-well format [12]. This setup has been tailor made for high throughput molecular lipidomics targeting quantification of the absolute molecular lipid concentrations and made applicable for handling large scale studies.

Proper sample handling is crucial for the success of any bioanalytical study. For lipidomic studies one should not only consider the storage of the samples but also sample preparation practises and processes throughout the workflow. Careful sample collection should be established. Samples should be quickly frozen and stored at the appropriate storage conditions if they cannot directly be subjected for lipidomic analysis. It has been shown that certain biological matrices can be safely stored for years at −80 °C [21]. However, the stability can dramatically vary depending on the type of lipid, type of sample and type of storage material. For instance, Hammad and colleagues recently showed that ethylenediaminetetraacetic acid (EDTA) is the preferred anticoagulant for sphingolipids [22]. Furthermore, other factors such as the number of freeze and thaw cycles can also influence the end result [23].

We routinely perform the robotic sample preparation at +4–8 °C and antioxidants are included prior to the lipid extraction to minimize any loss in sample stability. The recently developed MS technologies allow the analysis and production of comprehensive lipidomic outputs from minute sample amounts, for example, few microliters of plasma. However, the low sample and standard volumes also set higher demands on the overall pipetting precision. Manually pipetting low volumes at high precision can be difficult and cause unwanted variation in the results. This becomes challenging when highly volatile organic solvents such as chloroform, which is one of the most frequently used lipid extraction solvents, are to be pipetted. This challenge can be overcome by applying the recent robotic pipetting techniques that enable to pipette and transfer low volumes irrespective of sample matrix or type of solvent at very high precision.

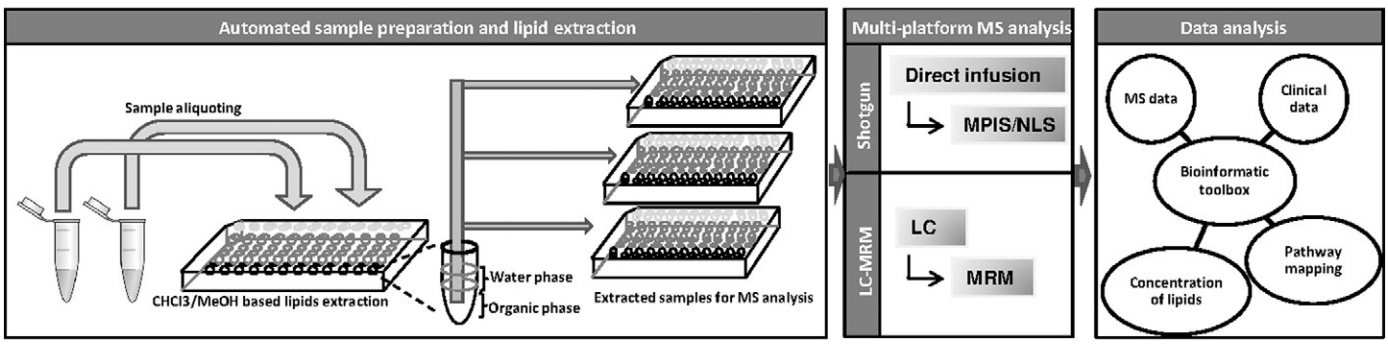

Fig. 1. Schematic outline of the high throughput lipidomic platform.