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REVIEW

Imaging and lipidomics methods for lipid analysis in metabolic and cardiovascular disease

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Cardiometabolic diseases exhibit changes in lipid biology, which is important as lipids have critical roles in membrane architecture, signalling, hormone synthesis, homoeostasis and metabolism. However, *Developmental Origins of Health and Disease* studies of cardiometabolic disease rarely include analysis of lipids. This short review highlights some examples of lipid pathology and then explores the technology available for analysing lipids, focussing on the need to develop imaging modalities for intracellular lipids. Analytical methods for studying interactions between the complex endocrine and intracellular signalling pathways that regulate lipid metabolism have been critical in expanding our understanding of how cardiometabolic diseases develop in association with obesity and dietary factors. Biochemical methods can be used to generate detailed lipid profiles to establish links between lifestyle factors and metabolic signalling pathways and determine how changes in specific lipid subtypes in plasma and homogenized tissue are associated with disease progression. New imaging modalities enable the specific visualization of intracellular lipid traffic and distribution *in situ*. These techniques provide a dynamic picture of the interactions between lipid storage, mobilization and signalling, which operate during normal cell function and are altered in many important diseases. The development of methods for imaging intracellular lipids can provide a dynamic real-time picture of how lipids are involved in complex signalling and other cell biology pathways; and how they ultimately regulate metabolic function/homoeostasis during early development. Some imaging modalities have the potential to be adapted for *in vivo* applications, and may enable the direct visualization of progression of pathogenesis of cardiometabolic disease after poor growth in early life.

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Introduction

All eukaryotes need to maintain a continuous flux of energy, which requires the ability to regulate storage of excess energy in a compact and stable form, such as fatty acids (FAs), for later use.[1,2] This comprises the uptake of non-esterified FAs from the bloodstream as well as their *de novo* synthesis in the cytosol of hepatocytes and adipocytes, trafficking via autophagosomes or the endoplasmic reticulum (ER), storage in lipid droplets (LDs) as esterified neutral lipids (e.g. triacylglycerols) and β-oxidation in mitochondria.[3–5] Other lipids, including phospholipids and triacylglycerides, are synthesized in association with the ER, which ensures that there is a continuous supply of lipids for structural/functional applications as well as an energy reserve for times when availability is limited, such as during starvation, prolonged exercise or during early development.[6–8] Adipocytes are specialized lipid-storage cells, with the specific role of neutral lipid storage for energy reserves, which can be mobilized out of cells into the bloodstream to provide energy for other tissues when glucose supply is diminished.[5] However, when dietary intake exceeds the storage capacity of the adipose tissue, there is spill-over of non-esterified FAs, which results in excess storage of these lipids in other tissues.[9] Most other cells, including hepatocytes, pancreatic cells, skeletal muscle cells, cardiomyocytes and macrophages have a capacity to sequester free FAs. Once this capacity is exceeded, there are consequences for cell function, but also whole body homoeostasis, which can lead to, for example, insulin resistance and dyslipidaemia.[3,10] In addition, the hypertrophy of adipocytes during obesity promotes a systemic pro-inflammatory state, which can further alter cellular function in other tissues.[11] The existing body of knowledge has relied on the ability to identify, localize and quantify intracellular lipids, using specialized biological and imaging techniques. This has contributed to our understanding of the pathways that regulate normal and pathological lipid storage in adipose and non-adipose cells/tissues.

The accumulation of lipids is not simply a response to adult lifestyle patterns in diet and exercise, with maternal nutritional status and fetal growth impacting on the risk for cardiometabolic disease later in life.[12–16] With almost half of mothers being obese and 5–14% of babies being born too small or too

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large,[17,18] our focus has shifted to understanding the links between growth in early life and the development of cardiometabolic disease in adult life. However, it may be most clinically relevant to develop tools to study the progression of cardiometabolic disease by characterizing the physiological and pathological deposition of lipids within organs. A range of techniques can be used to measure lipid content and their applicability to *Developmental Origins of Health and Disease* studies should be considered; but the advent of imaging technologies offers the potential to extend these observations to include dynamic real-time events.

Disease states such as obesity and insulin resistance lead to changes in intracellular lipid storage, which are evident in the altered amount and distribution of, for example, neutral lipids.[3,6,19] The control of intracellular lipids, and therefore systemic lipid homoeostasis, involves the interaction of multiple signalling pathways and the cooperation of several cellular structures and organelles. The coordinated functions of the ER, lysosomal network (including autophagosomes) and cytoskeleton provide a link between lipid synthesis, hydrolysis, storage, and both intracellular and extracellular transport.[20–24] Meanwhile, esterified neutral lipids destined for energy production or steroid and membrane synthesis, are primarily contained within intracellular membrane-bound LDs.[10,20] The generation of LD proteomes/lipidomes, using quantitative biochemical methods has provided vital information on their molecular, and specifically neutral lipid, composition.[25] Unfortunately, limited information is available on the organization and lipid flux within and from LDs. However, this is changing as new methods for imaging lipids in live cells are being developed, which will lead to more detailed information on the structural organization of lipids in biosynthetic/storage organelles and on the traffic of lipids through different regulatory networks.[10,19,21]

In the context of lifestyle and metabolic diseases, the focus has been mainly on altered lipid storage and the changes in neutral lipids that affect energy metabolism; however, a majority of lipids reside in cell membranes. Both cholesterol content[26] and the specific phospholipid composition[27] of membranes are important determinants of membrane fluidity and architecture, which in turn influences a cells' receptivity to membrane–receptor dependent signalling. For example, this could provide an explanation for the correlation between the ratio of specific unsaturated FAs from the diet and insulin resistance.[28] Membrane-derived lipid signalling molecules also provide a plausible link between obesity and inflammation, and are factors in insulin resistance. This is through their actions as second messengers in signalling cascades.[29,30] For example, the increased production of membrane second messengers, such as ceramide and sphingolipids, in obesity contributes to the development of pancreatic β-cell failure, atherosclerosis and cardiomyopathy.[29] Sphingolipids also have important roles in membrane–protein interactions through their organization into specific functional domains (often referred to as lipid rafts).[27]

Dyslipidaemia and lipid profiles: systemic lipid imbalances and the development of insulin resistance and cardiovascular disease

Biochemical methods have played an essential role in establishing links between metabolic disease, intracellular lipid storage and the pathological changes in blood lipid profiles, which are referred to as *dyslipidaemia.* For example, in prospective cohort studies, knowledge about the role of insulin resistance and dyslipidaemia in human cardiovascular disease has come from quantitative analysis of blood insulin.[31] In addition to altered insulin sensitivity, individuals with more rapid growth in early life are more likely to have dyslipidaemia.[15] Systems biology approaches are being used to study lipid metabolism and acquire quantitative data from sensitive mass spectrometry (MS) and chromatography methods.[30] This information has been modelled to determine how changes in serum lipoprotein composition and specific lipid species, rather than just total triglyceride levels, are associated with insulin resistance, non-alcoholic fatty liver disease, cardiomyopathy and atherosclerosis.[29,32]

Cellular and tissue lipid profiling has gained significance following the introduction of technology for high-throughput lipid analysis. In-depth reviews of biochemical methods for lipid analysis have been conducted elsewhere[30,33] and are summarized in Fig. 1. Here, we briefly discuss some of the most important developments in the quantitative analysis of lipids in human metabolic disease.

Gas chromatography (GC)

GC is used to quantify FAs in biological extracts either by simple peak integration or using a flame ionization detector.[34,35] The GC method can measure serum FAs, improving our understanding of their role in the early development of human cardiovascular and metabolic diseases.[34,36–38] For example, analysis of the EPIC-Norfolk cohort, using GC to measure FAs in plasma and erythrocyte-membrane phospholipid fractions, found that FA profiles are associated with an increased risk of developing type II diabetes.[38] High levels of palmitic acid (16:0), palmitoleic acid (16:1*n*-7), dihomo-γ-linolenic acid (20:3*n*-6) and low levels of heptadecanoic acid (17:0), vaccenic acid (18:1*n*-7), eicosenoic acid (20:1*n*-9), linoleic acid (18:2*n*-6) and eicosadienoic acid (20:2*n*-6) were predictive of type II diabetes.[38] Although both plasma and erythrocyte FAs showed a similar pattern, the plasma FAs demonstrated greater values for assessing the association with diabetes risk.[38] Therefore, the GC method can provide an important bridge between lipidomics and cohort studies defining factors that influence metabolic function, and can also lead to the concept of metabolic fingerprinting.[38,39]

The critical limitation of the GC method is that it can only be used to analyse volatile compounds; and therefore lipids such as FAs, phospholipids and sterols require derivatization before GC analysis.[30] Increasing the volatility of these lipids is generally achieved through esterification, which in the

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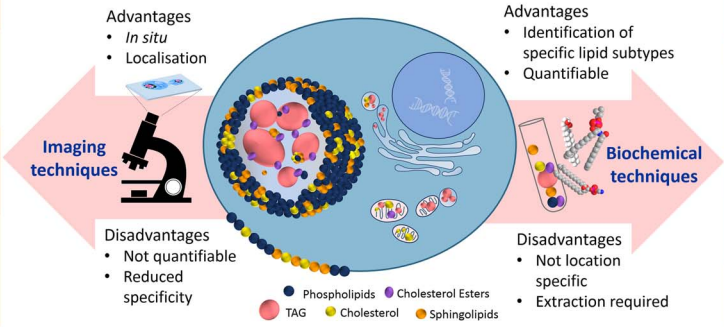
Method	Advantages	Disadvantages
Light microscopy	Affordable, uses standard equipment, fast data acquisition	Contrast agents require fixation, low resolution, low specificity
Electron microscopy	Very high resolution, can image membranes	Requires specialised equipment, sample fixation , toxic contrast agent
Coherent anti-Stokes Raman scattering spectroscopy	Contrast agents not required, can image live cells and tissues in situ	Requires specialised equipment
In situ mass spectrometric imaging	Highly specific identification based on chemical structure, can image live tissue in situ	Interference from reagents and endogenous compounds, expensive
Fluorescence imaging	Uses standard equipment, fast data acquisition, can image live cells and tissues, colocalisation with proteins and other structures	Requires labels, may require fixation, low sensitivity, low specificity with existing labels



Method	Advantages	Disadvantages
Thin-layer chromatography	Affordable, fast analysis, no specialised equipment needed	Low sensitivity, low resolution
Gas chromatography	High specificity for fatty acid subtypes	Requires derivatization for analysis of non-volatile compounds
Nuclear magnetic resonance spectroscopy	Non-destructive analysis, suitable for all lipid types	Low sensitivity
High-performance liquid chromatography	Analysis of a wide range of polar and nonpolar lipids, specific fatty acids	Requires specialized detectors due to low UV-Vis absorption of lipids
Mass spectrometry and GC or HPLC-MS	Highly specific and sensitive analysis of complex extracts	Expensive standards required for quantification

Fig. 1. There is a wide range of analytical techniques for studying lipids. Biochemical methods can be used for precise quantification of specific lipid subtypes/species, whereas emerging imaging modalities can permit the study of lipids in live cells.

case of phospholipids also requires enzymatic hydrolysis.[30,35] However, the process of lipid preparation for the GC method is time-consuming, inevitably incurs further complexity and increases the likelihood of artefacts and errors during analysis.[35]

MS

MS is arguably one of the most commonly utilized technologies in lipodomic studies as it can provide information about molecular mass, chemical structure and analyte concentration.[40,41] MS has led to several valuable findings in addressing cell biological questions, including demonstration of the significance of glycerophospholipid cardiolipin in mediating mitochondrial autophagy.[41] Moreover, MS methodology was used to perform lipid profiling to identify specific lipid metabolites in patients with myocardial infarction. Elevated serum levels of phosphatidylcholine, sphingomyelin, ceramide (d18:1/24:1) and glucer (d18:1/16:0) were detected in patients with myocardial infarction.[42] However, there are some drawbacks associated with MS methodology, particularly sample handling and lipid extraction, as interactions between lipids and solvents can alter the lipid fraction being isolated from the samples.[43] Some novel 'soft-ionization' techniques can eliminate the need for lipid extraction and enable the analysis of lipids *in situ* (a concept that is discussed in detail in section *In situ* mass spectrometry imaging (MSI)).[40,44] Nonetheless, many of these techniques are yet to be optimized for effective lipid analysis, and are not easily accessible to many researchers.[45]

More common ionization methods include electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI). Although ESI and MALDI-MS are well-established techniques in lipid analysis, analysis of complex biological samples is often hindered by suppression of ionization, which leads to an inability to detect low-abundance chemical species due to the presence of low-volatility compounds.[44,46] For ESI-MS, upfront separation via high-performance liquid chromatography (HPLC), GC or solid-phase extraction methods, offers a solution to this issue.[30,40,41]

HPLC and HPLC-MS

HPLC provides a relatively inexpensive and simple approach to analyse lipid classes. Normal and reverse-phase methods, using evaporative light-scattering, UV-Vis, fluorescence and refractive index detection, are utilized for the analysis of polar and neutral lipid classes, without the requirement for expensive internal standards to account for the matrix effects encountered in MS.[35] Depending on the detection method, this technique can separate lipid classes in complex extracts before more complex analyses.[35] For example, by using reverse-phase HPLC, it was found that the concentration of cholesteryl esters was reduced and the concentration of triacylglycerides was increased in low-density lipoproteins from type II diabetic patients, when compared with healthy individuals.[47] HPLC alone has a limited ability to resolve different lipid species within polar and neutral lipid classes, owing to their diversity within biological samples. In contrast, mass detectors offer substantially improved sensitivity and allow for the identification of individual lipid species based on their unique chemical structures.[35]

Imaging intracellular lipid distribution and architecture in real time

The challenge for analysing lipids is to determine the spatial and temporal changes in the intracellular localization of

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these molecules.[27] This information is lost by the homogenization and extraction procedures that are required for most quantitative biochemical analyses,[48] and therefore alternative approaches, such as imaging techniques are required to develop a comprehensive understanding of how intracellular lipid distribution is altered in major disease states. Moreover, imaging techniques can provide information on the dynamic activities of lipids and their interactions with other organelles (methods for visualizing lipids *in situ* are summarized in Fig. 1 and discussed below).

Light microscopy

Before the introduction of fluorescence imaging and other high-resolution techniques, conventional light microscopy was used to study neutral lipids; stained with Oil Red O and Sudan Black B.[49] In this type of microscopy, visible photons (λ = 400–700 nm) serve as the source of illumination, and by passing through the specimen, they are refracted through glass optical lenses to form a magnified view of the sample. Oil Red O is frequently used to visualize LDs in mammalian cells and tissues.[33,50] In particular, Oil Red O has been used to visualize the increased lipid accumulation that occurs in the liver, heart and skeletal muscle during obesity[1] and metabolic syndrome;[51] which are thought to contribute to the subsequent development of type II diabetes, non-alcoholic fatty liver disease[50] and cardiomyopathy.[52] Both Oil Red O and Sudan Black B require sample fixation and the use of ethanol as a solvent, which can result in lipid extraction.[33,53] However, this method only gives a pictorial of a static window in time. Furthermore, conventional light microscopy has a resolving power limited to ~230 nm, which restricts observations of LDs in many cells (in which they can range from 100 to 1000 nm in diameter)[49] and specifically cannot delineate their internal architecture.

Electron microscopy (EM)

The best resolution (<0.1 nm) is currently achieved using a beam of electrons rather than light as the source of illumination. EM is operated in a vacuum where scattered electrons pass through the specimen and are focussed by the electromagnetic lenses of the microscope.[54] In EM, the shorter wavelength of the electron (λ = 0.005 nm, 50 kV) is associated with the increased resolving power.[54] There are three commonly used types of EM: transmission electron microscopy (TEM), scanning electron microscopy (SEM) and scanning transmission electron microscopy (STEM), which differ fundamentally in their uses. Typical TEM images are two-dimensional projections of an ultrathin slice (<0.5 μm) of the specimen, which are collected by spreading the electron beam with energy between ~60 and 300 keV.[55] SEM generates an image with the help of backscattered or secondary electrons recorded by a focussed beam with energy between ~500 eV and 30 keV; this type of EM gives the impression of three dimensions.[55] STEM has features of both EMs and uses the SEM beam over the specimen.[55] In cell biology, EM has enabled high-resolution images of the association between LDs and ER membrane, overcoming one of the major limitations of light-based microscopy. Increased resolving power led to a proposed mechanism for LD formation that involves 'budding' from the ER,[56] and this was based on ultrastructural observations of LDs sharing a continuous membrane with the ER.[24] In addition, the association of LDs with both mitochondria and cytoskeletal filaments has been observed using EM in adrenal and Leydig cells.[20–22] For example, hormonal stimulation of normal rat adrenal tissue results in morphological changes to mitochondrial membranes, increasing the number of membrane protrusions into LDs to facilitate the transport of cholesterol and sterol esters into the inner mitochondrial membrane for steroid synthesis.[20,22] These membranous protrusions have also been observed using EM in yeast between LDs and peroxisomes, which are the site of β-oxidation.[57] Collectively, EM has been and continues to be an important tool in providing detailed maps of intracellular lipid distribution in relation to specific cellular structures. Moreover, EM has provided evidence for the proposed mechanism by which LDs are formed at the ER where they act as a reservoir for newly synthesized triacylglycerols, which can then be withdrawn for the synthesis of steroids in response to hormonal signals from the hypothalamus, or for energy mobilization via β-oxidation.[10,21,22,57]

While the application of conventional EM to lipid biology is potentially powerful there are a number of technological limitations. One limitation is the requirement for sample fixation, which restricts its application to imaging immobilized structures in fixed cells. Thus, EM cannot provide any spatial temporal information for the visualization of lipid dynamics; unless the cell biology experiments are performed on electron transparent membranes and imaged with a light microscope before fixation and then imaged with SEM.[55] Another limitation is that visualizing large areas requires the assembly of multiple images, which is a technically challenging process.[49] Finally, the contrast agent used to visualize lipids in EM, osmium tetroxide, is toxic.[33] However, osmium tetroxide can be used to stain for subcategories of lipids. For example, osmium oxide demonstrated co-staining with unsaturated C18 FAs as well as complementary localization to saturated (C14, C16 and C18) and unsaturated (C16) FAs in mouse adipose tissue.[58] Cryo-EM offers an alternative to chemical fixation methods, and has been used to elucidate the three-dimensional structure of human low-density lipoprotein particles.[59] However, this method still provides limited spatial temporal information. Focussed ion beam scanning electron microscopy (FIB-SEM) tomography represents a technology with a built-in gallium ion source, in which the specimen (thin as ~3 nm) is milled off with the Ga^+ ion beam and then with the electron beam to generate an image.[60] This method can allow the generation of volume data for three-dimensional analysis, and has been successfully used as cryo-FIB-SEM tomography on the mouse optic nerve.[61] Using this technology, lipid-rich structures such as membranes and myelin appeared dark on the micrographs, when compared with the cytoplasm.[61] Ultrahigh-resolution

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TEM, transmission electron aberration-corrected microscope, is a new technology that can provide an information limit of 0.05 nm at an accelerating voltage of 200 kV.[62] There are many new EM technologies, for example, four-dimensional ultrafast electron microscopy,[63] that have potential applications in lipid biology.

Coherent anti-Stokes Raman scattering (CARS) spectroscopy

CARS spectroscopy offers an effective alternative approach to visible light and fluorescence-based (vide infra) imaging techniques,[64] and does not require exogenous labels for the visualization of lipids in the biological specimens. Moreover, CARS spectroscopy allows the non-invasive quantification of lipids within live cells based on the detection of specific molecular bond vibrations,[65,66] such as those of C–H, C=O and P–O bonds, and can therefore be tuned to discriminate individual lipid species in complex samples.[67,68] This approach can also allow observations on cellular dynamics, to help identify key regulatory processes in lipid homoeostasis. For example, by using CARS spectroscopy, it was demonstrated that overexpression of Perilipin 5 results in increased sequestration of saturated FAs in LDs and an increased proportion of esterified FAs and cholesterol in skeletal muscle fibres.[69] The protein Perilipin 5 is involved in the translocation of hormone-sensitive lipase to LD membranes in response to protein kinase A signal induction, which allows cells to mobilize neutral lipids in response to catecholamines and β-adrenergic stimulation.[10,70] These results suggest that dysregulation of FA hydrolysis feedback mechanism in disease states might induce alterations in LD composition,[69] and that CARS spectroscopy is an effective method for observing these changes.

Notably, CARS spectroscopy permits imaging for extended period of time without a loss of signal;[49] this capability has been particularly effective for monitoring dynamic trafficking events that have a vital role in LD activities.[10,53,71] The active transport of LDs in Y-1 mouse adrenal cells in correlation with cell rounding, a characteristic feature of cells that are actively undergoing steroidogenesis, has been observed using CARS spectroscopy.[71] These active processes can be sensitive to laser effects and the binding of exogenous compounds,[10,53,71] and are therefore difficult to study using alternative live-cell imaging methods. However, the application of CARS spectroscopy in lipid biology is still limited, by its reliance on relatively weak signals[72] and its inability to differentiate between structurally similar lipid species.[49] Furthermore, this approach is time-consuming and requires specialized equipment, which is not widely accessible.[33]

In situ *mass spectrometry imaging (MSI)*

Another label-free imaging technique is *in situ* MSI, which has been used to study lipids.[73] This technique involves the acquisition of mass spectra from discrete areas, as small as 1 μm^2 within tissue sample, which can be compiled into high-resolution images to provide a detailed depiction of both the chemical composition and localization of cellular components.[49,74] This modality has been particularly amenable to the two- and three-dimensional mapping of lipids in large sections of brain tissues,[73,75,76] and has been effectively utilized for the identification and localization of a wide range of lipids, such as cholesterol,[73] saturated/unsaturated FAs,[73] phospholipids and sphingolipids.[75,76]

In contrast to CARS imaging, there is no need for specific calibration to detect target molecules using MSI.[73] However, data interpretation with this technique is complex because there is unavoidable simultaneous detection of multiple compounds; whereas exogenous labels are superfluous, cell culture media and reagents used in sample preservation/preparation and residual ice or water inevitably contribute to background signals.[74] Signals from endogenous biomolecules can also interfere with the analysis of specific compounds, particularly when target molecules exist at low concentrations. This issue can be overcome through modifications at the levels of ionization, detection and data analysis, but only at the expense of spatial temporal resolution.[73] Furthermore, resolution at the organelle level is limited and quantification of individual lipid species requires calibration with standard solutions of target analytes, making this procedure time-consuming and expensive.[49]

Confocal microscopy

Wide-field fluorescence, or epifluorescence, microscopy is often easily accessible and has the benefit of fast data acquisition. In high-resolution wide-field microscopy, focus and depth aberration in three-dimensional imaging have been corrected via incorporation of a large-throw deformable mirror; and this dramatically improved spatial resolution, peak intensities and deconvolution of the images.[77] In contrast, confocal microscopy, which excites samples using a scanning laser and detects fluorescence from a pinhole-sized focal point, can offer resolutions at 180 nm or less depending on the type of microscopy, making it suitable for imaging at the organelle level.[49] The spatial resolution can also be increased by overcoming the diffraction barrier (from 200–300 nm to 20–50 nm in the lateral dimensions), and this has been achieved with a number of super-resolution far-field microscopy techniques.[78] For instance, by using the single-molecule localization approach, three-dimensional stochastic optical reconstruction microscopy allowed visualization of nanoscopic cellular structures such as clathrin-coated pits (~150–200 nm) in BS-C-1 cells.[79] By employing non-linear effects to sharpen the point-spread function of the microscope, for example, in two-colour three-dimensional stimulated emission depletion microscopy, nanoscale imaging of mitochondria demonstrated predominant localization of Tom20 in clusters of the outer membrane of these organelles.[78,80] Compared with confocal laser scanning microscopes, spinning disk confocal microscopy (SDCM) has a high rate of frame acquisition (up to 2000 frames/s in theory), which is achieved by the use of a rapidly rotating disk with thousands of pinholes that scan the specimen by thousands of

points of light in parallel, and therefore this microscopy technique has an important advantage in live-cell imaging.[81] For example, the use of SDCM allowed investigation of passive transport of the fluorescent molecule PEG-8-NBD into giant unilamellar lipid vesicles.[82] Light-sheet fluorescence microscopy offers fast data acquisition and high intracellular resolution, and relies on the use of a thin sheet of light that optically section tissues or whole organism;[83–85] and while this offers significant advantages in imaging, this technology is not yet widely available.

Some of the most significant findings relating to lipid biology have only been possible because of the ability to visualize lipids and their associated proteins in cells and tissues with fluorescent dyes, antibodies and/or fluorescent lipid analogues.[33,49] Nile Red is a neutral lipid-imaging agent that is commonly used to visualize LDs in live- and fixed-cells and tissues.[33,86] The visualization of LD by Nile Red and imaged with confocal microscopy has identified cell populations with differences in the size and number of LDs in AML12 mouse hepatocytes and rat liver tissue.[23] These results suggest a protective mechanism for preventing hepatic lipid overload, in which a subpopulation of hepatocytes accumulates larger amounts of less toxic lipids.[23] Furthermore, this LD heterogeneity decreases following high or long-term FA exposure, which could reflect a similar loss of such protective mechanisms during obesity or exposure to high-fat diets, which leads to the onset of non-alcoholic liver disease.[23]

More recently, the lipid-binding dye boron dipyrromethene difluoride (BODIPY) 493/503 has been used to study LD morphology and activity, in live and fixed cells. Colocalization experiments have provided a link between LD numbers and autophagy, which is an additional mechanism for intracellular FA regulation.[87,88] Colocalization between LDs visualized with BODIPY and autolysosome markers, microtubule-associated protein 1A/1B light chain 3 and lysosome-associated membrane protein type 1, indicates an interaction between these organelles;[87,89] and identifies a process that is upregulated in response to starvation[87] and thyroid hormone stimulation,[88] but impaired after long-term exposure to a high-fat diet.[87,90] Fluorescent lipid analogues can also be used to monitor lipid uptake and/or transport in live cells and tissues. FA-, sphingolipid- and cholesterol-conjugated BODIPY analogues have been successfully utilized to study the uptake of neutral and polar lipids.[33,91,92]

An important consideration when visualizing lipids using fluorescent agents is their potential effect on native cell processes and structure.[49] For example, co-location of lysosomal stains with Nile Red and BODIPY-labelled FAs suggested that these dyes are also interacting with the endosomal/lysosomal network.[93] Filipin, which can be used to visualize free cholesterol, disrupts plasma membrane and this prevents its application in live cells.[49,94] Similarly, anti-lipid antibodies can affect membrane structure due to their relatively large size.[92] To overcome these issues, bio-orthogonal probes have been developed. These probes utilize modified ligands with biologically inert functional groups that are conjugated to fluorescent reagents after binding their target. This procedure has already been demonstrated using FA, cholesterol and sphingolipid probes.[49]

Perhaps one of the most significant problems for many existing fluorescent agents, which are based on organic fluorophores, is their propensity for photobleaching after prolonged exposure to light sources.[95,96] Lipophilic imaging agents with inorganic frameworks offer a promising solution to this issue.[95,97] These imaging agents have shown improved photostability, the potential to stain polar membrane lipids,[98] and label LDs.[95,96] Some of these imaging agents are also suitable for live-cell imaging, and therefore can be used to image disease pathogenesis. Moreover, lipophilic imaging agents have been successfully used for two-photon microscopy.[73] Two-photon microscopy is a high-resolution technique, which offers increased depth of penetration into biological specimens and reduced levels of photo-damage, compared with one-photon techniques.[99] Improved penetration has been achieved by the use of deep red and infrared excitation wavelengths as well as non-linear excitation.[99] In two-photon microscopy, a fluorophore is excited by the simultaneous absorption of two low-energy photons, typically from the same laser, minimizing photobleaching and phototoxicity.[99] Thus, the use of multiple reagents for lipid visualization has the potential to provide important spatial temporal information on lipid dynamics, which will be critical in defining metabolic and cardiovascular disease pathogenesis.

Summary

Changes in lipid metabolism in response to pathogenesis of cardiometabolic disease caused by adult lifestyle or growth in early life are multifactorial. The integration of findings from biochemical and imaging experiments, in a range of cellular and animal models, has provided a better understanding of the intrinsic processes altered in response to diet and the genetic manipulation of metabolic pathways. These innovations for studying lipid biology can now be applied to investigate the associations between plasma FA and LDs, and the progression of insulin resistance, diabetes and cardiovascular disease in individuals at increased risk due to suboptimal growth in early life. The most appropriate method for analysing intracellular and tissue lipids is dependent on the question under investigation and the sample being examined, and requires consideration of several factors, including but not limited to: sample type and preparation, such as the need for fixation and/or solvents,[33,53,100] selectivity for different lipid subtypes, and the effect on native cellular activity for imaging live cells.[49] In most cases, utilizing a suite of these techniques offers the most comprehensive approach for studying lipid distribution and composition, and their interactions with other cellular components, in response to internal and external factors. Continued advances in methods for labelling and quantifying lipids will provide a more complete picture of their roles in cell function and the progression to as well as frank cardiometabolic disease.

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Conflicts of Interest

The following authors, D.A.B. and S.E.P., are shareholders in ReZolve Scientific, a company that has the license to sell lipophilic probes with inorganic frameworks that are mentioned in line 305–308 in references in the review articles.[73–75]

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