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Title page

Long Title

Advances in understanding and in multi-disciplinary methodology used to assess lipid regulation of signalling cascades from the cancer cell plasma membrane

Running title

Membrane methods in cancer

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Abstract

The lipid bilayer is a functional component of cells, forming a stable platform for the initiation of key biological processes, including cell signalling. There are distinct changes in the lipid composition of cell membranes during oncogenic transformation resulting in aberrant activation and inactivation of signalling transduction pathways. Studying the role of the cell membrane in cell signalling is challenging, since techniques are often limited to by timescale, resolution, sensitivity, and averaging. To overcome these limitations, combining 'computational', 'wet-lab' and 'semi-dry' approaches offers the best opportunity to resolving complex biological processes involved in membrane organisation. In this review, we highlight analytical tools that have been applied for the study of cell signalling initiation from the cancer cell membranes through computational microscopy, biological assays, and membrane biophysics. The cancer therapeutic potential of extracellular membrane-modulating agents, such as cholesterol-reducing agents is also discussed, as is the need for future collaborative inter-disciplinary research for studying the role of the cell membrane and its components in cancer therapy.

1. Introduction

It is now a century since the cell membrane was shown to be composed of lipids, and recent advancements in 'computational', 'wet-lab' and 'semi-dry' methodologies have suggested additional novel concepts for the membrane lipids, such as lipid phases, lateral heterogeneity, membrane thickness, diffusion and flip-flop motion[1]. Figure 1 shows how various lipids contribute to membrane features. Concepts that are now emerging from the literature suggest that lipids within the membrane are not only central for modulating structural membrane organisation, but have key functional role in membrane-mediated initiation of cell signalling cascades[2]. Observations that lipid composition is altered in diseases such as cancer have led to studies identifying the functional roles in cancer signalling cascades for these membrane lipids[3-6]. Furthermore the hypothesis that short-lived[7] dynamic transient assemblies of cholesterol/SM/saturated lipid-rich domains exist in membranes[8] has been

functionally linked to various oncogenic processes such as cancer cell signalling[9, 10] and multi-drug resistance (MDR) [11, 12]. As a result, the range of applications for cancer lipidomic studies has rapidly expanded yielding an array of lipid-targeting cancer therapeutics[13-17] and diagnostic tools [18, 19]. This review details the range of disciplines needed to understand these biophysical drivers of membrane associated cancer cell biology with the cancer cell biologist in mind, and explores the fields of computational biology, molecular and cell biology and biophysics. Methodological complications and complexity are discussed, as is how inter-disciplinary collaborations bypass the disadvantages of using each method/discipline in isolation. A mixed method approach to understanding these processes will accelerate progress in the search for novel cancer preventive and therapeutic agents.

2. The cell membranes of healthy and tumour cells

The mammalian plasma membrane comprises of a mixture of lipids and proteins that form outer and inner leaflets of an asymmetric bilayer[20-22] (Figure 2A). The inner leaflet contains mostly phosphatidylserines (PS), phosphatidylethanolamines (PE), and phosphatidylinositols (PIs) while phosphatidylcholines (PC) and sphingomyelins (SM) predominate in the outer leaflet (Figure 2B). Functionally, these lipids species allow compartmentalised functions. The outer leaflet is afforded mostly structural and barrier roles, and provides a platform for sensing the extracellular environment, whilst the inner leaflet contains lipids that participate in and regulate signalling pathways.

2.1. The physical properties of membrane lipids and their role in tumorigenesis

The plasma membrane is made up of phospholipids which have hydrophobic tails that face each other, and hydrophilic heads that face the intra- and extra-cellular water-based environments. These amphipathic properties force natural (spontaneous) assembly into a bilayer, driven by the hydrophobic effect. This holds true in the plasma membrane of biological systems or in artificial conditions; simple water/lipid mixtures forming many self-assembled lyotropic liquid crystals, including liposomes. The self-organisation of phospholipids in living plasma membranes forms a ~5 nm wide double layer of lipids. In non-biological systems self-assembled lyotropic crystals can be

artificially synthesised ranging from a few <50 nm up to 100 µm. While most membrane phospholipids rarely (once in several hours) flip between the two bilayer leaflets (cholesterol and some other molecules are the exception to this rule and flip flop more rapidly), they also move laterally within each leaflet. Lipids may diffuse laterally (translational freedom), rotate, or have flexibility around bonds in the carbon backbone (configurational freedom/order) (Figure 1).

Lipid flip-flop is slow compared to lateral diffusion and typically takes 10^9 times longer to occur than it takes a lipid to laterally diffuse 50Å [23]. Lipid flip-flop is enzymatically regulated by flippases, floppases, and scramblases, all of which preserve an asymmetric contribution of lipids in the two membrane leaflets for long periods[24]. Lipid movement, lipid order and membrane fluidity are greatly affected by lipid composition with cholesterol playing a crucial role. The hydrophobic nature of the lipid tails prevents polar molecules from easily passing through the membrane into the cytoplasm, thus precisely maintaining the concentration of solutes of the cytosol, intracellular compartments and the extracellular fluid. Instead, import and export of polar molecules, ions, essential nutrients and metabolic waste products, are exquisitely controlled by specialised transmembrane proteins.

More than 30% of the mammalian proteome encodes transmembrane proteins, indicating the importance of membrane function[25]. These are proteins that are surrounded by the lipids of the membrane for their lifetime. Membrane proteins include transporters (carriers) and channels (aqueous pores) which transport molecules via passive transport or facilitated diffusion. ATP-binding cassette (ABC) transporters, which include P-glycoprotein (P-gp) comprise the largest family of membrane transporters and are clinically very important for drug resistance in cancer cells[26]. Membrane receptors are central to normal and oncogenic signal transduction and determine the behaviour and function of their target cell [27]. Overington *et al.* showed that about 60% of drug targets are membrane proteins[28]. Some of the most common cancer transmembrane proteins that are drug targets include the epidermal growth factor receptor (EGFR)[29], vascular endothelial growth factor, (VEGF)[30] and integrins[31].

The cellular adaptations that occur during tumorigenesis such as rapid cellular division confer new membrane biophysical characteristics to the cells [6, 32, 33]. Membranes delimit the cell space from its environment but also preserve the characteristics of membrane-enclosed organelles (endoplasmic reticulum (ER), mitochondria, Golgi apparatus) and the cytosol. Membranes are the immediate inter-cellular structures connecting healthy and cancer cells and form a structural barrier between the cell components and the microenvironment (e.g. other cells, matrix, growth factors, ions, hormones, cytokines) and thus control the transmission of signals between the extra-cellular environment to intracellular processes. While the structural features of membranes were studied for decades, lipid structures and their functions are only now emerging as therapeutic targets in oncogenesis (reviewed in [17, 22]). In cancer, changes in cell membrane lipid composition, packing, and fluidity, are widespread[6, 34, 35] (Figure 2C) and are functionally and mechanistically linked to oncogenic signalling[9, 10], metastasis[36, 37], angiogenesis[38, 39], drug resistance[11, 12], and even cancer immune surveillance[40, 41]. Although the role of the immune system in tumorigenesis is not the scope of this review, we direct the reader to reviews focusing on the crosstalk between immune and cancer cells, mechanistically linked to cancer immune surveillance (reviewed in [40, 41]). Immune cells, part of the tumour microenvironment, are influenced by their own lipid compositions and were reported to be regulated by lipid components. For example, compositional changes seen in cancer (see section 2.2), such as PS exposure on the outer cancer membranes, allow cancer cells to evade recognition and targeting by immune cells, like natural killer (NK cells)[42]. Targeting lipid reprogramming in immune cells and their effect on tumour cells is an emerging and additional field of research for lipid-targeted cancer immunotherapy is attracting more interest over the years[41].

2.2. Alterations of membrane lipid composition during tumorigenesis

Lipidomic studies on primary tissue and cancer cell lines provided insights into the alteration of membrane lipid composition in tumorigenesis. While primary cells retain the morphology and function of the tissue of origin, they are not appropriate models for studying lipidomic profiles in metastasis and multi-drug resistance. Models of

metastatic and MDR cell lines are in concordance and overcome primary cell limitations. A systematic evaluation of lipid membrane composition in cancer was performed and 29 papers were included in qualitative analysis. Our search methodology is reported in Supplementary Tables, and our findings are summarised in Figure 2D with full details of all lipid classes in every cancer/cell type we identified in all 29 papers of primary cell and cancer cell line studies. The lipid composition of cancer cell plasma membrane varies with the type of cancer[17, 43, 44] as well as the severity of the disease [45] and was shown to be associated with patient survival[45]. In breast cancer, higher levels of PE lipids [45-47], PC-saturated fatty acid rich lipids[45-48], SM lipids[45, 46], and of the lipid messengers, PIs [45-48], have been reported in comparison to non-cancerous tissue (Figure 2D). An increase in PI lipids was also observed in Ras-induced lymphoma cells[49]. In *Myc*-induced lymphoma cells PS, PE and PI lipids were decreased and phosphatidylglycerol lipids increased compared to normal cells [49]. Typically in healthy cells, PS lipids are asymmetrically localised in the inner layer of the plasma membrane[50] and this asymmetry is maintained by aminophosholipid translocases[51]. Loss of function of the protein machinery responsible for maintaining membrane asymmetry[52] results in PS translocation to the outer leaflet, a key stage in apoptosis. PS lipids move to the outer layer during transformation to cancer[53-56] (Figure 2C) and is associated with worse cancer outcomes[57]. This translocation has been found mediated by P-glycoprotein (ACBB1/MDR1/P-gp) [58] and also activates the coagulation pathway which enhances activity of pro-survival factors, again favouring oncogenesis[57, 59]. Similar findings have been reported for PE lipids[52]. These observations allowed subsequent mechanistic insight into the anti-cancer peptide Polybia-MP1; movement of both PS and PE lipids to the outer leaflet in cancer cells enhanced membrane poration due to the charged PS electrostatically attracting MP1, and the PE stabilising the formation of large pores. [60].

During metastasis a change in biophysical properties of the plasma membrane is required to allow the amoeboid like migration, extravasation, dissemination and extravasation[34-36]. Alterations to the lipid content as measured by cell polarisation[61], have been observed in cancer metastasis with increased SM and PE

concomitant with decreases in cholesterol in unpolarised cells during the relatively early stage of metastasis of epithelial-to-mesenchymal transition (EMT)[62]. While alterations in membrane lipid composition are cancer and disease stage specific, a trend for metastatic[62, 63] and blood cancer cells[64] to have lower cholesterol and MDR[4, 65, 66], greater membrane cholesterol has been observed in some situations (Figure 2C). Gene ontology analysis revealed that the cholesterol biosynthetic pathway is upregulated during acquisition of multi-drug resistance [67]. Wang *et al.* showed that inhibiting the enzyme that synthesises polyunsaturated phospholipids (lysophosphatidylcholine acyltransferase 3 [Lpcat3]) decreases membrane fluidity and induces cholesterol biosynthesis[68]. Increase in cellular cholesterol content was sufficient to independently enhance intestinal stem cell proliferation and tumorigenesis *in vivo* and *ex vivo*[68].

Loss of bilayer asymmetry also stimulates aberrant and oncogenic cell signalling[69-71]. Biophysical models of asymmetric membranes have indicated that formation of signalling regulatory domains, which are expected to preferentially form in one leaflet due to the asymmetric lipid composition, were actually suppressed by the asymmetric opposing leaflet which lacked domains [72]. Scrambling of the leaflets, thus resulting in symmetrical lipid layers, causes formation of cholesterol-rich domains that are predictable based on the lipid composition of the bilayer [72]. The forces linking phase behaviour in the opposing leaflets of a bilayer are not currently understood and represent an important gap in the understanding[72].

Repressing cholesterol availability can promote metastasis in breast cancer[73]. Moreover, palmitoylation of the surface adhesion receptor stem cell marker CD44, which facilitated its localisation to cholesterol-rich membrane domains, resulted in limiting metastasis[37, 74]. These findings indicate that decrease in membrane fluidity by localisation of the protein CD44 from low- to rich-cholesterol regions had an anti-migratory effect.

2.3 Membrane cholesterol regulates membrane function

Cholesterol is an important component of the membrane comprising up to 30-40% of the membrane lipids in specialised cells[20, 75]. Cholesterol is also a main component of specialised membrane domains. The increase in cholesterol in some cancer cells [65, 68, 76, 77], is linked to elevated levels of cholesterol-rich membrane domains[78, 79] that are involved in oncogenic signalling[78, 79]. These domains are a result of preferential lipid/cholesterol interactions, which drive lateral heterogeneity in membranes and the formation of distinct lipid phases, the liquid ordered (L_o) and liquid disordered (L_d) phases[80] (see section 2.4). In 1997 cholesterol-rich domains were first defined as 10-200nm dynamic-ordered transient assemblies enriched in cholesterol and sphingolipids or saturated lipids, and were defined as lipid rafts[8]. Even-though the lipids of lipid raft domains are more ordered, L_o phases and lipid rafts are not interchangeable terms.

Alterations in cholesterol concentration results in an array of biophysical changes that affect the behaviour of cancer cells. For example, cholesterol-driven lateral membrane heterogeneity is linked to membrane protein pump opening and closing, critically diminishing the cells' ability to maintain gradients. As an example, the ABC transporters are organised into distinct cholesterol-rich domains[81], and are surrounded by a region enriched with cholesterol and PC lipids[82]. This local environment creates multiple non-specific interactions between the lipids and the transporter proteins that impact conformation and its efflux capacity[11]. Mechanosensitive ion channels such as PIEZO1, which has been linked to several cancers[83], is also functionally dependent on membrane lateral organisation and the presence of cholesterol-rich domains[84].

Increase in membrane rigidity and thickness driven by increased saturated lipids with long fatty acyl chains and elevated cholesterol, reduces passive diffusion and permeability to amphipathic and hydrophilic drugs (Figure 1)[12, 26, 85]. Increase in cholesterol concentration results in increased hydrogen bonding between headgroups of lipids and cholesterol[86] and subsequently to a tighter and thicker membrane with a reduced number of empty spaces, thus creating a greater distance and a greater barrier in the membrane for the influx of drugs[12, 26, 85]. MDR breast[65],

leukemic[4] and ovarian[66] cancers have all been found to contain significantly greater cholesterol content, and higher membrane lipid order[87] than their drug-sensitive counterparts. Peetla *et al.* suggested that this cholesterol-driven increase in membrane rigidity results in the chemotherapy drug being trapped in the membrane[65] (Figure 1) and Rivel *et al.* even provided evidence that membrane permeability to cisplatin was proportional to cholesterol concentration [88, 89]. Depleting membrane cholesterol through cholesterol-reducing agents leads to decreased P-gp activity[12] and P-gp transport function[85], indicating intra-cellular concentration of the many anti-cancer therapeutic substrates of this pump is at least partly dependent on membrane cholesterol concentration.

Cholesterol is a modulator of lateral mobility and diffusion of membrane lipids and was shown to linearly decrease, or increase with increase or decrease of cholesterol concentration, respectively[90]. Similar effects of cholesterol concentration were also observed for other membrane components, such as membrane proteins (Glycosylphosphatidylinositol-linked and native major histocompatibility complex class III-I-E^k)[91]. Lateral diffusion is an important property for membrane-associated proteins and has been suggested to impact the clustering of lipids essential for binding specific proteins and thus increasing their binding affinity. For example, the FERM domain of the ezrin receptor, which is responsible for binding of the protein to the bilayer, was suggested to slow down the lateral diffusion of phosphatidylinositol 4,5 bisphosphate ($PI(4,5)P_2$)[92] . This resulted in clustering of $PI(4,5)P_2$ that increased its local density around the protein and resulted in greater binding affinity to the membrane compared to the absence of protein[92].

Cholesterol-rich domains in cancer cell membranes have been suggested to act as hot-spots for cancer cell signalling cascades, through the physical segregation of some proteins in these domains that results in modulation of their accessibility to their target molecules[93, 94]. It is suggested that various proteins associate with rafts[95-97] (and are “raftophilic”) or are otherwise excluded from lipid raft domains[98]. The physical co-localisation of some proteins in these domains might bring them in close proximity favouring their interaction and enhancing the sensitivity to cellular signalling

cascades[99]. Apart from protein-protein interactions, lipid-protein interactions were shown to facilitate the over expression of some membrane-receptors (such as EGFR[100], CD44[37]) and to mediate survival of oncogenic peripheral proteins, such as Akt[101].

2.4. The biophysics of lateral heterogeneity in cancer cells

The lipid raft hypothesis has been a controversial topic in modern membrane biology for many years and only recently, following improvements in methodologies with the required temporal and spatial resolution (Figure 3), stronger direct evidence of lipid rafts has been obtained in live cells[102, 103]. Although controversial, the self-organising capability of eukaryotic membranes is undeniable[80] and there is therefore still a lot to be discovered and understood about lipid rafts and their function in tumorigenesis using appropriate techniques. Lipid rafts likely play crucial roles in cellular bioactivity including membrane-recruitment of lipids and proteins, and importantly in oncogenesis, cell signalling initiation[8].

The plasma membrane is home to more than 1000 different lipid species[104], which participate in lipid-lipid interactions and drive lateral heterogeneity and phase separation[1, 105, 106]. Cholesterol preferentially interacts with saturated (rather than unsaturated) lipids[107, 108] since saturated chains permit maximal lipid-cholesterol interaction, via Van der Waals attraction, whereas the “kinks” in unsaturated lipid tails form from double bonds, making close packing difficult. As a result of this binding, cholesterol’s rigid ring induces a high degree of saturated lipid acyl chain conformational order, drives phase separation, and subsequently makes the cholesterol/saturated fat/sphingolipid-rich domains more packed and highly ordered[109]. Phase separation is observed in ternary mixtures of model membranes, where preferential lipid-lipid and lipid-cholesterol interactions occur. These preferential interactions result in lateral heterogeneity and domain formation of liquid-ordered (L_o) rich domains (cholesterol abundant) and liquid-disordered (L_d) rich domains (cholesterol scarce)[20]. In the L_o phase, lipids with a high melting temperature (T_m) (saturated lipids) cluster with themselves and/or cholesterols and are separated by clusters of low melting temperature (T_m) lipids (unsaturated lipids) that

cluster on their own. Cholesterol reduces the conformational freedom of the lipid chains resulting in a thicker and more rigid bilayer in the L_o phase compared to the more fluid L_d phase [110, 111]. The L_o phase closely resembles the gel phase for having high molecular ordering while being laterally highly mobile, two to three times slower like the L_d phase. The L_o and L_d fluid phases co-exist in the membranes under suitable conditions (lipid content, temperature, pressure), which are met in biological membranes. These observations of lateral heterogeneity at the sub-micron level were made through biomimetic model membrane techniques and led to the generation of the lipid raft hypothesis[8].

Fluorescence based-methodologies have provided convincing evidence that lipid rafts are enriched in cancer [78, 79, 108, 112, 113]. Li *et al.* showed that several cancer cell lines contain more L_o membrane domains and are more sensitive to cholesterol depletion than their normal counterparts[78]. Hyper-sensitivity of cancer cells to cholesterol depletion was corroborated in colon cancer cell cultures, when inhibitors of cholesterol biosynthesis, such as statins, slowed proliferation and enhanced apoptosis[78]. A number of studies show that L_o phases comprise a biologically meaningful portion of the plasma membrane in several cell types [114, 115] including HeLa cells derived from the cervical cancer that killed Henrietta Lacks [116, 117]. Levental *et al.* studied the role of palmitoylation in regulating integral protein affinity for L_o domains[118]. This group suggested that approximately 30% of plasma membrane proteins were clustered in L_o domains of giant plasma membrane vehicles[118]. On the other hand, methodologies involving membrane-order-sensitive dyes and fluorescence lifetime shifts on intact cells, suggested the existence of sub-resolution mixture of 76% ordered and 24% disordered lipid domains suggesting that L_o domains might dominate the plasma membrane. These observations suggest that while 76% of actual biological membrane is in L_o phase, only 33% of membrane proteins are clustered in L_o domains. Extrapolation of these data indicate that 66% of all membrane proteins are restricted to the 30% of the membrane comprising the L_d phase. While many studies argue about the biological importance of the L_o phase for membrane proteins, this observation indicates that most proteins would rather be overcrowded into the small amount of L_d. Furthermore, this perhaps indicates

inconsistencies between different methodologies and that we do not really understand the biological importance of the membrane properties and its domains, especially at the stage of disease. Decades of research with model membranes allowed researchers to understand the physical chemistry behind domain formation. However, few studies were able to provide direct evidence that the living mammalian plasma membrane can self-organise. Giant plasma membrane vesicles (GPMVs) provide an intermediate biological system as they are isolated from live cells [105], preserving the lipid and protein diversity and complexity and the natural properties of the living cell plasma membrane[105, 119], but maintain the resilience of model membranes[105, 120, 121]. As a result, these natural membranes allow observation of biological plasma membrane phenomena, such as phase separation. Very recently, direct evidence of nanodomains in biomimetic and biological membranes[102, 103] has been found adding further support to the hypothesis that lateral heterogeneity has vital physiological significance [122]. Recent methodological developments have provided direct evidence that lateral heterogeneity exists in cell membranes, meaning prior debate was most likely stimulated by limitations of previous methodologies unable to resolve the dynamic and nanoscopic nature of membrane domains and membrane properties. Therefore focus on more appropriate interdisciplinary methods should be given to understand the biological significance of rafts in cancer[122].

3. Molecular and Cell Biology

3.1. From detergents to dyes

Non-ionic detergents played a significant role for decades in the study of the plasma membrane and the proteins that reside within it. Detergent-based methods were some of the first methods to suggest membrane domains organise signals from the plasma membrane. Separating membrane proteins from the hydrophobic lipids in which they are embedded has historically been achieved through exploiting the hydrophobic properties of lipids. Highly compartmentalised membrane regions are separable using non-ionic detergents such as Triton X-100 or CHAPS at low temperatures[9, 123, 124]. These detergent-based methods have been widely used for decades to separate

detergent-resistant membrane fractions with similar components to rafts being rich in cholesterol, associated protein markers and sphingolipids[100, 125-127]. However, detergent based methods have many limitations for representing L_o phases in cells. Detergent based methods require low temperatures that render the native membrane asymmetry undetectable and that may independently induce phase changes[128, 129]. The composition of membrane protein partitioning into detergent and non-detergent regions was suggested to be depended on the type concentration of detergent used for isolation[130]. Detergent-resistant membranes (DRMs) have been observed as artefacts resulting from methods that fail to completely solubilise membranes [122, 129-131] and that could be a result of leaflet-specific detergent sensitivity[132]. Detergent-based techniques are still used, and they do provide valuable information about protein-lipid interactions, but methodological effects driven by detergents must be controlled and accounted for[129]. Detergent-based techniques are still commonly employed as they are a simple, fast, and inexpensive method to observe if the membrane has the propensity to form domains and provide estimates of protein-lipid interactions. Throughout the years, the caveats arising from the use of these techniques has become well-known and a shift towards the use of fluorescent dyes has occurred.

Fluorescent probes allow quantitative analysis of cholesterol concentration, lipid/lipid and lipid/protein molecular aggregation, compartmentalisation, and lateral diffusion. Fluorescent probes are highly sensitive and can be applied in simple model membranes to complex cellular systems alone or in combination with techniques to achieve spatial and/or temporal resolution. Environmentally sensitive fluorophores are affected by the polarity of their native environments particularly by water penetration into the bilayer and the dipolar relaxation effect [133, 134]and so are good probes for the study of lipid packing and membrane fluidity. Lipids contributing to L_o phases have less hydrated headgroups and higher lipid order compared to L_d phases, since they use more hydrogen bonds to bind together, leaving less available for binding to free water [135-137]. Greater lipid order results in lower polarity meaning sufficiently sensitive probes distinguish phase changes of their local environments. However, as with detergents, dyes have limitations. Lipid labels may be bulky, hydrophobic, and reduce the affinity

for liquid ordered phases[122] leading to artefactual changes in lipid partitioning[138]. For example, fluorescence labelling of lipids normally found in L_o domains [138] with the 3-HF probe, F2N8, were nearly impossible to be labelled in the L_o phase in coexisting L_d/L_o bilayers, since the probe affected the properties of the unlabelled native lipids [139]. Probe specificity for the different phases, with negligible flip-flop between the two bilayer leaflets, is also an important consideration. In typical plasma membranes, probes that label L_o should be specific to the high cholesterol, SM, and saturated lipid regions of the outer bilayer, and therefore reveal bilayer asymmetry.

The potentiometric styryl dye di-4-ANEPPDHQ was first developed and applied in neuronal studies as a fluorescent sensor of transmembrane potential[140]. As di-4-ANEPPDHQ's emission spectrum is partially related to cholesterol concentration it has also been used to visualise phase separation in cell membranes[141] (Figure 4). Di-4-ANEPPDHQ is excited at 488nm and shifts its emission from a peak at about 560 nM (green) in the L_o phase, to 620nm (red) in the L_d phase[142], and dissociation of lipid domains can be visualised in cholesterol depletion experiments[141]. This dye is unique compared to other potentiometric dyes in that it has low cellular toxicity; it is water-soluble, with a short rate of internalisation into the membrane[141]. Di-4-ANEPPDHQ is non-fluorescent until bound to membranes and, it only shows slight fluorescence in water, minimising background fluorescent influences. Study of membrane lipid domains with di-4-ANEPPDHQ, has been previously performed using vesicles made of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), egg n-palmitoyl-sphingomyelin (PSM), DPPC and different cholesterol concentrations or in live cells through direct addition to the culture medium[141, 143]. di-4-ANEPPDHQ’s excitation and emission spectra can be assessed with a spectrofluorometer and when combined with confocal fluorescence microscopy provides detailed information on membrane structure and colocalization with proteins that have themselves been tagged, with for example GFP [143]. Di-4-ANEPPDHQ staining in live epithelial (HEK293) cells can also be combined with fluorescence lifetime imaging (FLIM) to study increased order in the plasma membrane and appears to be a robust tool for identifying membrane microdomain formation and function in live cells[143].

Laurdan is another environmentally sensitive fluorophore that allows the study of lipid packing and membrane fluidity[133, 134] (Figure 4). Laurdan is excited by UV and is sensitive to lipid phase changes, emitting fluorescence at around 500nm (blue) when the lipids are in the L_d phase and shifted at about 450nm (violet) when the lipids are more packed and in the L_o phase[115, 142]. Importantly for the cell biologist, Laurdan can be applied not just in in model membranes, but also in live and fixed cells, and has even been utilised in whole organisms (live zebrafish embryos) without affecting native membrane properties[142]. Sanchez *et al.* used Laurdan with scanning fluorescence correlation spectroscopy (FCS) (instead of single-point FCS) to study membrane heterogeneity and membrane fluidity in intact live rabbit erythrocytes and Chinese hamster ovary cells. This group observed generalized polarization (GP) fluctuations in the cell membranes of live cells and proposed that these fluctuations represented the existence of tightly packed micro-domains moving in a fluid background phase [115]. The GP quantitatively measures the emission shift of the dye and in this study gave a high-GP, tightly packed L_o structures diffusing in a background of less packed lipids with lower-GP[115]. Disadvantages of Laurdan however include phototoxicity towards live cells caused by UV illumination[144] and low solubility of Laurdan in water. To address this, C-Laurdan has been designed with a carboxymethyl group replacing a methyl group (in the headgroup) and so shows improved solubility and provides brighter fluorescence signals, thus improving its applicability to live cells[145]. Recently, Salinas *et al.* combined C-Laurdan and di-4-ANEPPDHQ to reveal that the plasma membrane might be a central mechanistic link between obesity and colorectal cancer[146]. AdipoRon, an inhibitor of the adipocyte secreted factor adiponectin, was observed to reduce plasma membrane free cholesterol and rigidity resulting in suppression of oncogenic Wnt signalling in colon cancer [146]. Wnt-receptor binding occurred selectively in L_o membrane domains[147]. With the use of environment-sensitive fluorescent probes the biophysical properties of membranes in live cells in relation to tumorigenesis can be studied and provide insights to develop novel cancer membrane-targeted therapies.

Detergents have been used to reveal crucial information regarding the biological signalling functions of the plasma membrane, but the emergence of dyes has

exacerbated the limitations of detergents. Careful consideration as to which dye to use should still be given, as some may influence lipid partitioning and others have been further developed to improve utility in the study of live cells.

3.2. Antibodies

Hyperphosphorylation and expression of oncogenic proteins indicates enhanced cell signalling initiation, usually synonymous with cancer hallmarks characteristics. Directly detecting changes in protein levels and phosphorylation using antibodies remain common methodologies to measure signalling initiation[148]. Immunoblotting and immunofluorescence alone, and in combination with confocal microscopy or immunoprecipitation, all utilise antibodies raised against the antigen of interest for measuring activity of cell signalling pathways. These are well-established antibody-based techniques that quantify and localise (coarsely) proteins and determine the abundance and variety of post-translational modifications (PTMs)[149, 150]. Protein-protein interactions that indicate signal transduction, can be detected using co-immunoprecipitation[149] or with fluorescent methods such as FRET (described in detail below).

Antibody-based techniques are widely used for studying signalling mechanisms that are sensitive to changes in membrane lipid concentration and functions. A lot of the initial evidence on cholesterol sensitivity of oncogenic protein expression and the role of cholesterol-rich domains in localising some membrane and peripheral proteins has been obtained through antibody-related techniques. In many cases, antibody-based approaches were used in combination with detergent-based extractions and sucrose gradient ultracentrifugation[151], or with non-detergent based biochemical raft fractionation methods[126, 152] to separate “raft” and “non-raft” membrane fractions. Adam *et al*. following detergent-based isolation of membrane fractions (by sucrose gradient ultracentrifugation and detergent extraction with Triton) of prostate cancer cells, used immunoblotting, immunoprecipitation and immunofluorescence to show partitioning of oncogenic Akt in membrane domains[125]. Oncogenic Akt was overrepresented in cholesterol-rich (raft) microdomains compared to wild type Akt[125]. Depletion of cholesterol by methyl-beta-cyclodextrin (cholesterol-binding

agent) inhibited Akt signalling originating from raft domains and reduced oncogenic Akt phosphorylation, leading to an overall decrease in cell survival signalling[125]. Similar findings were observed by Chun-Li *et al.* with immunoblotting, immunofluorescence, and confocal microscopy for detection of Akt, pro-apoptotic proteins, and raft markers (Cholera toxin B subunit (CTB), filipin, monosialotetrahexosylganglioside (GM1)) in prostate and breast cancer lines without the use of detergents[78]. CTB binds GM1 lipids that are concentrated in cholesterol-rich domains much like lipid rafts[153]. Membrane cholesterol depletion resulted in reduced expression of raft levels along with down-regulation of Akt activity and upregulation of pro-apoptotic proteins (Bcl-xL, caspase-3)[78]. Using a non-detergent based raft isolation (lysis of breast cancer cells using a calcium and magnesium isotonic buffer and floatation in an OptiPrep gradient), immunoblotting and immunoprecipitation, Irwin *et al.* showed that EGFR and c-Src co-localised to raft membrane domains[100]. Co-localisation of EGFR and c-Src in the membrane raft fraction was also observed when breast cancer cells were immunostained with Alexa fluor labelled EGFR, c-Src and CTB antibodies[100]. Using immunoprecipitation, immunoblotting and immunofluorescence. Donatello *et al.* studied the role of cholesterol-rich domains in the promotion of breast cancer cell migration[37]. Following both detergent and non-detergent-based extraction methods of membrane domains from migrating and non-migrating breast cancer cells this group suggested that in non-migrating conditions CD44 is mainly localised in raft fractions and ezrin mainly localised in non-raft fractions[37]. Following the induction of migration, CD44 is retained in non-raft fractions and coprecipitated strongly with ezrin[37]. Depleting cholesterol and subsequently disrupting rafts resulted in increased coprecipitation and colocalisation of CD44/ezrin in the non-raft fractions during migration[37]. This study using various antibody-based techniques suggested that raft domains might be involved in the regulation of CD44-dependent breast cancer cell migration[37]. Similar findings were observed by a recent study that showed less CD44 localisation into lipid rafts in highly metastatic hepatocellular carcinoma (HCC) cells than normal liver cells[154]. Treatment of cholesterol promoted the formation of lipid rafts, the translocation of CD44 into lipid rafts, decrease in CD44/ezrin interaction and thus inhibition of migration and invasion[154]. These findings were confirmed in clinical

HCC tumour samples of patients with high or low serum cholesterol and in a mice liver metastasis model with or without hypercholesterolemia[154]. *In vitro* and *in vivo* data using a combination of antibody-based techniques such as immunoprecipitation, immunofluorescence and immunoblotting as well as raft isolation techniques such as detergent-based or fluorescent-based (with raft markers) were in agreement[154].

Antibody-based techniques have been and are still widely used for the study of membrane properties and membrane associated signalling in cancer. While their applications were originally attributed to molecular biology assays, their use has been extended to biophysics and super resolution microscopy. In combination to fluorescence-based applications antibody-based methods are powerful tools in understanding the complexity of the plasma membrane and the lipid-lipid or protein-lipid interaction in cancer membrane bioactivity.

3.3. Lipidomics

Lipidomics is the study of the structure and function of the lipidome, which includes the complete set of lipids in a cell. It provides information about chain length, headgroup chemistry and quantification of lipids and thus providing important information on molecular mechanisms that characterise tumorigenesis[155]. Lipidomics has been used to provide insights into global membrane lipid composition and dynamic changes during tumorigenesis, which emphasise the potential of lipid-centric therapeutics in cancer[3]. Determining the membrane lipid composition of cancer cells is critical in understanding the biological properties of tumours. Mass spectrometry (MS) methodologies suggested specific lipid types to be markers for cancer diagnosis. MS is a label free technique which can explore the lipid composition of healthy cells/non-cancerous tissue compared to cancer cells/tissue.

Maciel *et al.* studied the lipid remodelling in human melanoma cells upon UVA exposure using gas or liquid chromatography (GC or LC) coupled to MS. This study observed an increase of monounsaturated fatty acids (MUFA), stearic acid (FA:18:0) and PI lipids and a decrease of palmitic acid (FA16:0) and PC lipids following 24 hours of irradiation[156]. Cifkova *et al.* used liquid chromatography in combination to

electrospray ionization mass spectrometry quantification to study the lipidomic characterisation of breast cancer and the surrounding normal tissue of the same patient[46]. Significant increases in PI, PE and PC lipids were observed in breast cancer tissues compared to the normal tissue[46]. Conventional mass spectrometry methods usually involve lipid extraction, separation by chromatography before mass spectrometry detection. More recent applications have employed mass spectrometric detection without the requirement for lipid extraction and separation, but using *in situ* detection of membrane lipids, that maintains cell integrity[157]. He *et al.* used such method to study the membrane lipid profiling of an epithelial breast cell line compared to the lipid profiling of six different breast cancer cell lines linked to their metastatic ability using matrix-assisted laser desorption/ionization (MALDI)-Fourier transform ion cyclotron resonance mass spectroscopy[157]. This method is based on the circular movement of charged particles in a strong magnetic field, named the cyclotron movement of which its frequency directly depends on the mass-to-ratio of the ions[158]. Alterations in the levels of lipids were associated with the types of the different breast cancer cell lines[157]. In total, levels of eight membrane lipids were found to differentiate the healthy cell line from the six breast cancer cell lines. Furthermore 15 lipids were different between the six breast cancer cell line types[157]. Significant increase of monounsaturated lipids was also associated with the degree of malignancy in the breast cancer cells[157]. Frisz *et al.* used a high-resolution chemical imaging approach to detect sphingolipid-enriched plasma membrane domains in mouse fibroblasts[159]. Sphingolipids within the plasma membrane of mouse fibroblasts were isotopically labelled and their plasma membrane distributions were mapped by secondary ion mass spectrometry[159]. Detection of spatially dependent variations in the analysis or lipid clustering was achieved by global labelling of most lipids with carbon-13 and nitrogen-15 labelling of sphingolipids and its precursors (SM, ceramide, glycosphingolipids)[159]. Scanning electron microscopy was first used to identify well-reserved cells with intact micro-extensions and with normal morphologies before analysis by secondary ion mass spectrometry[159]. Sphingolipids were found to be enriched within membrane microdomains of about 200nm in diameter, larger than what is expected for rafts in cells[159]. Although the sphingolipid domains organisation

and abundance were influenced by cholesterol, they were mainly altered by cytoskeleton disruption, thus domains were proposed to not resemble lipid rafts but rather distinct sphingolipid assemblies [159].

Mass spectrometry methodologies allow the identification of lipidomic membrane changes during tumorigenesis. Changes in lipid composition cause structural and functional alterations in cell signalling that originate at the plasma membrane that are detectable with mass spectrometry. More lipidomic studies are required to fully understand the cellular composition and the architecture of cancer cells, especially given the differences seen in different cancer cell types and stages, and under different treatment strategies (for example drug resistance and metastasis). The rapidly developing interest for studying cancer membranes and their properties as potential lipid-targeted therapies, is at least in part due to these mass spectrometry techniques that have demonstrated measurable alterations of cancer membrane lipid composition (Figure 2). Once these compositional changes are well established, methodologies with higher spatial resolution (Figure 3) should be fully exploited to identify and link membrane properties (driven by composition changes) to the oncogenic signalling pathways that are affected.

4. Computational Biophysics

4.1. Molecular Dynamic Simulations

Computational methods such as molecular dynamics (MD) simulations have been used to study the dynamics, interactions and functions of membranes and of membrane proteins[160, 161] (Figure 4). MD techniques are considered as a computational microscope, providing details for membrane organisation/dynamics at the molecular level. Atomistic molecular dynamics (AT-MD) simulations, whilst more detailed, can be computationally expensive and thus lower resolution simulation techniques e.g. coarse-grained molecular dynamics (CG-MD) simulations[162] are also used. In CG-MD simulations rather than simulating every atom, groups of atoms are simulated as a bead and are given the physico-chemical properties of that group, in a process termed parametrisation. In this way larger or more complex systems can be simulated for a

longer time course, at the expense of losing information on every atomic interaction. CG-MD simulations were widely used to provide insights into cancer related protein-lipid and lipid-lipid interactions and dynamics.

4.2. MD studies of complex membranes and with lateral heterogeneity

MD simulations can provide useful molecular insights into membranes as well as their properties e.g. lipid-lipid interactions and fluidity (Figure 4). While most studies employ vastly simplified model membranes because much of the complex behaviour of membrane can be recreated with just three species in both experiment and simulation (namely high and low T_m lipids and cholesterol) there may be unforeseen subtleties that are lost. Simulation can recreate the complexity of the plasma membrane. Studying the complexity and the asymmetric nature of the plasma membrane is particularly important, especially since, in cancer membrane asymmetry is lost. MD simulations can study the structural changes seen in tumorigenesis in an atomic level and for a fixed period of time to understand in detail the membrane properties involved in these changes. Furthermore, as previously discussed, an increase in cholesterol content is observed in some cancer cells/tissues and subsequently there is an enrichment of cholesterol-rich membrane domains and lateral heterogeneity. The details of lipid-lipid interactions that drive lateral heterogeneity, which is seen in cancer cells, can be studied with simulations over time in controlled conditions.

Recently, MD simulations of multicomponent systems that more closely resemble membranes found in various cell types and organisms at different cell stages have been performed[160]. Ingolfsson *et al.* simulated an asymmetric plasma membrane model consisting of 63 different lipid species[163]. This study suggested enrichment of cholesterol and ganglioside nanodomain formation in the outer leaflet, and phosphoinositide clustering in the inner leaflet of the plasma membrane. L_o domain formation and disappearance was observed at the microsecond time-scale[163]. Simulations of a model of the asymmetric human brain plasma membrane showed that although the neuronal plasma membrane has a different lipid composition and is enriched in cholesterol, its bilayer properties were similar with the previously-simulated average plasma membrane[164]. Andoh *et al.* used MD to simulate two

complex PM models of the normal and the cancer thymocyte membrane and observed changes in membrane properties following cancer development[165]. Koldsø *et al.* used MD simulations to study an asymmetric plasma membrane consisting of 1500 lipids and observed the formation of lipid nano-clusters of glycolipids and to a lesser extent of PI(4,5)P_2 lipids[166]. Furthermore, the same study showed that cytokine receptor gp130 co-clusters with glycolipids (GM3)[166]. Other simulation studies also showed formation of glycolipids/protein nano-domains[167].

Lateral heterogeneity and the coexistence of L_o and L_d phases in membranes, their properties and the effects of such heterogeneity in oncogenic protein function has been widely studied by MD simulations. AT-MD simulations also showed that the membrane permeability of the chemotherapy drug cisplatin is decreased upon increase in cholesterol concentration or when the membrane asymmetry is lost[88]. Sodt *et al.* used AT-MD to elucidate the molecular structure of the L_o phase[168]. CG-MD simulation studies showed that an increase in steroid headgroup hydrophobicity move them from the canonical upright orientation, towards the centre of the bilayer in a vertical orientation, thus disrupting domain formation[169]. Moiset *et al.* also used a CG-MD simulation approach to study how disaccharides can destabilise phase separation accompanied by other experimental techniques. In this study, liquid-ordered domains were disrupted by non-reducing disaccharides (sucrose, trehalose), but not by monosaccharides (glucose) resulting in uniformly mixed membranes[170]. CG-MD simulations were also used to explore equimolar binary mixtures of a saturated lipid with di-unsaturated lipids of differing chain lengths to provide information about their mixing behaviour and local composition-induced changes in their molecular structure. Rosetti *et al.* showed that saturated lipids tails become less ordered in di-unsaturated-rich lipid regions. Also, longer unsaturated lipid tail length induces a better mixing with saturated lipids[171]. A later study also focused on unsaturated lipids suggesting that the greater the unsaturation the better the stability of lipid domains[172].

Cholesterol not only affects the ordering, fluidity, and thickness of the plasma membrane, but may also have an amplifying role in signal transduction across the two

membrane leaflets. MD simulations revealed that cholesterol flip-flop impacts nanodomains registration demonstrating that it may plays a role in the transfer of information between the leaflets[173]. Cholesterol undergoes oxidative modification, through reactive oxygen species (ROS) to form tail-oxidised sterols, named oxysterols[174]. Kulig *et al.* used AT-MD simulations to suggest a mechanism by which sterols rapidly translocate in cellular compartments, referred to as bobbing[175]. Bobbing was observed only with a sterol that contained additional hydroxyl group at the sterol tail. They suggested that this occurs at the nanosecond timescale and is potentially responsible for facilitating rapid translocation of different sterols from cellular compartments to their receptors, without changing their orientation.

MD simulations of lipid-only systems provided detailed information on the structure and function of the plasma membrane. Understanding the structural properties of membrane lipids is particularly important for studies that involve oncogenic proteins that sit in the lipid environment. Knowing the effects of lipid-only systems of complex and asymmetric membranes and their properties could help estimate and understand the relationship between protein-lipid interactions later on.

4.3. MD simulations of signalling receptors

Molecular dynamics simulations have also been used to provide insights into the function and interactions of signalling receptors e.g. to study lipid interactions with receptor tyrosine kinases (RTK)[176] as well as the oligomerization of transmembrane helices[177]. RTKs reside in mammalian cell membranes and play a significant role in initiating signalling cascades and de-regulated oncogenic cell growth. Hedger *et al.* studied all 58 known human RTKs using a multiscale computational simulation approach and observed that the juxtamembrane (JM) regions of RTKs interact with anionic lipids[176]. More specifically PIP_2 lipids had the highest contacts with JM residues, particularly the N-terminal end of the JM regions, followed by PS and PC lipids[176].

CG- and AT-MD simulations have helped reveal the mechanism of dimerization and activation of the ephrin (Eph) receptor family, the largest of RTKs, and critical to cell

migration. Chavent *et al.* studied the changes in the transmembrane region of EphA that happen during EphA activation[178]. Their simulations revealed 2 distinct states of the EphA transmembrane helix dimer as well as possible paths from one state to another[179]. Understanding the mechanistic dynamic behaviour of RTKs is important for cancer drug design that target these receptors[178]. MD simulation studies also showed the importance of phosphatidylinositol phosphates (PIPs) in facilitating kinase domain-membrane interaction at the cytoplasmic surface of the cell membrane[179]. For example, CG simulations showed that the kinase and juxtamembrane domain region of the EphA2 receptor, interact with PIP lipids, resulting in nanoclustering of these lipids in the vicinity of the receptor. This PIP nanoclustering around the receptor potentially results in recruiting more receptors and facilitating autophosphorylation of the kinase domains of adjacent receptor dimers[179]. Additionally, a number of MD simulation studies focused on the association of the TM region of ErbB and the effect of lipids on this association[180-184].

Halim *et al.* showed that PIP_2 lipids interact strongly with the EGFR juxtamembrane (JM) domain[185]. This may explain why epidermal growth factor (EGF)-induced EGFR activity decreases in membranes containing fewer PIP_2 membrane lipids[185, 186]. Another study showed that 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS) lipids interact with basic residues in the (JM) segments of the EGFR stabilising the JM helix dimer[187]. This stabilisation strengthened the coupling between the extracellular and the intracellular modules of the EGFR[187]. Using AT-MD simulations Kaszuba *et al.* showed that *N*-glycosylation of the EGFR ectodomain significantly affects its orientation and its interactions with the membrane[188]. This observation was also supported by Arkhipov *et al.* which suggested that the flexibility of glycans permitted the EGFR ectodomains to rest on the membrane[189]. MD simulations of the fibroblast growth factor receptor 3 (FGFR3) suggested a shift of the mutant dimer (G380R in its transmembrane domain) towards the outer membrane leaflet to allow arginine sidechain/ phosphate lipid head group interactions[190].

The family of heterodimeric receptors, integrins, are potential cancer therapeutic targets through their involvement in various signalling pathways, including tumour

metastasis[191, 192]. MD methodologies revealed that cholesterol and PS lipids cluster around the integrin/talin complex, potentially facilitating the receptor activity and signal transduction events[193]. More specifically Kalli *et al.* performed CG-MD simulations of an asymmetrical model membrane that consisted of five different lipid types and showed that integrin changed the lipid in its vicinity[193]. This simulation showed elevated density of cholesterol and PS lipids close to the integrin receptor due to interactions of lipids with the integrin/talin F2-F3 complex[193]. PIP_2 lipids were also suggested to play an important role in stabilising an integrin/talin heterocomplex[194]. Other studies used MD simulations to study the mechanism for integrin activation[195, 196] and the role of integrin-lipid interaction[196]. Guo *et al.* suggested that the ionic interplay of a Lys residue in the integrin $\alpha L\beta 2$ with acidic phospholipids and calcium ions in T cells regulates integrin function[196].

4.4. MD simulations of peripheral membrane proteins involved in signalling

The interaction of peripheral membrane proteins (PMPs) with lipids at the membrane surface is critical for cellular functions, including cell signalling[197, 198]. This interaction is facilitated by lipid-binding modules located on many PMPs, which bind to specific membrane lipids, such as PIPs[199]. The pleckstrin homology (PH) domain is one such example. Yamamoto *et al.* studied the formation of the PH/PIP complexes of 13 different PH domains, including the Akt-PH domain that is important for cancer cell signalling. PH domains were found to associate with PIP lipids via a highly positively charged loop and that clustering of PIP molecules occurs upon binding of the PH domain to the lipid bilayer[199]. Comparison with experimental PH/PIP structures showed that the predicted PH/PIP complexes are in good agreement with NMR and X-ray crystallography data of PH/PIP headgroup complexes[199]. Buyan *et al.* explored the formation of the Dok PH/membrane complex, a protein that is required along with membrane lipids to activate MuSK receptor tyrosine kinase (MuSK)[200]. Using CG- and AT-MD simulations, this study suggested that DOK7-PH domain associates with PIP containing membranes, via a positively charged surface on the PH domain that interacts with the headgroups of PIP lipids. Clustering of PIP molecules was also observed[200]. MD simulations also provided insights into the localisation and diffusion

of PH domains on PIP-containing lipid bilayers suggesting that the diffusivity of the PH domains is regulated by the number of PIP lipids that are bound to the PH domain[198, 201]. Furthermore, using MD simulations and mechanochemical network simulations forces from the PIP_2-containing membranes and from the cytoskeleton were found to activate focal adhesion kinase (FAK)[202].

K-Ras is another example of a peripheral membrane protein that is attached to the inner leaflet of the plasma membrane. K-Ras regulates numerous signalling pathways and its somatic mutations are linked to about 15-20% of all human cancers. Prakash *et al.* used MD simulations in conjunction with biological assays, to study K-Ras and their association with a negatively-charged bilayer, rich in PS lipids[203]. Their study revealed that a constitutively active oncogenic mutant on K-Ras directly interacts with the membrane in multiple orientations[203]. In two orientations that are more prominent the catalytic domain interacts with the bilayer[203]. Janosi *et al.* suggested that 4-10 H-Ras molecules assemble and segregate into clusters in domain-forming mixed bilayers or non-domain-forming bilayers. This group noticed that clusters of H-Ras were larger and more stable in the domain-forming than the non-domain-forming bilayers, and that localisation occurred at the L_o/L_d domain interphase[204]. Cholesterol was found to enhance H-Ras nanocluster domain stability[205] but it was not required for their formation. Other CG-MD simulations also indicated that in phase-separating membranes, H-Ras nano-or/and large clusters mainly concentrate at the domain boundary[205]. A CG-MD simulation by Jefferys *et al.* observed similar localisation for N-Ras and suggested that the protein slows the rate of the formation of membrane domains[206]. Additionally, Li *et al.* suggested using models representing the active or the inactive state of Ras molecules, indicate a single large aggregate, but protein-protein interactions are different depending on activity state [207].

Arcario *et al.* used MD simulations with a highly mobile membrane mimetic model (HMMM) to study the protein talin, which is essential for integrin receptor activation at the plasma membrane. They observed a conformational change within the talin F2-F3 domain upon binding to the membrane [208]. Simulations with the complete talin head domain showed that it adopts a V-shaped configuration upon binding to the

membrane that may stabilise its interactions with the membrane[209]. MD simulations revealed important information about molecular mechanisms and specific protein/membrane lipid interactions, of other peripheral membrane proteins like the phosphatase and the tumour suppressor tensin homologue (PTEN) [210].

4.5. Quantification of membrane proteins interaction with lipids

Measuring interactions between lipids and proteins provides valuable information regarding which lipid species are strong candidate drug targets. MD simulations of free energy can be used to quantify the strength of lipid/protein interaction[211].

Hedger *et al.* compared the free energy of the interactions between the TM helix dimer of wild type and mutant EGFR receptor with GM3 and PIP_2 lipids with potential of mean force (PMF) calculations. Mutations at modulatory sites of the EGFR decreased the free energy of GM3 and of PIP_2 lipids[212]. Chan *et al.* used PMF calculations to show that PIP_2 molecules bind stronger to two binding sites of ACAP1-PH domain compared to POPS[213]. Naughton *et al.* used CG-MD simulations in conjunction with PMF calculations to quantify the free energy of binding of the GRP1 PH domain to PIP_2 and PIP_3 lipid molecules and showed preferential binding of GRP1 PH to PIP_3 compared to PIP_2 lipids. In the presence of mutations in the PIP binding site, the primary orientation of the PH domain to the bilayer was not observed[211], indicating that PIP lipids may be important for GRP1 protein binding and signalling pathway initiation. In a recent study the same methodologies were used to study the strength of protein/lipid interactions of 12 PH domains with PIP_2 and PIP_3 lipids[214]. Lu *et al.* used Molecular Mechanics-Poisson-Boltzmann Surface Area (MM/PBSA) calculations to measure binding free energy of protein/ligand systems and explore the interaction behaviour of wild-type and 11 mutants of the PH domain in Bruton's tyrosine kinase (Btk) protein with inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P4)[215]. These free energy calculations distinguished the mutations in two groups, the "folding" and "functional" mutations, the former resulting in the loss of function of the PH domain and the latter disrupting the binding affinities between the Btk/Ins(1,3,4,5)P4 interactions, but without affecting the overall fold[215]. The same approach was used by Rosen *et al.* to understand the affinity of PKB PH domain to inositol phosphate head-groups that play

a role in the PI3K pathway[216]. This MD study agreed with the 3-phosphorylated phosphoinositide selectivity observed *in vivo*[216]. They also proposed that Ins (1,3,5) P_3 lipids, that are produced from PI(4,5)P_2, lipids and were shown to not bind Akt *in vivo,* can bind Akt if their inositol ring is flipped with respect to their parent lipid and the 3-phosphorylated inositol phosphate ligands and if not bonded to a phosphatidate tail[216].

Simulations provided insightful information about transmembrane proteins, which are extremely difficult to crystallise from aqueous solution. Not only MD simulations provide structural predictions about proteins, they also provide information about how these proteins interact with their lipid environment over time. MD simulations are reliable prediction tools for studying various aspects of the membrane in detail not accessible by typical wet-lab, or semi-dry experimental set-ups.

5. Membrane Biophysics

5.1. Membrane dynamics and interactions

Techniques for measuring lipid diffusion, membrane dynamics and interactions include fluorescence correlation spectroscopy (FSC) (single molecule or several molecules), Förster Resonance Energy Transfer (FRET) (can be ensemble or single-molecule-based) and single particle tracking (SPT), a single-molecule method. Single-molecule based techniques have been used for assessing molecular heterogeneities in biological membranes through the incorporation of single particles, such as labelled proteins and tracking their behaviour[217]. They are often fluorescence based and are extensively used for the study of membrane proteins and their interactions in live cells. Single-molecule methods allow observations of individual interactions in high mechanistic detail, rather than the effect of millions of interactions in an ensemble average. Single-molecule approaches are useful in studying how individual molecules behave over time and how they change in conformation and properties. Unlike population techniques, single-molecule approaches provide details regarding intermolecular variations (static disorder) of individual molecules (such as protein folding)[218] and allow tracking of the random and stochastic dynamics for various processes[219]. Furthermore, the

molecular structure of macromolecules as well as their structural and functional response to mechanical manipulation of molecules and how they change their conformation or properties can be studied with single-molecule methodologies[218].

5.1.1. Fluorescence Correlation Spectroscopy

FCS coupled to confocal microscopy measures fluorescence intensity fluctuations in the small confocal volume as a function of time to generate diffusion coefficients and particle concentrations with high temporal resolution[220](Figure 3). The overall fluorescence intensity and the particle number fluctuate around an average value thus providing information on the particle dynamics, states of aggregation and molecular interactions[221] (Figure 4). FCS techniques have been applied to study membrane receptor dynamics of Rous sarcoma virus membrane interactions in living cells by analysing the interactions between labelled Lyn kinase and FCERI receptor after stimulation with fluorescently labelled IgE[222]. FCS was also used to study the contribution of lateral membrane organisations in the PI3K/Akt signalling pathway activation. This study suggested that membrane nanodomains potentially contribute to the majority of PI3K mediated signalling events and that PIP_3-containing nanodomains are only formed when PIP_3 binds to PH domain[223]. Gerken *et al.* used FCS to study the membrane dynamics of two plasma membrane receptors, tumor necrosis factor receptor 1 and 2, TNFR1and TNFR2, respectively[224]. This study observed enhanced diffusion of TNFR1 upon cholesterol depletion and no changes at diffusion constant of TNFR2 upon cholesterol depletion suggesting potential microcompartment-plasma membrane topological segregation of the two receptors[224]. While FCS has been widely used for studying protein and lipid dynamics in model membranes or *in vivo*, it has a relatively lower spatial resolution (diffraction limited) compared to other techniques, like Single Particle Tracking (SPT)[225] (Figure 3).

5.1.2. Single Particle Tracking

SPT is a single-molecule technique that traces the path of multiple particles down to a precision of nanometres, tracking their detailed positions and velocities. The high

858 temporal and spatial resolution of SPT (Figure 3) make it a powerful technique for
859 studying dynamic membrane events such as particle aggregation/dissociation and
860 lateral diffusion (Figure 4). SPT has been applied to study Lck tyrosine kinase localisation
861 to membrane microdomains in T cells and their role in cell signalling; phosphorylation
862 and dephosphorylation balance[226], as well as EGFR related cell signalling[227].
863 Furthermore SPT has been used for identifying changes in diffusion of proteins (EGFR,
864 HER2, I-Ek) upon modulating membrane cholesterol concentration[228] and Ras
865 activation following EGF and insulin activation[229]. Lommerse *et al.* studied the
866 relationship between the activation of H-Ras, which is part of Ras family of proteins
867 that regulate cancer cell growth and membrane microdomain localisation in live cells
868 using single-molecule fluorescence microscopy[229]. In this study a constitutively
869 inactive mutant H-Ras and a constitutively inactive mutant were fused to enhanced
870 yellow fluorescent protein (eYFP)[229].Both forms showed a major fast-diffusing
871 population and a minor low-diffusing population of H-Ras. The slow-diffusing
872 population of the active mutant was restricted to 200nm membrane domains, which
873 were calculated by mobility analysis of populations of molecules [229]. While this was
874 not observed for the inactive mutant, upon insulin-activation the slow-diffusing
875 population of H-Ras in the wild-type was also confined to the membrane domains[229].
876 This study showed that there is a relationship between membrane microdomains and
877 H-Ras activation [229].

878 ### 5.1.3. Förster Resonance Energy Transfer

879 FRET based techniques have been applied to model membranes and live cells for
880 studying interactions of species such as docking, protein-protein interactions, protein-
881 lipid interactions and signalling on a membrane surface[230] (Figure 4) by using
882 fluorophores that are coupled to the proteins of interest. FRET is a powerful technique
883 that can reveal nanodomains that are not visible by light microscopy[231] (Figure 3)
884 and utilises transfer of excited-emission from the donor to the sensitised emission of
885 the acceptor; the efficiency of transfer informs on the distance between the donor and
886 acceptor[232]. The Förster expression predicts that the efficiency of this transfer is
887 inversely proportional to the 6^{th} power of this distance ($1/R^6$), which typically indicates

888 that resolution on the scale of 1-10nm are possible[233]. FRET approach allows
889 studying the spatial relationships of molecules including nanoscale protein interactions
890 and changes in protein conformational states (Figure 4).

891 While variations in the sample intensity can make it difficult to determine the transfer
892 efficiency based on the donor emission, this artefact, can be eliminated with the use of
893 Fluorescence Lifetime Imaging (FLIM) in conjunction with FRET (discussed later). FRET
894 was used to study the activity of a genetically encodable fluorescent kinase reporter,
895 AktAR in different microdomains of the plasma membrane of live cells[234]. Activation
896 of Akt and its downward signalling cascades were enriched in lipid raft domains in
897 comparison to non-lipid raft domains of the plasma membrane[234]. This genetically
898 encoded Akt activity reporter could be detected by FRET in a large dynamic range, and
899 was sensitive in detecting changes in Akt activity upon treatment of Akt inhibitors[234].
900 The green fluorescent protein (GFP) has been used in association to FRET to visualise
901 the flow of signalling cascades in live cells through monitoring protein-protein
902 interactions and conformational changes in living cells[235]. More specifically FRET was
903 applied for the visualisation of Rac[236], and Ras protein activation, which are involved
904 in cancer cell proliferation using bimolecular fluorescent indicators[237]. In a similar
905 approach, Murakoshi *et al*. observed activated Ras molecules that immobilised in the
906 cell membrane and the potential formation of a signalling complex of activated Ras in
907 a subline of the ubiquitous keratin-forming tumour cells[238], in agreement to single-
908 molecule techniques; SPT[229]. In combination with phospho-specific antibodies, GFP
909 fusion proteins were used as donors in FRET to study autophosphorylation of protein
910 kinase C alpha (PKCα)[239] and EGFR activation [222, 240] in the plasma membrane of
911 fixed cells. Issiki *et al.* used FRET to show that caveolae are involved in signal
912 transduction pathways in living cells by using genetically engineered Ca^{2+} probes,
913 named cameleon proteins. This engineered calcium sensor yellow cameleon was
914 shown to target the plasma membrane caveolae in preference to the endoplasmic
915 reticulum, indicating Ca^{2+} controlled signalling machinery to be functionally organised
916 in the L_o of the plasma membrane; caveolae[241]. Tyrosine kinases and their
917 phosphorylation status are good markers of cell signalling activation and their
918 phosphorylation is often a marker of increased cell signalling and cell growth in cancer.

Unimolecular indicators constructed from an appropriate phosphorylation substrate peptide and a phospho-amino acid binding domain were genetically linked to form a hybrid protein and were then observed by fluorescent mutants[231, 242]. In combination to confocal microscopy, FRET was utilised to determine the efficiency of osteopontin (OPN-R3) in inhibiting the tumorigenesis inducing OPN binding to its cell surface receptors (CD44, avb3 integrin) in breast cancer cells[243].

Homo-FRET is a less utilized form of FRET, where two identical fluorophores are used that overlap between excitation and emission spectra but differ in fluorescence polarisation. Only a single fluorescent probe is required, with the ability to monitor intracellular events in real time with a microplate reader in the nanometre scale. Homo-FRET in association with fluorescence anisotropy measurements has been used to assess the oligomerization state of biological macromolecules in their native environment, which plays a fundamental role in signal transduction. In particular, studies on glycosyl-phosphatidylinositol-anchored proteins (GPI-APs) clustering used homo-FRET to observe the modulation of GPI-anchoring upon depletion of cholesterol and sphingolipids that resulted in loss of homo-FRET in live cells. Using the same approach, they suggested the presence of small clusters of proteins that act as platforms for GPI-APs and suggested their potential of being crucial for signal transduction[244, 245]. GPIs are evenly distributed in the surface of resting cells and are enriched in DRMs suggesting that clustering of cell surface receptors may induce L_o microdomain formation. These methodologies were able to show that small alterations in protein or lipid aggregation are able to highly influence membrane domain organisation, and facilitation of protein-protein interactions.

FCS, SPT and FRET methods provided useful information on the mobility of molecules in their membrane environments. In combination with fluorescence dyes SPT and FRET are capable of operating in complex biological systems such as live cancer cells in a single molecule sensitivity rather than an ensemble average. Alterations in diffusion constants in membranes indirectly depict lateral heterogeneities, which interact with

948 oncogenic proteins, findings which can be directly seen using from microscopes and
949 super resolution.

950 ## 5.2. Microscopies

951 Biophysical changes in molecular packing and membrane phase separation indicates
952 changes in cell functioning. Thus, physical methods for studying the whole cell
953 membrane, including diffusion, bending (curvature), fluidity, rigidity and how they
954 change in various conditions and external stresses have been extensively applied
955 (Figure 4).

956 ### 5.2.1. Fluorescence Lifetime Imaging Microscopy

957 FLIM microscopy maps the spatial distribution of fluorescence lifetimes at the
958 nanosecond level. FLIM has been previously used for measuring membrane properties
959 using environment-sensitive probes, like solvatochromic dyes, which change their
960 colour in response to change in polarity, viscosity and order[117, 246]. Combined
961 FRET/FLIM allows for detecting spatiotemporal protein-protein interactions and has
962 been employed as an improved method for the assessment of FRET in association with
963 multiphoton microscopy[247]. Multiphoton microscopy gives a better axial sectioning
964 compared to other optical imaging approaches (wide-field, confocal microscopy),
965 improves z resolution and allows penetration into deep samples like tissue, with less
966 toxicity[247]. Multiphoton microscopy with combined FRET/FLIM has been widely
967 applied for studying invasion and metastasis with sub-cellular resolution in vivo[247]
968 (Figure 4).

969 Chemokine Receptor Type 4 (CXCR4) promotes metastasis in breast, prostate,
970 pancreatic and hematopoietic origin tumours and is consistently upregulated in human
971 breast cancer cells[248]. Peter *et al.* used FRET detection by multiphoton FLIM to
972 characterise ligand binding of the CXCR4 chemokine receptor and protein kinase C
973 (PKC) of MDA-MB-231 breast cancer carcinoma cells using EGFP (donor) and mRFP11
974 (acceptor) as their fluorophores, respectively [249]. Ahmed and colleagues also
975 employed FRET/FLIM (on a multiphoton microscope) in prostate cancer cells and

observed that p21 activated kinase (PAK4- Rho family GTPases) binds to and phosphorylates LIM kinase 1 (LIMK1), downstream of hepatocyte growth factor (HGF) to increase cell migration speed[250]. As with the previous group Mrfp1 and GFP were used as donor and acceptor, respectively.

5.2.2. Atomic Force Microscopy

Atomic Force Microscopy (AFM) provides sub-nanometre resolution and can be used to image biological systems with minimum sample preparation and with no requirement for fluorescent dye incorporation (although the use of dyes is commonly utilised)[251]. A sharp probe approaches the sample that sits on an atomically flat surface and provides information on the distance-dependent interaction forces, generating 3D topography maps of biological samples in air or in their native liquid environment. As a result, information for the biological's specimen morphology and structure is generated. AFM has been used extensively for the study of lateral heterogeneities in membranes, since it can provide information on the height of nanodomains[251, 252] (Figure 4).

Giocondi *et al.* were the first to use AFM to study the effect of membrane phase separation on protein partitioning and the association with cell signalling[253]. In agreement to later findings[153], they observed clustering and filament formation in the SM and cholesterol rich domains, upon the cholera toxin receptor addition to the model membrane system showing complete insolubility to the L_o phase. AFM techniques also showed the accumulation of cholera toxin protein in L_o domains[239]. Yuan *et al.* also linked the behaviour of lipid phases in model membranes and function in real cells by visualising the effect of ganglioside G_{M1} distribution on the overall phase structure[254, 255]. In agreement with the other studies, it was observed that G_{M1} localised in the L_o phase and thus G_{M1} were considered to be a marker for L_o domains. These domains were too small to be observed with fluorescence microscopy since they are in the nanometre scale, thus making the AFM approach very useful.

Membrane phase separation has been observed when a combination of AFM and FCS was applied for studying membrane phase separation[236, 256] and membrane

proteins within L_o and L_d phase compartments. Orsini *et al.* extracted membrane patches of human triple negative breast cancer cells (MDA-MB-231) by Triton X-100 and sucrose gradient ultracentrifugation and used AFM and antibody-based techniques to study membrane domains in breast cancer cells. AFM provided evidence of microdomains with lateral dimensions of 100-500nm, extending from the membrane patches by 1-2nm with a greater roughness compared to the smoother surrounding membrane[257]. Treatment of samples antibodies against flotillin-1 (lipid-raft marker) showed increase in micro-domain surface area suggesting the presence of flotillin-1 in the AFM visualised microdomains[257]. These results were further supported by immunoblotting and high-performance thin liquid chromatography that also detected high cholesterol, SM and flottilin-1 content in the membrane microdomains of breast cancer cells [257]. The authors discussed the use of detergents for the membrane patches extraction and stated that their observations are in agreement with Pathak *et al.* (a FRET study) that showed detergents to increase domain size by coalescing pre-existing domains rather than inducing their formation[258]. The authors also suggested that AFM applications could greatly contribute in the study of membrane microdomains in healthy and cancer membranes providing high spatial resolution, in physiological-like conditions, without the need for fixation, staining or labelling of cells[257]. Lamprecht *et al.* used AFM to study the mechanisms of the plasma membrane interaction of Hsp70-1A with PS lipids in the presence and absence of cholesterol, which was previously linked to resistance to cancer radiotherapy, metastasis and poor prognosis[259]. This study highlighted a strong association between the plasma membrane Hsp70-1A and the negatively charged saturated PS lipids[259, 260], which are enriched in cancer cells[259]. PS lipids were sufficient for Hsp70-1A insertion to the membrane[259]. In the presence of cholesterol, the L_o phase emerged, which was about 1.2nm greater in height than the surrounding lipid phase[259]. Upon cholesterol addition, Hsp70-1A protein was found to be clustering in the L_o phase[259]. Increase of protein concentration in the presence of cholesterol resulted in in the onset of membrane blebbing[259]. According to the authors[259] the formation of membrane blebs that burst at higher concentrations are potentially linked

to the previously proposed non-classical pathway for the export of Hsp70-1A by tumour cells[261].

In combination with biological assays, Pommier *et al.* used AFM to study the role of Liver X Receptors (LXRs) in regulating cholesterol homeostasis in prostate cancer cells and observed smaller and thinner L_o domains post LXR stimulation[124]. Furthermore, this study suggested cholesterol to be an important modulator domain formation, since cell membrane replenishment with cholesterol reversed the LXR-induced results on the L_o domains[124]. Rivera *et al.* used a new fast scanning quantitative dynamic AFM method to observe rapid changes in the cytoskeletal architecture at the cell periphery of breast cancer cells and the activity of Syk protein tyrosine kinase[262]. This technique allowed studying of the time varying heterogeneous physical properties of live cells, by using the cantilever mean deflection as feedback signal rather than standard amplitude reduction, resulting in increased temporal resolution to capture dynamic changes in cellular properties[262]. As already mentioned, leaking of PS[54] and PE[52] lipids from the inner to the outer membrane leaflet in cancer results in loss of membrane asymmetry in cancer cells. In combination with fluorescence confocal microscopy Leite *et al.* used AFM to study the membrane interaction with the antimicrobial peptide Polybia-MP1, which shows anti-cancerous effects[60]. They observed PS lipids to significantly facilitate binding of MP1 peptide to the membrane through charge interactions and the role of PE lipids in potentiating the formation of larger transmembrane pores due to the particular shape of PE molecule stabilising the pores[60]. Alvares *et al.* also suggested synergy between PS lipids and L_o domains and proposed that PS is required for the Polybia-MP1 to apply its membrane lytic action, while membrane order can influence this activity and facilitate its binding to the membrane[263].

Quintela *et al.* used AFM in live cells along with biological assays to define the molecular function of histone acetyltransferase (HBO1), which is highly expressed in ovarian cancer[264]. This group showed that HBO1 increases the cell membrane elasticity in ovarian cancer cells[264]. A recent study explored the interactions of the GDP-bound form of K-RAS4b, a proto-oncogene with anionic lipids using AFM and MD

simulations[265]. K-RAS4b-GDP appeared to be more loosely associated with the PS-containing membrane, compared to the PIP_2-containing membrane[265]. Another AFM study explored the regulatory mechanisms of destruxin resistance phenotypes, which has anticancer activity[266]. This study observed hyper-activity of the mevalonate pathway and de-novo cholesterol synthesis, associated with increased cell membrane adhesiveness of the resistant lines compared to the parental cell lines[266]. Sakai *et al.* used AFM and showed that the targeted breast cancer drug Trastuzumab has a significant effect on the biological membrane by modulating the membrane fluidity and the formation of phases within the membrane of different fluidity levels[267]. A more in depth analysis of how AFM has been applied to cancer research and cancer cell morphology, elasticity and adhesion properties is available in a recent expert review (see review [268]).

Powerful microscopy techniques provide a new opportunity for fluorescence-based techniques resolving structures in high spatial resolution, while maintaining the capabilities of optical fluorescence microscopy for complex biological structures. AFM and FLIM, combined together or with other techniques (AFM/FLIM and FRET/FLIM) provided significant progress in studying important properties of the plasma membrane including lateral heterogeneity, molecular aggregation, diffusion, fluidity, thickness in cancer cells and identified alterations in cancer cell morphology that relate to the membrane composition.

5.3. Label Free Techniques

Label Free techniques overcome various limitations of fluorescence-based techniques, explaining why their use is exponentially increasing. As already mentioned, if not chosen wisely fluorescent probes can affect the native environment of the labelled molecule, alter its conformation and the function of the labelled protein, or oligomerize and fluoresce non-specifically[269].

5.3.1. Surface plasmon resonance microscopy

Surface plasmon resonance microscopy (SPRM) label free technique, which can measure protein-binding kinetics with the use of a sensor chip coated with membranes to prevent the disruption of the native environment of membrane proteins[270]. SRPM ensures intact native conformations of the plasma membrane and can also be coupled with fluorescence techniques allowing for optical and fluorescence imaging of the same sample, thus having the advantages of both label-free and label based methodologies in one experiment[270]. Wang *et al.* used SPRM simultaneously with optical transmission and immunofluorescence microscopy of the same sample to study how membrane glycoproteins and nicotinic acetylcholine (nAChRs) interact with their ligands; lectin in single cells and their polarization during chemotaxis[270]. Chemotaxis is involved in invasion, intravasation, extravasation of carcinoma cells, which are crucial steps in cell dissemination and metastasis [271].

5.3.2. Small-angle x-ray scattering

The effect of bilayer properties and cholesterol on cell signalling initiation and its potential role in biological disease such as cancer explains the use of indirect techniques on studying membrane biology. Small-angle x-ray scattering (SAXS) is a powerful technique, appropriate for examining the structure as well as the elastic parameters of fluid membranes under near-physiological conditions. Over the years, it provided critical insights to the current lipid membrane structure knowledge. It is based on X-ray diffraction, where a beam of X-rays exposes the membrane and scatters its electrons in all directions[272]. Several SAXS studies contributed important information into phase separation and the formation of L_o domains, suggesting their potential contribution in providing an additional environment for the assembly of membrane proteins[273-277]. SAXS provided insights to the extent of structural changes in receptor tyrosine kinase EGFR dimerization upon the presence or the absence of its ligand and suggested that its activation is not necessarily ligand dependent[278]. Hodzic *et al.* applied SAXS to investigate the responses of cholesterol incorporation in model bilayers in comparison to phytosterols, which are compounds that are known to decrease cholesterol concentration in the plasma membrane and good candidates to

be used as therapeutic agents in cancer. In comparison to cholesterol, stigmasterol and sitosterol differ by an additional double bond and ethyl group, respectively, showed a reduced condensing effect indicating that small variations in the bilayer sterol structure could modulate the lipid bilayer composition in a biologically meaningful manner[279]. This indirectly opens a way to research cancer therapeutics involving substances that are capable to modulate cholesterol concentration and other membrane parameters.

5.3.3. Electron microscopy and cryo-EM

Electron microscopy has provided important insights for nanoscopic lipid domains[118, 280]. Prior *et al,* used electron microscopy to directly visualise Ras proteins in spatially distinct cell surface domains and suggested that wild-type H-Ras is distributed in a dynamic equilibrium between cholesterol rich-ordered domains and other non-cholesterol-dependent microdomains[281]. As already mentioned, similar findings were found by MD simulations[204]. They also found an inner-plasma membrane raft marker (GFP-tH) to display cholesterol-dependent clustering in membrane microdomains[281]. The latest advancements in cryogenic electron microscopy (cryoEM) in intact cells may provide a reliable methodology for studying the biological significance of lateral organisation in biological membranes. Cryo-EM like SAXS, allows for sub-angstrom level resolution, but provides direct imaging instead. Heberle *et al.* recently used cryoEM to image nanodomains in model membranes and isolated intact plasma membrane vesicles (GPMV) from rat-basophilic leukaemia cells and showed similar nanoscale lateral heterogeneities between the two[103]. Although the use of cryo-EM in studying membrane biology holds promising, more studies are required to validate these findings using this technique.

Label free techniques complement some high-resolution fluorescent-based microscopy methods by maintaining a high spatial resolution without having risks of affecting the native environment of the sample. Comparison of label-free and label-based techniques can indicate appropriate probes that are non-invasive to the native structure of the membrane or the membrane protein being studied.

6. Conclusion

Biological membrane properties and membrane organisation at the nanometre scale are fundamental in cellular bioactivity via lipid-lipid and lipid-protein interactions and have critical roles in tumorigenesis. For this reason, a range of anti-cancer lipid-targeting drugs have been in clinical or pre-clinical drug developmental stages. Some examples include Perifosine that targets the cell membrane and membrane domain organisation[282] (Phase I-III clinical trials for solid and non-solid tumours), Nelfinavir that disrupts lipid homeostasis (Phase II for glioblastoma, pancreatic, lung cancers)[43, 283] and the PS-targeting antibody Bavituximab for cancer detection and cancer immunotherapy[284] (Phase I-III for advanced solid tumours non-small cell lung cancer, breast, pancreatic and hepatocellular cancers). While effective in pre-clinical work, their effectiveness in clinical trials requires a better lipid-centered perspective and understanding on their mechanism and action in cancer cells. Unlike protein and nucleic-acid targeting drugs, drugs targeting lipogenesis are newer and still entail less understanding in their mode of action to prove their effectiveness. Overall, cholesterol-rich membrane domains can both worsen (multi-drug resistance) and improve (aggressive metastasis) cancer outcomes, highlighting an unmet clinical need for more studies identifying the role of the plasma membrane and lateral heterogeneity in various stages, severity and types of cancer. Techniques widely used in the past, such as detergent resistance membranes, although being a useful empirical tool, do not precisely identify membrane domains, and are prone to induce experimental artefacts. The advancement in methodologies with high temporal and spatial resolution allows researchers to acquire direct evidence and expand the findings of previous methodologies. The combination of direct label-free techniques, like cryo-EM, non-direct label-free techniques (SPRM, SAXS) and the collaborative use of fluorescent labelling in whole cells or GPMVs, with quantitative diffusion measurement techniques (FCS, SPT, FRET), microscopy (AFM, FLIM) and computational biology will be critical in understanding the roles of membrane properties in cancer and lipid-targeted therapies. The potential application of some of these techniques, like AFM in the clinical setting and diagnosis for determining ultrastructure and mechanical properties of the tumours and cells such as the morphology, elasticity, rigidity and fluidity has been

reviewed recently[268, 285, 286]. The severity of tumours in clinic is mostly qualitatively examined by immunostaining and optical microscopy, [287] where tissue is fixed and loses its mechanical property characteristics. The capability of AFM in complementing histopathological data based on rheological properties in advanced colon cancer tissues was recently evaluated[288]. The application of AFM for future clinical diagnosis of metastatic and chemoresistant live cells guided by the properties of the cell membrane could open new opportunities, provide both quantitative and qualitative information in early detection and improve cancer prognosis. In addition, simulations provide mechanistic detail at the molecular level but require strong experimental evidence to validate its findings. A good temporal resolution allows tracking of biological dynamic processes, accurately time localising rapid changes and a good spatial resolution allows for direct detection of the dynamic membrane properties. This is especially important since domains in model membranes seem to be more easily detected being in the microscopic scale, whereas in live cells remain nanoscopic[122]. All techniques have their strengths and weaknesses, operating over different time and length scales, and the combination of two or more usually essential to verify the findings and bridge the biophysical observations to the biological function. It is required that cancer biologists studying the membrane collaborate and utilise high-resolution biophysical techniques along with biological assays to answer critical biological questions on membrane role in cancer as it is required by the nature of these dynamic processes. To conclude, membranes and their lipids must be studied using collaborative research to accurately and rapidly advance understanding of the cell membrane and its components in lipid-targeted cancer therapeutics.

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Figure Legends

Figure 1 The structure and physical properties of the membrane. Properties such as membrane thickness, lateral diffusion (translational freedom), bond rotation (configuration freedom or lipid ordering), lipid flip-flop are shown in red lines/arrows. L_o; liquid-ordered domains, L_d; liquid-disordered domains.

Figure 2 The plasma membrane. A) The asymmetric distribution of lipids in the plasma membrane. B) Coarse-Grained and all-atom representation of a phosphatidylcholine lipid and the major lipids comprising the plasma membrane. C) Membrane asymmetry in healthy and cancer cells. D) Overall changes in lipid components of the membrane in different cancer types. Full details are found in supplementary tables. SM; Sphingomyelin, PC; Phosphatidylcholine, PE; Phosphatidylethanolamine, PS; Phosphatidylserine, PI; Phosphatidylinositol, CHOL; Cholesterol, MDR; Multi-Drug Resistance.

Figure 3 The spatiotemporal resolution of methodologies used to study the cancer plasma membrane and the relevant biological processes involved in signaling through the plasma membrane. AT-MD; Atomistic Molecular Dynamic Simulations, CG-MD; Coarse-Grained Molecular Dynamic Simulations, FCS; Fluorescence Correlation Spectroscopy; SPT; Single-Particle Tracking, SPRM; Surface Plasmon Resonance Microscopy, FRET; Förster resonance energy transfer, AFM; Atomic Force Microscopy, SAXS; Small Angle X-ray Scattering, EM; Electron Microscopy, cryo-EM; Cryogenic-Electron Microscopy

Figure 4 Properties of the plasma membrane that are involved in cancer and the methodologies that can be used for each. MD; Molecular Dynamics Simulations, IF; Immunofluorescence, WB; Western Blotting, IP; Immunoprecipitation, MS; Mass Spectrometry, FCS; Fluorescence Correlation Spectroscopy; SPT; Single-Particle Tracking, AFM; Atomic Force Microscopy, FRET; Förster resonance energy transfer, FLIM; Fluorescence Lifetime Imaging Microscopy, SPRM; Surface Plasmon Resonance Microscopy, SAXS; Small Angle X-ray Scattering, EM; Electron Microscopy, cryo-EM; Cryogenic-Electron Microscopy

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