

Thematic Review Series: Proteomics

Contributions of quantitative proteomics to understanding membrane microdomains

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Abstract Membrane microdomains, e.g., lipid rafts and caveolae, are crucial cell surface organelles responsible for many cellular signaling and communication events, which makes the characterization of their proteomes both interesting and valuable. They are large cellular complexes comprised of specific proteins and lipids, yet they are simple enough in composition to be amenable to modern LC/MS/MS methods for proteomics. However, the proteomic characterization of membrane microdomains by traditional qualitative mass spectrometry is insufficient for distinguishing true components of the microdomains from copurifying contaminants or for evaluating dynamic changes in the proteome compositions. In this review, we discuss the contributions quantitative proteomics has made to our understanding of the biology of membrane microdomains.—Zheng, Y. Z., and L. J. Foster. Contributions of quantitative proteomics to understanding membrane microdomains. *J. Lipid Res.* 2009. 50: 1976–1985.

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Our view of biological membranes has changed from the original fluid mosaic model (1), which suggested a homogenous distribution of proteins and lipids across the two-dimensional space, to the recent model in which membranes are compartmentalized as a result of an uneven distribution of specific lipids and/or proteins into various microdomains (2). Formed on the basis of aggregation of specific lipids and/or proteins, membrane microdomains are cellular functional units of biological membranes that

are different from other protein complexes in several ways: 1) Their localization on the cell surface puts them precisely in the interface between cells and external environments, including other cells. Many receptors and cell signaling proteins are located on membrane microdomains; the membranes are thought to provide a platform for the initiation and coordination of several cell signaling and trafficking events (3–5). 2) Unlike other cytosolic protein complexes, membrane microdomains are formed not only on the basis of protein-protein interaction but also through lipid-lipid and lipid-protein interactions. 3) The size of these microdomains is generally larger than other protein complexes, ranging up to microns in diameter (6, 7), and they can be very abundant, occupying up to 30% of the total membrane area in certain cells (8). 4) They are highly dynamic, constantly recruiting and displacing proteins as demands require (9, 10).

Lipid rafts and caveolae are perhaps the best-studied and thus most well-understood membrane microdomains, although our understanding of them is still far from complete. Lipid rafts are planar microdomains enriched in cholesterol and sphingolipids, creating a tight packing lipid-ordered phase that is different from the rest of the plasma membrane (11–14); thus, certain proteins will naturally partition into this environment. Rafts have been shown to be membrane reaction centers essential for many cellular processes with the most recognized being signaling and trafficking (3). Due to their plasma membrane localization, rafts are also considered to be the entry points of certain intracellular pathogens (15–18); when raft integrity is disrupted by cholesterol chelating drugs like methyl- β -cyclodextrin (M β CD), virus infection is inhibited and this inhibition is reserved as the cholesterol level is

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Abbreviations: BCR, B-cell receptor; 2DGE, two-dimensional gel electrophoresis; DRM, detergent-resistant membrane; ICAT, Isotope-Coded Affinity Tags; M β CD, methyl- β -cyclodextrin; PDGF, platelet-derived growth factor; SILAC, Stable Isotope Labeling of Amino acids in Cell culture; TCR, T-cell receptor.

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restored (19). The planar nature of rafts make them indistinguishable from the surrounding membranes by traditional visualization tools like electron microscopy but recent experiments have observed rafts in synthetic vesicles and by immunofluorescence microscopy and scanning atomic force microscopy (20–25). These very compelling results demonstrate that rafts do indeed exist *in vivo* and will hopefully finally lay to rest the arguments that rafts are artifacts of detergent extraction (26–28), allowing membrane biologists to move on to more pressing questions.

Caveolae, unlike rafts, are classically pictured as flask-shaped, stable invaginations of the plasma membrane. Because of their unique morphology, caveolae can be easily seen under electron microscopy as small caves if observing the external surface of the membrane (3, 29). Caveolin proteins are the structural proteins of caveolae; their presence is important for the biogenesis of caveolae and maintaining the correct structure and function of these microdomains (30). Caveolin proteins are adaptor/scaffold proteins; they do not have enzymatic activities, rather they homo- or hetero-oligomerize with other caveolins to form a coat that stabilizes caveolae and forces its concave structure. At the same time, caveolins interact with other membrane proteins and recruit specific cytosolic proteins to caveolae through their scaffolding domains (31–34). Caveolae are involved in signal transduction (35–39), in vesicle transport through caveolae-mediated endocytosis (40), in host-pathogen interactions such as with human immunodeficiency virus (41), and in various disease pathologies as witnessed by mislocalizing mutations of caveolins that are linked to cancers in humans (42–45).

Biochemically, membrane microdomains are detergent insoluble, low buoyant density membrane fractions of cells, often referred to as detergent-resistant membranes (DRMs). They can be purified by certain nonionic detergents at low temperature, which act by solubilizing less structured membranes away and leaving DRMs intact, followed by subsequent flotation by density gradient centrifugation (14, 46). Proteomics, in the loose sense,² is the identification, characterization, and quantitation of all or most of the proteins in a complex sample and biochemically-enriched membrane microdomains are often the subject of such studies (47). However, there are several challenges facing such efforts, such as the generally low abundance of membrane proteins and signaling factors, as well as the difficulties in analyzing transmembrane domain proteins by conventional LC-MS/MS. Challenges in basic biochemistry still plague membrane microdomain proteomics as well, as imperfect purification techniques require stringent controls that are often not included. Modern mass spectrometers are exquisitely sensitive and can easily identify hundreds or thousands of proteins in a biochemically-enriched fraction. The problem is that there is currently no other orthogonal technique for quickly

verifying or validating this many proteins, so in many cases, the proteins identified in a membrane microdomain proteome are taken at face value as components of whichever domain was enriched. To our knowledge, no one has purified any membrane microdomain to homogeneity, however, so it is false to state, e.g., that all proteins identified in lipid raft-enriched fractions are lipid raft proteins.

BIOCHEMICAL APPROACHES FOR MEMBRANE MICRODOMAIN PROTEOMICS

The proteomic analysis of any subcellular entity requires the enrichment of the object of interest away from the rest of the components of the cell in order to reduce the complexity of the sample as much as possible; thus, biochemistry is a necessary upstream component of any membrane microdomain proteomics study. Most membrane microdomain proteomics studies have isolated the target membranes as an insoluble membrane fraction in cold, nonionic detergents (e.g., Triton X-100), using their buoyant density to float them upwards in a density gradient (4, 14, 48). Various detergents have been used to solubilize the undesired regions of the membrane, such as Triton X-100, NP-40, CHAPS, Tween, Lubrol WX and several of the Brij series (9, 49–53), although the differences imparted by the use of these detergents when isolating membrane microdomains is not completely clear. Studies have reported that different detergents could result in different microdomain protein compositions; e.g., Blonder et al. (49) compared the DRM proteome when extracted using Brij-96 versus Triton X-100 and concluded that Triton X-100 extracted more DRM material than Brij-96. Other factors that contribute to the proteome variability are certainly the ability of detergents to effectively solubilize non-microdomain lipids and to break up some protein-protein interactions. It is likely that Triton X-100 is slightly less effective at solubilizing nonmicrodomain lipids as more DRM protein was recovered when using Triton X-100 versus Brij-96; additionally, more contaminating proteins, e.g., ribosome components, were observed with Triton X-100. Furthermore, it is becoming clear that lipid rafts and caveolae are two different membrane microdomains and that they are each only a subset of DRMs (54); due to the limitation of detergent methods used to purify them, they have not yet been enriched to homogeneity in any of the proteomics studies reported so far.

In response to some of the early suspicions that lipid rafts were artifacts of detergent extraction, detergent-free methods were also developed to isolate membrane microdomains. The initial step in all these processes is always mechanical disruption (e.g., Dounce homogenizer or sonication) of cells, sometimes in high salt or alkaline pH conditions, followed by ultracentrifugation on a sucrose or Optiprep density gradient (55–61). This alternative isolation procedure was designed to circumvent any criticism of detergent-induced artifacts and also should help to preserve weaker protein-protein interactions that might have been disrupted by detergents; however, the procedure assumes that mechanical disruption would somehow specifi-

² The strict definition of proteomics would be: the identification, characterization and quantitation of all proteins in a cell, organ, tissue or organism, i.e., where the minimal unit is a whole cell.

cally break the membranes up right at the boundaries of the microdomains, a completely unsupported and unrealistic expectation. In fact, evidence suggests that microdomain fractions prepared in this manner are even less enriched than those prepared with detergents (54). That is, a larger fraction of the proteins in detergent-free preparations are not sensitive to M β CD, the gold standard for raft association. Regardless of the procedure, it is important to note that all the detergent-free and detergent-based enrichment procedures mentioned above cannot demonstrate, or at least have not demonstrated, the ability to separate lipid rafts from caveolae or vice versa. However, in a more recent study, Yao et al. (62) developed a modified sucrose gradient that can separate the cholesterol-dependent rafts from caveolin-dependent caveolae, allowing the separation of the two in one experiment.

Besides detergent and detergent-free methods, membrane surface labeling and affinity purification have also been used to isolate membrane pieces containing particular protein populations. Immunisolating rafts has proven to be very challenging because of the choice of a suitable bait, but for caveolae, silica coating and immunoisolation have been used to first coat intact cells with dense, positively charged silica particles (63), which function to increase the density of the plasma membrane for easier isolation and then immunoisolation with the aid of a monoclonal Cav-1 antibody or by anti-Cav-1-coated magnetic beads (64–66).

QUALITATIVE AND QUANTITATIVE PROTEOMICS

Proteomics is the study of all proteins in a cell, an organelle, or an isolated complex system, including their expression, three-dimensional structure, localization(s), interactions, and modifications (as reviewed in Ref. 67). An organism's proteome is in part determined directly by the genome encoding the primary amino acid sequences. Other factors affecting a proteome include alternative splicing to produce different transcripts from one gene and posttranslational modifications (e.g., phosphorylation, ubiquitylation). Thus, the number of possible proteinaceous molecular species in a cell is far greater than the number of genes. Moreover, the proteome of a cell is not static, as it changes in response to the external environments and internal cellular states.

The techniques used in proteomics include antibody-based methods such as parallelized immunoblotting, enzyme-linked immunosorbent assays, and immunofluorescence microscopy (68), as well as spectroscopic methods such as X-ray crystallography and nuclear magnetic resonance (NMR) for determining and probing the high-resolution structure of all proteins in an organism. Both types of approaches focus on a single protein in each assay, or possibly two, three, or four, in the case of immunofluorescence. MS-based proteomics is a much higher content technology; with a fully sequenced genome of the organism being studied and more discerning software tools, thousands of proteins can easily be identified and quanti-

fied from a few micrograms of protein sample with modern mass spectrometers (69).

Mass spectrometers do not measure mass per se, they measure the mass-to-charge ratio of ions and although certain types of mass spectrometers can detect whole proteins, in general, this is an inefficient and insensitive process. Smaller peptides in the range of 7 to 30 amino acids in length are detected with much higher sensitivity and are better behaved in the gas phase. Trypsin is the favored enzyme for generating such peptides, not only because the sizes are optimal but also because of the basic Arg or Lys left on the carboxy-terminus of the peptide, which assists ionization and helps to direct fragmentation. Measuring only the mass of such peptides, however, is not sufficient to unambiguously identify them in most cases because there can be many peptides of a similar or identical mass in the entire complement of proteins encoded in a genome, e.g., anagramic peptide sequences. Instead, peptides can usually be unambiguously identified by interpreting the masses of ions that result from fragmenting the original peptide ion, a process known as tandem mass spectrometry or MS/MS. De novo interpretation of such MS/MS spectra to arrive at the sequence of a peptide is challenging and often unsuccessful. On the other hand, if 'all' possible peptides from an organism are known from a genome sequence then it is a relatively simple matter to compare the fragment spectra with theoretical fragment spectra predicted from a database of all possible peptides in that organism to determine the identity of the observed peptide. It is much more difficult to identify proteins in organisms whose genomes are not sequenced because de novo sequencing of peptides is required. More extensive reviews on mass spectrometric analysis of peptides can be found elsewhere (70, 71).

Previous limits on how quickly spectra could be acquired meant that only a single analyte could be analyzed at a time. Mass spectrometer technology has advanced considerably but even so, very complex protein samples are separated before MS analysis using a variety of one, two, and three dimensions of separation to reduce the complexity of the sample prior to the peptides entering the mass spectrometer. SDS-PAGE, isoelectric focusing, and various chromatographic methods are used most frequently in combination with reversed phase HPLC as the final separation method prior to the peptides being electrosprayed into the mass spectrometer (72).

Historically, this approach has been largely qualitative, simply being used to identify which protein(s) are present in a sample, but quantitative proteomics approaches are gaining in popularity, allowing investigators to get at the specificity and functionality of the protein components in their samples. In a mass spectrum, the intensity of any given peptide (which is a proxy for a specific protein or proteins) is proportional to the concentration of that peptide in the sample. However, the ionizability of the peptide also has an enormous contribution to the signal measured for it, meaning that it is difficult to directly compare the intensities of two different peptides. Stable isotope dilution methods are the most accurate methods for quantita-

tive proteomics and the current choice for most groups with a serious interest in quantitative proteomics. However, two-dimensional gel electrophoresis (2DGE), with its unparalleled resolution, was favored historically and semi-quantitative methods such as spectral counting have gained some popularity more recently (73).

Most quantitative proteomics approaches measure relative protein abundances between two or more samples (74), as opposed to determining absolute levels of proteins through the spiking of known amounts of isotope-labeled standards (75). The wide range of technologies employed in quantitative proteomics has been reviewed extensively elsewhere (76–78) so we only briefly describe the three approaches that are necessary to understand with respect to the membrane microdomain proteomics literature: 1) 2DGE, 2) SILAC (Stable Isotope Labeling by Amino acids in Cell culture), and 3) chemically-introduced isotope labels such as ICAT (Isotope-Coded Affinity Tags). In 2DGE, the proteomes to be compared are each resolved by pI and molecular weight and detected using one of a variety of protein stains. The staining intensity of each spot is proportional to the amount of that protein allowing an easy method of quantifying the hundreds to thousands of protein spots visualized. Some of the pitfalls of 2DGE include the resolution of protein isoforms, which makes quantitation challenging, and the difficulties in obtaining protein identifications from many spots. In SILAC, the proteomes to be compared are mass tagged by growing the cells initially in media containing specific amino acids enriched in various isotopes, e.g., media containing either normal isotopic abundance arginine or arginine with all six carbon atoms replaced with carbon-13. The two samples can then be combined prior to isolation of any membrane microdomains so that all downstream sample handling is carried out on the combined samples. The two forms of the peptides are easily resolved by the mass spectrometer and the relative intensities of the light and heavy peptides give a measure of the relative amounts of the original protein between the two samples (79). SILAC and other metabolic labeling methods can normally only be applied to cultured cells. Chemical derivatization to introduce isotope labels is similar in principal to SILAC in that the peptides from the two different conditions are encoded with different masses. The difference lies in how the isotopes are introduced; typically one or two types of functional groups on proteins or peptides are targeted with specific chemistry to introduce a new moiety containing an isotope label (80). Chemical derivatization can be applied to any sample but is based on the assumption that each sample is completely and specifically labeled, often an unsupported assumption for complex biological samples. Stable isotope dilution methods, such as SILAC or chemical approaches, can be more expensive than 2DGE but the additional information obtained (i.e., protein IDs, more proteins quantified) is usually judged to be worth the cost. Although each method has its advantages and disadvantages, 2DGE is no longer very popular, whereas SILAC and some chemical derivatizations remain the most commonly used stable isotope dilution methods.

QUANTITATIVE PROTEOMICS APPLIED TO MEMBRANE MICRODOMAINS

General challenges in organelle proteomics

In any organelle proteomics study, the main challenge is to isolate a homogenous sample free of all nonspecific proteins or contaminants. In reality, it is impossible to purify any organelle to homogeneity (81, 82), although many biochemical approaches have been developed in order to maximize the percentage of authentic organelle components in a sample (e.g., multi-step biochemical preparations or immunoenrichment) and to minimize the nonspecific contaminants (46). Because the latter cannot be reduced to zero, however, there is always some doubt as to whether any protein identified in an organelle proteomics experiment is a real component of the enriched compartment or not. Thus, in a qualitative proteomics experiment, identified proteins really should be validated by an orthogonal method to confirm that they are localized to the subcellular location in question. The most common validation tool for organelle proteomics studies is fluorescence microscopy, of course, but this requires 'gold standard' markers and is only good for validating a small number of proteins due to the cost and availability of detection reagents, e.g., antibodies. Several quantitative proteomics approaches have been designed to achieve much more accurate assignments of general subcellular localization (as reviewed in Ref. 83). In addition, work from us and others has led to quantitative methods for determining the proteome of membrane microdomains and how they change in response to stimuli (54) (see below).

Quantitative proteomics approaches to more accurately determine the protein complement of lipid rafts

Taking advantage of their detergent resistance is a simple and effective way to enrich lipid rafts and caveolae but DRM preparations are notoriously dirty, containing copurifying proteins from a wide variety of locations within the cell besides membrane microdomains. This fact has long been recognized by cell biologists studying lipid rafts and, as a result, the field has come to require that for a protein to be considered a bona fide component of rafts, its presence in DRMs must be shown to be sensitive to cholesterol disruption (84, 85) because the liquid crystal state of lipid rafts is completely dependent on the rigid lipid packing provided by cholesterol intercalation between phospholipid tails. Despite this standard in the lipid raft field, many proteomics groups have tried to equate the proteins identified in DRMs with lipid raft proteins and, as a result, the lipid raft community has largely ignored the proteomic studies of lipid rafts; much of the data simply contains far too high a rate of false-positive assignments to be of any use. There are now at least four proteomics studies of DRMs that have also tested the sensitivity of each identified protein to cholesterol perturbation using M β CD. Initially, Bini et al. (9) used 2DGE to measure, among other things, the effects of M β CD on proteins in DRMs isolated from Jurkat T-cells. The authors observed that most pro-

teins, including several unexpected proteins such as mitochondria-resident proteins, were sensitive to cholesterol disruption. As will be discussed, later studies have found that these proteins in fact are not sensitive to cholesterol disruption and closer examination of the original methodology suggests a possible reason for the discrepancy: Bini et al. normalized their preparations based on equal amounts of protein but this was only after the DRM preparations had been made, which assumes that the treatment itself, i.e., M β CD, does not have a marked effect on the yield of DRM. The same year as the Bini study, however, we reported a study where we used SILAC to quantify the impact of M β CD on 392 proteins identified in epithelium-derived HeLa DRM preparation (54). One additional finding of this study was that M β CD has an enormous effect on the DRM yield, explaining the discrepancy between our data and that of Bini (later confirmed by us and others). According to the known sensitivity of lipid raft proteins to this drug, we were able to classify three differentially sensitive groups into 'raft proteins', 'raft-associated proteins' and 'co-purifying proteins or contaminants', significantly, the nonraft group including mitochondrial and endoplasmic reticulum (ER) proteins that had been incorrectly assigned to rafts by DRM proteomics studies before. By using the quantitative proteomics approach, we overcame the inability to biochemically purify rafts and eliminated the contaminants that copurified with rafts in DRMs (Fig. 1). The greater specificity afforded by this approach also allowed us to demonstrate in an unbiased manner that lipid rafts are enriched in signaling proteins. This confirmed what most people already thought but until that point had not been demonstrated in an unbiased manner. There was a multitude of anecdotal reports of signaling proteins being enriched in rafts, of course, but no demonstration that this was a general phenomenon.

In response to several claims that mitochondrial proteins are in rafts, we expanded these studies to two different cell lines, 3T3 mouse fibroblasts and human Jurkat T lymphocytes, and found similar results: mitochondrial proteins in DRMs are insensitive to cholesterol depletion and are thus not components of rafts (86). As before, rafts are enriched with signaling molecules; major cytoskeletal components show an intermediate sensitivity to cholesterol disruption and are thus likely associated with rafts. We further probed the question of why mitochondrial proteins such as the voltage-dependent anion-selective channels and F₁/F₀ ATPase subunits are in DRMs, testing the degree to which such proteins are enriched in DRMs versus a whole cell membrane preparation or versus a mitochondrial preparation. Voltage-dependent anion-selective channels and ATPase subunits, as well as other mitochondrial proteins, are not even enriched in DRMs, again suggesting that they are simply contaminants. Finally, using high-resolution linear density gradients to better resolve the components of DRMs, classical lipid raft proteins and mitochondrial components showed different distribution profiles across the gradient, lending more support to the thesis that mitochondrial proteins are copurifying contaminants of the normal DRM preparation.

Similar results in plant membranes have been confirmed by Kierszniowska et al. (87) using ¹⁵N metabolic labeling and M β CD to define the sterol-dependent raft proteins in DRMs. In that study, they found many signaling proteins enriched in rafts, as well as cell wall-related proteins, suggesting that, in plants, rafts are anchored through the plant skeleton to the cell wall. In a study by Yu et al. (88), the change caused by ceramide-induced cholesterol displacement of immortalized Schwann cells was examined by SILAC. Here, the authors found ceramide-induced cholesterol depletion only partially decreased the association of caveolin-1 with rafts and had a minimal effect on changing the abundance of other lipid raft proteins such as flotillin-1 and G-proteins that are normally sensitive to M β CD treatment. However, the association of ATP synthase β -subunit with DRMs was increased by the treatment, confirming that it is not a raft-resident protein. M β CD is the favored and most effective pharmacological agent for disrupting rafts, but does disruption with other compounds lead to the same conclusions? Ledesma et al. (89) compared the effect of sphingomyelin depletion by fumonisin B1 to cholesterol depletion by M β CD on hippocampal neuron DRMs and quantified the differences using 2DGE. They concluded that the two drugs induced a similar decrease of raft protein content. The authors also identified proteins such as enolase, annexin, and Thy-1 membrane glycoprotein to be in cholesterol-dependent rafts, contrary to other reports of the insensitivity of such proteins to cholesterol depletion (54).

Comparative proteomics of caveolae

As an object for biochemical study, caveolae are more tractable than lipid rafts because they can be easily seen by electron microscopy and their formation is dependent on the structural protein caveolin. Neither detergent-based nor detergent-free methods yield pure caveolae and although immunoisolation of caveolae via caveolin can achieve a higher enrichment, it too leaves room for improvement. Along another vein, methods for resolving caveolae from cholesterol-rich rafts has only been described very recently (62). Nonetheless, two groups have reported the application of quantitative proteomics to study caveolae composition: Hill et al. (90) used 2DGE to distinguish differences in the DRM proteome of mouse embryonic fibroblasts from both wild-type and Cav-1 knockout mice. Because *cav1*^{-/-} cells are devoid of identifiable caveolae by microscopy, proteins that are absent in *cav1*^{-/-} DRMs compared with wild-type DRMs are reasonably expected to be components of caveolae. The 2DGE approach used by Hill et al. allowed the unbiased assignment of seven proteins to be unique to caveolae and one, Cavin/Ptrf, was determined to be a coat protein of caveolae. In another study, Oh et al. (91) first identified aminopeptidase P to be associated with caveolin by silica coating and immunoisolation of caveolin-associated partners. Then, proteomic analysis of the isolated luminal endothelial cell plasma membranes that contain caveolae with the lung homogenates further revealed aminopeptidase P is particularly concentrated in caveolae. In the future, the use of more

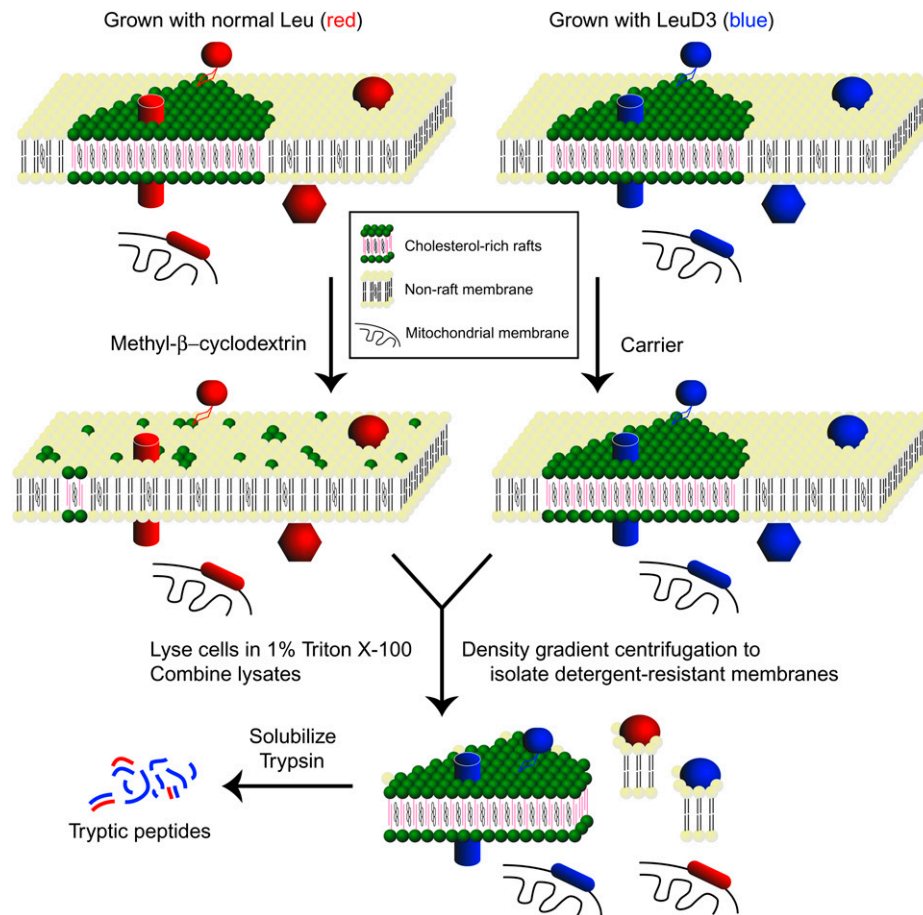


Fig. 1. The use of SILAC and cholesterol depleting drug M β CD to determine the true components of lipid rafts. Figure adapted from Foster et al. (54). Two populations of HeLa cells were grown in normal isotopic abundance leucine (red) or $^2\text{H}_3$ -leucine (blue) and then treated with carrier (blue) or M β CD (red) to disrupt rafts. The cells were then solubilized in ice cold Triton X-100 and equal amounts of protein from the two samples were mixed together prior to isolation of DRMs by equilibrium density gradient centrifugation and LC-MS/MS. Peptides from proteins that are true components of lipid rafts should then present in the mass spectrometer with the heavy version (blue) much more abundant than the light (red), whereas copurifying nonraft proteins should present in roughly equal levels of light and heavy. In this scheme, mitochondrial proteins, endoplasmic reticulum components, and most cytosolic housekeeping proteins presented with roughly equal ratios, indicating that they are contaminants of the raft preparation.

sensitive and higher-throughput quantitative proteomics approaches should enable a deeper coverage of the proteome of caveolae.

Using proteomics to understand membrane microdomain dynamics

The ultimate application of quantitative proteomics to the study of membrane microdomains is not simply to catalog their contents but rather to measure how their contents change during various dynamic processes. Although microdomains such as caveolae are also likely to be quite dynamic, lipid rafts remain the quintessential microdomain for such studies. In the case of lipid rafts, the focus is typically on identifying proteins that are recruited to rafts during signal transduction, especially in response to agonists that act to cluster rafts.

T-cell activation is closely connected with raft dynamics as the T-cell receptor (TCR) is a component of rafts and it recruits other proteins to these small platforms upon ligand

engagement (92–95). Bini et al. (9) used 2DGE to follow the changes in the DRM proteome when TCR was activated by ligation with anti-CD3 monoclonal antibody cross-linking. They identified a small number of spots on their gels that increased during activation and these included known proteins involved in T-cell activation signaling pathway such as ZAP-70, Grb2, and phospholipase C. They also clustered raft-associated proteins based on their temporal raft association by examining the protein spot intensities at different time points after T-cell activation; the data strongly suggested that rafts are highly dynamic structures.

In a similar study by Tu et al. (96), normal Jurkat DRMs were compared with DRMs from anti-CD3/CD28 costimulated cells using 2DGE. The authors were particularly interested in the inducible association of activated I κ B kinase complexes with rafts; they demonstrated that treatment of Jurkat cells with M β CD disrupted the assembly and activation of this raft complex and also interfered in anti-CD3/CD28-induced activation of a NF κ B response element in

the IL-2 promoter. A small number of proteins were found to be recruited to rafts after CD3/CD28 costimulation, e.g., heat shock proteins, vimentin, calmodulin, and a Rho guanine nucleotide dissociation inhibitor; however, these proteins are not well-known members of the TCR signaling pathways and the role they play once recruited to rafts remains unclear.

Von Haller et al. (97, 98) also used ICAT to quantify isolated DRMs from control, unstimulated cells and TCR/CD28 cross-linked cells separately and then the two samples were labeled with light and heavy ICAT reagents at the protein level before combining and digestion. The authors identified several recruited proteins but unfortunately left it up to others to follow these up and validate them. Another study, by Gupta et al. (10), also used ICAT to study the change of B-cell receptor (BCR) when ligated by anti-IgM. Here, ICAT labeling was performed at the peptide level where all Cys-containing peptides were labeled. Only four proteins identified showed altered localization, including two cytoskeletal proteins that suggested that the aggregation of rafts might be controlled by cytoskeletal remodeling and another structural protein, ezrin, was dissociated from rafts. Besides identifying proteins recruited to or displaced from rafts upon ligation, this study also looked at the change of posttranslational modification of ezrin and they found threonine was dephosphorylated upon BCR ligation. Ezrin was released from the underlying actin cytoskeleton when BCR activated, indicating a transient uncoupling of lipid rafts from the actin cytoskeleton.

Quantitative proteomics has also been used to study other dynamic processes involving lipid rafts. Yanagida et al. (99) used spectral counting to investigate the changes in protein composition of DRMs during DMSO-induced differentiation of the human leukemia cell line HL-60 cells into neutrophilic lineage. They identified a group of proteins that were upregulated during differentiation, including known cell differentiation proteins such as CD11b/CD18 subunits of β_2 -integrin MAC-1, CD35, and GPI-80, as well as other proteins such as flotillins and a group whose expression was downregulated like G proteins, heat shock proteins. The authors then further quantified the absolute amount of nine DRM proteins using spiked, isotope-labeled internal standards and concluded that the protein amounts nearly corresponded to the result obtained by spectra counting. Blonder et al. also reported the use of trypsin-mediated $^{18}\text{O}/^{16}\text{O}$ differential stable isotope labeling to compare the untreated DRMs and Iota-b toxin induced DRMs in Vero cells to explore the effects of Iota b binding and Iota a uptake through rafts (100). Although markers of DRMs like flotillins and caveolins were identified and quantified with ratios close to one, meaning that their levels remain unchanged in response to the toxin, several other proteins were observed to change. The functional significance of these changes was not pursued.

Finally, the changes imposed on raft protein composition by platelet-derived growth factor (PDGF) stimulation was studied by Maclellan et al. (101) wherein they used both 2DGE analysis and ICAT labeling on primary smooth

muscle cells to quantify the changes. Following a 15 min exposure to PDGF, 23 proteins were increased in protein abundance in rafts, whereas raft localization of only three proteins increased after 12 h of stimulation. The proteins recruited to rafts included GPI-anchored proteins, cytoskeletal proteins like actin, and endocytosis-related proteins such as clathrin, suggesting a role for rafts in regulation of PDGF-stimulated changes in the cytoskeleton.

CONCLUSIONS

The first significant contribution that quantitative proteomics made to lipid raft biology was most certainly the unbiased demonstration that signaling proteins in general are enriched in rafts (54). From a more technical point of view, quantitative proteomic studies have been able to debunk some of the misleading data generated from qualitative approaches (9, 54, 86, 87). Such qualitative data, going against much that was known in the lipid raft field about what defines a lipid raft protein, resulted in proteomics in general having a disproportionately small impact on the lipid raft field, especially compared with the large number of lipid raft proteomics reports (102). By demonstrating the utility of measuring the M β CD sensitivity as one dimension of proteomic analysis of DRMs, these studies have set a standard in the field that we hope others will begin to follow. Certainly, there are numerous accessible quantitative proteomic technologies available to researchers now so there is no need to waste effort on a qualitative study of DRM proteomes.

Proteomics has also made several more specific contributions to lipid raft biology. In TCR signaling, a number of important components of the complex pathways downstream of receptor engagement have been revealed through proteomics, particularly by the work of Bini et al. (9) and Tu et al. (96). Likewise, the elegant work of Gupta et al. (10) demonstrated the importance of ezrin and its dephosphorylation in BCR signaling. These and many other studies have identified many other components of DRMs that appear to change with various types of stimulation but the impact of these studies on the larger raft field has been mitigated by the lack of follow-up work done on those proteins to demonstrate their functional significance, a more general problem in proteomics.

Despite the successes discussed above, several challenges still face this field. First of all, the effect of different detergents on DRM composition is still not clear. Does one detergent yield 'better' lipid rafts than others? Does one preserve raft-interacting proteins better than the others? Should one detergent be the 'gold standard'? Second, cholesterol-enriched lipid rafts and caveolin-dependent caveolae are two distinct microdomains that both enriched in DRMs, with some debate still about whether caveolae should be viewed as specialized rafts. Despite this, most proteomics studies have failed to acknowledge that the M β CD sensitive proteins (if this response was measured at all) they have identified in DRMs are likely coming from rafts and caveolae in their preparations. As of this time, we know of no studies reporting the separate characterization

of raft and caveolae proteomes together in one cell type but such a feat is conceivable with the aid of caveolae-deficient cell lines and various quantitative approaches. Third, there are likely to be several distinct subpopulations of membrane microdomains in any given class, e.g., different kinds of lipid rafts, caveolae, etc., but current biochemical techniques are not effective at resolving these, making most current microdomain proteomes an average view of several subpopulations. In reality, different subclasses could have distinctly different proteomes, and therefore, functions, if only we were able to resolve them. Future work in this area will need to focus on higher resolution techniques combined with quantitative proteomic approaches to gain any insight into the potential roles of microdomain subpopulations. Finally, many quantitative proteomics microdomain studies are now moving to look at the dynamic changes to the proteome upon various types of stimulations. More so than any other microdomain proteomic goal, such studies get directly at the function of the microdomains and the proteins in them, so we anticipate seeing analyses of how microdomain composition changes during the plethora of different signaling pathways in which such domains are implicated. These efforts should also start to incorporate analysis of posttranslational modifications, especially the highly dynamic ones such as phosphorylation and the microdomain-targeting ones such as glycosylphosphatidylinositol (103, 104). With the increasing accessibility of quantitative proteomics approaches and more affordable mass spectrometers, future proteomic analyses of membrane microdomains should include some level of quantitation.

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