

Role of the actin cytoskeleton in regulating LYVE-1 lateral diffusion and function



Dissertation submitted for the degree of *Doctor of Philosophy* by

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Declaration

I hereby declare that except where specific reference is made to the work of others, the contents of this dissertation are original and have not been submitted in whole or in part for consideration for any other degree or qualification in this, or any other university. This dissertation is my own work and contains nothing which is the outcome of work done in collaboration with others, except as specified in the text and mentioned below. The design, cloning and generation of the following hLYVE-1 plasmid constructs for stable lentiviral transductions was carried out by Dr. Suneale Banerji.

- Human dermal lymphatic endothelial cells (HDLECs) stably expressing human LYVE-1 (hLYVE-1) GFP construct.
- The design of ACP-hLYVE-1 and hLYVE-1 HaloTag[®] in PHR vectors for the use of stable lentiviral transductions.
- Generation of hLYVE-1 truncation mutants Δ 263 and Δ 263 GFP, full length (FL) and FL - GFP hLYVE-1 plasmid constructs for lentiviral transductions.
- Generation of biotinylated hyaluronan.

The MATLAB script for analysis of scanning FCS data was written by a fellow D.Phil student, Falk Schneider.

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Abstract

The lymphatic endothelial receptor LYVE-1 investigated here is a weak binder to its ligand hyaluronan (HA) and uses receptor clustering to achieve the avidity required for stable interaction to HA. These clustering based interactions are highly dynamic and are often controlled by several molecular players that contribute to receptor functional changes. One such contributor is the cortical actin cytoskeleton. As the actin cytoskeleton lies immediately below the membrane surface, it creates compartments on the membrane that control the receptor organisation, dynamics and allows physical interaction of the receptors with actin, which either hinder or enhance their functions. In this study, I investigate the role of the actin cytoskeleton in regulating the dynamics and function of LYVE-1. At first, the native organisation of LYVE-1 and cross-linked clustered state of LYVE-1 (that are known to cause avidity induced increase in HA binding), on primary human dermal lymphatic endothelial cells (HDLECs), were identified by confocal and super-resolution STED microscopy. Following which the effects of actin disassembly on LYVE-1:HA binding function and cross-linked clustering of LYVE-1 were investigated using FACS and STED microscopy. The dynamics of LYVE-1 during actin steady state or disassembly was studied using diffusion measurements on primary and cytoplasmic tail truncated hLYVE-1 transfected HDLECs. The results from the diffusion experiments suggest that LYVE-1 mobility decreases after actin depletion and correlates with the increase in HA interaction as determined by FACS during actin disassembly, although there was no change in the surface organisation of LYVE-1. Further diffusion measurements using STED-FCS showed that LYVE-1 was 'trapped' or confined on the membrane surface of HDLECs in native conditions. This data along with biochemical assays show a hitherto unknown direct link of the actin cytoskeleton regulating LYVE-1:HA binding and dynamics, giving an indication of how LYVE-1 *in vivo* may modulate binding to HA.

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List of acronyms

ACP acyl carrier protein

ADP adenosine diphosphate

ASB actin stabilising buffer

ATP adenosine triphosphate

bLYVE-1 biotinylated LYVE-1

bPhal biotinylated phalloidin

CD31 platelet endothelial cell adhesion molecule

C-ERMAD C-terminal ERM-associated domain

co-ip co-immunoprecipitation

Cyto D Cytochalasin D

D diffusion coefficient

DARPP-32 dopamine and cyclic adenosine 3, 5 - monophosphate-regulated phosphoprotein

DC dendritic cells

ECM extracellular matrix component

EDTA ethylenediaminetetraacetic acid

EGFR epidermal growth factor receptor

ERM Ezrin, radixin and moesin

ESAM endothelial cell-selective adhesion molecule

FACS fluorescence-Activated Cell Sorting

F-actin filamentous actin

FCS Fluorescence Correlation Spectroscopy

FGF-2 fibroblast growth factor-2

FRAP fluorescence recovery after photobleaching

FWHM full-width-at-half-maximum

GA glutaraldehyde

G-actin globular actin

GPI	glycosylphosphatidylinositol
HA	hyaluronan
HDLECs	human dermal lymphatic endothelial cells
HMWHA	high molecular weight HA
hLYVE-1	human LYVE-1
Hsp	heat shock proteins
i.p	immunoprecipitation
Lat B	Latrunculin B
LECs	lymphatic endothelial cells
LPS	lipopolysaccharide
LYVE-1	Lymphatic vessel endothelial hyaluronan receptor-1
mAb	monoclonal antibody
MFI	median fluorescence intensity
MHC-1	major histocompatibility complex I
nAChRs	nicotinic acetylcholine receptor
NaOH	sodium hydroxide
NPF	Nucleation-promoting factor
PALM	Photo-activated localisation microscopy
PBS	phosphate buffered saline
PDGF-BB	platelet derived growth factor-BB
PD-L1	programmed death ligand-1
PFA	paraformaldehyde
Prox-1	prospero-related homeodomain transcription factor-1
ROCK	Rho-associated protein kinase
RT	room temperature
S/N	supernatant
SAXS	small angle X-ray scattering
sFCS	Scanning FCS
SLB	Supported lipid bilayer

SPT Single Particle Tracking

STED Stimulated Emission Depletion

STORM Stochastic optical reconstruction microscopy

TIRF-SIM Total internal reflection fluorescence-structured illumination microscopy

TLR toll-like receptors

TR transferrin receptor

TSG-6 tumor necrosis factor-inducible gene 6 protein

VE-cadherin vascular endothelial cadherin

VEGF-A vascular endothelial growth factor-A

VEGF-C vascular endothelial growth factor-C

VEGFR-3 vascular endothelial growth factor receptor-3

WCL Whole cell lysate

xsHMWHA excess high molecular weight HA

ZO-1 zona occludin-1

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Preface

Lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) is a transmembrane receptor that is abundantly, and almost exclusively, expressed on the endothelial cells of the lymphatic system. The most well studied interactions of LYVE-1 are to the extracellular matrix component (ECM), hyaluronan (HA). LYVE-1, and its interaction with HA has been widely implicated in several functions, such as HA turnover [1], transmigration of dendritic cells (DC) and T cells [2, 3] to the lymph node, dissemination of bacteria [4], and tumour metastasis [5]. Thus, LYVE-1 plays a crucial role in mediating these functions *via* the lymphatic system.

Much of the recent discovery on LYVE-1 function has been linked to receptor avidity, induced by the clustering of the receptors on the surface of the membrane [6]. Receptor clustering based functions is a mechanism often controlled by the actin cytoskeleton [7, 8], which is a complex dynamic structure that lies close to the plasma membrane. This gives it the ability to control receptor lateral dynamics and clustering, and holds the key to many molecular events that contribute to receptor function [9, 10]. In this thesis, I work towards understanding the mechanism of LYVE-1 function contributed by the clustering of the receptor, and in particular I look at the role played by the actin cytoskeleton in controlling the lateral dynamics and receptor function.

This thesis is divided into this preface followed by the six chapters. In chapter 1, I will introduce the lymphatic system which is central to understanding the importance of the receptor of interest, LYVE-1, and its interaction with HA. Next, I will also include a description on the actin cytoskeletal system and its general role in receptor functioning. This will provide the necessary background to understand the biological motivation of this thesis. Finally, I will present an overview of the fluorescent biophysical techniques used to study LYVE-1 receptor organisation and lateral diffusion.

Chapter 2 contains detailed instructions of all the cell lines generated, cell labelling

for imaging and FACS, details of image and statistical analysis, and all other materials and methods that have been used to perform the experiments for this thesis.

In chapter 3, I first characterise the native distribution of LYVE-1 on the surface of lymphatic endothelial cells (LECs). Studying the clustering of receptors in a fixed state is a challenging process with increased chances of studying artefacts. Hence, I optimise the labelling and fixation conditions needed to study the nanoscale clustering of membrane receptors. I briefly introduce the methods employed for fixing cells to study receptor clustering. I use super-resolution Stimulated Emission Depletion (STED) microscopy to resolve the nanoscale clusters formed by LYVE-1, and analyse the clusters using advanced image analysis tools such as Huygens professional.

In chapter 4, I investigate the actin turnover in primary human LECs and its effect on the clustering of LYVE-1, using the optimised protocol established for STED imaging in the previous chapter. I then focus on studying the HA binding effect of LYVE-1 upon depolymerisation of the actin cytoskeleton using FACS.

In chapter 5, I focus on LYVE-1 lateral mobility on the surface of primary LECs modulated by the actin cytoskeleton. The changes in lateral mobility during actin turnover is investigated by using actin depolymerisation drugs and by using advanced fluorescent techniques such as Fluorescence Correlation Spectroscopy (FCS) and fluorescence recovery after photobleaching (FRAP) and STED-FCS to deduced the interaction with actin and LYVE-1. I also provide evidence for a direct interaction between actin and LYVE-1 using co-ip experiments.

Chapter 6 is the final conclusion containing a summary of the results in this thesis and connecting it to a bigger biological picture. It also contains future direction of this project with experiments that are still pending.

Chapter 1

Introduction

1.1 The lymphatic system

The lymphatic system is mostly known for its role in maintaining tissue homeostasis, where it handles the uptake of interstitial fluid that leak from blood into the tissues and returns it back into circulation. Any failure to do so leads to chronic, disabling condition called lymphedema [11]. The lymphatic system is part of the circulatory system and comprises of highly vascular lymphatic vessels (Fig. 1.1 A) and lymphoid organs such as the bone marrow, thymus, tonsils, lymph nodes, spleen, peyers patches and the appendix [12, 13]. The function of the lymphatic system varies depending on the location of the capillaries for example, lymphatics in the intestinal villi are involved in the absorption of lipids from the intestines [12–14] and clearance of dietary induced infections [15, 16]. Most importantly, lymphatic vessels are the principle conduits for soluble antigens, trafficking leukocytes, lymphocytes, and antigen-presenting cells [1, 3, 11, 17] that allow local immune surveillance and efficient immune response to invading pathogens [18]. In the following sections the structure of the lymphatic system along with the functions coordinated by the lymphatics and receptor of interest, LYVE-1, will be introduced.

1.1.1 Architecture of the lymphatic system

The lymphatic vessels are found in almost every organ containing blood supply, including the eye, and the central nervous system [19, 20] but are not present in avascular structures such as hair, nails, cartilage, and epidermis [11]. They form thin walled, blind-ended capillaries called initial lymphatics and continue to form larger pre-collectors and collecting vessels with valves (Fig. 1.1 B). The initial lymphatic capillaries form blind ended, bulbous sacs, and constitute a single layer of endothelial

cells, collectively called as lymphatic endothelial cells (LECs) (Fig. 1.1 C, upper panel). The initial lymphatics lack a continuous basal membrane, pericytes (connective tissue cells), smooth muscles around the vessels and valve structures [17]. The LECs of the initial lymphatics have a characteristic oak leaf morphology with overlapping flaps, which are held together loosely at the initial lymphatic capillaries by specialised interendothelial button-like junctions (Fig. 1.1 D) composed of adherens junctions and tight junctions such as vascular endothelial cadherin (VE-cadherin), endothelial cell-selective adhesion molecule (ESAM), zona occludin-1 (ZO-1), and occludin. These junctions also express the cell adhesion molecule, platelet endothelial cell adhesion molecule (CD31), and LYVE-1 (explained further in this section) at the flaps which are crucial site for leukocyte entry and lymph flow (Fig. 1.1 C, lower panel and D, upper panel). The diameter of the initial lymphatic capillary is approximately 1 - 60 μm having a wall thickness of 50 - 100 nm. They are supported by anchoring filaments (6-10 nm), that keep them from collapsing when the interstitial pressure increases by anchoring it to collagen of the ECM [18, 21]. The lymphatic capillaries are much larger in diameter and also feature the selective and abundant expression of the protein LYVE-1. These characteristic features distinguish them from the blood endothelial cells [12, 16, 22–24].

The initial lymphatics continue to become pre-collectors and larger collecting vessels that are held tightly by zipper-like junctions [3, 22], are surrounded by a continuous smooth muscle, an adventitial layer, and contain valves that prevent backflow during pumping action of the vessels (Fig. 1.1 B & C (upper panel)) [11, 23, 25]. These larger vessels then connect to the lymph nodes, which then contribute to immune surveillance due to soluble antigens carried in the lymph fluid. The collecting lymphatics (50 - 200 μm diameter) contain a single layer of LECs surrounded by a continuous basement membrane, muscle cells, pericytes, and endothelial valves. The muscle layer performs contractile activity that provides a unidirectional flow for the lymph and prevents retrograde flow of the lymph [26].

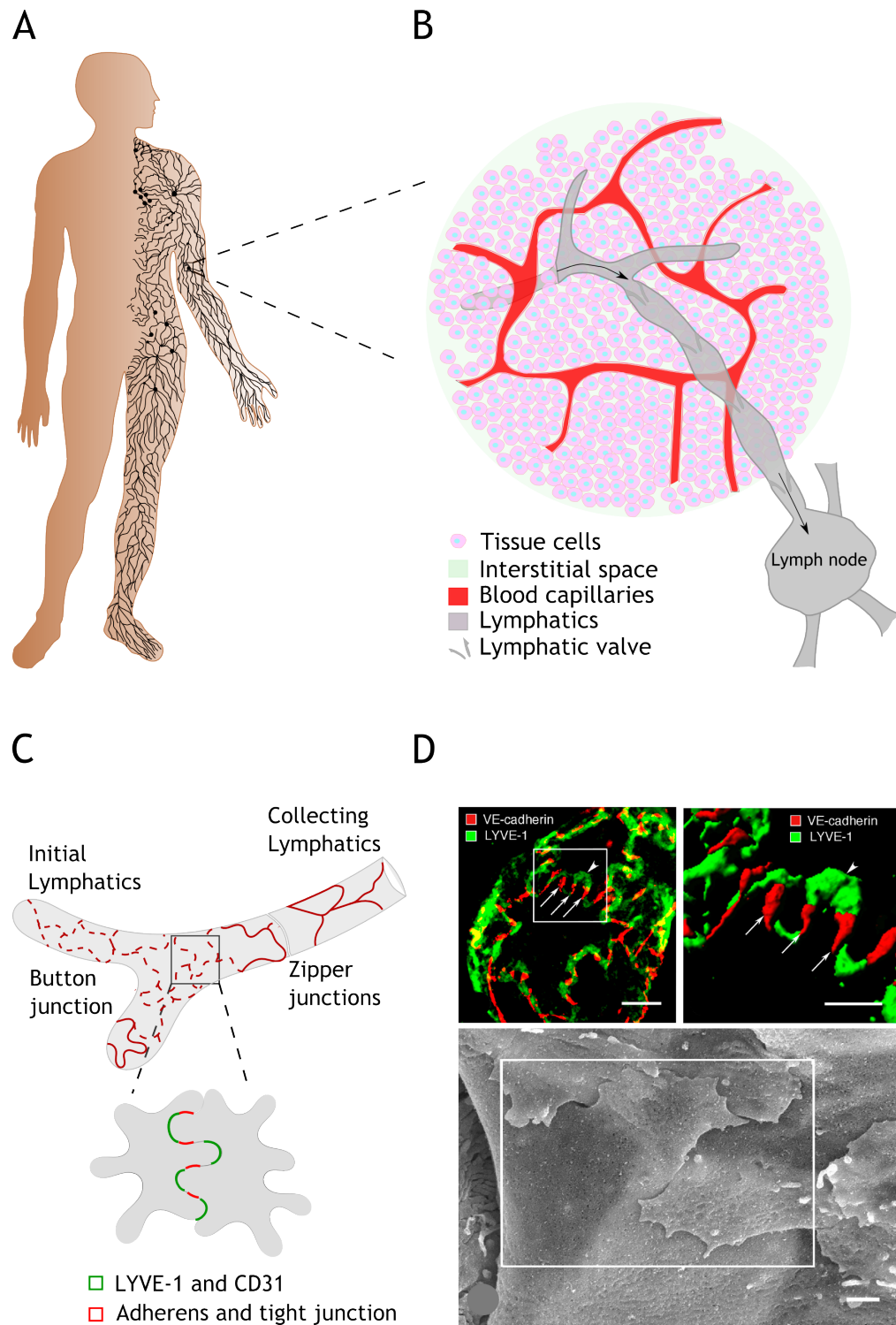


Figure 1.1: The lymphatic system. A) Diagram of the lymphatic capillaries in the human skin. B) Lymphatic capillaries along with blood capillaries (red) draining from tissue to the lymph nodes. C) Lymphatic capillaries showing initial lymphatics and collecting vessels held together by button like junction at the initial end and zipper-like junctions at the collecting ends (upper panel). D) Confocal images of the oak leaf shaped endothelial cells of initial lymphatic showing VE- cadherin at buttons (arrows) and LYVE-1 between buttons (arrowhead) at the borders (left). Enlarged isosurface rendered confocal image stack of boxed region (right). Scanning electron microscopic image showing external surface of overlapping flaps at the junction of endothelial cells of initial lymphatic (below). Scale bar 10 μm (above), 5 μm (below). Images adapted from [11, 22, 27].

The development of the lymphatic system starts at day 9 - 9.5 of the embryonic development. A sub-population of endothelial cells of the anterior cardinal vein commit to the lymphatic lineage by turning on the expression of the transcription factor, prospero-related homeodomain transcription factor-1 (Prox-1) [28, 29]. These cells distinguish itself by expression of several markers that are characteristic to LECs. They include the above mentioned Prox-1, LYVE-1 is also one of the earliest and specific marker expressed by LECs. Apart from these, the cells constitutively express several molecules that distinguish them from blood vessels such as, vascular endothelial growth factor receptor-3 (VEGFR-3), CD31, membrane glycoprotein podoplanin and lack the presence of CD44 [22, 25, 30].

1.1.2 Functions of the lymphatic system

LECs at different anatomical locations such as the lymph node or tumour, express several immunomodulatory molecules on the surface that alter the function and phenotype of the cell upon interaction. LECs of the initial lymphatic regions, produce chemokines such as CCL21, CCL19, CCL27 and adhesion molecules such as ICAM and VCAM that strongly recruit immune cells like mature DC, T cells, neutrophils and M1 polarised macrophages, that lead to the elicitation of the appropriate immune response [1, 2, 31, 32].

The lymph fluid collected from the interstitium contains the soluble self antigens that circulate from the tissue of origin to the lymph nodes [29]. The LECs present in the lymph node sinus function as antigen presenting cells with the ability to present peripheral tissue antigens *via* its major histocompatibility complex I (MHC-1). They also express high levels of programmed death ligand-1 (PD-L1) and low levels of co-stimulatory molecules. This combination leads to the effective elimination of CD8 + T cells that recognise peripheral tissue antigens present on MHC-1 molecule [33–36]. The circulating antigens in the lymph can also be taken up by the immature DCs which then cross present these self antigens to T cells that induce self-tolerance by activating regulatory T cells [12, 16]. Therefore, the lymphatic system also boosts self

tolerance of T cells [12, 16].

Tumour cells often hijack the lymphatic system to disseminate to other tissues and organs. The tumour microenvironment enriched with chemokines and cytokines is capable of tumour-induced lymphangiogenesis thereby contributing to the metastatic potential of the tumours *via* the lymphatic system [13]. The solid tumour cells and circulating immune cells secrete abundant growth factors such as vascular endothelial growth factor-C (VEGF-C). VEGFR-3 on the LECs interact with VEGF-C that lead to generating new lymphatic vessels at the tumour periphery (peritumoral lymphatic vessels) [37, 38]. The high interstitial pressure inside the tumour and the lymphatic vessels, partly play a role in enabling tumour cells to enter lymphatic vessels and to metastasise [25]. Chemokines CCL21 and CXCL12 secreted by the LECs in the tumour microenvironment allow the adhesion, migration and growth of tumour cells by interacting with its cognate receptors CCR7 and CXCR4 [39, 40].

In all the above mentioned scenarios, it is important to understand that the LECs express the protein LYVE-1 abundantly hence potentially play an important role in several of these functions. LYVE-1 interactions with its ligand HA contributes to trafficking of DCs to the LN either from normal peripheral or inflamed tissue for antigen presentation and eliciting an immune response or tumour cells for metastasis [2, 5, 17, 24, 41]. In the following sections, I will introduce the structure and function of LYVE-1, specifically looking at the regulation of LYVE-1:HA interactions.

1.1.3 LYVE-1 structure

LYVE-1, is a 322 amino acid long type-I transmembrane glycoprotein, consisting of a HA binding domain, a glycosylated stalk and a short cytoplasmic domain. They largely exist as dimers and monomers on the surface of LECs and bind to the extracellular matrix component HA (Fig. 1.2 A). It is also expressed on lymph node sinuses, liver and spleen sinusoids and subsets of macrophages. It is expressed on both the luminal and basolateral side of the lymphatic endothelium [23, 41–45].

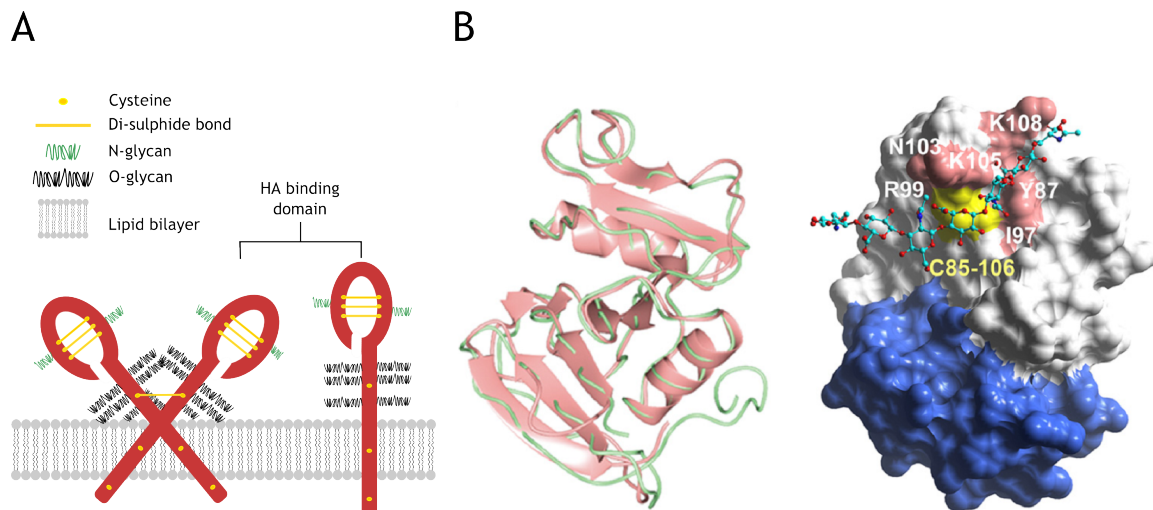


Figure 1.2: LYVE-1 Structure. A) Schematic representation of type 1 integral transmembrane glycoprotein LYVE-1 as dimers (left) held together by a di-sulphide bond at the stalk region and monomer (right), on the membrane surface consisting of HA binding domain with two N-glycosylations (green), a heavily O-glycosylated (black) stalk, transmembrane and cytoplasmic domain. B) Overlay of human CD44 (green) and human LYVE-1 (pink) ribbon representation of the hLYVE-1 HABD modelled using coordinates of the mouse CD44 crystal structure (left) and space-filled representation of the LYVE-1 HABD model with predicted ligand-binding surface (pink and yellow) with a HA octasaccharide docked onto the putative binding site. B adapted from [46].

LYVE-1 was primarily identified as a homologue to CD44, the main HA receptor on leukocytes, by sequence homology using the CD44 amino acid sequence. It has a molecular mass between 60-70 kDa and belongs to the Link module superfamily of C-type lectins that include HA binding proteins [47, 48]. LYVE-1 has a single N-terminal HA-binding domain that comprises the extended link module flanked by two N-glycans and contains six crucial cysteine residues that cross-link to form the HA binding domain. The glycosylated stalk is flanked by several O-glycans and a cysteine residue that is crucial for homodimerisation of the receptor. The transmembrane region also contains a cysteine, but is not involved in receptor clustering and its functional role is less explored. Following this is the short cytoplasmic C-terminus tail comprising of 64 amino acids [48, 49]. The main difference between LYVE-1 and CD44 is the shorter cytoplasmic tail of LYVE-1 and the presence of only 2 sialylated N-glycan instead of 5 present in CD44 [41]. The crystal structure of LYVE-1, especially, the HA binding domain has only recently been solved (Banerji, S., et al., unpublished) but considerable amount of knowledge of the structure of LYVE-1 was deduced using truncation mutations and site-directed mutagenesis of the receptor [46]. *In silico* modelling and secondary structure predictions using the crystal structure

of CD44 were generated for the HA binding domain that helped elucidate the HA binding regions on the domain (Fig. 1.2 B).

1.1.4 LYVE-1 and its interactions

LYVE-1 is known to interact with an array of ligands, such as fibroblast growth factor-2 (FGF-2), vascular endothelial growth factor-A (VEGF-A), VEGF-C, platelet derived growth factor-BB (PDGF-BB), fibronectin and HA [50–54]. Here, the function of LYVE-1 varies depending on the ligand with which it interacts. For instance, binding to FGF-2 causes LYVE-1 to act as a regulatory loop to generate more surface LYVE-1 or when interacting with VEGF-A, C, D and PDGF-BB leads to lymphangiogenic functions often involved in generation of new tumour lymphatic vessels [16, 55].

Apart from these interactions, LYVE-1 predominately interacts with its main ligand, HA *via* the N-terminal HA binding domain. It mediates the uptake and transport of the HA for final degradation to the lymph node in a clathrin/ caveolin independent manner [1, 47]. The interaction between LYVE-1 and HA are the most well characterised and are implicated in numerous functions. These functions specifically elicited by LYVE-1 and its interacting partners, particularly HA, will be introduced in the next sections which is the primary focus of this thesis.

1.1.5 Hyaluronan

Hyaluronan (HA) is a large glycosaminoglycan polymer consisting of linear, repeating disaccharide units [β (1,4) D - glucuronic acid - β (1,3) N - acetyl - D - glucosamine] (Fig. 1.3 A). It is synthesised by transmembrane HA synthases as long unbranched chains (Fig. 1.3 B) and have a molecular weight of 10^5 - 10^7 Da [41, 56]. The turnover rate of HA varies dramatically between tissues, from 2 - 5 minutes to as long as 1 - 3 weeks [57]. It is a major component of the ECM with the total HA content of ~ 15 g, almost 50 % of which is present in the skin. The function elicited by HA largely varies depending on the protein with which it associates such as

LYVE-1, CD44, tumor necrosis factor-inducible gene 6 protein (TSG-6), Aggrecan, Brevican and also the size and chain length of the HA molecule [58–60]. Due to this, the functions of HA are diverse, ranging from space-filling and scaffolding roles in the ECM to the regulation of cell behaviour and progression of disease [61, 62].

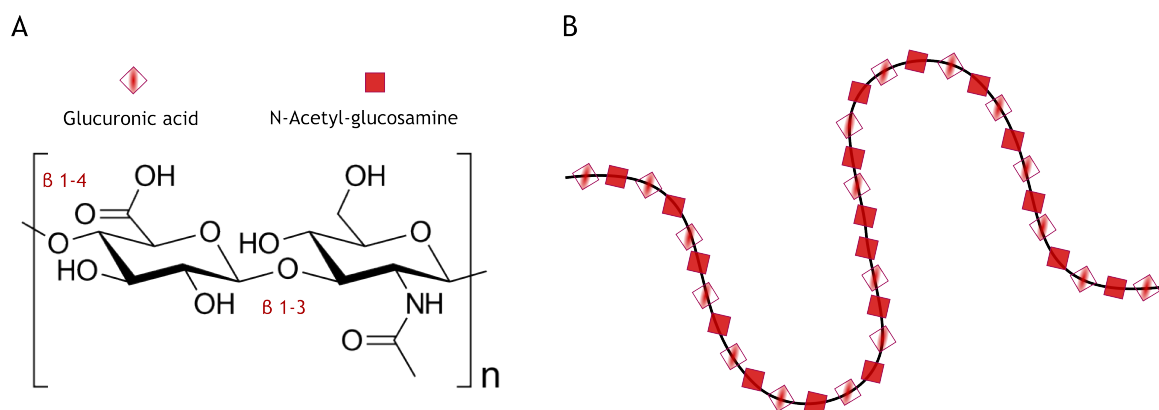


Figure 1.3: Hyaluronan. A) Molecular structure of hyaluronan showing a disaccharide unit of D-glucuronic acid and N-Acetyl glucosamine which are joined together by alternating β 1-3 and β 1-4 linkages to make the HA polymer. B) A native linear chain of hyaluronan with repeating disaccharide units. Images adapted from [23, 63].

The size of the HA molecule generated can induce different cascades of events. The high molecular weight HA present at resting conditions is often broken down to low molecular weight HA and HA oligomers during an injury or inflammatory response [60]. These include fragments, oligomer and complexes of HA. HA fragments that are 500 kDa and below often indicate signs of damage and inflammation. These are mostly recorded in lung and airway inflammation injury. HA oligomers are normally only present in the lysosomes as part of its turnover mechanism, hence finding these suggests a severe inflammatory environment that is accompanied by cell death. These HA oligomers may activate DCs when binding to CD44 along with toll-like receptors (TLR), (TLR 2 and 4) [60, 64]. Alternatively, HA can form complexes that exist as long cables with inter-alpha-trypsin inhibitor (I α I) [65], which are associated with inflammatory bowel disease, or as complexes with TSG-6 that occur during inflammation and tissue remodelling. TSG-6 complexed with HA is persistent in lung inflammation *in vivo* and this complex increases affinity to CD44 and LYVE-1 [6, 60, 61, 66, 67].

Immune cells and tumour cells are activated by proinflammatory cytokines and

inflammatory stimuli which cause an increase in CD44 expression and binding to HA [60]. Thus, HA bound to CD44 can mediate the leukocyte and tumour cells trafficking [5, 41, 58] *via* interaction with LYVE-1 on the surface of LECs. Finally, during the resolution of inflammation HA is partially degraded by macrophages or fibroblasts [60] before being terminally degraded in the LN with the aid of the LECs [47].

1.1.6 Regulation of LYVE-1 and HA interaction

The interaction between LYVE-1 and HA is tightly regulated by several factors such as glycosylation and end terminal sialylation (addition of a sialic acid at the end of the glycans) and receptor conformations [68, 69]. Studies to uncover LYVE-1 regulation and HA binding functions have been done using model systems such as human LYVE-1 (hLYVE-1) transduced cell lines, primary human dermal lymphatic endothelial cells (HDLECs), LYVE-1 knockout murine models and primary mouse LECs (mLECs).

When binding to HA, LYVE-1 directs its uptake and clearance, causes HA mediated lymphangiogenesis or transmigration of immune cells through the lymphatic endothelial cells (LECs) [2, 6, 55, 58, 59, 69]. The functional effect can also vary depending on the size of the HA polymer or the higher order structural form of HA binding to LYVE-1 [6, 23, 41, 58, 70]. Although the function of the receptor binding to HA has been well studied, the mechanism that triggers LYVE-1 function is still under investigation.

LYVE-1 has weak affinity to its ligand HA (125 μ M). Nightingale et al., had identified that affinity to HA was masked by the endothelial mediated sialylation of LYVE-1, thereby suppressing the receptor function in native LECs [68]. Although the LYVE-1 de-sialylation can lead to increase in receptor function *in vitro*, unlike its homologue CD44, there is no mechanism of activating the receptor function by de-sialylation *in vivo*, leaving the receptor in a constitutively silenced state. The glycosylation states of LYVE-1 also contributes to changes in HA binding. Removing the glycosylation on the stalk region (O-glycans) or the cell type used for expression

of LYVE-1 has shown to increase binding to HA to LYVE-1 [46, 68]. This silenced state of LYVE-1 in native conditions due to sialylation or glycosylation has since been attributed to the orientation of the receptor. Using small angle X-ray scattering (SAXS) the LYVE-1 dimer ectodomain was demonstrated to have an open scissors conformation that could contribute to the charged repulsion of ligand binding in native conditions [69].

Recent *in vitro* studies on primary and transfected HDLECs have shown that LYVE-1 binding to HA relies on the receptors clustering on the cell surface. Increasing the overall surface density of LYVE-1 by lentiviral transductions of LYVE-1 (Fig. 1.4 A), or by inducing a local increase in receptor density using cross-linking monoclonal antibody (mAb) 8C (Fig. 1.4 B), lead to an increase in the receptor binding to HA. This receptor avidity induced increase in function, was also achieved if the HA polymer was introduced as a complex (using biotinylated HA and streptavidin or HA and physiologically relevant TSG-6) rather than its native linear form [6].

LYVE-1 HA interactions by clustering was also emulated by HA bound macrophages [6] and in group A streptococcus (GAS) where the surface LYVE-1 clusters encapsulate the HA coat around the microbe *in vitro* in primary HDLECs (Fig. 1.4 D) [4]. More recently, using primary cells isolated from murine skin it has been demonstrated that DCs use LYVE-1 on the surface of mouse LECs to form trans-migratory cups (Fig. 1.4 C) when adhering to the cell surface as a pre-step before ensuing transmigration. These transmigration cups show LYVE-1 clustered around the DCs [3]. These receptor clusters have been speculated to be formed from multiple LYVE-1 homodimers, as they were found as the majority on the surface of HDLECs. Monomeric LYVE-1 were much lower in number and were found to be largely incapable of binding to the ligand HA, even in the presence of cross-linking agents. This allows for the explanation that the receptor avidity and also the configurations could contribute to LYVE-1 function [69].

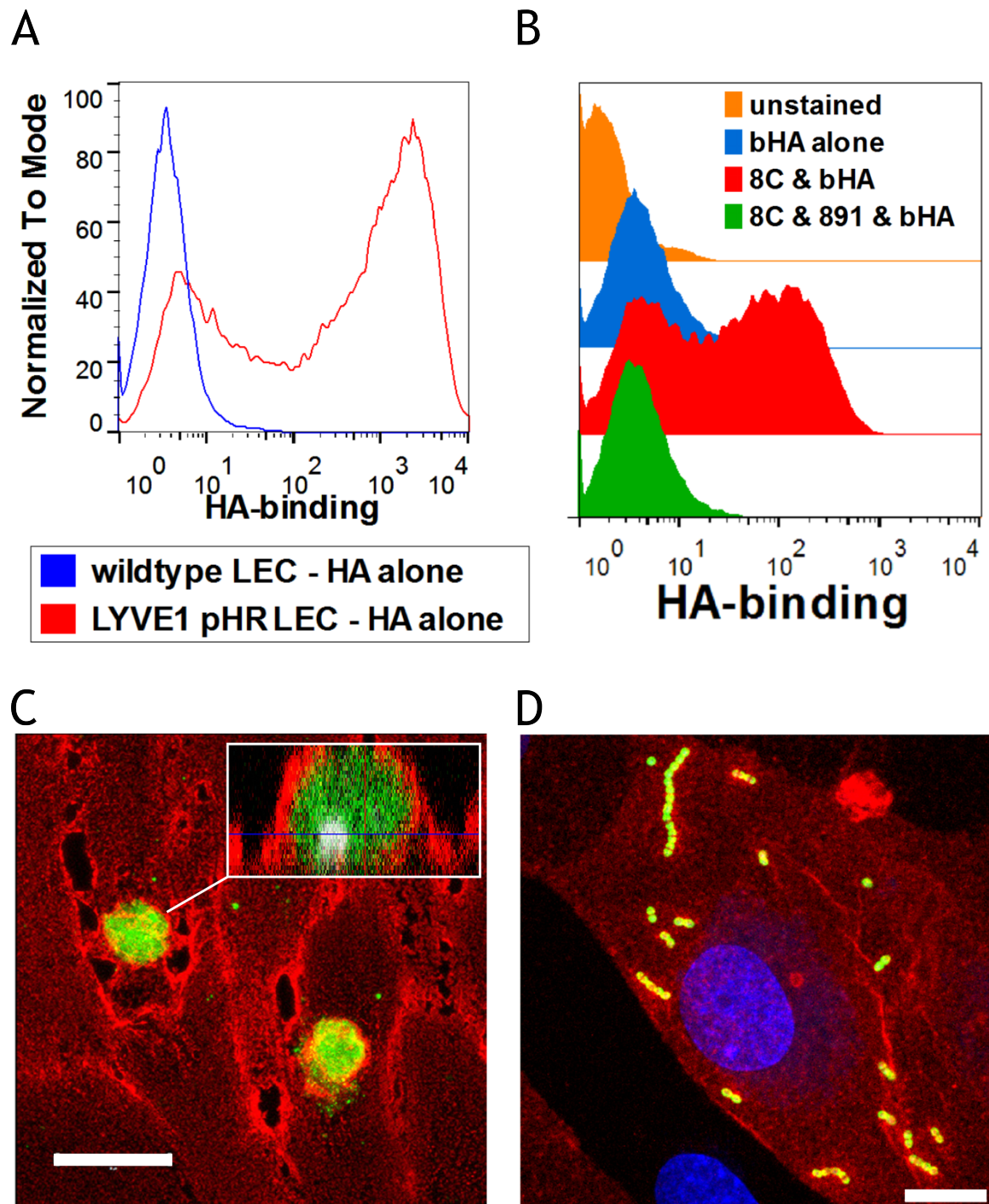


Figure 1.4: LYVE-1 HA Interactions. A) Increase in HA binding, as seen in the FACS histograms, due to increase in overall surface density in LYVE-1 PHR transfected HDLECs compared to WT native cells. B) Local increase in surface density by mAb 8C cross-linking showing an increase in bHA binding in WT HDLECs compared to treatment with control IgG, bHA alone and with a LYVE-1 function blocking antibody. C) Confocal images of mouse LECs showing adherence of mouse DCs (green) showing adherence on LYVE-1 stained (red) mouse LECs. The LYVE-1⁺ LECs forming trans migratory cups around the DCs (inset) after 3 hours on incubation. D) Confocal images of Group A streptococcus (green) being encapsulated *via* its HA coat on LYVE-1 positive WT HDLECs (red). Scale bars: 20 μ m (C) and 10 μ m (D). Images adapted from [1, 4, 6].

The ability of LYVE-1 to form clusters on the surface of the cells indicates that the receptors are dynamic on the membrane surface. Often, the dynamics of receptor and its function are directed by the cortical actin network [71, 72]. The homologue

to LYVE-1, CD44 has been shown to alter its clustering and dynamic properties during actin depolymerisation [8]. It can be speculated that LYVE-1 receptor clustering and dynamics could be similarly influenced by the actin cytoskeleton. This promoted the question of whether the lateral dynamics of the receptors contribute to the avidity induced increase in functions. In order to understand this, the next section will cover an introduction to the actin cytoskeleton and its general role in receptor functions.

1.2 Actin cytoskeleton

The cell cytoskeleton consists of tightly woven mesh of actin filaments (cortical actin and cytoplasmic cytoskeleton), intermediate filaments, actin-binding proteins and microtubules (Fig. 1.5 A), which provide structural integrity to a cell. The cortical actin which lies beneath the membrane is largely composed of F-actin filaments. These filaments are very dynamic and actively turning over thus maintaining the cell in a highly dynamic state. They form a highly dense network of actin filaments that closely interact with the membrane proteins (Fig. 1.5 B) [73]. The filamentous actin scaffold is about 100 - 500 nm thick [9] and has been shown to be involved in a multitude of receptor ligand interactions which lead to changes in cell functions; endocytosis, signalling cascades, receptor organisation, dynamics, and in contributing to motility, cell shape, cell migration and generating or resisting mechanical forces, and stress [74–76].

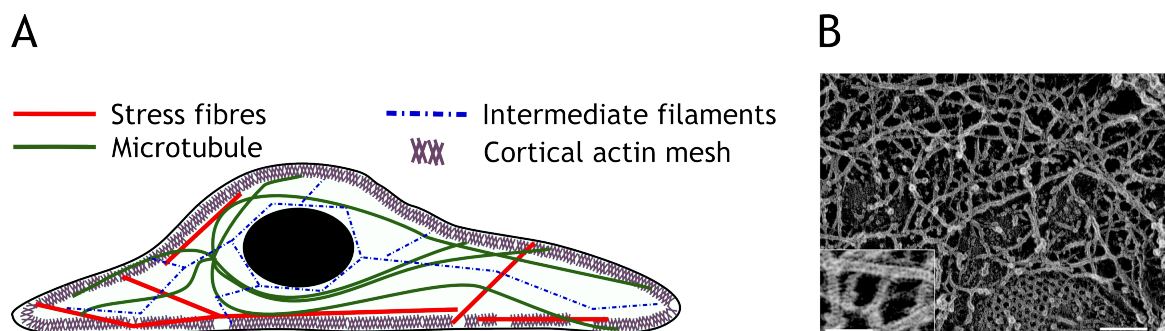


Figure 1.5: Actin Cytoskeleton. A) Diagram of the cell cytoskeleton showing the cortical actin network (maroon), stress fibres (red), microtubules (dark green) and intermediate filaments (blue). B) Electron micrograph images showing dense cortical cytoskeleton below the cytoplasmic surface of Fetal Rat Keratinizing Epidermal Cells (FRSK Cells). The inset shows stripped banding pattern (5.5 nm intervals) of the actin filaments. Scale bar 100 nm (inset, 50 nm). Images adapted from [73, 77]

The actin dynamics in endothelial cells crucially controls the regulation of the endothelium such that it can effectively respond to changes in the barrier functions [76]. Barrier functions can be compromised during an inflammatory response or bacterial infection [76]. This causes the actin filaments to quickly rearrange (1 - 10 seconds to 1 minute) in response to stimulations such as wound healing and inflammation [75, 78, 79]. The actin dynamics controlling endothelial functions are largely studied from cells of the blood endothelium but these mechanisms can also be applied to understand the lymphatic endothelium.

1.2.1 Structure and dynamics

Actin is a 42 kDa protein that is abundantly expressed in all eukaryotic cells and it exists in cells as globular actin (G-actin) and filamentous actin (F-actin) [80, 81]. The actin filaments exist as double stranded helices that contain a barbed end (plus end) and a pointed end (minus end) and are formed by the addition of G-actin monomers. The filament formations (polymerisation) occur in three phases: nucleation, elongation and steady state turnover. G-actin monomers spontaneously come together *via* their adenosine triphosphate (ATP) binding site and form dimers followed by the addition of a third monomer to form a stable nucleation site (Fig. 1.6 A) [82]. Further addition of monomers to this nucleation site contributes to the elongation of the F-actin filaments (Fig. 1.6 C). The G-actin monomers bind to the barbed end and hydrolyse the ATP to adenosine diphosphate (ADP) releasing a phosphate group thus stabilising the filament [83]. The monomer disassociates from the pointed end by the aid of ADF/cofilin and releases the ADP to bind to a new ATP and continue polymerisation [84, 85]. Eventually, once the monomer addition and removal reach a balance at both ends, a steady state of actin turnover is reached which is known as tread-milling [86]. The resulting actin filaments are about 5 -7 nm in diameter [76].

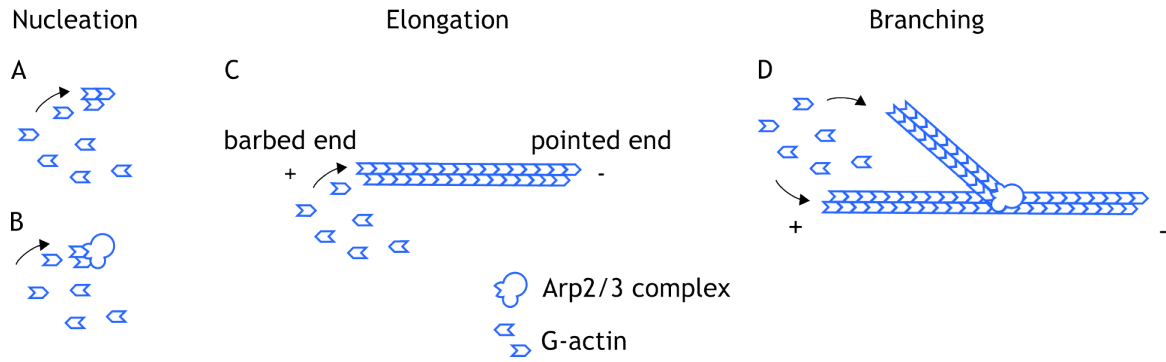


Figure 1.6: Actin formation. A,B) Nucleation occurring either by spontaneous nucleation (A) of G-actin monomers or in the presence of actin nucleator Arp 2/3 (B). C) Elongation at the barbed end by addition of G-actin. D) Branching of actin filaments by binding of Arp 2/3 onto existing F-actin filaments.

The spontaneous formation of actin filaments is energetically unfavourable and so exists a more common process where the G-actin monomers bind specialised proteins called nucleators. There are two forms of nucleators: pointed end nucleators such as Arp 2/3 complex, leiomodin and spire family proteins, and barbed end nucleators such as formins [87]. Pointed end nucleators such as Arp 2/3 generate actin seeds to which monomers are spontaneously added to the barbed end whereas formins create an actin nucleator at the barbed end and assist in the elongation of the filament (Fig. 1.6 B) [88].

The actin nucleator Arp 2/3 is a large 220 kDa complex that consists of seven stably associated subunits. These include the actin related protein Arp 2 and Arp 3 and five actin related complexes Arp C1 - Arp C5 [89]. Arp 2/3 complex generates the actin seeds from the side of a pre-existing actin filament thereby creating a 70° Y-branch (Fig. 1.6 D). This is facilitated by a Nucleation-promoting factor (NPF) that binds to the inactive Arp 2/3 complex leading to a change in the conformation that allows ATP binding [89] hence, stabilising the complex. Once stabilised, it remains at this point forming a branch point and continues the polymerisation and elongation of the actin filaments at the free barbed end [87, 88, 90]. In endothelial cells the functional integrity is maintained by the Arp2/3 complex [76].

F-actin filaments in the cortex exist as two sub-populations both of which have recently been identified to have kinetically different turnover rates. Sub-population one of the actin filament is abundant, making up two thirds of all the filaments. These

filaments are shorter in length and are independent of formin mediated elongation at the barbed ends. The short nature of these filaments allows faster turnover for this sub-population. The second sub-population of F-actin filaments are less abundant, longer and undergo elongation mediated by formins bound to the barbed end. They exhibit a slower turnover despite having elongation mediated by the faster formins [75].

There are several other proteins that constitute the cortical actin which regulate and cross-link the filaments and provide structural integrity and mechanics to the cells. Actin regulators such as F-actin capping proteins (CapZ and tropomodulin) prevent the addition of actin monomers and shift the equilibrium between F-actin assembly and disassembly [91, 92]. Proteins that cross-link the F-actin such as alpha-actinin and myosin II motors organise the actin filaments into dynamic filament network that imparts the mechanical properties to the cell [82].

Other actin filament linkers are the proteins that link the cellular actin cortex to the plasma membrane. Two such crucial proteins that tether the cytoskeleton to the membrane is the ERM family proteins [93] and filamins [94]. Ezrin, radixin and moesin (ERM), known as the ERM proteins, are capable of binding directly or indirectly to the plasma membrane. They interact with the cytoskeleton and transmembrane protein to facilitate membrane organisation and F-actin regulation. They consist of an N-terminal FERM domain through which they bind to the membrane and a C-terminal ERM-associated domain (C-ERMAD) that aid in anchoring to the actin filaments [95]. The FERM domain is capable of binding indirectly to the membrane by associating with transmembrane receptors and membrane proteins [93, 96].

1.2.2 Regulation of receptor functions

The cortical actin cytoskeleton is closely associated with the plasma membrane. The distance between the actin cortex and the membrane, measured in Jurkats and Ptk2 cells, is very small ($<10 - 20$ nm) [9, 97]. This close proximity induces partitioning of membrane, affecting the diffusion of membrane molecules [73]. In normal

conditions, the compartmentalisation formed by the cortical actin, organises the membrane into small compartments of 50 - 500 nm. Thus, the actin cytoskeleton beneath the membrane is intimately involved with the membrane proteins and lipids, allowing it to be a crucial system for controlling receptor signalling [74]. Due to this architecture, the cytoplasmic ends of proteins or receptors might be hindered or tethered to the actin cytoskeleton, thus preventing the molecule from diffusing freely. [72, 78, 97, 98]. Often, in response to cell stimulus, the actin cytoskeleton undergoes rearrangements which contribute to changes in the membrane receptor organisation and dynamics. During this phase, the size of the compartments are constantly changing due to the turnover of the filamentous actin. Therefore, it was well established that the cortical cytoskeleton provides a barrier to free diffusion of membrane proteins *via* steric hindrance [72].

The highly dynamic nature of the actin cytoskeleton allows changes in the distance between the cortex and membrane, initiate structural remodulation, or continuously undergo disassociation-association that alter the filament length and mesh size of the cortex, which allows the membrane proteins to temporarily be released from actin cortex [9, 72]. This highly dynamic shifts generated by the actin modulates lateral diffusion of membrane receptors by, for example, inducing the formation of multi-protein assemblies with lipids and protein, and shift their free diffusive behaviour into confined, compartmentalized, or hop-diffusion [73].

The regulation of actin on receptor functions are commonly studied by observing the nanoscale changes on the surface of receptors and by measuring the mobility and dynamics of the molecule. Often, transmembrane receptors and membrane proteins and lipids are confined within the dense cortical meshwork that contributes, for example, to the receptor clustering. Interactions of membrane proteins with the cortical actin cytoskeleton can be transient interactions that either make the proteins immobile or move slowly. They can also undergo hop diffusion induced by confinement within the cortical actin meshwork which is frequently the most direct reason for receptor hindrance [73, 99–101].

Numerous receptor functions that occur due to receptor clustering are now being linked to the actin cytoskeleton. MHC class I and II [102, 103], IL-1 receptor α -subunit, transferrin receptor (TR) [72], Fc ϵ RI [104], CD1d [7], BCR - IgM, IgD, CD19 [74] and TCR are a few such receptors whose functions are controlled by the actin cytoskeleton. The clustering and dynamics of these receptors controlled by the cytoskeleton often facilitates or inhibits their interaction. Another example for actin regulating receptor dynamics and clustering is the LYVE-1 homologue, CD44. The lateral mobility of CD44, on activated epithelial cells, was determined to be slow when the cholesterol and actin were intact, but increased dramatically when the actin was removed. CD44 was also found to be associated with membrane cholesterol and co-clustered with annexin II in actin rich bundles [105]. Hence, the mechanism of LYVE-1 function and dynamics may be realised by studying the actin cytoskeleton using similar approaches.

1.3 Optical microscopy of receptor dynamics and organisation

Formations of clusters or domains initiated by interactions between proteins, lipids, and protein-lipid complexes indicate that the membrane architecture is highly heterogeneous. The molecular heterogeneities on the surface of cells are dynamic both spatially and temporally [106]. Diffusion dynamics in the cell membrane is usually restricted and hindered due to structural heterogeneity contributed by the membrane curvature, membrane molecules or actin cytoskeleton [101, 107, 108]. This heterogeneity in the membrane can be investigated by measuring spatial protein organisation and the diffusion dynamics of the proteins or lipids in the membrane using optical fluorescence techniques.

An important feature in characterising membrane diffusion of receptors and proteins that contribute to heterogeneity is the diffusion coefficient. Optical techniques such as, Single Particle Tracking (SPT) [72, 109], Fluorescence Recovery af-

ter Photobleaching (FRAP) [75, 110], Fluorescence Correlation Spectroscopy (FCS) [71, 99, 111] are prominently used to measure the diffusion coefficient. Using these techniques, the binding dynamics of the membrane receptors influenced by the transient interaction with cytoskeleton can be studied, which will help identify the molecular paths taken up by receptors such as LYVE-1 to full-fill its functional role.

But all microscopes that use light are limited in their spatial resolution by a physical barrier called the diffraction limit. Light of wavelength λ , focused by a lens with a numerical aperture $NA = n \sin\alpha < 1$ (α being the angle of focused light and n being the refractive index of the medium), cannot discern alike objects that are closer together than a distance of $d = \lambda/(2n \sin\alpha)$ as the diffraction limit causes the images of the objects to merge into a blur [112–114]. For visible light, this lower limit is about 200 nm. In general, the spatial resolution of an optical system is approximately equal to half the wavelength of the excitation light and emission. Hence, objects closer together than 200 nm cannot be distinguished and this is the resolution limit of all conventional far-field microscopes [106, 108].

The size of most of the receptor organisations that form on the cell surface are well below the diffraction limit of the confocal and conventional microscopes. These nanoscale structures thus cannot be resolved, which limits our understanding of the proteins. Other challenges include those of immunolabelling and fixations [115, 116] when studying fixed samples which will be introduced in chapter 3. In order to overcome the limitation of diffraction limit several super-resolution imaging techniques, such as stimulated emission depletion (STED), Photo-activated localisation microscopy (PALM)/ Stochastic optical reconstruction microscopy (STORM), Total internal reflection fluorescence-structured illumination microscopy (TIRF-SIM) have been used to study the nanoscale organisation of the proteins while linking it to their physiological function [7, 117–119]. Each of which have their advantages and disadvantages requiring the use of multiple techniques for complete understanding of the nanoscale dynamics and function [106].

In the following sections, the techniques engaged to study LYVE-1 receptor or-

ganisation and lateral dynamics such as STED, FRAP and FCS will be introduced, highlighting the principle of the methods and its influence in current day biological functioning.

1.3.1 Stimulated Emission Depletion Microscopy

Optical fluorescence microscopy provides a method of studying receptor organization with minimal invasion. However, visualizing the spatial organisation of fluorescently labelled proteins and receptors is limited by the diffraction of light [113]. Recent development of super-resolution far-field fluorescence microscopy (or nanoscopy) surpasses the diffraction limit imposed by light thereby improving the spatial resolution, making it a versatile tool for studying biological interactions. The principle for most super resolution techniques is based around the fluorophore's excited state. A fluorophore is brought from its ground state, S_0 to its fluorescent singlet state S_1 when excited by light of a certain wavelength as shown in the Jablonski diagram [Fig. 1.7 A]. Super resolution imaging takes advantage of reversible switching of the fluorescence states. One such technique is STED which applies stimulated emission to reversibly silence the fluorophores. In STED, the de-excitation of the fluorophore is stimulated by light causing it to transit from the fluorescent 'on' state, S_1 to the dark 'off' state, S_0 , thereby inhibiting the detected spontaneous fluorescence emission [Fig. 1.7 A] [108, 112, 113]).

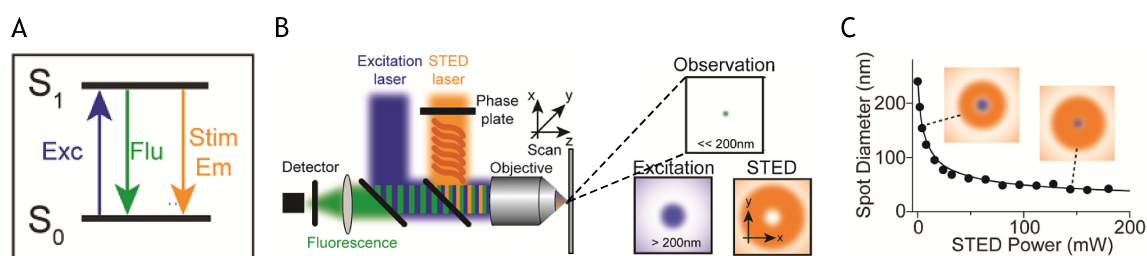


Figure 1.7: Principle of a STED microscope. A) Jablonski diagram of a fluorescence label showing the excitation event (Exc) moving an electron from the ground state S_0 to the excited state S_1 and emission of fluorescence on returning back to S_0 . The STED laser inhibits this spontaneous emission by de-exciting the excited state to S_0 by stimulated emission. B) Schematic drawing of a STED microscope with the objective lens that focuses the excitation light (blue) and STED laser (orange), and detectors that collect the spontaneous fluorescence signal (green). The STED beam passes through a phase plate that generates an intensity distribution with a central zero intensity in the shape of a doughnut thereby creating a sub-diffraction fluorescence observation spot. C) Increase in the power of the STED laser increases the resolution by decreasing the area in which spontaneous emission is still allowed. Images adapted from [113].

The STED laser is chosen such that the wavelength is at the tail end of the fluorophore's emission spectrum and is usually introduced into a scanning confocal system. Its focal intensity distribution has a characteristic doughnut shaped beam (due to a wavefront modifying optical element or phase plate in the beam path) with a central zero intensity. Increasing the intensity of the STED laser leads to a reduction in the area where spontaneous fluorescence emission is still allowed, thus resulting in an increase in spatial resolution [Fig. 1.7 B, C]. This allows two molecules that would otherwise be blurred into one image to be revealed. Therefore, with STED we can obtain images with a sub-diffraction resolution down to about 20 nm in cells [108].

The nanoscale resolution and fast image acquisition provided by STED with minimal to no image processing post image acquisition marks this as a valuable super-resolution technique when compared to the other techniques available [106]. Nevertheless, there are a few limitations that need to be considered with this technique. Due to the use of the high power STED depletion laser most of the conventionally used fluorophores are easily bleached [106]. Another major concern is the high levels of phototoxicity that may be induced by the high power of the STED laser when imaging live cells. The first limitation can be easily overcome by using commercially available photo-stable dyes (such as Alexa fluor, Atto and Abberior Star dyes). Newer protein labelling strategies such as SNAP-, HALO-, CLIP-labelling use organic dyes that are linked to small active groups (O6-benzylguanine derivatives for the SNAP-tag, O2-benzylcytosine derivatives for the CLIP-tag, and primary alkylhalides for the HaloTag[®]), which can easily be attached to the proteins that carry the SNAP, HALO or CLIP tag during expression in cells [120–122]. The second limitation can be circumvented by using slightly lower powers of the STED laser and time-gated STED microscopy or changing immunolabelling strategies [106, 123].

The applications of this techniques is widely popular and has been used in investigating distributions of various proteins, inner cellular structures such as peroxisomes and mitochondria [117, 124]. STED has, for example, been applied to show the organisation of tetraspanins that form functional higher-order complexes, dopamine and

cyclic adenosine 3, 5 - monophosphate-regulated phosphoprotein (DARPP-32) and nicotinic acetylcholine receptor (nAChRs) organisation on membrane surface of cells [125–127]. It has also been used to decipher maturation-induced clustering of Env proteins on HIV virus that depended on the Gag-interacting Env tail [128]. Adhesion *via* VE-cadherin is shown to occur through clustering of VE-cadherin in an actin dependent manner. They exist as clusters on the junction and fuse together to increase barrier function as seen by STED microscopy [76]. Hence, using this technique, the organisation of LYVE-1 clusters on HDLECs can be studied.

1.3.2 Fluorescence Recovery after Photobleaching

Fluorescence Recovery after Photobleaching (FRAP) is one of the commonly used techniques that allows determining the protein turnover of diffusing molecules. In FRAP, a region of interest, of about 1 micrometre in diameter, on the cell is photobleached by high laser intensity and the recovery of fluorescence from surrounding unbleached fluorophores is recorded over time (Fig. 1.8 A). From the recovery curve (Fig. 1.8 B), the half time $t_{1/2}$, of the fluorescence recovery gives the transit time of the molecules from which the diffusion coefficients can be determined. This technique has the added advantage of detecting immobile fractions of membrane proteins [108, 110].

The shape of the recovery curve generates information on diffusive and/ or reactive processes that occur. Diffusive processes occur due to constant protein turnover leading to association and disassociation of the protein while reactive processes are more complex and involve interactions with other proteins that may lead to changes in protein conformations. Using advanced curve fitting function and experimental setup the two processes can be delineated [75, 110]. The only disadvantage here is that the technique offers only a bulk or average mobility of the molecules [129]. Details of the analysis are described in Chapter 2.

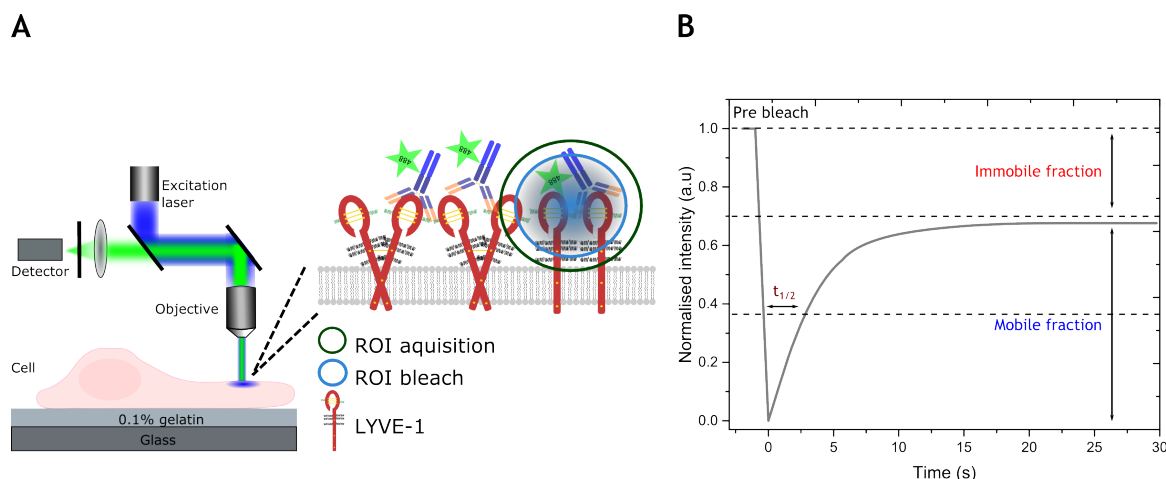


Figure 1.8: Principle of Fluorescence Recovery after Photobleaching (FRAP). A) Schematic diagram of FRAP experiments. Confocal scanning microscope with excitation laser (blue), objective lens, collected fluorescence (green), fluorescence detector and sample (lymphatic cell with labelled LYVE-1 in the plasma membrane grown on a gelatin layer on top of the microscope cover glass, inset): first fluorescent labels are photobleached in a region-of-interest (ROI bleach, blue circle, focus on apical plasma membrane) by intense laser irradiation and then mobility of the labelled molecules measured as recovery of fluorescence signal due to influx of un-bleached molecules (ROI acquisition, green circle) using low laser irradiation. B) An exemplary FRAP recovery curve where the normalised fluorescence intensity is plotted against the recovery time. Initial recovery of fluorescence is rapid followed by a plateau phase. The half-life, $t_{1/2}$ of the fluorescent recovery obtained during the rapid recovery provides the transit time of the molecule. This fraction is termed as the mobile fraction. The difference in recovery from the initial fluorescent state of the molecule, before photobleaching, to that which is fully recovered provides the immobile fraction. Figures adapted from [110, 130]

Several molecular interaction of membrane receptors and proteins were studied using FRAP. For example, transferring lipopolysaccharide (LPS) from CD14 to heat shock proteins (Hsp) (Hsp 70 and 90) [131], movement of TLR receptors (2 and 4) to specialised membrane domains upon bacterial encounter [132] and as previously introduced, CD44 interaction with cholesterol and annexin II occurring in an actin dependent manner [105].

1.3.3 Fluorescence Correlation Spectroscopy

Among the spectroscopic techniques available, fluorescence correlation spectroscopy (FCS) is an efficient tool to determine concentrations, diffusion coefficients, diffusion modes or binding parameters of membrane proteins and receptors. It is frequently applied to various biological systems *via* confocal laser scanning microscopy [133–136].

In FCS, fluorescent molecules diffuse in and out through a single point in the focal volume of the objective (Fig. 1.9 A) at a time. This random diffusion of molecules results in time-dependent changes in the detected fluorescent signal called

intensity fluctuations that arise due to the multiple excitation and emission cycles of diffusing fluorescent molecule. FCS performs the statistical analysis of these intensity fluctuations, which were recorded over time by sensitive detectors, to determine the amplitude and the frequency of the fluctuations (Fig. 1.9 B). For example, in other words, it compares the fluorescent intensity at a given time $F(t)$ with the signal intensity at a later time, $F(t + \tau)$. If the diffusion is fast, then $F(t)$ and $F(t + \tau)$ (Fig. 1.9 B) are similar for smaller τ values. The product of $F(t)$ and $F(t + \tau)$ is averaged over several time points and describes the auto-correlation function $G(t)$.

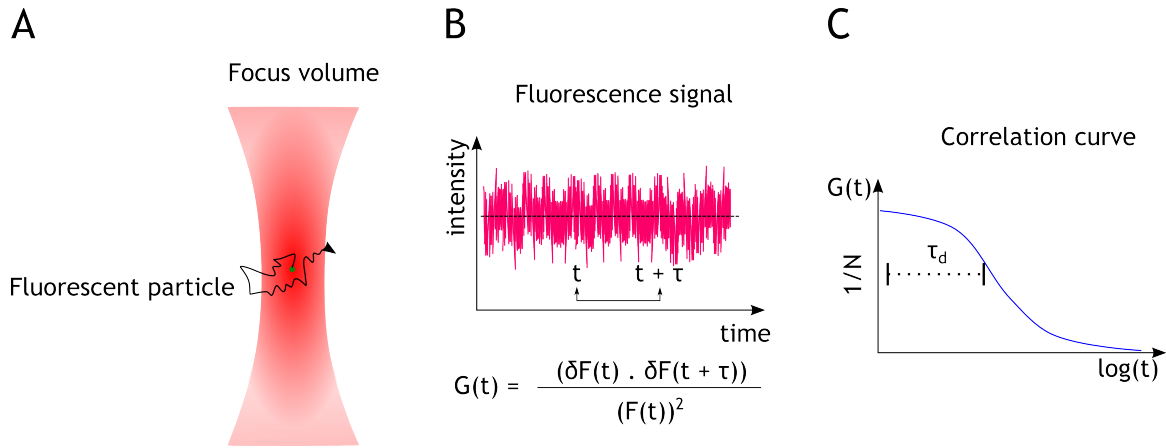


Figure 1.9: Principles of Fluorescence Correlation Spectroscopy (FCS). A, B) Fluorescent molecules (green) traversing through a diffraction limited focal spot (red) (A) which results in characteristic fluorescence intensity fluctuations recorded over time (B). C) The auto-correlation curve generated from the intensity fluctuations showing the amplitude of the curve plotted against time, the half time of this curve can be analysed to give the diffusion coefficient of the molecule. Figures adapted from [134, 137].

This correlation can be described as self-similarity of the signal over time. From the measured intensity trace $F(t)$, an auto-correlation curve of time $G(t)$ can be determined as,

$$G(t) = ((\delta F(t) \cdot \delta F(t + \tau)) / F(t)^2)$$

This auto-correlation curve (Fig. 1.9 C) can be fitted with a mathematical model for functions (for instance, a 2D free diffusion model) with which the average transit time of the molecule can be determined. The amplitude of the autocorrelation curve is inversely proportional to the concentration of molecules ($1/N$). This can often be used to determine the quality of the curves obtained. From this transit time, the diffusion coefficient of the molecule can be determined using the formula, $D = \omega^2 / 8 \cdot \ln \tau_d$, where ω is the full-width-at-half-maximum (FWHM) diameter of the focal volume (i.e 250

-200 nm in confocal setup) and τ_d is the determined transit time of the molecule [99, 135, 138–140].

Some of the main applications of FCS include the study of the diffusion and concentration of molecules, interactions on membrane bilayers [99], and binding of free ligands on membrane bound receptors [141]. The limitations and drawbacks often associated with FCS are easily overcome as FCS can be modified by means of scanning a line rather than measuring a single focal spot or coupling it with super-resolution techniques [108, 142] as described in the following sections.

Scanning-FCS

Scanning FCS (sFCS) is variation of FCS and is ideal for slow diffusing molecules that occur on membrane surface [143]. In sFCS, rather than recording diffusion in a single point, FCS data is recorded over multiple points (as a line or circle). A major advantage is the extended spatial measurement with different time scales that can be assessed. The measurements are carried out pixel by pixel, over time, on the membrane, resulting in multiple points observed simultaneously. The intensity time-series at each point is then correlated and parametrised by fitting using similar models as done with point FCS. This allows acquisition of multiple correlation curves hence improving the statistics of the acquired data (Fig. 1.10 upper panel) [135, 144]. The simultaneous measurement in sFCS between different locations provides information on the heterogeneities exhibited by membrane molecules in the correlation carpet, hence, excellent spatial and temporal resolution (Fig. 1.10 lower panel) [142, 144, 145].

In standard confocal FCS, the long residence time of the fluorophores in the detection area may lead to photobleaching, even for moderate laser powers. This is a major disadvantage of single point FCS. This depletion of fluorophores leads to a decrease of the concentration over time and serious distortions of the correlation curves, giving rise to systemic errors with reduced apparent transit times and larger diffusion coefficients [142]. In sFCS, these artefacts can be easily avoided as the measurements are carried out in different regions at faster scan speed, minimising photobleaching.

Therefore, slow diffusion can be measured accurately within very short measurement times making this technique ideal for measuring dynamics in live cells [135, 142, 144].

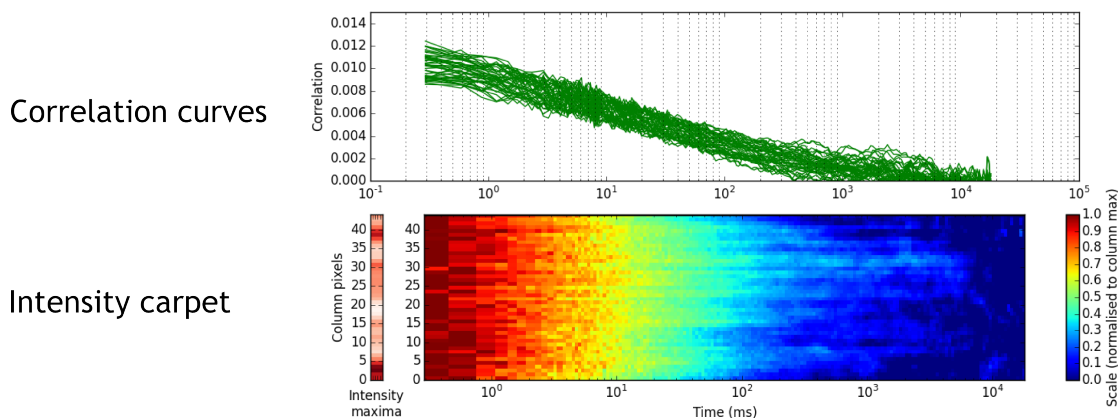


Figure 1.10: Principle of Scanning-FCS (sFCS). Top panel showing multiple correlation curves (green) obtained from scanning a line of 40 μm giving 40 pixels plotted against the time of measurement. Bottom panel shows the correlation carpet plotted against time, which can give information on the heterogeneity of the diffusing molecule. Exemplary sFCS data of LYVE-1 labelled with fluorescent fab conjugate.

STED-FCS

Imaging by confocal techniques is limited by the spatial resolution of the microscope to 200 nm due to the diffraction limit of light as detailed earlier. As a consequence, dynamic molecular events captured by FCS or FRAP gives an average over the diffusion characteristics of the molecules. By combining two versatile techniques, FCS and STED microscopy, a fluorescent technique that can overcome this issue and which gives diffusion information on spatial scales below 200 nm was introduced [100, 108]. Most compartmentalised plasma membrane processes occur at spatial resolutions well below the conventional confocal microscope due to the obstructive nature of the membrane and cytoskeletal components. Thus, a prominent approach to reveal molecular interactions in the plasma membrane of living mammalian cells is studying diffusion of lipids and proteins using STED-FCS.

Molecules in the membrane not only diffuse with varied diffusion constants but also follow different diffusion modes [146]. The diffusion modes can simply be defined as how the diffusion coefficient of a certain molecule changes in dependence of the size

of the observation spot [106, 147]. In STED-FCS, the observation spot of the focal volume is tuned to below 250 nm of standard confocal setting (Fig. 1.11 A, B) by tuning the STED laser power i.e., the higher the STED power, the smaller the spot diameter. The determined diffusion coefficient (D) of the molecule is dependent on the diameter (d) of the observation spot used for measuring. This $D(d)$ dependency measured at varying STED laser powers reveals the diffusion pattern of the molecule of interest [100, 108, 148]

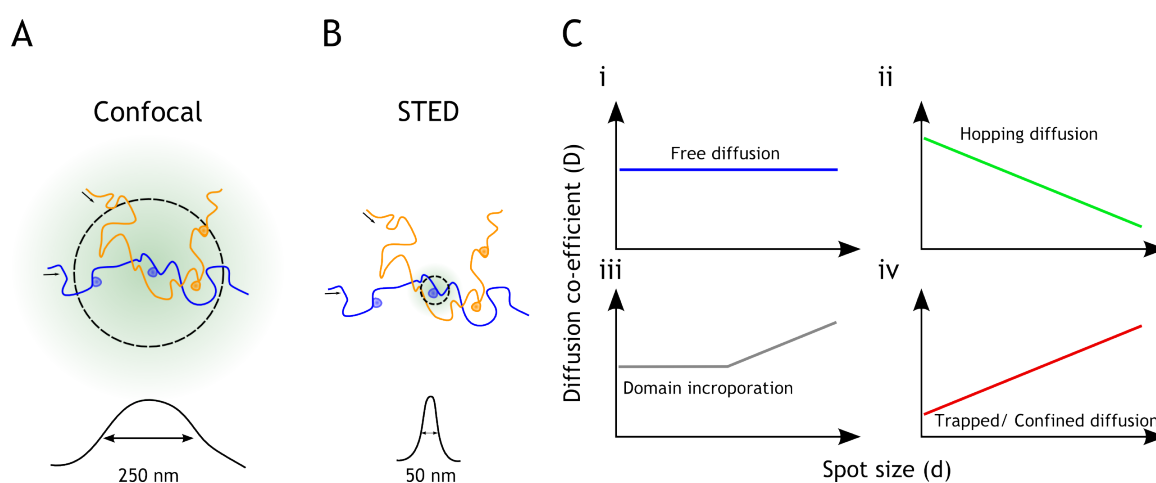


Figure 1.11: Principle of STED-FCS. A, B) Scheme of fluorescent molecules moving through a confocal observation spot (A) unable to discern nanoscale details and through a STED observation spot (B) that can identify hindered diffusion coefficient (D) of these molecules. C) The different diffusion modes that can be determined by tuning the STED observation focal spot to smaller diameters (d). The dependence of $D(d)$ for free Brownian movement (blue), hopping diffusion (green), domain incorporation (gray) and transient trapping or confinement (red). Figures adapted from [100, 108].

For a molecule undergoing Brownian (free) diffusion, the determined D is not dependent on the size of the observation spot. That means the D value will remain constant no matter the size of the observation spot (Fig. 1.11 C (i)). Recent studies have shown that most of the plasma membrane components do not exhibit free diffusion but largely undergo a trapped (confined) mode of diffusion. In this circumstance, the mobility of the receptor is halted due to confinement within the compartment, so the D values decline with decreasing spot size (Fig. 1.11 C (iv)) [100, 101, 140]. They can also exhibit hopping diffusion, especially when they interact very closely with the cortical actin meshwork, which tends to cause the diffusion coefficient to increase with smaller observation spots [71, 113] (Fig. 1.11 C (ii)). Another mode of diffusion occurs as domain based diffusion. Here, the diffusion coefficient only decreases to a

certain point after which it becomes constant. This is due to the molecule halting for a short while as it is incorporated into a domain and the hindrances within the domain are minimum (Fig. 1.11 C (iii)) leading the molecule to diffuse freely [99, 146]. This provides the ultimate tool to study the direct interaction of LYVE-1 with the membrane or cortical actin and its diffusive properties.

1.4 Aims of this thesis

The functions of LYVE-1 have been attributed to the receptor clustering on the membrane surface to create the avidity required to generate a stable interaction to its ligand HA. Clustering of receptors is an active process that often triggers several signalling events and that mostly involves the underlying cortical actin. The receptors use the actin and membrane as a framework to organise themselves to be able to effectively respond to the signalling events. The size and nanoscale organisation of the receptors thus provides vital information on the active states of the receptors and its function. Thus, the native state of LYVE-1 and organisation of LYVE-1 clusters that potentiate HA binding are important to characterise (see Chapter 3).

The dynamics of the actin cytoskeleton is often the main regulator for transmembrane receptor functions. The changes in the filament lengths and mesh size might contribute to the changes in the receptor organisation and hence change the receptor functions. A key aim in this thesis is to determine the role played by the actin cytoskeleton in modulating the LYVE-1 organisation and function on primary cells. Hence, the changes in the clustering of LYVE-1 and its HA binding functions influenced by actin cytoskeleton turnover will be investigated (Chapter 4).

The function of many transmembrane receptors has also been regulated by the lateral diffusion on the membrane surface. This lateral mobility of receptors is also largely influenced by the dynamics of the actin cytoskeleton. The network of cortical actin filaments creates compartments on the membrane that either cause hindrance or stabilisation of the receptors. This can often result in how the receptor changes its function based on its organisation and lateral mobility. Thus, there is potential to study a hitherto unexplored mechanism of actin and LYVE-1 interaction using advanced super-resolution techniques to identify the diffusion mode on LYVE-1 on the membrane. With biochemical assays like co-immunoprecipitation, the direct interaction between the two will also be probed (Chapter 5).

Hence, the main aims in this thesis:

- Identify the nanoscale organisation of LYVE-1 that potentiates HA binding.
- Investigate the influence of the actin cytoskeleton on LYVE-1 organisation and HA binding function.
- Explore the influence of LYVE-1 lateral mobility modulated by the actin cytoskeleton on the membrane surface of primary HDLECs.

Chapter 2

Materials and Methods

2.1 Chemicals

2.1.1 Commercial drugs and chemicals

Cytochalasin D (# C8273, Sigma Aldrich), Latrunculin B (# L5288, Sigma Aldrich), CK-666 (# 182515, Merck Millipore), Jasplakinolide (# 420127-50UG, Merck), Y-27632 (# 688000, Calbiochem Millipore Merck Chemicals), Blebbistatin (# 203391, Calbiochem Millipore Merck Chemicals), Sodium Azide (# S8032, Sigma Aldrich)

2.1.2 Preparation of paraformaldehyde

Paraformaldehyde (PFA) was prepared from powdered PFA (#P6148-500G, Sigma Aldrich, UK) and therefore is methanol free. A 4% stock concentration was prepared by dissolving 4 g of PFA in 50 ml of miliQ water. The mix is heated with constant stirring to 55-60° C (be careful not to exceed this temperature). When the temperature reached 55° C add drop-wise 10 M sodium hydroxide (NaOH) (#10396240, Fisher Scientific, UK) until the solution turns from white to transparent. Remove from heat and allow to cool to room temperature. Once cooled, pH the solution to 7.4 and make up the volume to 100 ml with 2x phosphate buffered saline (PBS). 1% PFA was prepared when required from this 4% stock.

2.2 Antibodies

Monoclonal anti - β - actin (# A5441, Sigma Aldrich), goat anti - human LYVE-1 antibody (# AF2089, R&D Systems), goat anti - human LYVE-1 biotinylated antibody (# BAF2089, R&D Systems), biotinylated phalloidin (# B7474, Molecular probes) and mouse anti- human β actin (# A5441-100UL, Sigma Aldrich).

Secondary antibodies goat anti-mouse Oregon Green[®] 488 (# 06380, Life techno-

logies, UK) and rabbit anti-mouse KK114 (KK114 = Abberior[®] Star Red, Abberior GmbH, Göttingen, Germany), streptavidin Alexa Flour 488 (# S11223 ,Life technologies, UK), IRDye 800cw Donkey anti-mouse (# P/N 925-32212, LI-COR Biotechnology, UK) and IRDye 800cw Donkey anti-goat (# P/N 925-32214, LI-COR Biotechnology, UK).

2.2.1 Production of LYVE-1 monoclonal antibodies

Monoclonal mouse anti-human LYVE-1 (8C) hybridoma cells were grown for 6 weeks and affinity purified as previously described in [1, 68]. 8C hybridoma cells were cultured in HT media (RPMI Media (# R878, Sigma), 1x HT supplement (# 41065012, Invitrogen), 1U/ml Penicillin /Streptomycin (# P0781, Sigma Aldrich), 2 mM Glutamine (# G7513, Sigma Aldrich)) decreasing concentration of fetal calf serum (FCS) from 20%, 10%, 5%, 2.5% and 1%. The cells were split every three days and finally were left in 1% FCS for 2 - 3 weeks after which they were harvested. All media supernatant (S/N) were collected into a 250 ml orange capped conical flasks. The flasks were spun 4000 rpm for about 10 minutes at 4° C. The S/N was filtered through a Steritop Millipore disposable bottle top filter (# SCGPT10RE, Millipore, Germany) and collected into a large Duran bottle (2L). The pH was adjusted using 2M Tris-HCL (to a final concentration of 20 mM). The filtered S/N was run through a Protein G column (# P3296-5ml, Sigma Aldrich) (2x) overnight for affinity purification. The column was then washed thoroughly with 1x PBS. The antibody is eluted with 0.1 M Glycine (pH 3) and collected into Eppendorf tubes containing Tris-HCL (200 mM final concentration, pH 8).

2.2.2 Generation of fluorescent LYVE-1 antibody

LYVE-1 mAbs generated were dialysed using SnakeSkin dialysis tubing (#68100, Sigma Aldrich) according to manufacturer's protocol to remove Tris-Glycine storage buffer. 100 µl of the dialysed antibody (at 2 mg/ml) was conjugated with Oregon Green[®] 488-X succinimidyl ester at a 1:3 Ab:molar dye ratio in the presence of 20 µl

0.1 M NaHCO_3 maintaining the total volume to 150 μl . The reaction was incubated for 1 hour at room temperature (RT) with constant shaking (800 rpm on Eppendorf table top mixer). Unbound dye was removed by filtration through an Amicon 50000 MWCO (3700g, 4000 rpm in the mid-range centrifuge) and washed several times with PBS. The resulting degree of labelling was 4 dyes per IgG molecule, as determined from the absorption spectrum of the labelled antibody, which was obtained using the NanoDrop ND-1000 [149].

2.2.3 Generation of fluorescent LYVE-1 fab antibody

LYVE-1 F(ab) fragments were generated as per manufacturer's instructions (mouse IgG₁ Fab preparation kit, #44980 Thermo Scientific, UK). The obtained LYVE-1 F(ab) was then purified using FPLC gel filtration, concentrated to 100 μl and conjugated as above with Oregon Green[®] 488-X succinimidyl ester and Abberior[®] Star Red NHS ester. After conjugation, the unbound dye was removed using FPLC gel filtration. The resulting fab was concentrated and the degree of labelling and concentration was determined using the NanoDrop as 1 dye per F(ab) molecule [149].

2.3 Cell culture

2.3.1 Isolation of primary human lymphatic endothelial cells

Primary HDLECs isolated from human skin were used for most experiments. The skin was obtained from healthy individuals undergoing plastic surgery at the John Radcliffe Hospital (Oxford, UK). The cells were isolated as per protocol published in [2]. The fat layer from the skin was cut and discarded from the dermal layer. The skin was then cut into 1 cm^2 pieces and placed into FALCON tubes containing 20 ml of Dispase (# 17105-041, GIBCO) solution and allowed to incubate overnight at 4° C in a rotator. The following day, the skin was transferred, epidermal side up, to a 15 cm^3 petri dish containing 15 ml of warm EGM-2 media. The epidermis was removed and discarded using a sterile curved forceps. Then using a blunt scalpel, the dermal layer of the skin was scraped until it lost elasticity. This process was repeated until

all the pieces of skin had been scraped into EGM-2.

A 70 μm FALCON cell strainer was pre wet with 2 ml EGM-2. The scrapped cells from the petri dish were passed through the strainer. The strainer was rinsed with 15 ml of media and then spun at 1500 rpm for 10 minutes. The cells were resuspended in 2 ml EGM-2 and added to a 0.1% gelatin coated T-75 containing 12 ml of EGM-2 to incubate overnight. The following day, the media was removed and the cells were washed cells with 1x PBS and replaced with fresh EGM-2. The growth of the cells was monitored until they grew confluent. Once confluent, the cells were lifted with Accutase (# A6964, Sigma Aldrich), then immunoselected with LYVE-1 mAb and MACS beads (Miltenyi Biotec) as previously described [2]. The resulting cells were cultured in 0.1% gelatin coated T-75 flasks with EGM-2. The cells were checked for expression of lymphatic endothelial cell markers - Prox-1, LYVE-1, Podoplanin, CD31, and an absence of CD44, at an early passage by flow cytometry and immunofluorescence microscopy.

2.3.2 HEK 293T Cells

HEK 293T cells were grown in T-75 flasks containing DMEM (# BE12-604F, Lonza), 10% FCS, 1U/ml Penicillin /Streptomycin and 2 mM glutamine. When required the cells were split using Trypsin EDTA (Thermo Fisher Scientific) for 5 minutes. The trypsin was neutralised using the media and spun at 1500 rpm for 5 minutes. The cells were resuspended in required volume of media and split into 6 well dishes for lentivirus generation or into T-75 for further use.

2.4 FACS staining

Monolayer of confluent primary or transfected HDLECs were rinsed in 1 x sterile PBS and then lifted with 5 ml of Accutase[®]. The Accutase[®] was neutralized using 5 ml of EGM-2, following which the cells were collected into a 14 ml FALCON tube and spun down at 1500 rpm for 3 minutes. The cells were then resuspended into the required volume of FACS wash and aliquoted into a 96 well U-bottom plates and

centrifuged as before. For bHA binding assays: The primary cells were treated with the varying concentrations of actin depolymerising drugs and allowed to incubate for 10-15 minutes at 37° C. The drugs were removed and cells were immediately rinsed once with FACS wash and incubated in 500 µl of FACS wash for 5 minutes. After the incubation, the cells were rinsed with 1x PBS and the cells were lifted with Accutase[®]. Once, the cells were lifted they were neutralised as before and added to 96 well U-bottomed plates. 5 µg/ml bHA was prepared in a 1:1 ratio with streptavidin 488 to form bHA - SA488 complex in FACS wash. This complex was then added to each treated or untreated cells. Once the bHA- SA488 complex was added, they were allowed to incubate overnight at 4° C in the shaker at 800 rpm. The following day, the cells were spun down as before and the supernatant flicked from the wells making sure the cell pellet is still intact. The cells were re-suspended and washed in cold FACS wash twice as above to remove any unbound complex. The cells were then fixed in FACS fix (2% formaldehyde and 0.02% sodium azide) and taken for cell sorting. The cells were run through Cyan (BD Biosciences) or Attune (Thermo Fisher Scientific) analysers and the results FACS plots were analysed using FlowJo Version 10 (FlowJo, LLC).

When labelling cells for checking cell surface expression, antibodies were used for FACS, polyclonal goat anti-human LYVE-1 or monoclonal mouse anti-human LYVE-1 (8C) were made up to a final concentration of 10 µg/ml and allowed to incubate as above at 4° C or for 30 minutes at RT. Next, the cells were washed twice with FACS wash followed by treatment with a fluorescent conjugate against goat or mouse for 20 minutes at RT. The cells were then washed as before and fixed with FACS fix before subjecting to analysis.

2.5 Molecular Biology

2.5.1 LYVE-1 fusion constructs for cell surface expression

LYVE-1 Halo fusion construct: The coding sequence was amplified from a full length cDNA using the high fidelity polymerase pfu Ultra AD (Agilent) with the

following primers:

hLYVE-1 -14 MluI F 5' GCGACGCGTGAAAGGGTAGGCACGATGGCCAGG

hLYVE-1 969 BamHI R 5' CGGGATCCAACTTCAGCTTCCAGGCATCGCAC

Segments of the primers that generate restriction sites are underlined.

ACP LYVE-1 fusion construct: The primers used to amplify a segment for the mature LYVE-1 polypeptide (minus signal peptide): Stop codon is in italics.

hLY 130F BamHI 5' GCGGGATCCTCTTTGCGTGCAGAAGAGC

hLY 969R NotI 5' GCGGCGGCCGCCTAAACTTCAGCTTCCAGGCATCGC

This was ligated into a BamHI/NotI cut pACP-tag(m)-2 to give an in-frame fusion with the ACP tag. This new construct was digested with EcoRI and NotI to give an insert for the fusion of ACP and LYVE-1 that was then ligated into the pACP ADR beta2 plasmid (supplied as a +ve transfection control for an ACP fusion of a plasma membrane receptor) to add the signal peptide (from the 5HT3A Serotonin Receptor) to the N-terminus. The entire assembly was then amplified with:

pACP ADRbeta2 F BglII 5' GCGAGATCTGGATCGTCGCTAGCACCATGC

hLY 969R NotI, as above

The amplified product of both was cloned into derivative of vector pHR Sin [150], carrying the gene for the Halo dehalogenase (# G771A, Promega, UK) or acyl Carrier Protein (ACP) (# N9322, New England Biolabs) (cut with BamHI (compatible with BglII) and NotI), such that the enzyme would form a fusion with the C-terminus or N-terminus of LYVE-1 respectively. The amplified products were then sequenced.

2.5.2 Generation of lentivirus for transduction

The fibroblast cell line HEK 293T were transiently transfected with the pHR Sin LYVE-1-Halo fusion construct plasmid together with pMD.G (encoding the VSV-G surface glycoprotein) and p8.91 (encoding gag and pol from HIV-1) in 6-well plates using Genejuice (Merck, Darmstadt, Germany) according to the manufacturer's instructions. Growth media had been changed immediately prior to transfection to EGM-2 MV medium (# CC-3202, Lonza, UK) for compatibility with primary HD-

LECs. Supernatant was harvested at 48 - 72 hrs post-transfection, passed through a 0.45 micron filter to remove cell debris. Transduction was achieved by adding virus-like particles in 2 ml of the appropriate supernatant to 2×10^2 primary HDLECs. Cells were incubated overnight before the supernatants were replaced with fresh growth medium.

2.5.3 Selection of stable transfected cells

The transfected cells were grown to confluency, they were then lifted using Accutase[®] and labelled using the HaloTag[®] ligand or ACP-tag ligand as described below. The cells were then sorted for high expression of ACP or Halo labelling and sorted into FALCON tubes. These cells were spun down and cultured in EGM-2 and used for experiments.

2.6 Immunolabelling

2.6.1 For fixed cells

Adherent primary or transfected HDLEC monolayers were washed with PBS followed by washing in PBS containing 10% FCS and 0.5% sodium azide (wash buffer). 10 µg/ml of unconjugated LYVE-1 mAb prepared in the same buffer was added to the cells and incubated for 15 minutes at RT. The cells were then washed 3 times in the wash buffer. A final rinse was carried out with PBS. The cells were then fixed with 1% PFA in PBS with or without 0.2% glutaraldehyde (50% in water, # 340855-25ml, Sigma Aldrich, UK) for 10 minutes at RT followed by rinsing with excess PBS. Fluorescent secondary antibody; goat anti-mouse Oregon Green[®] 488 or rabbit anti mouse Abberior[®] Star Red was added and incubated for 10 minutes at RT. The cells were then washed 3 times in PBS and then imaged in Leibovitz's (L-15) phenol red free media (# 21083027, Thermo Scientific, UK) using a confocal/STED microscope.

2.6.2 Phalloidin labelling

A monolayer of HDLECs were grown to confluency in 0.1% gelatin coated ibidi μ -Slide 8 Well Glass Bottom (# 80827, Ibidi, Germany), rinsed in PBS and fixed with 1% PFA for 15 minutes at RT. After fixation, the cells were rinsed with excess PBS and permeabilised with 0.1% Triton-X100 in PBS for 1 min. The cells were incubated with phalloidin Oregon Green[®] at a dilution of 1:100 in PBS for 15 min at RT. The cells were then rinsed with PBS three times and imaged by confocal microscopy.

2.6.3 Live-cell labelling with fluorescent LYVE-1 mAb

10 μ g/ml of the conjugated LYVE-1 Oregon Green[®] 488 were made up in EGM-2 medium and added to the HDLECs. The cells were labelled for 10 minutes at RT followed by 3 washes in PBS. Fresh EGM-2 or L-15 medium was then added to the cells and prepared for imaging. For measurements in fixed conditions, the cells were fixed with either 1% PFA or 1% PFA with 0.2% GA after labelling with the direct conjugates.

2.6.4 Live-cell HaloTag[®] labelling

hLYVE-1 HaloTag[®] expressing primary HDLECs were cultured to confluency in EGM-2 medium on 0.1% gelatin [# 1393-100ml, Sigma Aldrich, UK] coated 18 mm coverslips, WillCo-dish[®] Glass Bottom Dishes (# GWSB-3522, WillcoWells, Netherlands) or ibidi chamber wells prior to imaging. All cells were cultured at 37° C with 5% CO₂. HaloTag[®] Oregon Green[®] Ligand (# G2802, Promega, US) was mixed with EGM-2 medium at a dilution of 1:2000 from the stock. The mix was added to the cells and allowed to incubate at 37° C for 15 minutes followed by washing with PBS and further incubation in EGM-2 medium for 30 minutes. The media was replaced with warm L-15 before imaging.

2.6.5 Live-cell ACP-tag labelling

ACP FL hLYVE-1 HDLECs were plated onto 0.1% gelatin coated glass bottom WillCo-dishes and allowed to grow to confluency. The confluent cells were labelled as per the kits protocol. The enzyme mix with 1 mM CoA 488 (# S9348, NEB) , 1 mM MgCl_2 and ACP synthase (# P9301S, NEB) at 1:40 was prepared in at total volume of 1 ml EGM-2. This mix was added to the cells and allowed to incubate at 37° C with 5% CO_2 for 60 minutes. The cells were washed in sterile PBS times and 1 ml of sterile warm L-15 was added prior to imaging.

2.6.6 Live-cell labelling with fluorescent LYVE-1 fab

Primary HDLECs were grown to a monolayer on 0.1 % gelatin coated 18 mm coverslips. The cells were labelled with 10 $\mu\text{g}/\text{ml}$ of labelled LYVE-1 mAb in L-15 and incubated at 37° C for 10 minutes, with 5% CO_2 . After labelling, the cells were washed three times with warm L-15 and the cells were taken down for imaging in L-15.

2.6.7 Live-cell labelling actin turnover assay

A monolayer of primary HDLECs were grown on 0.1% gelatin coated glass bottomed WillCo dishes® and labelled with SiR actin in EGM-2 media for 1 hour at 37° C. After labelling, the media in the cells were removed and rinsed with warm L-15. It was then replaced with warm L-15 containing CellMask® (# C37608, Life Technologies) at 1:100 dilution and placed at the microscope for imaging. Images of the cells were acquired continuously every minute and after acquiring for a two minutes, either Cyto D or Lat B was added without disturbing the position of the cells and allowed to diffuse into the solution. The images were continuously recorded for 25 - 30 minutes.

2.7 Co-immunoprecipitation Assay

2.7.1 Preparation of actin stabilisation lysis buffer (ASB)

This protocol was modified from [151]. 0.5% Triton-X 100 (# 93443-100ml, Sigma Aldrich, UK), 0.1% SDS (# BP1311-1, Fisher Scientific, UK), 10 mM ethylenediaminetetraacetic acid (EDTA) (# 5134, Sigma Aldrich, UK), 0.5% sodium deoxycholate (# D6750, Sigma Aldrich, UK), 50 mM Tris-HCl, 150 mM NaCl, 0.5 mM ATP (A3377-1G, Sigma Aldrich, UK) was made up in ice cold milliQ water. Protease inhibitors (# 11873580001, Roche) + 200 μ M Sodium orthovanadate (# S6508, Sigma Aldrich, UK), 200 μ M sodium fluoride (# S6776, Sigma Aldrich, UK) and 0.5 mM ATP (# A3377, Sigma Aldrich, UK) were added just prior to lysing the cells.

2.7.2 Sample Preparation

Primary HDLECs were grown to confluency in 6 well plates and remained in culture for a total of 7-10 days. The cells were washed twice in ice cold PBS (1x) and lysed in 200 μ l ice cold ASB. The cell lysis was allowed to incubate for 20 minutes on ice (shaking occasionally). The cell supernatant (S/N) was collected in an Eppendorf and spun down at 15,000g for 15 minutes. The clear S/N was collected into a new Eppendorf. To each S/N fraction 2.5 μ g of b-phalloidin (b-phal) or b-LYVE-1 or 2x Lamelli buffer (LB) (to make up to 1x) was added and incubated overnight at 4° C.

The following day, the required volume of streptavidin sepharose beads (# 20359, Sigma) were washed with 500 μ l of ASB using the spin column at 4000g for 1 minute. Repeat once. Re-suspend the beads in ASB. 30 μ l of the beads were added to each S/N and incubate at 4° C for 4 hours by rotation. Spin the S/N at 4000g for 15 minutes. Discard S/N and wash the beads with cold ASB and spin at 4000g for 1 minute. Repeat wash for a total of 5 times. Finally, add 2x LB (to make a final conc. of 1x) to the beads. Boil the samples to denature at 95° C for 5 minutes. The samples were stored at -20° C to run an SDS page the following day.

2.7.3 SDS-PAGE and transfer

The denatured protein was run on 4-12% Bis-Tris polyacrylamide gels (# NP0322BOX LifeTEch) alongside Precision Plus Protein Standards (# 161-0373, Bio-Rad, UK) using 1x Nu Page MOPS running buffer (# NP0001, Life technologies), at 140 V and until the last protein marker ran out. The gel was rinsed and transferred onto PVDF membrane (Immobilon - FL Transfer membrane, # IPFL00010, Millipore) at 30 V for 90 minutes.

2.7.4 Western Blot

The transferred membrane was blocked with Odyssey blocking buffer (BB) (# P/N 927-40000, LI-COR Biotechnology, UK) at a ratio of 1:1 with PBS for 1 hour at RT. The primary antibodies, goat anti-human LYVE-1 (1:1000) or mouse anti-human β actin (1:5000) were incubated overnight at 4° C. The next day, the membrane was washed three times with PBS-T (1x PBS, 0.1% Tween (# P2287, Sigma Aldrich, UK)) and IRDye 800cw Donkey anti-mouse and IRDye 800cw Donkey anti-goat antibodies. It was allowed to incubate for 1 hour at RT. The membrane was washed as before and then scanned using the LiCor Odyssey Scanner (LiCor Biosciences).

2.8 Confocal Microscopy

Confocal imaging was performed on a Zeiss 780 LSM inverted confocal microscope equipped with a Plan Apochromat 63x oil immersion lens or 40x C-Apochromat NA 1.2 W Corr FCS water objective. Time lapse images were taken at the Leica SP8 TCP inverted microscope with a HCX PL APO 100x oil immersion lens with a numerical aperture of (NA=1.4). For fixed cells, the measurements were performed at RT while all live-cell imaging was performed at 37° C in the presence of 5% CO₂.

2.9 Super-resolution STED microscopy

Primary HDLECs were grown on 0.1% gelatin coated sterile glass ibidi chambers. The cells were grown to confluency for 10 days and were then treated for 15 minutes with 10 μ M Cyto D or 200 nM Lat B at 37° C. The drug CK-666 at 100 μ M was also used to treat a batch of cells. After incubation, the cells were rinsed in warm FACS buffer (1x PBS with 0.05% Sodium Azide and 10% FCS) and incubated for 5 minutes with the same at 37° C. The buffer was removed and the cells were treated with 10 μ g/ml of LYVE-1 mAb and allowed to incubate at RT for 10 minutes. The cells were then washed and rinsed in 1 x PBS following which they were fixed with the combination of 1% PFA and 0.2% GA. The cells were rinsed with excess PBS to remove the fixatives and allowed to incubate with a fluorescent secondary antibody (KK114) for 10 minutes at RT. The cells were then washed three times with PBS and taken for imaging in L-15 or PBS. The STED images were acquired at the Leica SP8 using gated STED at RT by exciting the sample with 633 nm and using the 775 STED depletion laser at 70%. For every treatment at least 10 images were acquired per repeat.

2.10 FRAP measurements

FRAP data acquisition was designed following [110]. FRAP experiments were performed on a Zeiss 780 scanning confocal inverted microscope using a Plan Apo-chromat 63x oil immersion lens. FRAP time-lapses were acquired in a circular imaging region of 1.4 μ m radius comprising a smaller circular region of interest (ROI) with a radius of 1 μ m within the imaging region at the apical cell membrane. The FRAP protocol consisted of three steps: 2 frames of acquisition, a 2 second photobleaching event, and a subsequent FRAP recovery recordings for 30-150 seconds at a rate of 1 frame per second. FRAP images were acquired using the 488 nm laser at 4-7% power. 100% of 488 nm laser power was used to bleach the ROI after 2 frames were acquired. The photobleaching control measurements were performed with the same settings but

without the photobleaching irradiation.

2.11 Fluorescence Correlation Spectroscopy

sFCS

2.11.1 Calibration

To a 0.1% gelatin coated 18 mm coverslip (no. 1.5 thickness) 300 μl of 10 nM of AlexaFluor 488 in water was added and allowed to equilibrate to 37 ° C at the microscope. The confocal sFCS measurements were taken on a Zeiss 780 LSM inverted confocal microscope (Zeiss) equipped with a 40x C-Apochromat NA 1.2 W Corr FCS water objective (Zeiss). Using a laser power of 5 μW . FCS measurement of 5 s in triplicates were taken of the solution and the diffusion time (τ_d) values were obtained by fitting the correlation curve with the 3D membrane diffusion fit in FoCus v.13 software [152]. The diffusion coefficient (D) of the dye can be obtained from the product data sheet (620 $\mu\text{m}^2/\text{s}$ for AlexaFluor 488) is used to calculate the focal diameter (ω) which is used to derived the D values for LYVE-1 diffusion.

2.11.2 sFCS measurement

The cells were allowed to equilibrate at the microscope for a few minutes prior to addition of the drug (Cyto D, Lat B, CK-666 or Jasplakinolide). The drug used was made up in 500 μl of L-15 and added to the chamber and allowed to incubate for 10 minutes. Once the incubation was complete sFCS measurement was initiated. The laser intensity was set to 1% to 2.5% for measuring. A line scan on 5.2 μm , with a pixel dwell time of 3.94 μs was carried out per measurement with a scan time of 472.73 μs . Around 10 - 14 cells were measured per experiment. The correlation curves obtained were fitted with the 2D membrane diffusion fit using FoCus v.13 of the software [152] to determine the transit time of LYVE-1.

STED-FCS

2.11.3 Supported Lipid Bilayer

Supported Lipid Bilayer Supported lipid bilayer (SLB) was prepared as previously described in [71, 99, 123]. A lipid mix of 1mg /ml DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine, Avanti Polar Lipids) and DHPE-AbberiorStar Red (dihexadecanoyl phosphatidylethanolamine, (Abberior GmbH)) at a ratio of 2000:1 were dissolved in Chloroform: Methanol mix (a ratio of 2:1 ratio (v/v) (Sigma Aldrich Co Ltd, UK)). The mix was vortexed vigorously and 20 μ l was dropped onto a Piranha acid (3:1 sulphuric acid and hydrogen peroxide, 15 minute incubation) cleaned microscope cover-glass (25 mm diameter, no. 1.5 thickness, # MIC3350, SLS Ltd) placed on top of a spin coater chuck (KW- 4A Spin-Coater, Chemat Technology). It was immediately spun at 3,200 rpm for 45 seconds. The cover glass was mounted onto a chamber and rehydrated with SLB buffer (10 mM HEPES (Lonza Biologics plc, Slough, UK) and 150 mM NaCl, pH 7.4).

2.11.4 Determination of Spot Diameter

The diameter (d) of the STED observation spot is tuned by the STED laser power (P_{STED}). STED-FCS measurements on the above DHPE- AbberiorStar Red SLB at different P_{STED} was acquired to accurately calibrate the $d(P_{\text{STED}})$ dependency. The STED spot diameter ($\text{FWHM}_{\text{STED}}$) was determined using the following equation,

$$\text{FWHM}_{\text{STED}} = \text{FWHM}_{\text{CONF}} \cdot \sqrt{(\tau_{\text{d,STED}} / \tau_{\text{d,CONF}})}.$$

$\tau_{\text{d,CONF}}$ and $(\tau_{\text{d,STED}})$ are the transit times of the fluorescent molecules through the observation spot in confocal and at varying STED laser power (15 -150 mW), respectively. The confocal observation spot ($\text{FWHM}_{\text{CONF}}$) was set to a diameter $d = 200$ nm. The FCS measurements on the SLBs were carried out for 5 s in triplicates at different spots.

2.11.5 STED-FCS measurement

Primary cells were grown on 0.1% gelatin coated coverslips and allowed to stay in culture for 7 days after confluency. They were then labelled for surface LYVE-1 using fluorescent fab as indicated above. The STED-FCS measurements was carried out for least 5-10 different cells and were measured at different spots for every cell at the different STED powers. The apparent diffusion coefficient (D) for each diameter was calculated according as following, $D = \omega^2 / 8 \ln \tau_d$, where ω determines the diameter of the observation spot and τ_d is the transit times at these varying diameters. This is used to plot the $D(d)$ dependency curve.

STED-FCS measurements were carried out using the commercial Leica SP8 TCP inverted microscope (Leica Microsystems, GmbH, Mannheim, Germany). This setup is equipped with a white-light-laser (WLL, NKT Photonics) for flexible choice of excitation wavelengths, and a high-power continuous-wave (CW) STED laser. The master power of the WLL was set to 30% (1 μ W). Sample excitation at 633 nm was used at 1 - 25% output power (total power of 1-5 μ W). FCS was carried out at STED laser power (15-150 mW), STED-FCS was acquired using a HCX PL APO 100x oil immersion lens with a numerical aperture of (NA=1.4) The images were collected onto a single-photon-counting avalanche photo-diode (APD; Micro Photon Devices, PicoQuant, Berlin, Germany) in the external port of the microscope. The APD signal was recorded with a time-correlated single-photon-counting (TCSPC) detection unit (PicoHarp 300, PicoQuant), which was fully integrated into the microscope, and saves the raw photon stream. The microscope software (SymPhoTime, PicoQuant), allows reconstruction of fluorescence lifetime decays as well as fast calculation of FCS data. The recordings were directly controlled by the Leica LAS AF software, which communicates with the PicoQuant SymPhoTime software as an already integrated FCS package in LAS AF.

2.12 Image Analysis

2.12.1 STED Cluster Analysis

Image cluster analysis of LYVE-1 was performed using Huygens Professional image analysis software (Scientific Volume Imaging, Netherlands). The images were first deconvolved and then further analysed by the object based analysis tool in Huygens which generates the surface area and lateral width of the clusters. Huygens software calculates lateral width as follows: the lateral width was calculated by first obtaining the length of the cluster along the three principal axes. The width of the object was calculated using a search algorithm that acted as a virtual calliper held perpendicular to the length of the object. The calliper was used to scan along the length to obtain several slices of the object. From this, the largest of the smallest width was reported which was represented as cluster size in this study. The lateral width of the clusters provided the size of the clusters that is less likely to be affected by the orientation of sampling.

2.12.2 SiR Actin Turnover Analysis

The recorded images were analysed using ImageJ and the average intensity of actin in all the frames was measured and plotted over time. Small rectangles (yellow) were drawn in several F-actin rich areas and the average intensity of actin was measured for every minute recorded. The average of the intensities from every recorded rectangle for a single time point was calculated and plotted against time.

2.12.3 FRAP analysis

Each individual FRAP curve was processed using the image analysis software, Fiji/ImageJ (RSB, NIH, USA) following analysis strategies as described in [110]. FRAP raw data were extracted for each FRAP recovery curve and plotted using OriginPro 9 (Origin Labs, USA). All curves were time aligned, normalised, plotted and fitted using OriginLab fitting function obtained from [110]. FRAP recovery was

achieved after 50 seconds in all cases, and thus FRAP curves are presented up to 100 seconds. Note, FRAP curves were not corrected for fluorescence background because of the overall low signal to noise ratio presented by the single LYVE-1 receptors. FRAP recovery curve fitting was performed following [75]. FRAP recovery curves $F(t)$ over time t showing free Brownian diffusion were fitted from the photobleaching event until full recovery was achieved using the power-law dependencies $F(t) = F(0) + F(\infty) \times (t/\tau) / (1 + (t/\tau))$, where τ is the characteristic recovery time [110, 116].

Fluorescence recovery is at a rate proportional to $1 - t/\tau$, where τ is $= r^2 / (\gamma D)$, where r is the radius of the photobleached ROI, γ is a constant that depends on dimensionality, i.e. 4 in the case of 2-dimensional diffusion on membranes, and D is the diffusion coefficient of the mobile molecules. Consequently, the two-dimensional diffusion coefficient for membranous diffusion is determined as $D = r^2 / (4 \tau)$ with values of τ obtained by fitting individual recovery curves with the above function. The theoretical time of recovery ($\tau_{\text{theoretical}}$) in Figure 4D was calculated for increasing ROI radii r from the diffusion coefficient of the normal live-cell FRAP data ($D = 0.11 \mu\text{m}^2/\text{s}$ recorded at $r = 1.4 \mu\text{m}$), and the corresponding experimental τ values directly determined from measurement with different ROI sizes [116].

2.12.4 sFCS analysis

The sFCS data were processed using the FoCuS-scan software v.13. Initial bleaching was removed by cropping the first 5-10 seconds of all measurements and bleaching was further corrected by applying a local averaging of 16 second intervals. The curves obtained from this were fitted using a single component 2D diffusion model as described in [144] as follows.

$$G(\tau) = \frac{1}{N} \cdot \frac{1}{(1 + \tau/\tau_D)^\alpha} + O_f \quad (1)$$

τ denotes the correlation time, the α -value was fixed to 1 (no anomalous sub-diffusion), offset O_f was left to vary, amplitude (given as inverse average number of particles in the observation volume) $1/N$ and transit time τ_D were the fitting parameters. The

fitting was performed in the range of 0.5 ms to 4000 ms and the data generated were exported and saved as Excel-sheets.

These were then run through the MATLAB (MathWorks, USA) script written by fellow DPhil student Falk Schneider (Schneider F., et al, unpublished). The analysis of the sFCS data was performed on the basis of the finding that all histograms of transit times were lognormally distributed [144]. The lognormal nature of the sFCS data was a consequence of an inherent bias during the data correlation that results in a larger error for longer transit times along with a distorted point-spread-function due to the scanning process.

The Lognormal distributed data are defined by the Lognormal function that exists in three analytical forms - linear, cumulative, and logarithmic representation. This statistical analysis exploits these three representations. First, the data was read into MATLAB and pre-processed by applying a cut-off value to remove very large transit time values, accounting for bleaching and detector noise. To compute the sFCS histograms, the data sets were binned into 50 equally distributed bins, and normalized to either probability distribution functions for the linear and logarithmic representations, or to cumulative distribution functions in the case of the cumulative representation. The midpoints of the histogram data bars for all bins were extracted to perform the histogram fitting. To increase fitting accuracy, the transit time histograms were fitted in all three representations. First, the cumulative histogram, then the histogram of the logarithmic data and finally the linear histogram were fitted to the respective form of the lognormal distribution. From these fitted values a determined median transit time is obtained from the sFCS data. The diffusion coefficient is then calculated as explained in FCS section. The median values from each replicate were plotted into Graph Pad for determining statistical significance.

2.13 Statistical Analysis

GraphPad Prism 6.0 (GraphPad Software, Inc, USA) was used to perform statistics. The mean cluster size and width from the deconvolved STED images were

plotted in GraphPad and statistical significance was obtained using analysis of variance (ANOVA). A follow-up Dunnett's multiple comparison test was also performed to show a significance between the different fixation conditions, drug treatments, and bHA:SA488 binding assay. The diffusion coefficients obtained from individual FRAP curves were also plotted in GraphPad and an unpaired student t-test analysis was performed to obtain statistical significance. An unpaired t-test was also performed for comparing significance in the co-ip experiments. STED-FCS, sFCS diffusion coefficients were pooled from three repeats following which statistical significance was obtained using ANOVA with Tukey's multiple comparison.

Chapter 3

Surface organisation of the native and cross-linked state of LYVE-1 in HDLECs

Background

Surface proteins or receptors use the cell membrane bilayer and the cortical actin cytoskeleton as a framework to organise themselves so as to respond to various extracellular cues [153–155]. Hence changes in the spatial organisation of these molecules such as clustering are often considered as signs of cellular activation [119, 156–160]. The limited temporal resolution of fluorescent microscopes, insufficient fluorescent labelling, and the transient nature of the clusters, compromise direct visualisation of clustered and non-clustered states [148]. Investigating the diffusion dynamics of the receptors can highlight such clustering and molecular interactions [8, 145, 161] but do not permit direct visualisation of clusters.

One remedy for this problem is to “freeze” cells in a specific state by chemical fixation, thus preserving the true state of receptor clusters for visualisation by optimal immunolabelling. However, in recent years, owing to advanced fluorescence microscopy and labelling techniques, there has been a growing obligation to eradicate the potential artefacts formed during imaging of receptor clustering using immunolabelling, and in particular molecular redistribution induced by the processes used for sample preparation [115, 162–164].

It has been previously demonstrated that antibody crosslinking of CD44 receptors (the LYVE-1 homologue) converts it from a non-HA binding state to a HA binding state. A change in the conformation and/ or microdistribution of CD44 in relation to each other and with other membrane proteins was suggested as a possible mechanism for this increase in HA binding to CD44 [165]. During the course of my DPhil studies it was shown that native LYVE-1 can be induced to form clusters on the surface of

HDLECs by antibody cross-linking. This was found to be a critical event in initiating an increase in HA binding to LYVE-1 [6], suggesting that the distribution of LYVE-1 is crucial for receptor function. Thus, it is important to know whether the LYVE-1 clusters, observed during microscopy are indeed genuine and not artefacts generated during sample preparation. In this chapter, the suitable immunolabelling conditions to study the native and cross-linked clustered states of LYVE-1 distribution were investigated. To achieve this, hLYVE-1 supertransfected HDLECs were treated using one of the LYVE-1 antibodies, 8C, as a primary antibody to cross-link LYVE-1 receptors following which the cells were subject to two fixation conditions and secondary antibody labelling. Fluorescently conjugated monovalent 8C fab fragments, full length bivalent 8C mAb fluorescent conjugates and live cell labelling were performed to determine the native distribution of LYVE-1. 8C is a function potentiating antibody, that specifically binds to the HA binding domain of LYVE-1 which then leads to an increase in HA binding to LYVE-1 [6].

Fixation for immunolabelling is commonly attained using crosslinking aldehydes such as paraformaldehyde (PFA) or glutaraldehyde (GA), or organic solvents such as ice-cold methanol. PFA fixation alone is frequently used and is often assumed to retain the native distribution of the receptors and proteins on the cell surface. However, it is now becoming apparent that redistribution of proteins can occur if this fixation procedure does not completely immobilise the proteins [166], causing artefacts as a consequence due to antibody-induced crosslinking of molecules [115, 153, 167]. During fixation, monomeric formaldehyde (methylene hydrate, $\text{H}_2\text{C}=\text{O}$) reacts *via* its aldehyde group with proteins that are nearby to form $(-\text{CH}_2)$ methylene bridge adducts. The modified proteins form a matrix within which the membrane and lipids are trapped and a natural clustering state is stabilised [168]. In contrast, GA has two aldehyde groups separated by a sequence of three methylene groups ($\text{HCO}-(\text{CH}_2)_3-\text{CHO}$), and being bifunctional, has the potential to covalently crosslink proteins and stabilise clustered states over variable distances [168–171]. The combination of PFA and GA takes advantage of the crosslinking properties of both aldehydes. The small

PFA molecules crosslink proteins rapidly at short distances, while GA molecules promote slow crosslinking over longer distances. This initiates structural stabilisation and thorough stabilisation of protein states [115, 168, 170].

The combination of PFA and GA has been found to be sufficient to fully fix different transmembrane and lipid-anchored proteins, as well as membrane lipids, whereas PFA alone, was found to be inadequate [115]. While it is obvious that residual mobility from membrane receptors after fixation may induce artefacts in fluorescence imaging experiments when immunolabelling, the exact impact of improper fixation on the final images of nanoscale receptor organization has not been depicted so far. Hence, the effect of PFA and the combination with GA will be tested on the clustering of LYVE-1 by confocal imaging. Achieving complete stabilisation of the clustered state of LYVE-1 will be confirmed using fluorescence recovery after photobleaching (FRAP) in cells labelled with fluorescent conjugates of the LYVE-1 antibody (8C). Most receptor clustering events occur at the nanoscale level and conventional confocal microscopes are limited by diffraction to resolve these clusters. Optimal visualisation of clusters therefore requires the application of more sophisticated techniques such as super-resolution STED microscopy. Hence, in light of reports indicating that LYVE-1 can undergo clustering in the plasma membrane as part of their physiological functions [6], the nanoscale spatial organisation of cross-linked membrane LYVE-1 will be investigated using super-resolution STED imaging.

Aims

1. Identify the native state of LYVE-1 on the surface on HDLECs.
2. Optimise fixation conditions to study clustered state of LYVE-1 for STED microscopy.
3. Image and analyse LYVE-1 clusters on HDLECs using the optimised conditions.

3.1 Identifying the surface distribution of LYVE-1

LYVE-1 receptors have a low affinity for the ligand HA, thus requiring high avidity induced by antibody cross-linking or increased surface density of LYVE-1 [6] (see Introduction) marking LYVE-1 clustering an important phenomena to investigate. For this, hLYVE-1 with a C-terminal Halotag[®] plasmid was stably transfected into primary HDLECs (see Methods). First, the native distribution of LYVE-1 and the LYVE-1 antibody induced crosslinked state was investigated. To identify the surface distribution of LYVE-1 before and after 8C antibody induced cross-linking, hLYVE-1 transfected HDLEC monolayer were labelled with monovalent LYVE-1 (8C) fab, or bivalent LYVE-1 (8C) mAb, conjugated with OG 488 NHS ester dye, followed by fixation with 1% (w/v) PFA. From here on, these will be referred to as fluorescent LYVE-1 fab or LYVE-1 mAb respectively. The labelled cells were imaged by conventional confocal microscopy. This study revealed a change of receptor organisation from an unperturbed state using fluorescent LYVE-1 fab antibody (Fig. 3.1 A) to a punctate clustered state (Fig. 3.1 B) after cross-linking with fluorescent LYVE-1 mAb. The unperturbed state was also apparent when LYVE-1 C-terminal Halo tagged HDLECs were labelled with membrane permeable HaloTag[®] OG 488 ligand in live, unfixed conditions (Fig. 3.1 C), as was the presence of some larger LYVE-1 loaded intracellular vesicles (white arrow heads).

Next, to study the avidity induced receptor function, the LYVE-1 clusters formed by primary antibody cross-linking using LYVE-1 bivalent mAb was imaged using super resolution STED microscopy. The cells were incubated with bivalent LYVE-1 mAb 8C and 1% PFA fixation as before and then exposed to fluorescently conjugated secondary antibody (Fig. 3.1 D) to achieve the amplification often required for STED microscopy. Surprisingly however, the resulting confocal and STED images revealed an altered distribution of LYVE-1, with larger receptor clusters on the membrane surface than when cross-linked with fluorescent LYVE-1 mAb (Fig. 3.1 E).

These results suggested that native LYVE-1 shows an almost homogeneous dis-

tribution on the surface of HDLECs. Fixing the cells with 1% PFA fixation to study cross-linked LYVE-1 clusters had not completely fixed the surface LYVE-1 after initial antibody incubation, leading to an artefactual post-fixation clustering by the secondary antibody [116].

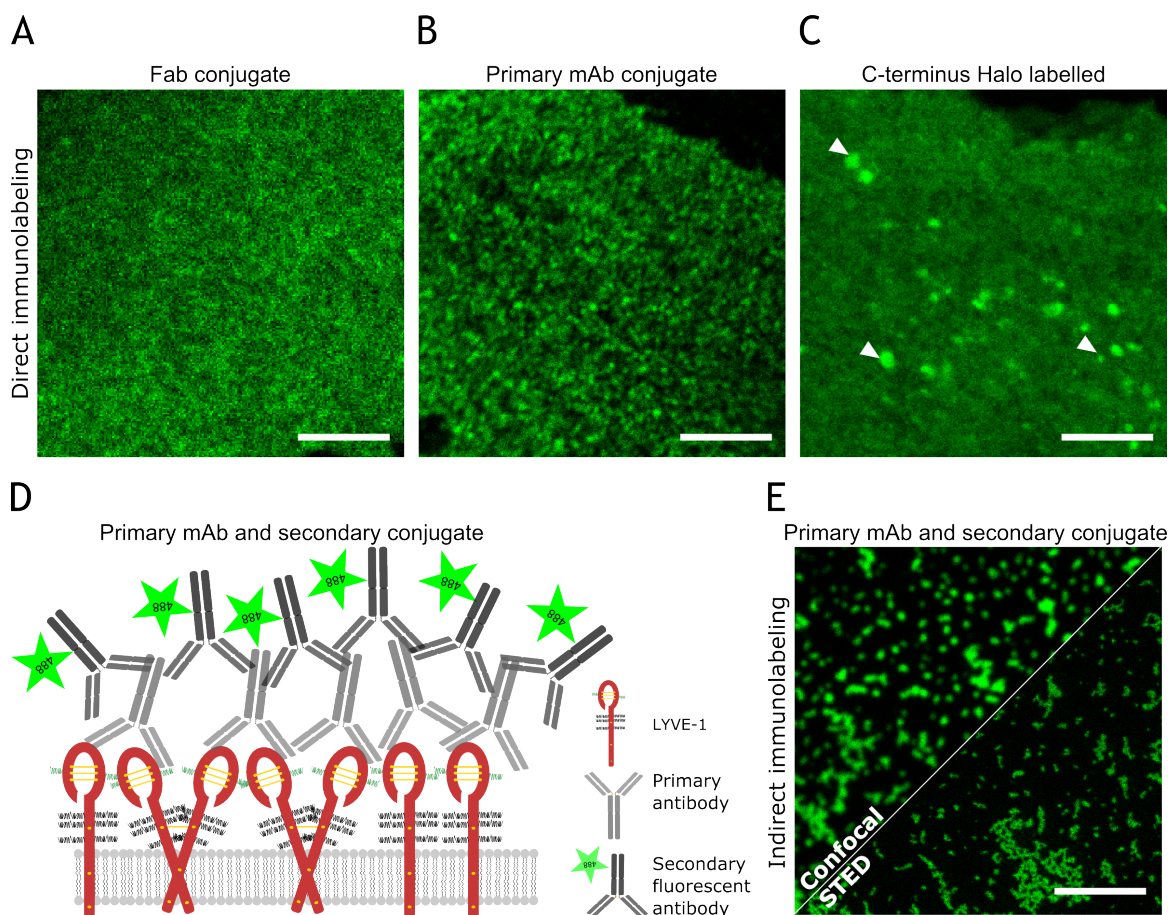


Figure 3.1: Fluorescence microscopy of LYVE-1 surface distribution in C-terminus HaloTag® transfected HDLECs. A-C) Confocal recording of LYVE-1 visualised with Oregon Green® 488 LYVE-1 fab fragment (A), Oregon Green® 488 LYVE-1 bivalent mAb (B) added prior to fixation with 1% PFA, and in live cells using Oregon Green® 488 membrane permeable HaloTag® ligand (C). D) Scheme of indirect immunolabelling of LYVE-1: monomeric or dimeric transmembrane LYVE-1 (red, glycosylation sites drawn as black curly lines) is first cross-linked by a primary antibody (grey), fixed and then targeted by a fluorescently tagged (green) secondary antibody (black). E) Confocal (upper left) and STED (lower right) recordings of LYVE-1 on the surface of HDLECs that had been incubated with primary LYVE-1 mAb, then fixed in 1% (w/v) PFA before addition of Oregon Green® 488 secondary antibody. Scale bar, 5µm. The images are representative of 3 replicate experiments. Images from [116].

3.2 Optimising fixation conditions to study the clustered state of surface LYVE-1

Super-resolution studies on mitochondrial protein, Tom 20, have shown that using PFA alone when immunolabelling causes an artefactual distribution of the protein resulting in an enlarged mitochondria which was abrogated when combining the fixative

with GA [164]. Hence, whether this fixation method could prevent the clustering artefacts encountered with LYVE-1 was investigated. Figure 3.2 compares confocal images of LYVE-1 in HDLECs that have been labelled with primary LYVE-1 mAb antibody, then fixed with 1% or 4% (w/v) PFA either alone (Fig. 3.2 A, upper panel) or in combination with 0.2% (w/v) GA (Fig. 3.2 A, lower panel), prior to detection with a fluorescent secondary antibody. Cells fixed with PFA in the presence of GA displayed clusters that were similar in occurrence to the ones observed after incubation with direct labelling using fluorescent LYVE-1 mAb (Fig. 3.1 B), unlike the altered distribution of clusters formed in cells receiving the bivalent mAb, fixed with PFA alone and treated with fluorescent secondary antibody. These results highlight that artefactual clustering induced by the secondary antibody can be prevented by use of additional fixatives like GA.

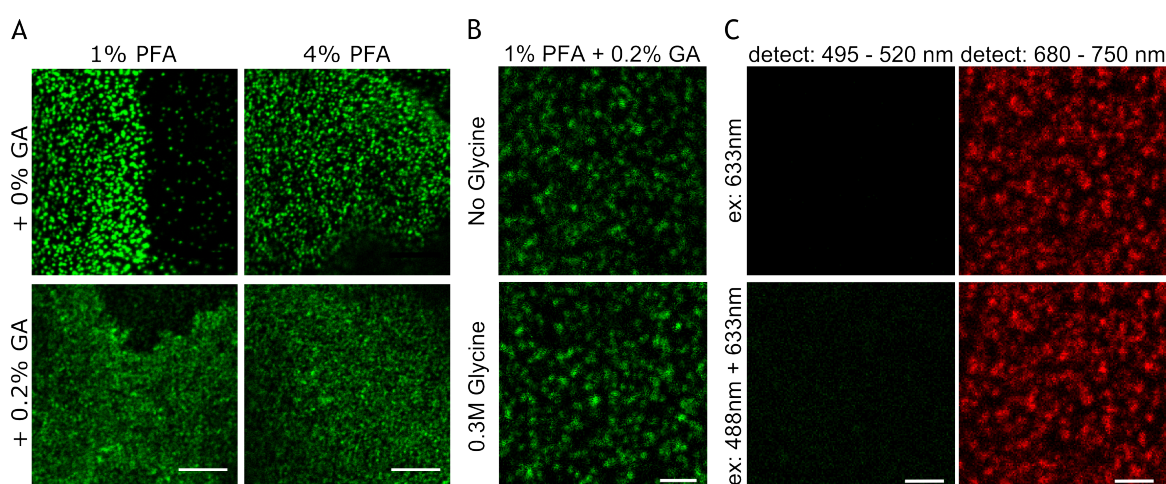


Figure 3.2: Confocal fluorescence microscopy of LYVE-1 surface distribution in HDLECs. A) Images of LYVE-1 surface distribution in HDLEC visualised with LYVE-1 primary mAb and Oregon Green® 488 conjugated secondary Ab added after fixation with 1% (left panel) and 4% PFA (right panel) in the absence (upper panels) and presence of 0.2% (w/v) GA (lower panels). B) Effect of quenching without (upper panel) and with 0.3 M glycine (lower panel) on surface distribution of LYVE-1. C) Detection of autofluorescence by imaging far red labelled LYVE-1 clusters in two detection ranges of 495-520 nm (left) and 680-750 nm (right) after excitation with 633 nm alone (upper panel) or 488 nm and 633 nm (lower panel). Scale bars, 5µm

Fixation with GA has been reported to generate autofluorescence [164], therefore, to test if the cells exhibited autofluorescence after GA fixation two experiments were performed. Primary HDLECs were incubated with LYVE-1 antibody and fixed as above in the presence of GA followed by either only washing in 1x PBS (Fig. 3.2; B upper panel) or followed by with quenching using 0.3 M glycine (Fig. 3.2 B; lower panel) before adding the secondary antibody. In both conditions, there was no

evident changes in LYVE-1 clustering. For LYVE-1 labelling, glycine treatment did not improve the single to noise ratio as indicated by Whelan et al [164]. Alternatively, the cells were labelled as before (without glycine) but a secondary antibody with a far red dye such as KK114 was used and confocal images were recorded. To detect the autofluorescence, the cells were either excited with 633 nm wavelength light (Fig. 3.2 C, upper panel) or a combination of 488 nm and 633 nm (Fig. 3.2 C, lower panel) with detection gates set at 495 - 520 nm (Fig. 3.2 C, right panel) and at 650 - 750 nm (Fig. 3.2 C, left panel). In both excitation conditions, signal was detected only in the far red detection range 650 - 750 nm with no signal in the range between 495 - 520 nm suggesting there was no autofluorescent signal emitted by the GA fixed cells in the observed wavelength ranges.

To further corroborate the effect of GA on complete fixation of membrane LYVE-1, the effect of PFA and GA on the mobility of LYVE-1 was evaluated using fluorescence recovery after photobleaching (FRAP). The mobility of LYVE-1 on either live cells, cells fixed with 1% PFA alone, or cells fixed with both 1% PFA and 0.2% GA were investigated by labelling the cells with the fluorescently conjugate bivalent antibody. Figure 3.3 A shows the confocal images of LYVE-1 within the selected region of interest (ROI) at different time points during acquisition showing a complete recovery by 100 seconds. Fluorescence recovery curve obtained from this data was plotted as a function of time with the fraction of mobile receptors undergoing complete recovery of fluorescence while immobile receptors stay at the photobleached level. For all conditions, the bleaching control was obtained in the same cells as separate measurements without the preceding photobleaching irradiation.

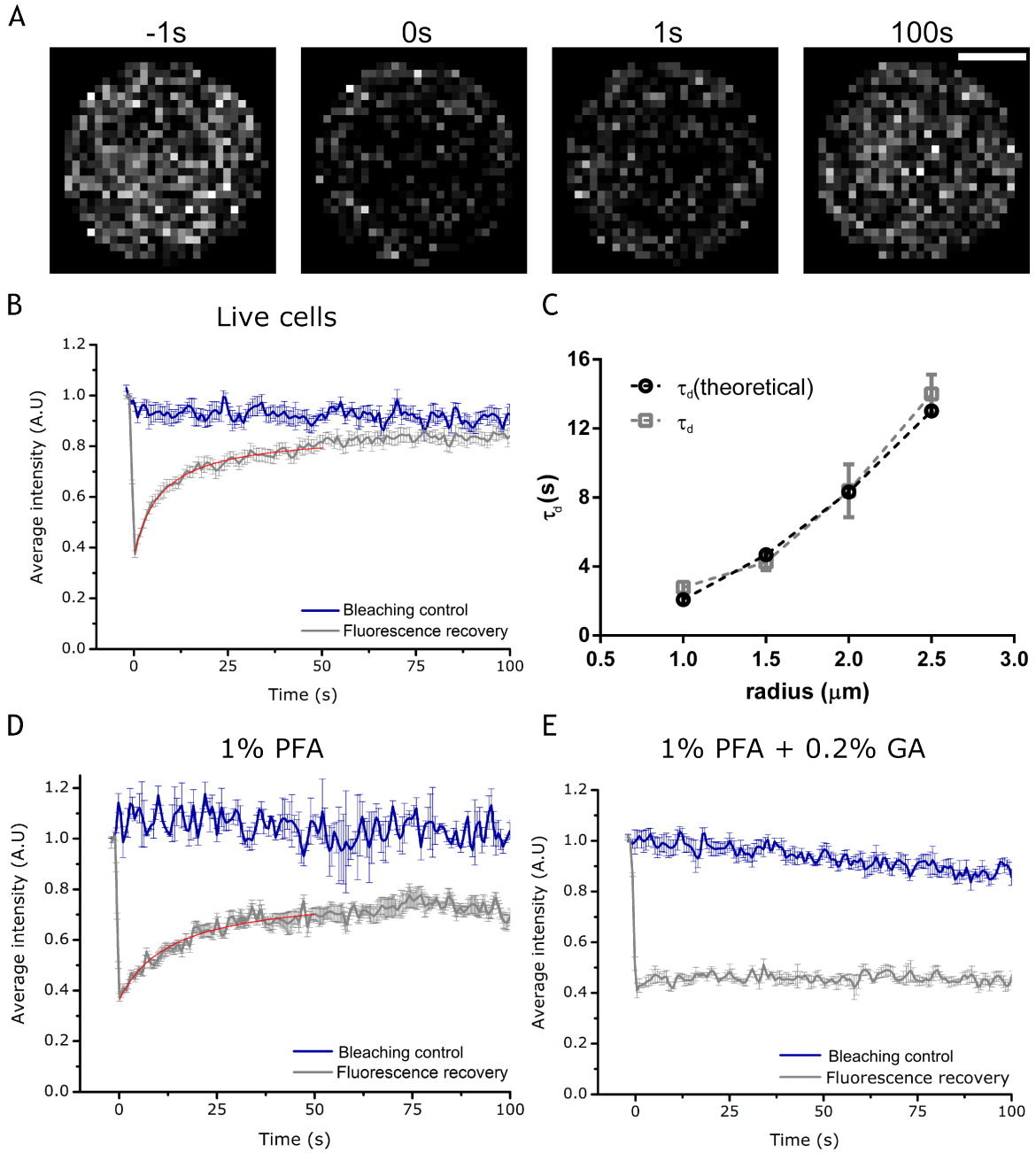


Figure 3.3: FRAP analysis of LYVE-1 in HDLECs labelled with fluorescent (Oregon Green[®]488) primary antibody mAb. A) Confocal images of LYVE-1 in living cells within the ROI at different time points with respect to the photobleaching (as labelled) depicting the recovery of fluorescence signal after the photobleaching. B) Fluorescence recovery of LYVE-1 as a function of time in live cells. C) Fluorescence recovery time τ of LYVE-1 in live cells (grey) determined for different radii of the ROI, depicting a quadratic dependence as expected for diffusion (theoretical in black). D) Fluorescence recovery of LYVE-1 as a function of time in cells fixed with D) 1%PFA and E) 1%PFA + 0.2% GA. Grey curve indicates original fluorescence recovery data as average over at least 14 measurements (live cells) and 5 measurements (fixed cells) at different sample positions and with error bars depicting the standard deviation of the mean, red curve represent fit to the data (see methods), and blue curve bleaching control data taken without photobleaching pulse depicting photobleaching during observation. Scale bar, 1 μm .

In live unfixed cells (Fig. 3.3 C), a complete recovery of fluorescence was achieved in less than 100 seconds, resulting in 100% mobility for LYVE-1 receptors with an average diffusion coefficient of $0.11 \pm 0.05 \mu\text{m}^2/\text{s}$. Diffusion time obtained from the recovery curves can give information on the diffusion process of the molecule (i.e, freely

diffusing on the cell surface or undergoing a reactive process (See Introduction). This can be visualised by recorded by measuring fluorescence recovery at varying radii [110]. FRAP data of LYVE-1 was recorded at increasing radii from 1 μm to 2.5 μm to ensure that the diffusion seen in (Fig. 3.3 B) is a freely diffusing process. The dependence of fluorescence recovery of time, τ , against the ROI was quadratic, as expected for free diffusion [110] (Fig. 3.3 D). Consistent with the images, FRAP data taken for cells fixed with 1% PFA alone show that more than 70% of LYVE-1 receptors were still mobile with a diffusion coefficient of $0.07 \pm 0.02 \mu\text{m}^2/\text{s}$ that was not significantly different to live cells (Unpaired student t-test; $P=0.064$). Strikingly, the lateral mobility of LYVE-1 was completely abolished upon fixation with 1% PFA and 0.2% GA (Fig. 3.3 E). Thus, it can be concluded in the indirect immunolabelling experiments artefactual clustering of LYVE-1 was caused by accretion of the unfixed LYVE-1 receptors and the added secondary antibody.

3.3 Analysis of LYVE-1 clusters using STED microscopy

Having established that GA is required for complete fixation of surface LYVE-1, the next aim was to quantify LYVE-1 mAb induced cross-linked clusters that potentiate HA interaction. hLYVE-1 Halo transfected HDLECs were incubated with 10 $\mu\text{g}/\text{ml}$ of LYVE-1 antibody followed by fixation with 1% PFA (w/v) and increasing concentrations of GA (0% - 0.2%), prior to adding the fluorescent secondary conjugate. Figure 3.4 A depicts STED images of cross-linked LYVE-1 that were acquired and the analysis of deconvolved STED images, using Huygens Professional Software, as described in the methods section. The analysis of the deconvolved STED images show a co-ordinate reduction in cluster size, decreasing from 100 nm^2 to 35 nm^2 and in width from 165 nm to 105 nm with increasing GA concentration ($P = <0.0001$; 1-way ANOVA with Dunnett's multiple comparisons) (Fig. 3.4 B) confirming the effects of improper fixation.

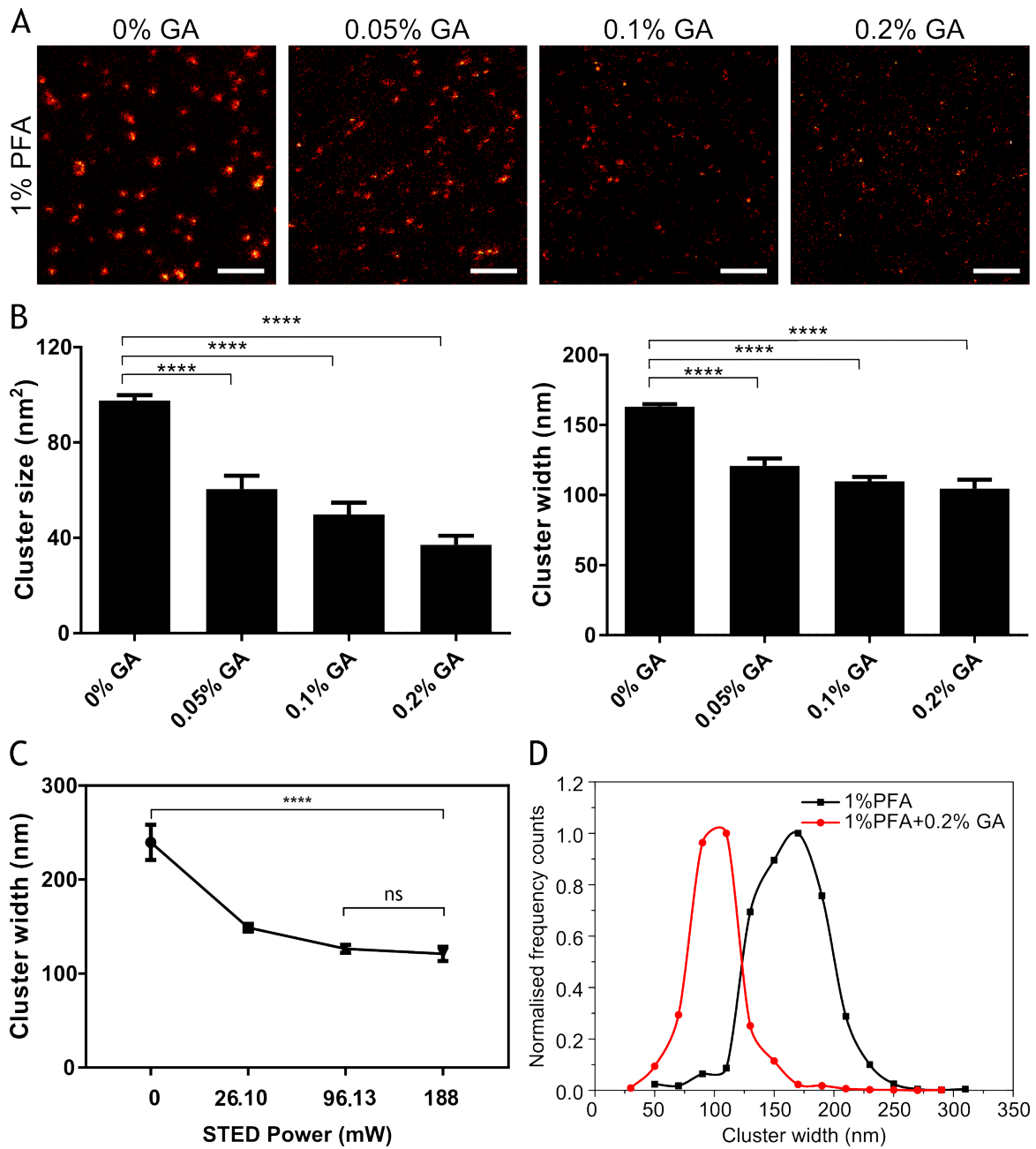


Figure 3.4: Analysis of cross-linked LYVE-1 clusters obtained with STED imaging. A) STED images of LYVE-1 with 1% PFA and varying concentrations of GA. B) Huygens deconvolved analysis of LYVE-1 cluster sizes as imaged by STED microscopy following 1% PFA and GA fixation protocols: cluster size (left) and width (right), depicting a reduction in size with increasing in GA concentration. Error bars: standard error of the mean. Average cluster size and surface area from 10 cells. ($P = <0.0001$; 1-way ANOVA with Dunnett's multiple comparisons. C) Effect of STED power on the observed cluster width of LYVE-1 with no significant difference obtained at higher powers (ANOVA with Tukey's multiple comparisons test; $P = 0.7016$). D) Clusters generated after cross-linking and fixation with 1%PFA + 0.2% GA showing clusters with a median width of 105 nm when compared to those fixed with 1% PFA alone showing 165 nm. Scale bar, 1 μm .

As highlighted in the methods section, the variation of STED power used alters the spatial resolution of the microscope and thus the observed cluster size, i.e, the higher the STED laser power the higher the spatial resolution in the resulting images [113]. Therefore, the STED power used may also contribute to the changes in cluster sizes

observed. To determine the effect of the STED power on the observed LYVE-1 cluster size, STED images of LYVE-1 labelled as before with 1% PFA and 0.2% GA were acquired at varying STED powers. The analysis of the STED deconvolved images show a consistent decrease of cluster width with increasing STED power, reaching a plateau where the cluster size does not change significantly anymore (ANOVA with Tukey's multiple comparisons test; $P = 0.7016$) when using higher STED laser powers (Fig. 3.4 C), indicating the true size of the clusters to be ~ 100 nm. In accordance with our previous results, fixation with PFA alone generated the largest clusters but upon using the optimised fixation conditions and STED laser powers the correct cluster sizes of crosslinked LYVE-1 were determined. From these results it can be concluded that LYVE-1 antibody cross-linked clusters that have been indicated to potentiate HA binding [6] had organised into clusters falling into a range of 50 - 150 nm, with a mean of 100 nm in diameter (Fig. 3.4 D) on the surface of HDLECs.

3.4 Discussion

Immunolabelling techniques used to study the distribution of membrane receptors require fixation, which ideally results in the preservation of their *in vivo* distribution. However capturing this dynamic organisation in a fixed state is problematic. For example, Whelan et al., demonstrated that labelling intracellular mitochondrial protein, Tom20, in COS-7 cells with PFA fixation alone lead to artefactual enlargement of the mitochondria, while combination of PFA and GA resulted in a more homogeneous distribution with a lower degree of clustering on the mitochondrial surface [164]. Similarly, in our experiments the observed distribution and organisation of membrane LYVE-1 in lymphatic endothelial cells was hugely different when using PFA alone or in combination with 0.2% GA for fixation. A distribution of LYVE-1 similar to that detected in cells labelled with conjugated primary antibodies was observed with PFA and GA combination, whereas without GA larger aggregates or aggregates with altered distribution were apparent, indicating that the secondary antibody is responsible for the clustering. These fixation-related artefacts observed when PFA is used

alone are not limited to LYVE-1, as similar effects were observed for CD31 and CD44 transmembrane receptor in the presence or absence of GA [116].

It should be noted that GA, at concentrations higher than 0.25% may dampen fluorescence signal [172], and may also lead to high background autofluorescence, due to the reactive aldehyde groups that are not blocked [162]. The use of reductants such as sodium borohydride or glycine [164] can effectively block the reactive aldehydes and remove any background autofluorescence. Referring back to LYVE-1, fixation with 1% PFA and 0.2% GA had not altered the fluorescence signal with or without glycine treatment. Alternatively, autofluorescence can be tested by exciting the fixed sample at 488 nm. Thus, LYVE-1 labelled using a far red dye after fixation was excited at 488 nm and detected at both ranges (495 -520 nm and 680 -750 nm). This also indicated that autofluorescence had not interfered with LYVE-1 cluster imaging.

Considering that the cluster organisation of the indirectly immunolabelled LYVE-1 is comparable to that of LYVE-1 cross-linked by a fluorescent conjugate mAb, it was still unclear if the receptors were truly fixed and immobile. In the study conducted by Tanaka et al., transmembrane and glycosylphosphatidylinositol (GPI)-anchored proteins and lipids exhibited a lateral diffusion after chemical fixation with PFA alone, but addition of 0.2% GA rendered more than 80% of molecules immobile. Hence, a similar study was extrapolated for LYVE-1, where by using FRAP, the residual mobility of the membrane receptors was highlighted in the case of PFA fixation alone (with a diffusion coefficient $D = 0.07 \pm 0.02 \mu^2/s$ compared to $D = 0.11 \pm 0.05 \mu^2/s$ in living cells), with none observed upon addition of 0.2% (w/v) GA. These results confirm that aggregation of incompletely immobilised receptors by the secondary antibody was responsible for the artefactual clustering observed when primary antibody labelled cells were fixed with PFA alone.

Recent years have seen a rise in the use of super-resolution optical microscopy techniques for studying receptor clustering. Hence, when using super-resolution techniques like STED, it is important to understand that artefacts can arise, and to circumvent them, one must optimise not only the antibody or cell fixation conditions but

also the STED power used. As seen from the results of LYVE-1 cluster sizes, using a lower power of STED shows larger clusters whereas, the two higher STED laser powers used revealed cluster sizes that were not significantly different (ANOVA with Tukey's multiple comparisons; $P = 0.7016$). Finally, to identify the size of LYVE-1 clusters that potentiate HA binding, clusters were imaged using higher STED laser powers at varying concentrations of GA (0% to 0.2%) along with 1% PFA. With the added advantage of STED, the nanoscale organisation of LYVE-1 clusters was resolved and was found to be organised on the surface of HDLECs as 100 nm clusters. From these optimised results, it can be concluded that reorganisation of LYVE-1 into clusters upon antibody treatment, lead to the formation of LYVE-1 clusters ranging from 50 - 150 nm. This implies that during antibody cross-linking induced potentiating of HA binding that has been previously shown, LYVE-1 organises into 100 nm clusters on the surface in fixed conditions.

This nanoscale clustering of LYVE-1 falls within the identified size of actin compartments beneath the plasma membrane between 50 - 100 nm [78, 97, 98]. Hence, there is potential for the cortical framework to direct the molecular process of clustering and therefore its function, which will be investigated in the next chapter.

Chapter 4

Role of the actin cytoskeleton in LYVE-1 clustering and HA binding function

Background

Several studies of the LYVE-1 homologue, CD44, have shown that clustering and functioning of CD44 on the membrane surface is mediated by the actin cytoskeleton [8, 173]. This is facilitated by the presence of an ERM and Ankyrin binding motif on the cytoplasmic tail of CD44 [93]. LYVE-1 has an 'ERM-like' binding site at the C-terminus (Wang, YJ et al., unpublished studies) thus indicating that LYVE-1 could interact *via* ERM proteins to the actin cytoskeleton. As the actin cytoskeleton has been shown to play a role in the formation or maintenance of membrane receptors [73], this could signify that the clustering of LYVE-1 may be influenced and limited by the cortical actin cytoskeleton. For this reason, the potential influence of the actin cytoskeleton on the organisation and HA binding function of surface LYVE-1 will be tested using pharmacological drugs that affect the cortical actin turnover.

As previously introduced, there are two sub-populations of F-actin filaments in the cortex with distinct turnover kinetics which can be studied by pharmacological treatments [75]. The three drugs used here, Cytochalasin D (Cyto D), Latrunculin B (Lat B) and CK-666, act on the cortical actin turnover but function differently at a kinetic level. Cyto D is a fungal metabolite that binds to the barbed end (or growing end) of the F-actin filament and actin nuclei and prevents the addition of actin monomers (G-actin) [174] (Fig. 4.1 A). This drug initially acts on the free barbed ends on the shorter F-actin filaments. Cyto D binds competitively with formin to the barbed end of the actin filaments. Formin continues to form filaments (see Introduction) until Cyto D outcompetes formin, eventually leading to complete disassembly of the actin cytoskeleton [75]. Lat B is a toxin purified from red sea sponge and is known to

sequester all the available G-actin in the cell thus preventing the elongation of F-actin [8, 175] (Fig. 4.1 B). As it sequesters all the G-actin, both the sub-populations of F-actin turnover are targeted equally. Lastly, the drug CK-666 is a small molecule that binds to actin nucleating site, Arp 2/3 complex, and prevents the branching of the cortical actin, creating larger cortical mesh sizes [176] (Fig. 4.1 C).

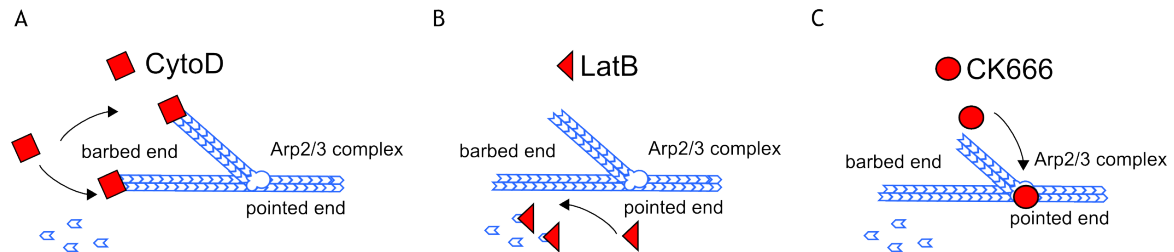


Figure 4.1: Site of action of actin depolymerisation drugs. A) Cytochalasin D (CytoD) capping the barbed ends of the F-actin filaments preventing addition of monomeric G-actin. B) Latrunculin B (LatB) binding to global G-actin monomers, preventing polymerisation. C) CK-666 binding to Arp 2/3 nucleating sites preventing branching of the actin filaments.

Using these drugs, the rate of actin turnover in primary HDLECs will be investigated by time-lapse imaging, to identify the time frame in which the cells are structurally intact after depolymerisation. Next, changes in the organisation of cross-linked LYVE-1 before and after Cyto D, Lat B and CK-666 treatment of the cells will be determined using STED microscopy. LYVE-1 clusters formed will be quantified, after deconvolution, using Huygens software, as done in the previous chapter.

Having established the reasons for clustering, the next aim in this chapter is to identify whether the cortical actin cytoskeleton can modulate LYVE-1 HA binding. As previously introduced, the actin cytoskeleton has surmounted its initial role of only providing structural integrity to being a crucial regulator of receptor clustering and function [74]. As observed for other membrane receptors, the actin cytoskeleton could provide a hitherto unexplored mechanism for mediating LYVE-1 interaction with HA during actin turnover.

In order to test this hypothesis, the direct effect of actin turnover on the HA binding of LYVE-1 will be tested. First, the optimal concentrations and incubation times of Cyto D and Lat B will be tested on primary HDLECs. Following which, LYVE-1 binding to HA will be analysed using FACS and FlowJo. The functional

effect will be confirmed with the optimised conditions along with competing ligand controls by FACS whereas, the structural integrity of the cells will be examined by confocal imaging of F-actin using phalloidin.

Aims

1. Establish the optimised conditions for drug treatments in primary HDLECs.
2. Detect the effect of actin depolymerisation on LYVE-1 clustering by STED microscopy.
3. Detect the effect of actin depolymerisation on LYVE-1 binding to complexed HA by FACS.

4.1 Effect of actin depolymerisation drugs on F-actin disassembly in HDLECs

The constant extension and removal of actin monomers to the actin filament make the cortex a highly dynamic structure undergoing perpetual turnover. This turnover rate of actin often varies depending on the cell type, and occur at two kinetically different rates that have so far been identified [75, 78, 177]. There is a fast turnover mechanism of filaments that are more abundant, and mediated by the Arp2/3 complex and a slower, formin mediated turnover. By interfering with the turnover rate using chemical treatments the influence of the actin cytoskeleton on receptor functions can be studied. Hence, it is important to identify the appropriate actin depolymerisation drugs, and incubation times that conserve the structural integrity of the cell yet maintain the steady state of actin disassembly.

In order to study this, the actin in the primary cells were labelled with SiR actin as described in the methods section and time lapse images of the actin depolymerisation during drug treatment was recorded. SiR actin is a fluorogenic probe, derived from the drug jasplakinolide and bound to silicon-rhodamine derivative, that labels predominantly intact F-actin filaments [178], hence, confocal time-lapse images taken during drug treatments would reveal the disassembly of the actin cytoskeleton as indicated by the reduction in overall signal intensity per area.

Fig. 4.2 A shows confocal images of the SiR actin labelled cells that are treated with 100 nM Lat B before treatment (right) and after the treatment (left). The averaged intensity measured from several regions (yellow boxes) in this treatment is plotted against time in Fig. 4.2 B which shows a steady decline in the intensity of actin after Lat B treatment, in a similar manner as characterised by Fritzsche et. al [75]. The resulting curve is fitted with an exponential decay (red) to indicate the declining levels of actin. From the resulting curve it can be identified that after 15 minutes of drug treatment, the cortical actin has been depolymerised.

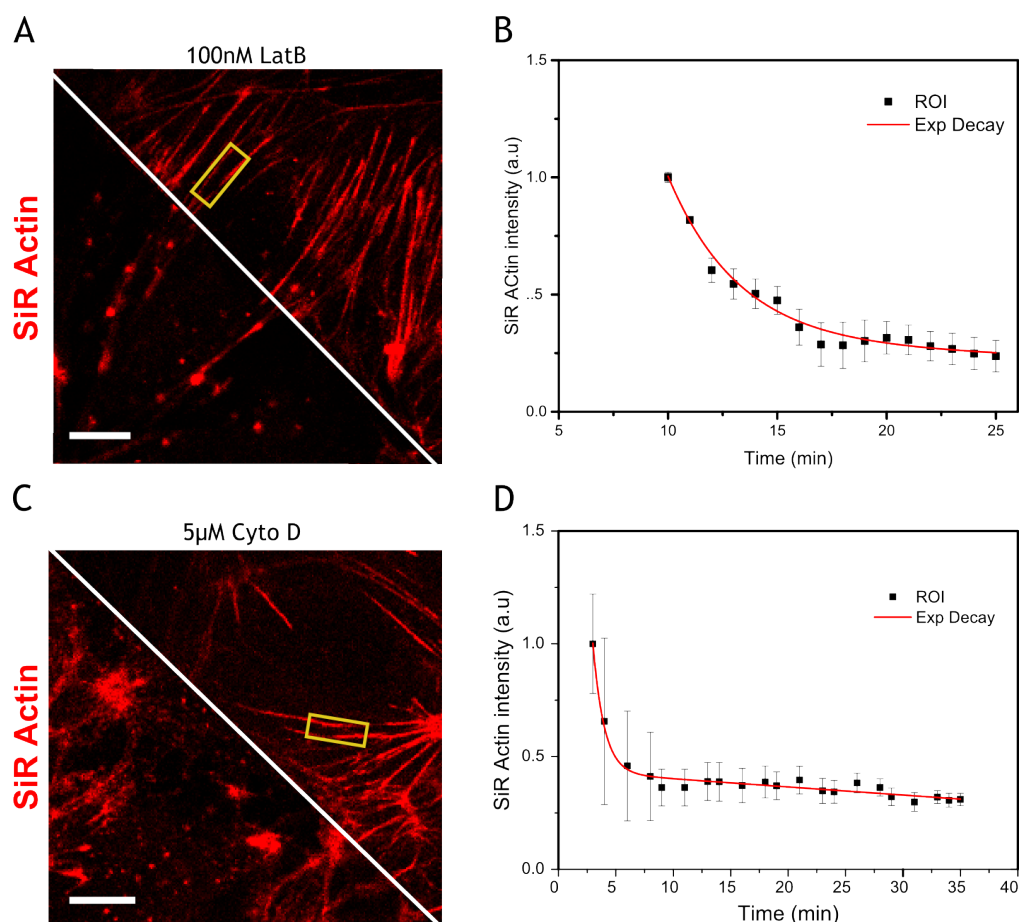


Figure 4.2: Effect of Latrunculin B and Cytochalasin D on F-actin depolymerisation in primary HDLECs. A, C) Confocal time-lapse images of F-actin in primary HDLECs labelled with Sir Actin (red) before (right) and after (left) treatment with 100 nM Lat B (A) or 5 μ M Cyto D (B). B, D) Average intensity of F-actin over time from 5 ROI (yellow box in A or B) plotted against acquisition time and fitted with an exponential decay fit for both treatments. Scale bar 10 μ m

Similarly, Fig. 4.2 C shows the effect of 5 μ M Cyto D on F-actin in primary HDLECs with the confocal image before treatment on the right and after the final time point on the left. Again, the averaged intensities measured from the individual time points shown in Fig. 4.2 D depict a rapid decline in the drug treatment within three minutes of receiving Cyto D followed by a steady decline. The exponential decay fit shows that the decline tails off after eight or nine minutes after having been treated with the drug. This accelerated drop and steady decline of actin depolymerisation corresponds with previous studies when using Cyto D exhibiting the dual kinetic turnover rates of F-actin depolymerisation [75]. From these titrations, it can be estimated that after 10 or 15 minutes most of the actin is depleted from HDLECs and acquiring data at earlier time points may still have some influence of the intact actin.

4.2 Investigating the effect of actin depolymerisation on the clustering of LYVE-1

Using the above experimental data on actin turnover in primary HDLECs, the next step was to identify nanoscale changes on the organisation of LYVE-1 due to actin turnover. To study this, primary cells were treated with 10 μ M Cyto D, 200 nM Lat B or 100 μ M CK-666 for 15 minutes, after which LYVE-1 was cross-linked with LYVE-1 mAb, fixed with 1 % PFA and 0.2 % GA and imaged by STED microscopy as described in the methods section.

Figure 4.3 A depicts STED images of cross-linked, clustered LYVE-1 comparing untreated and the different drug treated conditions. In order to be able to detect the nanoscale changes in clustering, the STED images were deconvolved and analysed to obtain the cluster size and length (width) (see Methods). Treatment with the actin depolymerisation drugs resulted in only a small change in LYVE-1 cross-linked cluster size (Fig. 4.3 B) or width (Fig. 4.3 C) for Cyto D and Lat B treated cells and a slight decrease when treated with CK-666 was noted, but statistically insignificant in all cases (one-way ANOVA with Dunnett's multiple comparison; $P = 0.7172$ and 0.6050 respectively). The average values of three replicates for cluster size and width are depicted in Table 1. These results indicated that the actin cytoskeleton disassembly does not significantly alter the nanoscale organisation of mAb cross-linked LYVE-1 on the surface of primary HDLECs.

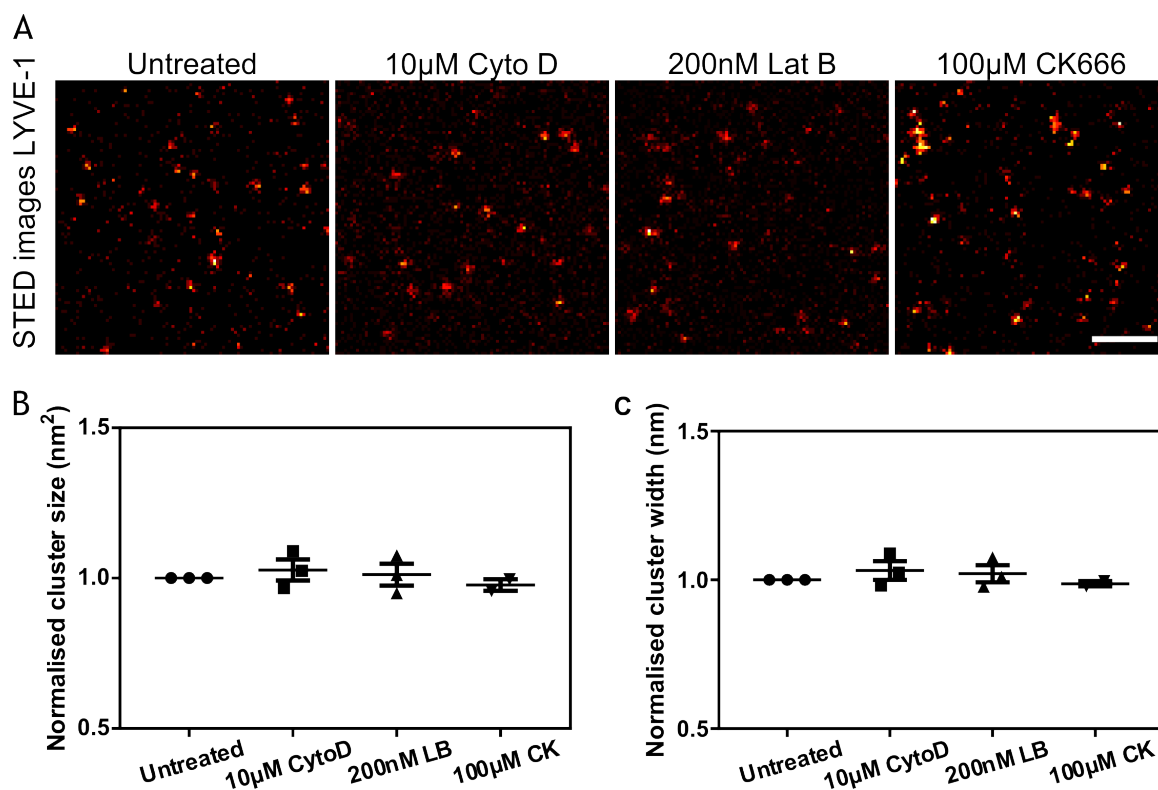


Figure 4.3: Quantification of LYVE-1 surface clusters before and after actin depolymerisation. A) STED images of cross-linked LYVE-1 clusters before and after drug treatments with CytoD, LatB and CK-666 using correct fixation technique. B and C) Quantification of LYVE-1 clusters showing only a small but not significant increase in cluster size (B) or cluster width (C). Biological replicates $n=3$, error bars indicate SEM. Scale bar 0.5µm

Cluster size (nm ²)			
Untreated	Cyto D	Lat B	CK-666
120 ± 35	125 ± 40	120 ± 40	80 ± 10

Cluster width (nm)			
Untreated	Cyto D	Lat B	CK-666
130 ± 35	140 ± 40	140 ± 40	100 ± 30

Table 1: Average cluster size and width from deconvolved STED images. Table on the right depicts the average cluster size and table on the left depicts the average cluster width.

4.3 Optimisation of actin depolymerisation drugs for FACS-based measurements of HA binding function of LYVE-1

As shown in the previous chapter, actin depolymerisation of HDLECs resulted in no change in the membrane organisation of LYVE-1. Other transmembrane receptors such as CD22 have also shown a lack of change in receptor organisation after actin

depolymerisation but had led to changes in the signalling functions of the receptor [179]. A similar mechanism could be envisaged by LYVE-1 in the primary HDLECs wherein the function of the receptor alters without a change in the receptor organisation during actin turnover. In order to test this, monolayers of primary HDLECs were treated with the appropriate concentration of cytoskeletal drugs (50 nM to 5 μ M for Lat B and 500 nM to 20 μ M Cyto D) and incubated from 10 minutes to 45 minutes. The binding of HA as a complex (bHA:SA488) was tested using FACS. The cells were prepared for FACS labelling as described in the methods section.

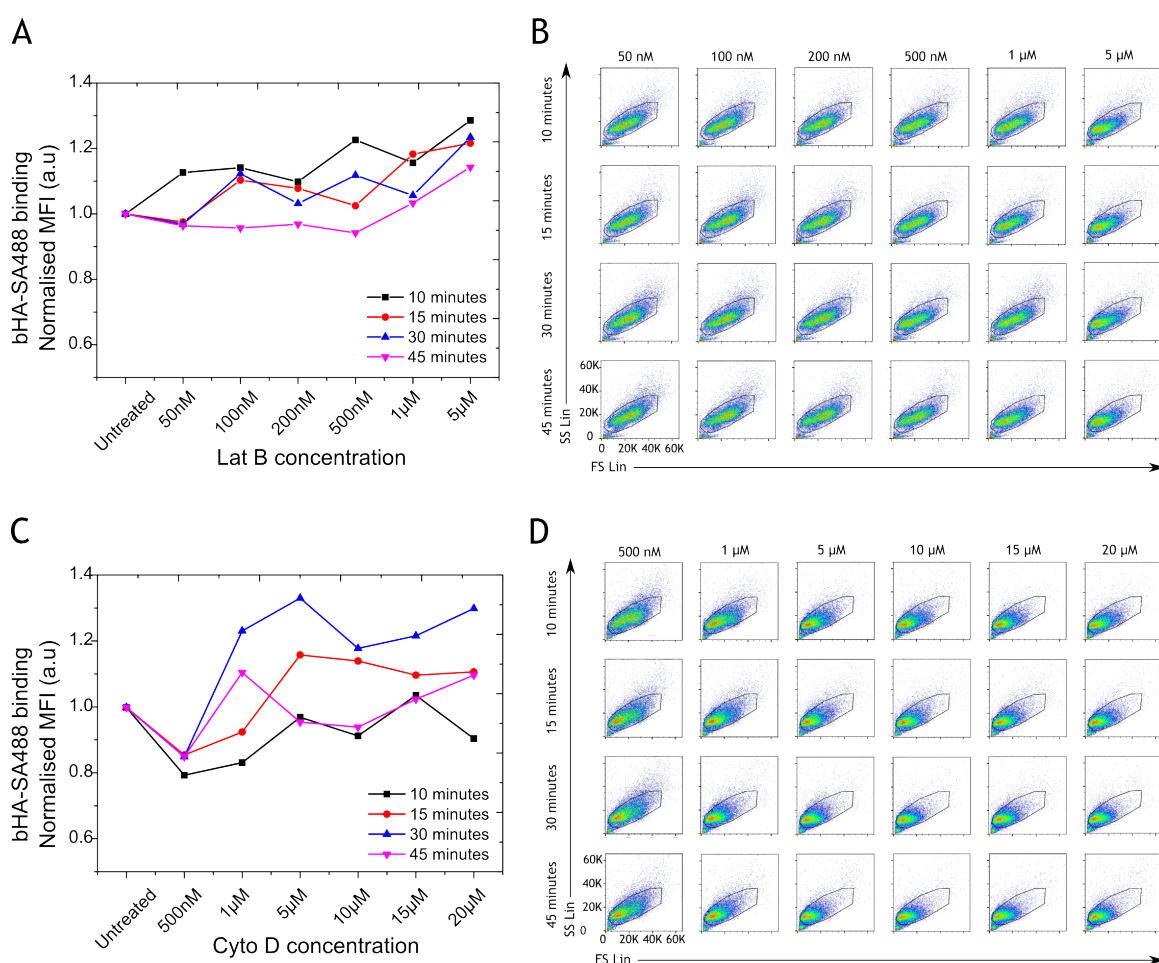


Figure 4.4: Titration of actin depolymerisation drugs. A, C) Normalised median fluorescence intensity plot of HA:SA488 binding to LYVE-1 from FACS analysis at varying incubation length and concentrations of LatB (A) or CytoD (C). B, C) Forward scatter (FS) and side scatter (SS) plots of the primary HDLECs after the drug treatments with LatB (B) or CytoD (D) showing the effect of the drugs at varying incubation length and concentrations on the phenotype of the cells.

The normalised median fluorescence intensity of bHA:SA488 binding to LYVE-1 in Lat B and Cyto D treated HDLECs is plotted against the drug concentration (Fig. 4.4 A, C). Fig. 4.4 A shows the results obtained for bHA:SA488 binding after Lat B

treatment of primary HDLECs at varying incubation times and concentrations. Data for incubation for 10 minutes (black), 15 minutes (red) and 30 minutes (blue) indicated a trend towards increased bHA:SA488 complex binding to LYVE-1 on HDLECs at rising concentrations of Lat B. Data for treatment with Lat B at lower concentrations (50 - 500 nM) for 45 minutes revealed no increase in HA binding most likely due to a still intact cortex. Cyto D treatment revealed much more variation in bHA:SA488 binding at 10 and 45 minutes. At 15 and 30 minutes an increase was observed with increasing concentrations of Cyto D (1 μ M to 20 μ M) (Fig. 4.4 C).

The forward scatter (FS) and side scatter (SS) give information on the size and granularity of the cells, hence providing information on the morphological changes with the drug treatments. The cells closer to the zero axis were smaller in size and those further away were larger in size and granularity. The data from the FS and SS dot plots obtained from FACS of the primary HDLECs after drug treatment and depicted the effect of the drug Lat B (Fig. 4.4 B) and Cyto D (Fig. 4.4 D) on the shape of the cells. It should be noted that the higher concentrations of Cyto D or Lat B seemed to cause a change in the shape of the cells although the function in Lat B treated cells was not affected in most cases (Fig. 4.4 B). This effect was found to be more drastic in Cyto D treated cells where cells receiving high concentrations and longer incubations affected the cell shape. This effect is most likely due to the cells losing the structure provided by the cytoskeleton, therefore for the functional analysis two mid-range drug concentrations that have minimum effects on the cell shape were used for further experiments.

4.4 Effect of F-actin depolymerisation on HA binding to LYVE-1⁺ HDLECs

To study the effect of actin depolymerisation on LYVE-1 function, primary HDLECs were treated with 5 μ M and 10 μ M of Cyto D, or 100 nM and 200 nM Lat B, or 50 μ M and 100 μ M CK-666, for either 10 minutes or 15 minutes (following the above optimised steps). The cells were then prepared for FACS labelling as done above.

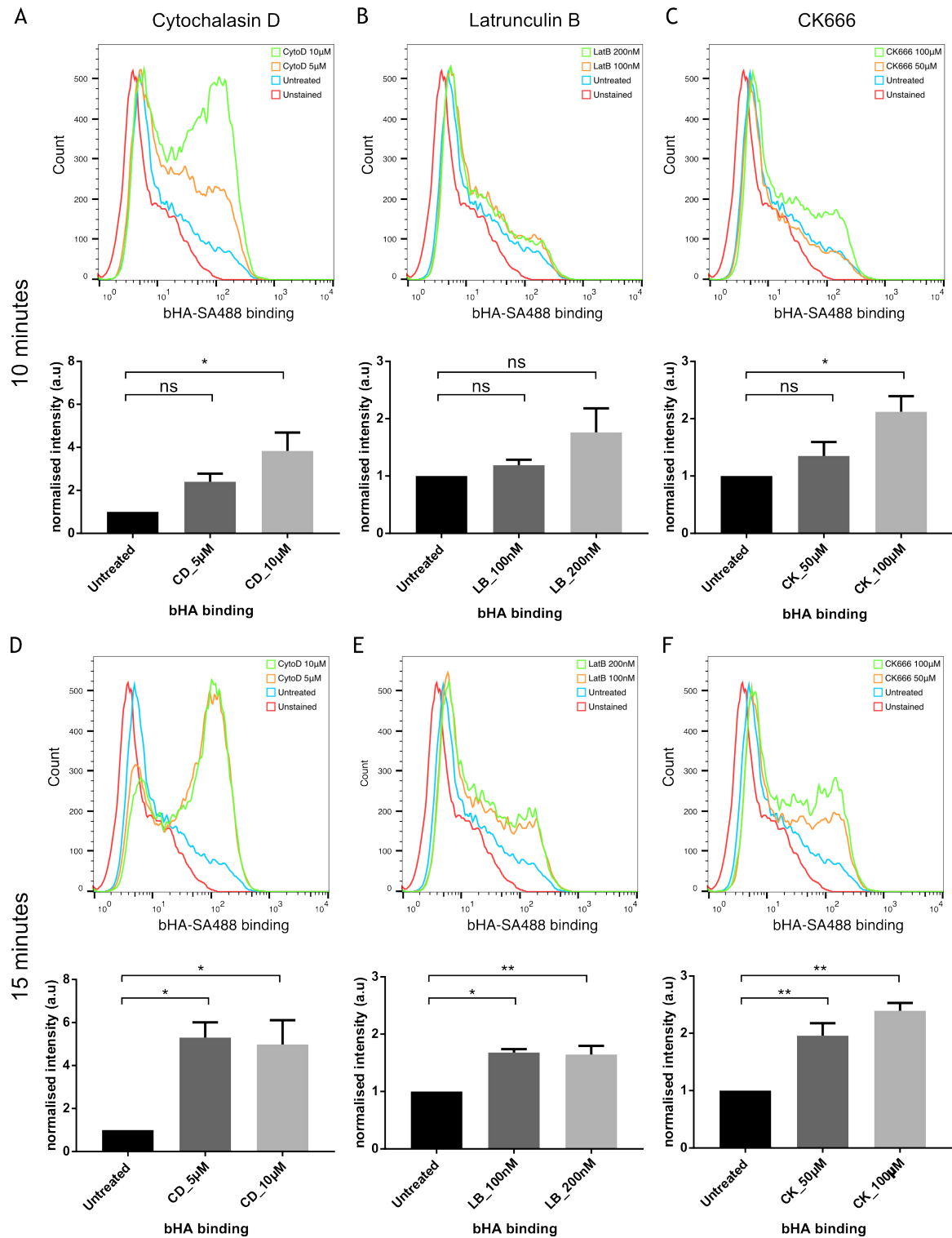


Figure 4.5: HA binding affinity of LYVE-1 in HDLECs increases with actin depolymerisation. A - F) FACS histograms (upper panels) of cells treated with Cyto D (A, D), Lat B (B, E) or CK-666 (C, F) for 10 minutes (A - C) and 15 minutes (D - F) showing an increase in HA:SA488 binding (orange and green) when compared to untreated cells (blue). The median fluorescent intensity plots (lower panels), normalised to untreated conditions, showing a significant increase in HA binding to LYVE-1+ primary HDLECs at higher concentrations when incubated for 10 minutes and almost all significant when treated for 15 minutes at both lower and higher concentrations. The bar plot shows the mean from 3 replicate experiments with error bars indicating SEM. CD = Cyto D, LB = Lat B, CK = CK-666.

Figure 4.5 shows the normalised FACS histograms (upper panels) and median fluorescent intensity graphs (lower panels) of bHA:SA488 complex binding to the primary HDLECs at 10 minutes and 15 minutes.

At the 10 minute time-point, significant changes of LYVE-1 HA binding was only observed for the highest concentrations of Cyto D (10 μ M) and CK-666 (100 μ M) (One-way ANOVA with Dunnett's multiple comparison; $P = 0.0276$ and 0.0228 respectively) (Fig. 4.5 A & C), while LatB did not show any significant changes (One-way ANOVA with Dunnett's multiple comparison; $P = 0.1566$) (Fig. 4.5 B). The capacity to bind bHA:SA488 showed a significant improvement when the cells were treated for 15 minutes using the same concentration of drugs as above (One-way ANOVA with Dunnett's multiple comparison; $P = 0.0211$ for Cyto D, $P = 0.0080$ for Lat B, $P = 0.0015$ for CK-666) (Fig. 4.5 D - F). This latter experiment gave the clearest indication of the increase in bHA binding to LYVE-1⁺ HDLECs after actin depolymerisation

In order to confirm that the observed effect was a true binding of HA to surface LYVE-1 on primary HDLECs, the drug treated cells were incubated with excess (of 200 fold) unlabelled high molecular weight HA (xsHMWHA) as a competing ligand, prior to adding the complexed bHA:SA488. The cells were then allowed to incubate overnight and then subject to the same FACS labelling protocol mentioned earlier. Figure 4.6 (A - C) shows the FACS histograms (upper panel) and median fluorescence intensity (lower panel) of LYVE-1 binding to bHA:SA488 with or without the presence of the competing HA. The results showed a reduction in bHA:SA488 binding to cells treated for 15 minutes with 10 μ M CytoD (Fig. 4.6 A), 200 nM LatB (Fig. 4.6 B) and 100 μ M CK-666 (Fig. 4.6 C) in the presence of the competing excess HA indicating that the fluorescent signal in the binding assay was not from internalised HA but from surface bound bHA:SA488 complex.

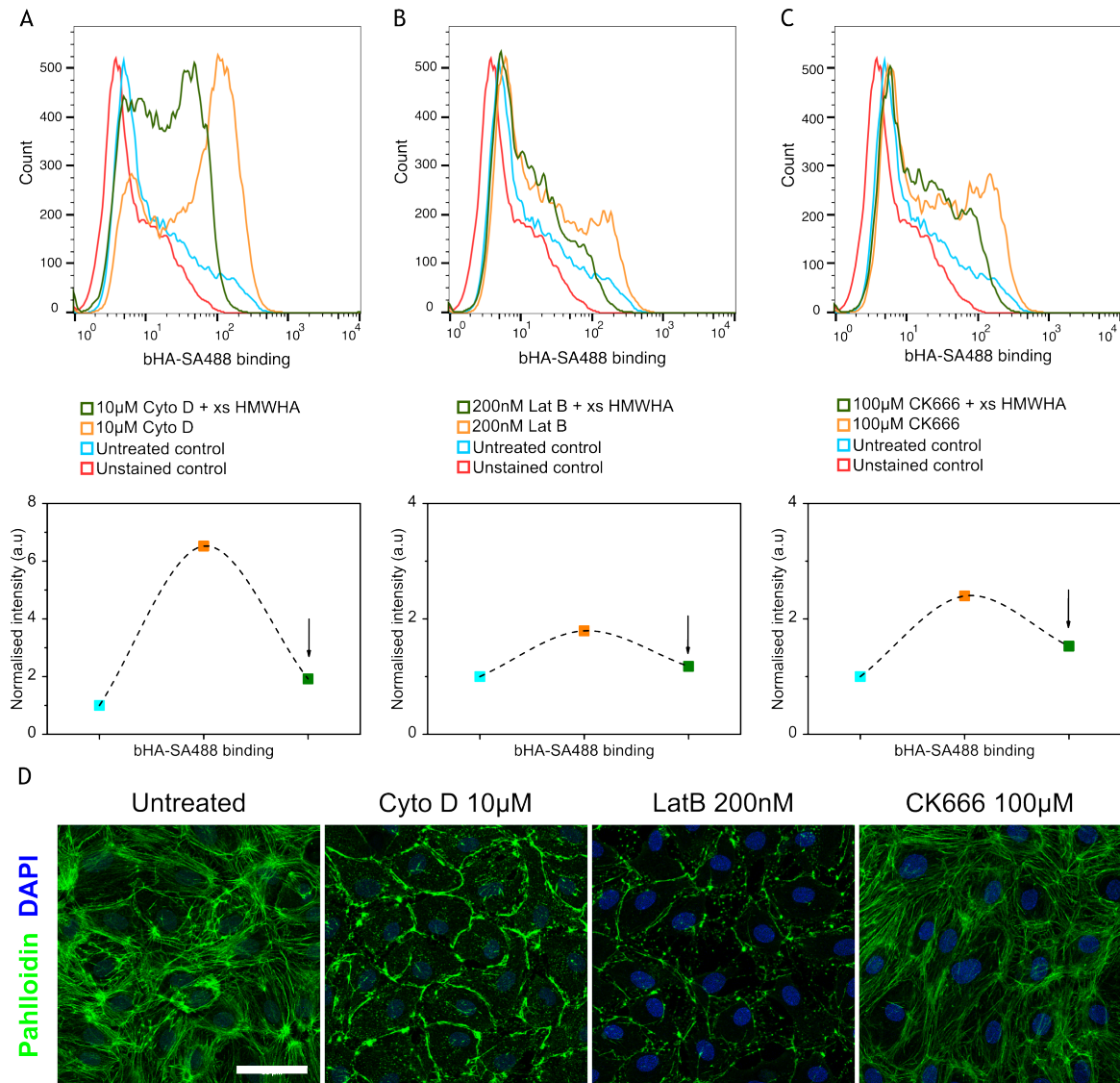


Figure 4.6: Competition with excess unlabelled high molecular weight HA (xsHMWHA) reduces the binding of bHA:SA488 to LYVE-1. A - C) FACS histograms (upper panels) of cells treated with CytoD (A), LatB (B) or CK-666 (C) for 15 minutes showing a reduction in bHA:SA488 binding (green) in the presence of xsHMWHA when compared to its absence (orange). Median intensity plot normalised to the untreated conditions show a representative graph from 2 replicates (lower panels). D) Confocal images of LYVE-1+ primary HDLECs untreated or treated with Cyto D, Lat B or CK 666 for 15 minutes showing that the actin cytoskeleton is depolymerised in these conditions. Phalloidin in green and nucleus in blue. Scale bar 50 μ m.

To confirm the depolymerised state of LYVE-1 at the time points used in the functional analysis, confocal images of the actin cytoskeleton were obtained before and after drug treatments in fixed conditions. Primary HDLECs grown to confluency and 10 μ M Cyto D, 200 nM Lat B and 100 μ M CK-666 were used to treat the cells for 15 minutes to depolymerise actin cytoskeleton as done for the FACS experiment. The cells were then fixed and labelled for F-actin using phalloidin as described in the methods section. As shown in Fig. 4.6 D, the cells treated with Cyto D and Lat B showed a reduction in actin filaments in the cell cortex when compared to untreated

conditions. Although CK-666 treated cells still retained some cortical actin, it appears to be far less compared to the untreated conditions. From these experiments it can be concluded that the bHA:SA488 complex binding to surface LYVE-1 increases during cortical actin turnover.

4.5 Discussion

In this chapter, the effects of the cortical actin disassembly on LYVE-1 clustering and HA binding were studied using confocal, and time-lapse imaging, super-resolution STED microscopy and FACS. The results indicated that the effects of actin turnover had different consequences on clustering and functional effects on membrane receptors. Live cell imaging of the cortical actin disassembly in the presence of actin depolymerisation drugs, Cyto D and Lat B gave crucial information on the actin turnover rates of primary HDLECs and provided information on the time-frame to carry out the drug treatments. Cyto D treatment revealed a sharp decline in actin filaments after receiving the drug within three minutes followed by a further steady reduction of actin to minimum levels. This is due to the characteristic dual kinetic nature of the actin dynamics with Cyto D as observed in melanoma cells [110]. The initial steep decline was due to the drug targeting the smaller formin independent F-actin filaments and the steady reduction that followed was due to Cyto D targeting the longer formin-dependent filaments [75, 110]. This eventually lead to the depolymerised state where most of the cortical actin was removed but the structural integrity was still maintained. In Lat B treated cells all the filaments were targeted equally, as all G-actin monomers were sequestered [110], leading to this steady linear decline.

When testing the clustering of LYVE-1 after actin depolymerisation, there was a slight decrease in LYVE-1 cluster sizes after CK 666 treatment and increase with Cyto D and Lat B, but neither of the sizes were significantly different ($P = 0.6050$ and 0.7172 respectively) to untreated LYVE-1 clusters. In this study, two parameters such as cluster size and lateral width were used to determine changes in LYVE-1 clusters before and after drug treatment. It could be possible that the density and proximity

of the clusters may have altered due to actin disassembly as seen for CD44 when using number and brightness analysis [8] which was not investigated in this thesis. Or it could be that the surface organisation remains the same after actin depolymerisation as seen for membrane receptor CD22 [74, 179]. Both of these could be possible explanations to why there was no significant change in LYVE-1 cross-linked clusters.

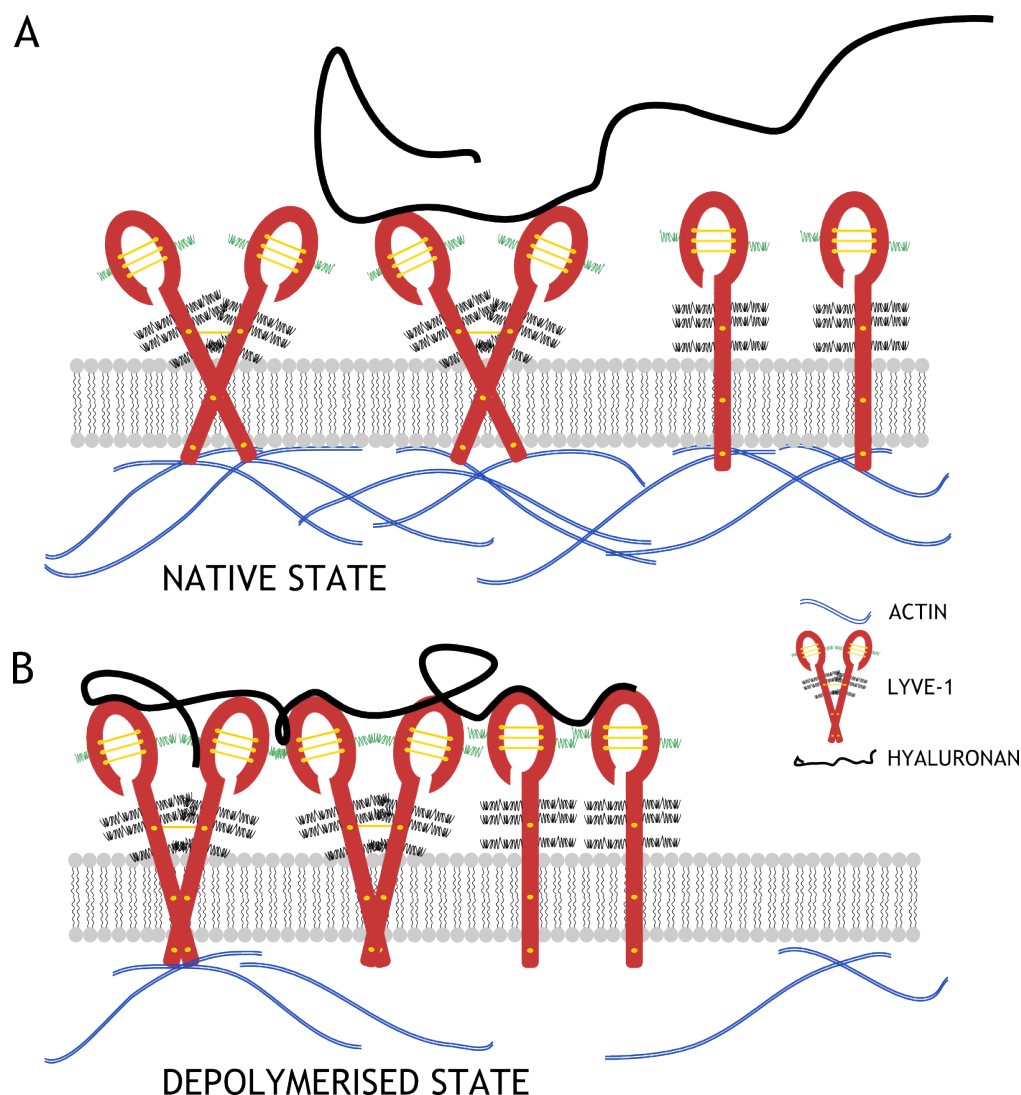


Figure 4.7: Hypothetical model of lateral diffusion contributing to increase in LYVE-1 interaction. A) HA binding in native cells restricted by the interaction of LYVE-1 with the cortical actin in steady state conditions. B) Increased interaction to HA due to a possible increase in lateral diffusion during actin turnover.

The results from the HA binding analysis using FACS showed an increase in HA binding to LYVE-1 after depolymerisation of the actin cytoskeleton. 15 minute incubations with Cyto D, Lat B, and CK-666 gave the best results in terms of ensuring ligand interaction (One-Way ANOVA with Dunnett's multiple comparison; $P = 0.0211$, $P = 0.0080$ and $P = 0.0015$ respectively). At this time point, most of the

actin filaments are depleted or resulted in larger mesh sizes by the drugs allowing the effective changes in HA binding. Controls using excess high molecular weight HA (HMWHA) indicated that the signal for bHA-SA488 binding is not from internalised HA but of surface HA. The effect of the drug was also tested by imaging the cells after drug treatment using a post fixation labelling with Phalloidin, which confirmed the potency of the drug on maintaining the integrity of the cells at the concentration and time frame used for the experiment (Fig. 4.6 D). Together these results confirm that although there was not an evident change in clustering, there was a functional effect observed during actin turnover as seen in the case of the transmembrane CD22 receptor on B-cells [74, 179].

From the functional assay, it can be concluded, that HA binding increased due to the absence of cytoskeletal restrictions, which otherwise hinders the ability of LYVE-1 interaction in the native state (Fig. 4.7 A). The release of LYVE-1 from the cortical actin could potentially lead to an increase in lateral diffusion as seen for other membrane receptors such as CD44, CD22, MHC I and other transmembrane receptors [8, 72, 72, 103, 179]. This increase in lateral diffusion during actin disassembly, as identified in other receptors, could allow the cross-linked HA polymer (HA:SA488) to effectively seize LYVE-1 receptors, providing the necessary avidity required for a stable interaction (Fig. 4.7 B) as seen in the HA binding assay.

Studying the highly dynamic state of LYVE-1 using a chemically fixed method might not have been an appropriate method for detecting changes in the organisation and interactions of the membrane receptors. The key to understanding this mechanism might be linked to the lateral diffusion of the receptors, hence, this mechanism of LYVE-1 receptor dynamics mediated by actin turnover will be investigated in detail in the following chapter.

Chapter 5

Exploring LYVE-1 dynamics and lateral diffusion in relation to the actin cytoskeleton

Background

The functional results of LYVE-1 in the previous chapter provided the evidence that the actin cytoskeleton does play a role in modulating the function of LYVE-1. The changes in the function resulting from the absence of the actin cytoskeleton indicate that LYVE-1 is interacting with actin and once the interaction is disabled it contributes to an increase in function. Work on membrane receptor functions over the years have long attributed the lateral mobility of the receptors, either as random or directed movements, to aid in the functional changes of the receptor. These movements are largely orchestrated by the dynamic turnover of the actin cytoskeleton and its interaction with the receptor [10, 102, 103, 107, 129, 131, 179, 180]. In the case of LYVE-1, it is yet unknown whether the actin cytoskeleton can contribute to a change in function by modulating the lateral dynamics of LYVE-1. In this chapter, the effect of actin turnover modulating LYVE-1 dynamics will be studied in detail.

In order to study the dynamics of LYVE-1, three hypothetical models of LYVE-1 interaction with cortical actin that could contribute to the change in the receptor dynamics, leading to increased HA binding, were proposed. These models were proposed based on previous work on actin and receptor interaction. Figure 5.1 shows a scheme of the proposed models of LYVE-1 diffusion on the membrane surface in a confluent monolayer of cells. Diffusion of many transmembrane receptors have been shown to be hindered by the actin network *via* their cytoplasmic tail end [10, 71, 102, 103, 180, 181]. As mentioned earlier, the cortical actin meshwork lies very close to the membrane which organises the membrane into compartments [9, 97]. The surface LYVE-1 lateral mobility may thus be hindered by the actin cytoskeleton, due to LYVE-1 being

confined within these compartments of the cortical meshwork, impeding ligand binding (Fig. 5.1, Model 1). To test this mechanism, diffusion of LYVE-1 was tested in the presence of CK-666 (detailed in Chapter 4). As this drug has the potential to open up the cortical actin meshwork [176], possible actin barriers that confine LYVE-1 dynamics would be removed. The increase in the mobility of LYVE-1 due to this drug treatment will be recorded using FRAP that will give an average change in the diffusion dynamics. sFCS will be employed to study any heterogeneity in the diffusion of the receptor.

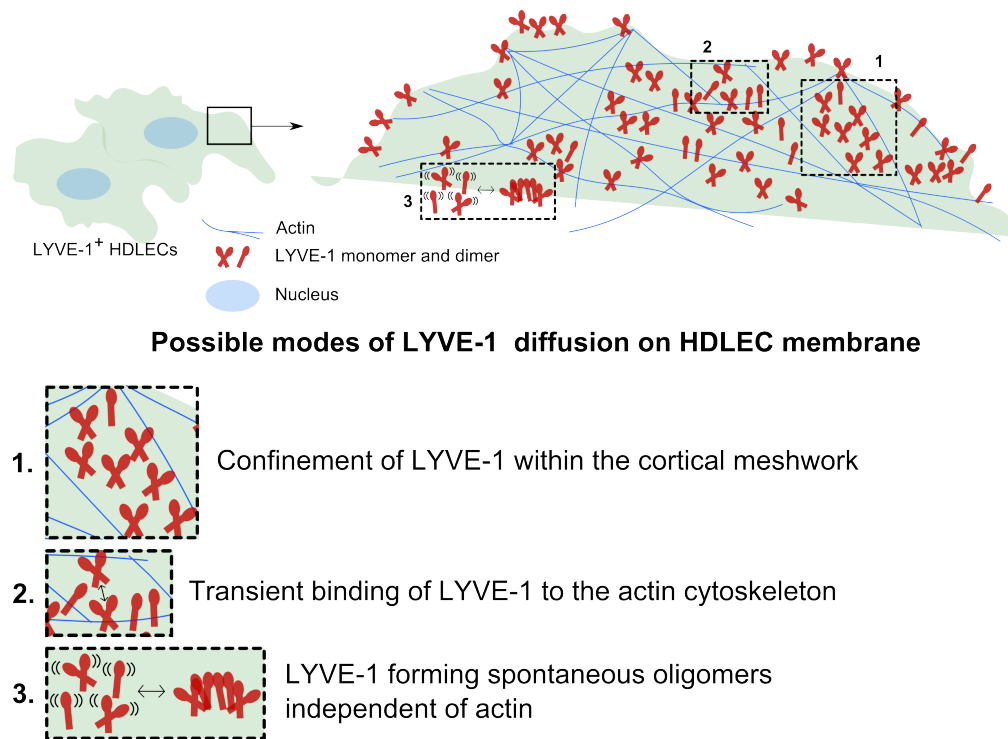


Figure 5.1: Proposed models of LYVE-1 dynamics on the membrane surface of HDLECs that influence ligand interaction. Confluent monolayer of cells with focus on LYVE-1 (red) interaction with the cortical actin (blue) (upper panel). 1) In the first model, LYVE-1 is assumed to be confined within the cortical actin network of the cell. 2) For the second mechanism, it is proposed that LYVE-1 is bound directly or indirectly to the actin filaments, and lastly 3) that LYVE-1 is completely independent of the cortical actin and spontaneously forms oligomers that aid in function.

For the second model, LYVE-1 can be bound to the cortical actin, hindering its interaction as seen for transmembrane glycoproteins and receptors [7, 8, 10] (Fig. 5.1, Model 2). The constant turnover of the filament could influence the binding and mobility of the receptors. In order to test this mechanism, previously introduced Cyto D and Lat B drugs will be employed to depolymerise the actin cytoskeleton and LYVE-1 dynamics will be measured with sFCS.

Direct interaction between LYVE-1 and the actin cytoskeleton were also determined using biochemical co-immunoprecipitation assays to pull down LYVE-1:actin complexes. As most interactions to the cytoskeleton are initiated by the cytoplasmic tail or adaptor proteins [103], short tail mutants of LYVE-1 and full length versions, transfected into primary HDLECs will be used to study dynamics and the interactions.

Finally, LYVE-1 dynamics might be completely independent of the actin cytoskeleton as seen for some transmembrane receptors [180], allowing LYVE-1 to spontaneously form oligomers on the cell surface (Fig. 5.1, Model 3) [182, 183]. However, based on the functional results showing evidence of increase HA binding only after actin depolymerisation in the previous chapter and by antibody induced clustering [6], this mechanism may be less likely to occur.

The final aim in this chapter is to further test the hypothesis put forth on LYVE-1 diffusion modes on the surface of primary HDLECs. To study this, a minimally invasive super-resolution STED-FCS technique will be used. This technique with its improved spatial resolution, allows probing the protein dynamics at various observation spot sizes with diameters from diffraction limited 200 nm to below 50 nm [71, 100, 101, 145, 148] that can confirm the mechanisms proposed for LYVE-1 being confined or binding to the cortical actin.

Aims

1. Optimise live cell labelling for dynamics studies of LYVE-1.
2. Identify the mechanisms of LYVE-1 interaction with the actin cytoskeleton using sFCS and FRAP.
3. Demonstrate the interaction between actin and LYVE-1 using co-immunoprecipitation.
4. Determine the diffusion mode of LYVE-1 on the surface of HDLEC using STED-FCS.

5.1 Optimisation of LYVE-1 labelling methods and imaging techniques

A number of intrinsic and extrinsic factors such as length of the time the cells were in culture (cell culture length), labelling method and imaging techniques can affect the diffusion of LYVE-1, therefore it is important to test these influences. Figure 5.2 shows the various controls performed in order to understand the experimental results. Experiments by colleagues from the Jackson lab using LYVE-1 GFP transfectants have shown that the expression of LYVE-1 increases with the length of time in culture after confluency (Wang, YJ. et al., unpublished). Hence, the effect of culture length after confluency on surface LYVE-1 expression was analysed using FACS.

Primary HDLECs were cultured in 0.1% gelatin coated 6 well plates to confluency and allowed to remain in culture for an additional ten days. From day one after confluency, cells were lifted using Accutase[®] and stained for FACS as mentioned in the methods section. The median fluorescence intensity (MFI) of surface LYVE-1 in HDLECs obtained from the FACS analysis was normalised to the MFI of the antibody isotype, IgG, control and plotted for each day as shown in Fig. 5.2 A. LYVE-1 expression increased exponentially up to day three and then starts to plateau until day ten. These results indicated, that the surface expression of LYVE-1 became fairly stable after an initial increase in LYVE-1 expression, with little to no variation after day four.

The next step was to determine the effect of this LYVE-1 surface expression due to cell culture length on the diffusion of LYVE-1 on the surface of the cells. To study this, confluent primary HDLECs were labelled as described in the methods and the diffusion of LYVE-1 was measured using sFCS. The diffusion coefficient (D) of each experiment was generated using the fitting and analysis code described in the methods section. Most importantly, the determined diffusion coefficient ($D = 0.2 \pm 0.02 \mu\text{m}^2/\text{s}$) of LYVE-1 did not vary much from day to day (Fig. 5.2 B) even though the level of surface LYVE-1 increased up to day three. These results help to conclude

that the changes in surface expression levels of LYVE-1 did not affect the diffusion of LYVE-1 on the membrane surface.

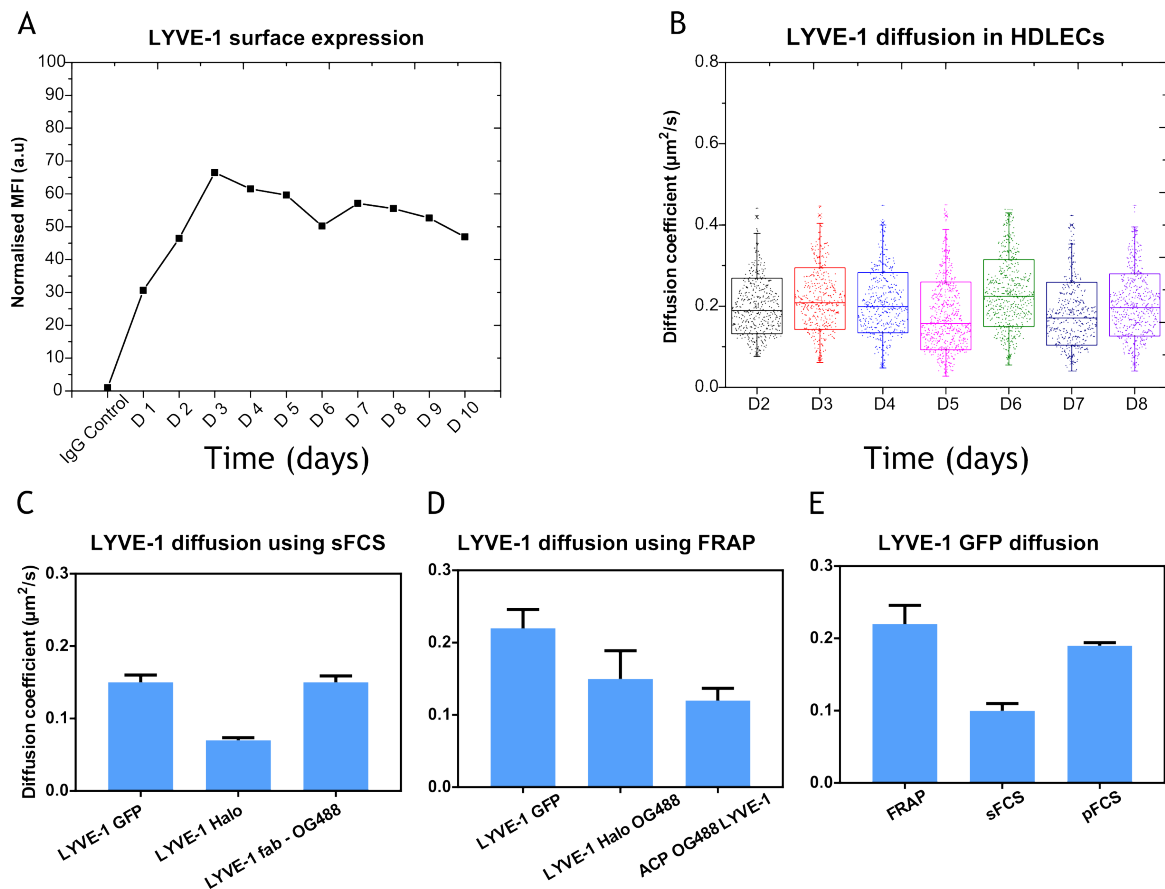


Figure 5.2: Effect of labelling and detection methods on the diffusion of LYVE-1 on the surface of HDLECs. A) Surface expression levels of LYVE-1, after confluency, over the number of days in culture plotted as the median fluorescent intensity (MFI) as determined by FACS. B) Average diffusion coefficient values of LYVE-1 on HDLECs up to day 8 after confluency detected by sFCS. C, D) Effect of labelling methods on diffusion of LYVE-1 detected by sFCS (C) and FRAP (D) and, E) dependency of determined average diffusion coefficient values on the observation techniques FRAP, point FCS (pFCS) and scanning FCS (sFCS).

Technique	Label	D (μm^2)
sFCS	LYVE-1 GFP	0.15 ± 0.01
	LYVE-1 Halo	0.07 ± 0.003
	LYVE-1 fab	0.15 ± 0.01
FRAP	LYVE-1 GFP	0.22 ± 0.03
	LYVE-1 Halo	0.15 ± 0.04
	ACP LYVE-1	0.12 ± 0.02

Table 2: Average diffusion coefficients (D) of LYVE-1 with different labelling techniques. Table depicts the determined D values obtained using sFCS (upper half) and from FRAP (lower half).

Label	Technique	D (μm^2)
LYVE-1 GFP	FRAP	0.22 ± 0.03
	sFCS	0.10 ± 0.01
	pFCS	0.19 ± 0.04

Table 3: Average diffusion coefficients (D) of LYVE-1 with different observation techniques. Table depicts the determined D values obtained using FRAP, sFCS and pFCS when measuring with LYVE-1 GFP.

Next, extrinsic factors such as labelling and observation techniques were tested. For this primary HDLECs were labelled with LYVE-1 fluorescent fabs or primary cells transfected with C-terminal HaloTag[®] and GFP tag, or N-terminal ACP-tag were labelled with its respective ligands as described in the methods (see Appendix fig. 6.4 for labelling sites). Average D values determined for LYVE-1 diffusion are plotted against labelling method, when comparing the different labelling techniques using either sFCS (Fig. 5.2 C) or FRAP (Fig. 5.2 D), and against the observation technique used to detect the change in diffusion (Fig. 5.2 E). The values of D are drawn up in Tables 2 and 3. In sFCS, the diffusion of LYVE-1 is found to be lower when labelled at the C-terminus with the HaloTag[®] ligand than for C-terminus GFP labelling and fluorescently conjugated fab antibody labelling at the N-terminus (Fig. 5.2 C). In FRAP, the determined D values between C-terminus GFP and HaloTag[®] differ similarly and they are also found to be higher than when labelled at N-terminus using ACP tag (Fig. 5.2 D). The different observation approaches yielded slightly different absolute values of D (Fig. 5.2 E and Table 2), which is in agreement with previous reports highlighting varying values of D between different observation approaches [184]. Hence, making comparisons between different observation and labelling techniques is difficult and it was noted that for direct comparison, only one respective approach was followed.

5.2 Investigating LYVE-1 confinement within the cortical actin meshwork

The effect of actin depolymerisation resulted in an increase in LYVE-1 HA binding function as shown in the previous chapter. The next step was to identify whether this process is mediated by changes in the lateral diffusion of LYVE-1 during actin turnover.

The first step was to test Model 1, i.e., the hypothesis of LYVE-1 interactions mediated *via* confinement within the cortical actin meshwork. Primary HDLECs were treated with a potent inhibitor of actin Arp 2/3 nucleation site, CK-666. CK-666 binding to Arp 2/3 results in larger cortical mesh sizes, possibly causing less confinement of LYVE-1.

Figure 5.3 shows the effect of this drug on the diffusion characteristic of membrane LYVE-1. In the first step, ACP-hLYVE-1 transfected HDLECs labelled at the N-terminus with the corresponding ACP ligand, acetyl CoA OG 488, were either left untreated or treated with 50 μ M CK-666 and the diffusion was measured using FRAP. Figure 5.3 A and B, show the fluorescence recovery curves (blue) fitted with a two component free diffusion model (red) in untreated and CK-666 treated conditions. The two average recovery times obtained from these fits and the calculated diffusion coefficients are plotted below (Fig. 5.3 C and D). The results indicated that LYVE-1 mobility increases after treatment with CK-666, consistent with that of a confinement in diffusion by the actin cytoskeleton ($0.12 \pm 0.02 \mu\text{m}^2/\text{s}$ to $0.18 \pm 0.04 \mu\text{m}^2/\text{s}$ and $0.02 \pm 0.003 \mu\text{m}^2/\text{s}$ to $0.03 \pm 0.01 \mu\text{m}^2/\text{s}$), with significant difference in both the components (unpaired student t-test; $P = 0.0002$ and $P = 0.0007$ respectively).

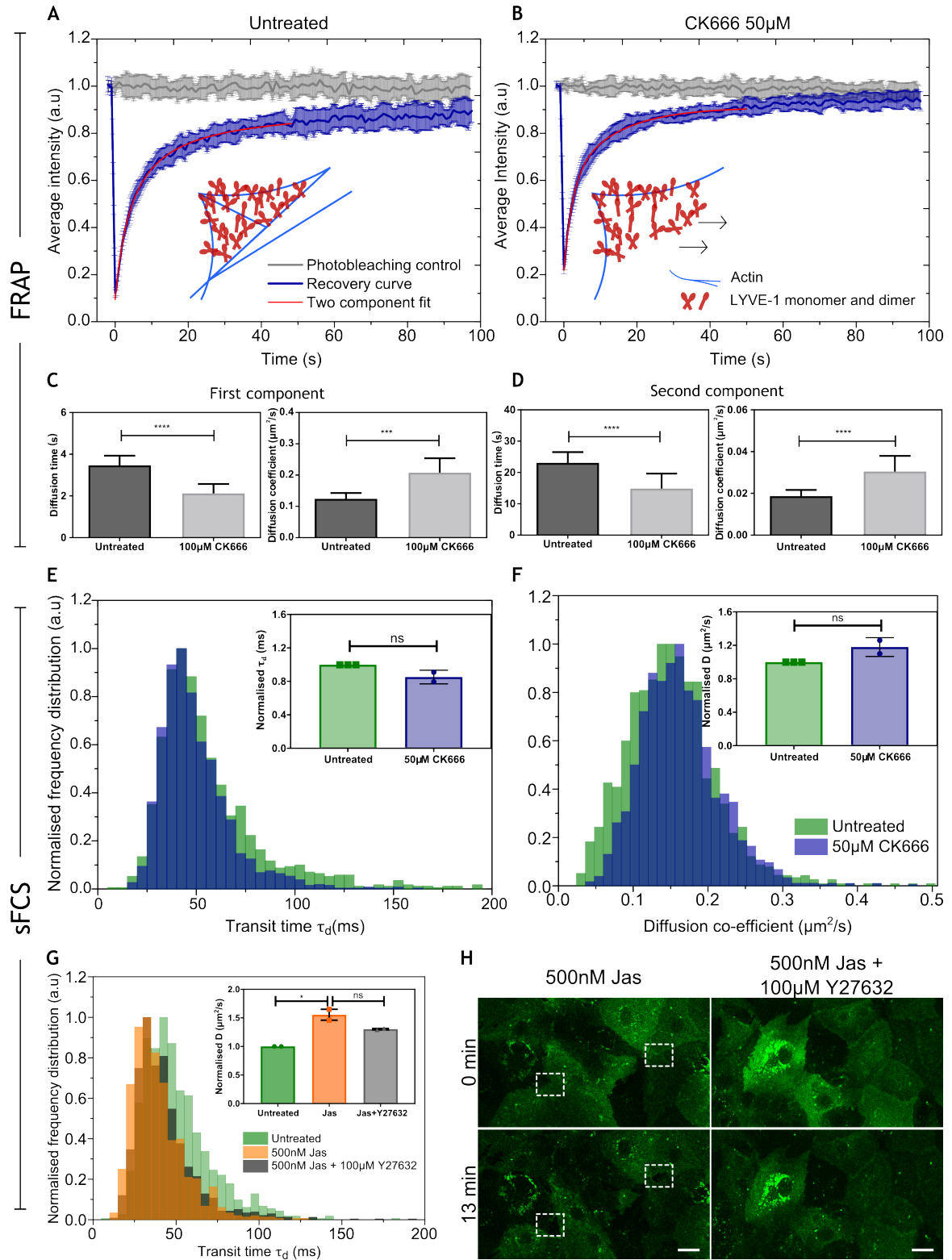


Figure 5.3: FRAP and sFCS measurements of LYVE-1 lateral diffusion to investigate confinement to the actin cytoskeleton. A-B) FRAP experiments on N-terminus ACP hLYVE-1 HDLECs before (A) and after (B) treatment with CK-666 showing the fluorescence recovery (blue) of the diffusing molecules fitted with a two-component free diffusing model. The recovery is compared to the photobleaching control (grey) for LYVE-1. C - D) Average recovery time and determined diffusion coefficient of the first (C) and the second (D) LYVE-1 component as determined from the two-component free diffusing fit of the FRAP curves. E, F) sFCS histograms showing transit time (E) and determined diffusion coefficient (F) of LYVE-1 in primary cells labelled with fluorescent fab conjugate, with (blue) and without (green) CK-666 treatment. The insets show the significance obtained from the replicates and error bar indicating SEM. G) sFCS histograms showing the effect of actin stabilising drug, jasplakinolide on LYVE-1 transit times in primary HDLECs labelled with fluorescent fab conjugates. Inset shows the determined diffusion coefficient with significance with SEM for error bars. H) Confocal images of the primary HDLECs labelled with fluorescent fab conjugates showing the effect of jasplakinolide before (upper panel) and after (lower panel) Y27632 treatment on the morphology of the cells. Scale bar 20 μm.

To confirm this characteristic mobility of LYVE-1 on HDLEC surface, LYVE-1 diffusion on the cells was also measured using sFCS and a different labelling approach. For this, confluent primary HDLECs were used after labelling with fluorescently conjugated LYVE-1 fab antibody as described in the methods. The average transit time (τ) and diffusion coefficient (D) obtained from the numerous individual correlation curves were plotted as histograms against the normalised frequency counts. The histograms obtained were fitted using a LogNormal fit as described in the methods section to obtain the median transit times of LYVE-1 (larger transit times indicate reduced or slower mobility). There was a slight but non-significant shift in the transit time (Fig. 5.3 E) (50 ± 7 ms to 45 ± 5 ms) and in the diffusion coefficient of LYVE-1 after treatment with CK-666 (Fig. 5.3 F) ($0.15 \pm 0.02 \mu\text{m}^2/\text{s}$ to $0.16 \pm 0.02 \mu\text{m}^2/\text{s}$), when compared to the untreated cells (insets, Unpaired t-test, $P = 0.01233$ and 0.1530 respectively), indicating the slight increase in mobility after CK-666 treatment.

Further, the primary cells were treated with an actin stabilising drug, jasplakinolide. This drug works by interacting with the growing monomers and stabilising the actin filament and preventing depolymerisation [185], thus enhancing the confinement of LYVE-1 within the cortex. Contrary to expectation, there is a huge shift in the transit time (with sFCS) with a significant increase (ANOVA with Tukey's multiple comparison $P = 0.0120$), instead of a decrease as would be detected from a potential confinement, in jasplakinolide treated cells (Fig. 5.3 G). Upon checking the phenotype of these cells by confocal time-lapse imaging, it was noted that the integrity of the cells was being compromised during jasplakinolide treatment (Fig. 5.3 H, left panel) as indicated in white dotted square comparing zero and 13 minutes of the treatment. To rectify this, the cells were treated with a combination of jasplakinolide and Y-27632, a Rho-associated protein kinase (ROCK) inhibitor that would disable myosin II, preventing contractility [186]. Although the treatment with Y-27632 rectified the phenotypic changes caused by jasplakinolide (Fig. 5.3 H, right panel), possibly by reducing the formation of actin stress fibres, but it did not alter the diffusion properties of LYVE-1 on the cell surface (Fig. 5.3 G, black).

The results from these experiments demonstrate that LYVE-1 is confined within the actin meshwork on the cell membrane of primary HDLECs.

5.3 Investigating the direct binding of LYVE-1 to the actin cytoskeleton

To test the hypothesis that LYVE-1 might interact with the actin cytoskeleton (Model 2), primary cells were labelled as before with a fluorescent LYVE-1 fab, followed by treatment with either Lat B or Cyto D, and then LYVE-1 diffusion was measured on the cells using sFCS. Figure 5.4 A - C shows the sFCS histograms of the determined values of transit times and diffusion coefficients, plotted against the normalised frequency and fitted with Log Normal fit as detailed in the methods section. The median τ values and D values from each experiment are plotted in Fig. 5.4 C. Remarkably, cells treated with Cyto D or Lat B showed reduced transit times (Fig. 5.4 A and B) (50 ± 7 ms to 44 ± 3 ms for Cyto D and 40 ± 5 ms for Lat B) leading to a consecutive increase in diffusion coefficient (Fig. 5.4 A and B lower panel) ($0.15 \pm 0.02 \mu\text{m}^2/\text{s}$ to $0.16 \pm 0.01 \mu\text{m}^2/\text{s}$ for Cyto D and $0.18 \pm 0.02 \mu\text{m}^2/\text{s}$). The change was found to be statistically significant (ANOVA with Dunnett's Multiple comparisons $P = 0.0099$ for Cyto D and 0.0018 for Lat B) when compared to diffusion of LYVE-1 in the untreated cells (Fig. 5.4 C upper and lower panel), showing a direct influence of LYVE-1 dynamics effected by the removal of the actin cytoskeleton.

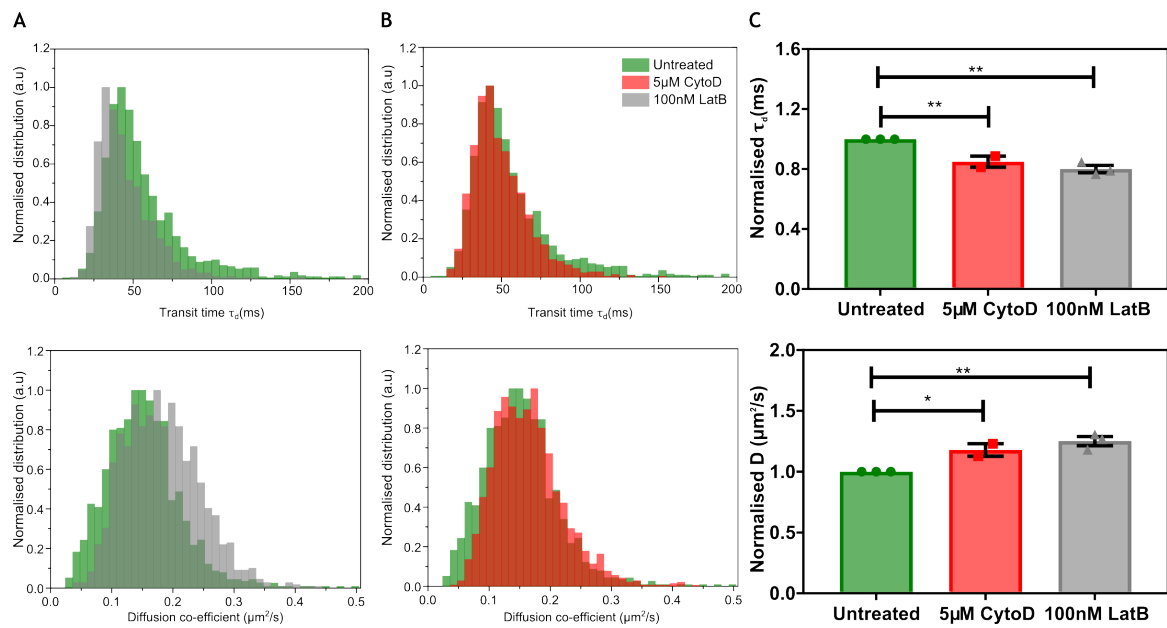


Figure 5.4: Lateral diffusion of LYVE-1 assessed by sFCS upon actin disassembly. A, B) sFCS histograms showing transit time (upper panel) and determined diffusion coefficient (lower panel) of LYVE-1 in primary cells labelled with fluorescent fab conjugate showing a shift in the histograms when treated with Lat B (A) in grey or Cyto D (B) in red. C) Bar graphs indicating the significant changes of the drug treatments for the normalised transit time (upper panel) and diffusion coefficient (lower panel). Error bars = SEM, n = 3.

Having observed a significant shift in the diffusion of LYVE-1 upon depolymerisation of actin, it is still unknown whether this is due to a direct interaction with actin. In order to look for evidence of a potential LYVE-1:actin interaction, a co-immunoprecipitation (co-ip) assay was performed on primary cells. LYVE-1 was immunoprecipitated using biotinylated LYVE-1 (bLYVE-1), run on an SDS-PAGE and blotted for actin to show the interaction of the two. The results from the western blot show that immunoprecipitation (i.p) of LYVE-1 indicated the presence of actin being pulled down along with it (Fig. 5.5 A). This co-ip result shows evidence of a direct LYVE-1 and actin interaction. The amount of actin pulled down with LYVE-1 is significantly lower (Unpaired student t-test, $P = 0.0001$) than that of the total actin in the WCL as quantified in Fig. 5.5 A (lower panel). An i.p in the reverse orientation, which acts as an important control for specificity, using biotinylated phalloidin (bPhal) also shows evidence of its interaction with LYVE-1 (Fig. 5.5 B). These results together provided the evidence of a direct interaction between LYVE-1 and actin, admittedly the amount of LYVE-1 interacting with the actin was significantly lower (Unpaired student t-test, $P = 0.0132$) compared to total LYVE-1 (Fig. 5.5 B upper panel). The lower pull down of LYVE-1:actin complex indicated that though there was direct interaction, it may be a transient interaction thereby resulting in less LYVE-1 or actin pulled down at a time.

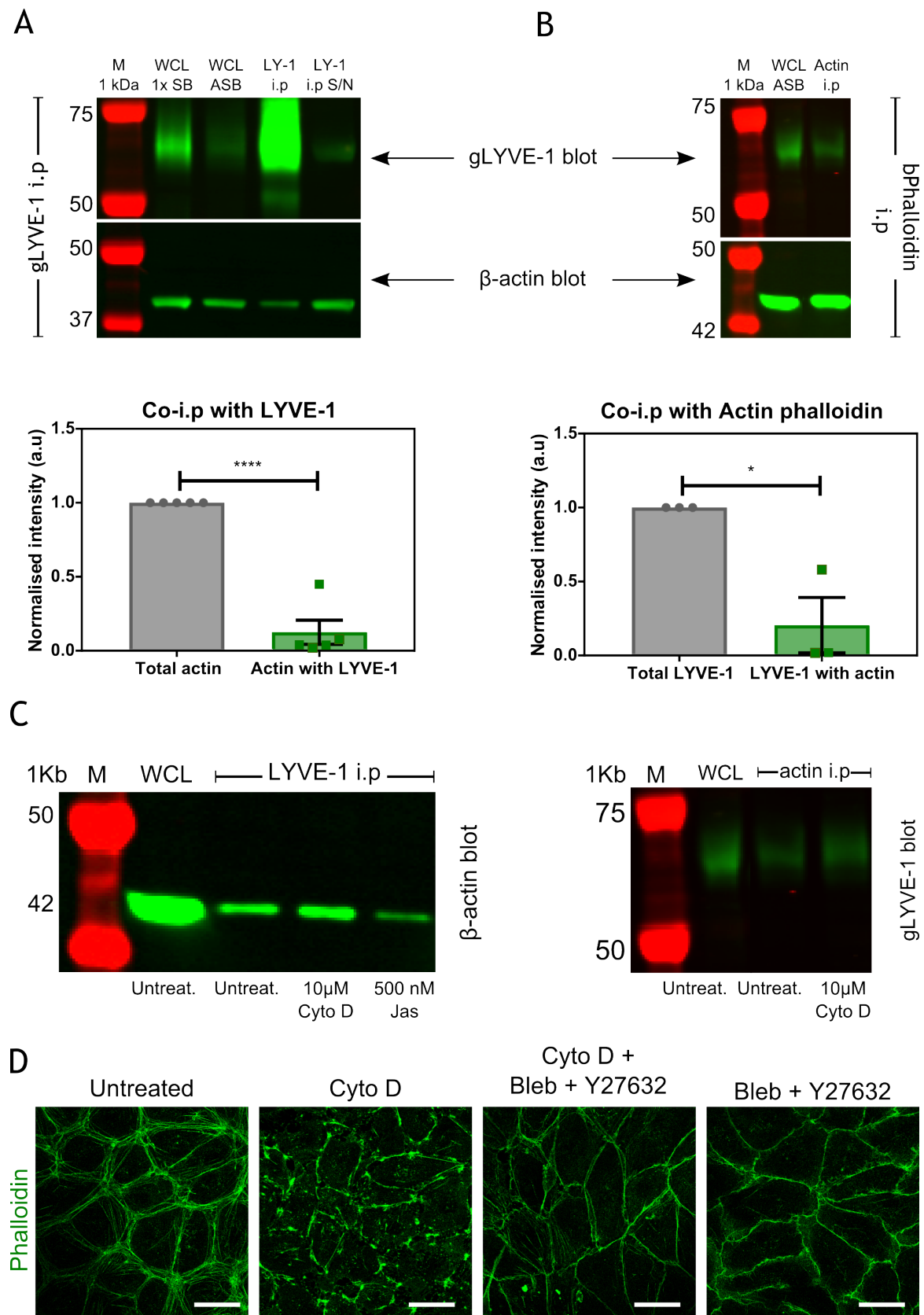


Figure 5.5: Co-immunoprecipitation of LYVE-1:actin complexes determined by western blot. A, B) Co-ip of LYVE-1:actin complexes pulled down using biotinylated LYVE-1 (bLYVE-1) (A) or biotinylated phalloidin (bPhal) (B) showing only small fraction of LYVE-1 and actin bound together (left panels). SB = Sample buffer, WCL = Whole cell lysate, ASB = Actin stabilising buffer, S/N = supernatant C) Co-ip of LYVE-1:actin complexes after actin depolymerisation of stabilisation with b-LYVE-1. D) Confocal images of primary HDLECs labelled for LYVE-1 with a fluorescent fab conjugate treated with Cyto D with or without a cocktail of blebbistatin and Y-27632 to show removal of actin clumps. Scale bar 20 μ m.

Next, to test the authenticity of the LYVE-1:actin interaction detected by co-ip, the experiment was repeated after prior disassembly or stabilisation of the cortical actin in drug treated cells. Accordingly, primary HDLECs were pretreated with Cyto D (destabilising), jasplakinolide (stabilising), or left untreated before lysing the cells, and performing co-ip with bLYVE-1 antibody. The results from the co-ip show that even though the cells were depolymerised with Cyto D or stabilised with jasplakinolide, results opposite to those expected were observed - with an increase in actin pulled down after Cyto D treatment and a decrease after jasplakinolide treatment (Fig. 5.5 C, right panel). The inverse with bPhal also showed an increase in LYVE-1 pull down (Fig. 5.5 C, right panel). Jasplakinolide was not included in this experiment as phalloidin and jasplakinolide bind competitively to the same binding site on F-actin [185, 187].

To assess whether the drug treated co-ip was an artefact of Cyto D treatment resulting in clumping of depolymerised actin monomers (Fig. 5.5 D, panel 2), primary HDLECs were treated with 10 μ M Cyto D, a cocktail of 10 μ M Cyto D, 100 μ M blebbistatin and 500 nM Y-27632, 100 μ M blebbistatin and 500 nM Y-27632 alone or left untreated. Blebbistatin blocks myosin II affinity to actin thus preventing the formation of actin clumps [188], while Y-27632 will contribute to keeping the architecture of the cell [186], thus the combination of the two will remove any clumping of the free monomeric actin. The cells were then labelled with a fluorescent conjugate of phalloidin for labelling actin and imaged with confocal microscopy. The images in Fig. 5.5 D revealed that clumping of actin monomers towards the borders of the cell in Cyto D treated cell, which were abolished in the cells treated with the cocktail of drugs (Fig. 5.5 D, panel 3 and 4). This helps us explain that the co-ip control with Cyto D treatment had not worked as expected because LYVE-1 might be sticking to the clumps of actin monomers that were formed during depletion, resulting in a false increase in the pull down during lysis.

The co-ip results showing the presence of LYVE-1:actin complexes, and the sFCS results indicating changes in LYVE-1 lateral mobility under drug treatment show a

direct, albeit a transient interaction of LYVE-1 with the actin cytoskeleton.

5.4 Interaction of LYVE-1 cytoplasmic tail truncated mutants with the cortical actin

As shown in the previous section, authenticating the LYVE-1:actin interaction *via* drug treatments in the co-ip experiments were complicated. To circumvent this issue, hLYVE-1 tail mutants were generated to study LYVE-1:actin interaction. Full length (FL) hLYVE-1 (Fig. 5.6 A) and C-terminus tail truncated version of hLYVE-1, Δ 263 (Fig. 5.6 B) were generated by Dr. Suneale Banerji. The hLYVE-1 Δ 263 truncation contained five amino acid residues at the cytoplasmic tail which bears a tyrosine phosphorylation site. Primary HDLECs were transduced with a FL version of hLYVE-1 or a C-terminus truncated hLYVE-1 Δ 263 for stable expression in HDLECs, as described in the methods. The transduced cells were analysed for LYVE-1 surface expression by FACS (Fig. 5.7 A) or whole cell expression by Western blot (Fig. 5.7 B).

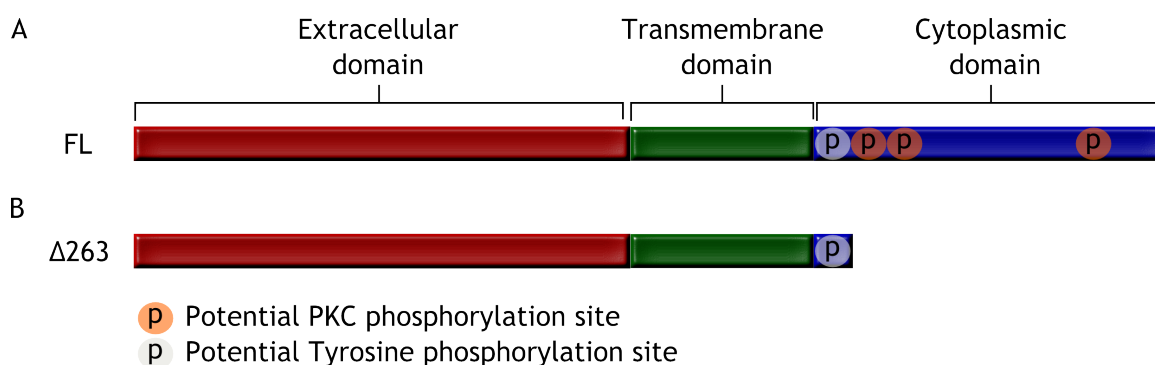


Figure 5.6: Schematics of full length LYVE-1 and cytoplasmic domain truncation Δ 263 mutant. A) Full length (FL) version of hLYVE-1 with 322 amino acids, showing the extracellular domain (red), transmembrane domain (green) and the cytoplasmic tail (blue) which harbour three potential Protein kinase C (PKC) phosphorylation sites and one tyrosine phosphorylation site. B) Cytoplasmic tail truncated version of hLYVE-1, Δ 263, with five remaining amino acids in the tail region with the presence of the potential tyrosine phosphorylation site. Figure adapted from unpublished data in the Jackson lab from *Thomas Nightingale's* thesis.

Figure 5.7 (A) shows FACS histograms of LYVE-1 surface expressions of hLYVE-1 FL (green), hLYVE-1 Δ 263 (orange) and the WT untransfected cells (blue), labelled for surface LYVE-1. hLYVE-1 Δ 263 and FL surface expression was increased compared to the WT cells as hLYVE-1 Δ 263 and FL were inserted into high expressing PHR vectors. The resulting blot from the SDS-PAGE of the whole cell lysates of the

cells (Fig. 5.7 B) shows the presence of the 65 kDa LYVE-1 in all three cell types. The truncated hLYVE-1 Δ 263 in lane two shows a slight shift in the migration pattern since this version of LYVE-1 is shorter than the FL and the native WT LYVE-1 by 59 amino acid residues.

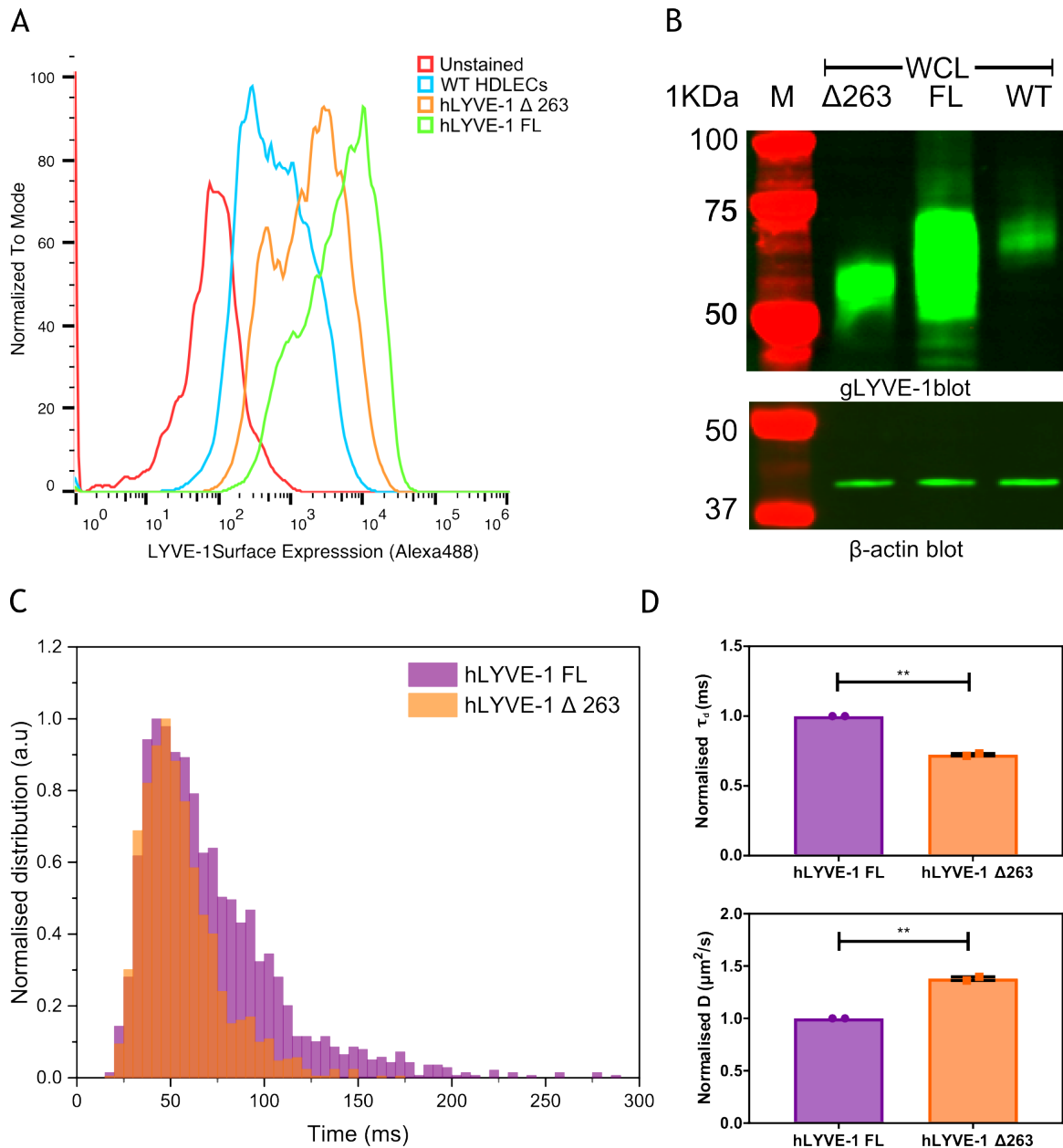
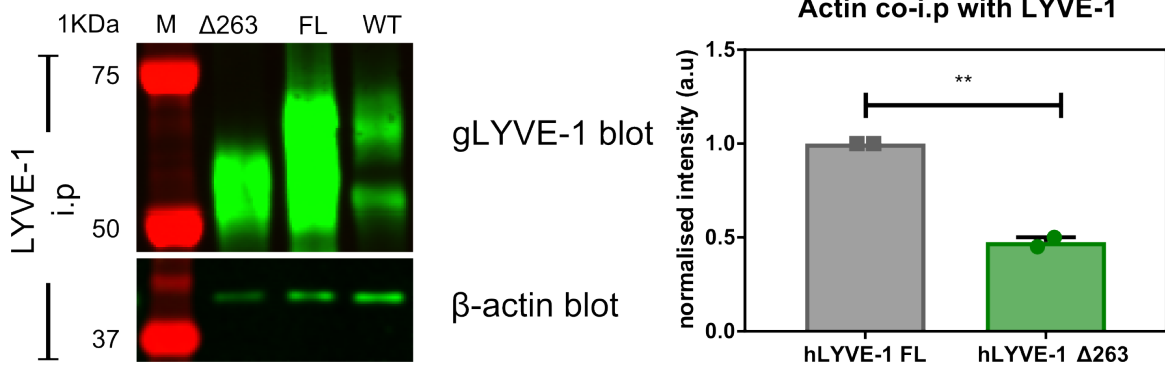


Figure 5.7: Interaction of LYVE-1 Δ 263 and full length (FL) on HDLECs with the cortical actin. A) FACS histograms showing the surface expression of wild type (blue), Δ 263 truncation (orange) and, FL (green) LYVE-1 on the surface of HDLECs labelled with LYVE-1 antibody. B) Total expression levels of Δ 263 truncation, FL LYVE-1 and wild type as assessed by SDS-PAGE. C) sFCS histograms of LYVE-1 transit time of Δ 263 (orange), FL LYVE-1 (purple) plotted against normalised frequency. D) The normalised transit times and determined diffusion coefficient from replicates plotted for significance showing SEM error bars.

A



B

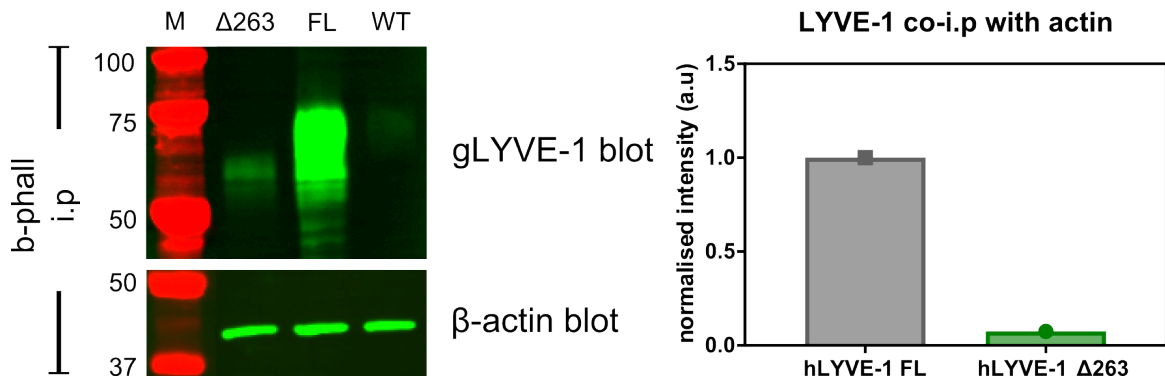


Figure 5.8: Interaction of Δ 263 and FL hLYVE-1 on HDLECs with the cortical actin. A, B) Co-ip of LYVE-1:actin complexes pulled down using b-LYVE-1 (A), or b-phalloidin (B), showing interaction of Δ 263 with the cortical actin.

The next step was to determine whether the truncations of the cytoplasmic tail had an effect on the diffusion properties of LYVE-1. hLYVE-1 FL and Δ 263 HDLECs were labelled with fluorescent LYVE-1 fab antibodies and the diffusion was measured with sFCS as before. Figure 5.7 C compares transit time of hLYVE-1 FL and Δ 263, plotted against the normalised frequency of distribution, indicating a faster transit time for hLYVE-1 Δ 263 when compared to the hLYVE-1 FL transfected cells (Fig. 5.7 C). The individual median values of transit times were calculated from the frequency histograms as before using the Log Normal fit and the results from the replicates show a significant change in the median transit time from 60 ± 15 ms in FL to 50 ± 4 ms in hLYVE-1 Δ 263 (Unpaired student t-test, $P = 0.0011$) and values of the diffusion coefficient from $0.12 \pm 0.03 \mu\text{m}^2/\text{s}$ to $0.14 \pm 0.01 \mu\text{m}^2/\text{s}$ (Unpaired student t-test, $P = 0.0020$) (Fig. 5.7 D), highlighting an increased mobility of the

cytoplasmic truncated mutant and indicating a reduction in interaction of LYVE-1 cytoplasmic tail with the cortical actin.

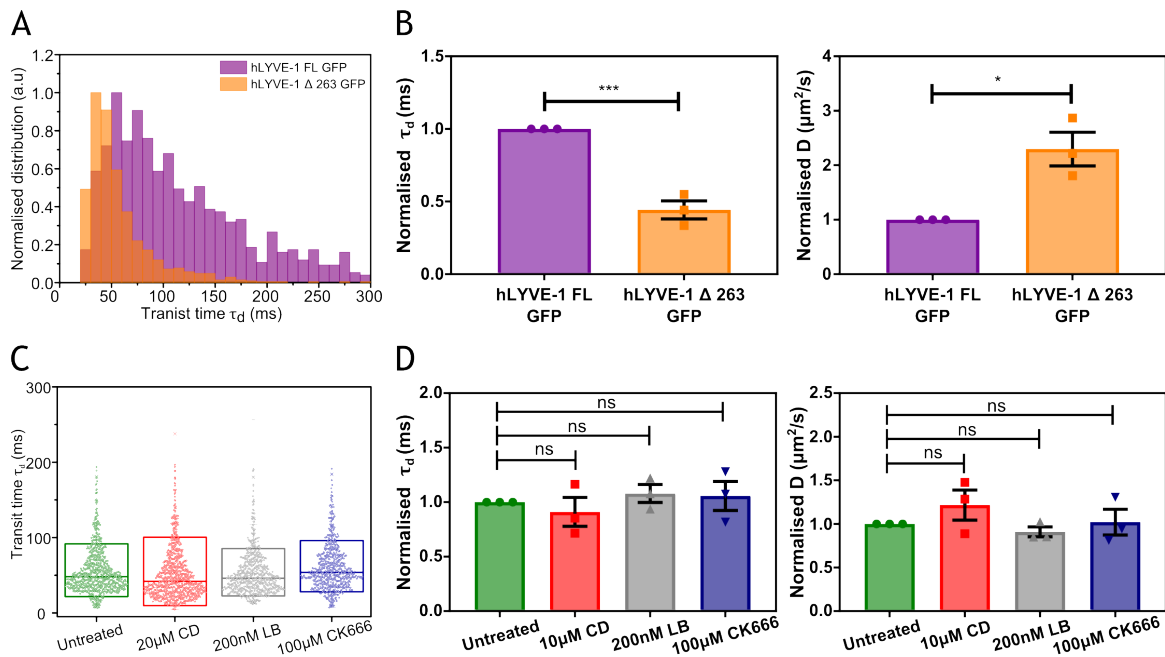


Figure 5.9: Effect of drug treatments on diffusion of Δ 263 hLYVE-1 GFP HDLECs. A,B) sFCS histograms of transit times showing a shift in the transit times of LYVE-1 Δ 263 GFP (orange) when compared to the FL GFP (purple). The significance obtained from replicates of the normalised transit time and diffusion coefficient is plotted in (B). C, D) Normalised sFCS box chart showing the transit times of LYVE-1 Δ 263 GFP after treatment with Cyto D (red), Lat B (grey), or CK-666 (blue) showing no evidence of interaction with actin. The significance obtained from replicates of the normalised transit time and diffusion coefficient is plotted in (D).

To confirm that there was no interaction between LYVE-1 and the actin cytoskeleton in the truncated version of LYVE-1, co-ip was performed as in the previous section (Fig. 5.8 A and B). The immunoprecipitation results, using bLYVE-1 antibody and bPhal shows the presence of LYVE-1:actin interaction in the truncated hLYVE-1 Δ 263 version (Fig. 5.8 A and B, right panels), indicating that hLYVE-1 Δ 263 truncated version still interacts with actin although much less compared to the hLYVE-1 FL version.

With the truncated mutant also showing a transient interaction with actin, it was important to determine if this interaction of hLYVE-1 Δ 263 with the actin cytoskeleton can be disrupted. In order to do so, primary HDLECs transfected with hLYVE-1 FL and Δ 263 with a C-terminus GFP-tag was used. First, the mobility of hLYVE-1 Δ 263 GFP was compared with the hLYVE-1 FL GFP to check that the mobility of the two in the presence of a GFP-tag exhibits the same trend as those

without the GFP tag. Figure 5.9 A and B show hLYVE-1 FL and Δ 263 GFP transit time histograms and median values from triplicates with a clear significant shift in the transit times (90 ± 12 ms in FL to 40 ± 5 ms in hLYVE-1 Δ 263; Unpaired student t-test, $P = 0.0008$) and a significant increase in diffusion coefficient ($0.07 \pm 0.01 \mu\text{m}^2/\text{s}$ to $0.18 \pm 0.02 \mu\text{m}^2/\text{s}$; Unpaired student t-test, $P = 0.0137$). Next, the hLYVE-1 Δ 263 GFP cells were subject to treatment with cytoskeletal depletion drugs as before which resulted in no effective change in the determined transit times or diffusion coefficient values (One way ANOVA $P = 0.6721$ and 0.3645 respectively) as seen in Fig. 5.9 (C and D). This indicated that the transient interaction of the truncated hLYVE-1 Δ 263 was not directly detectable using sFCS.

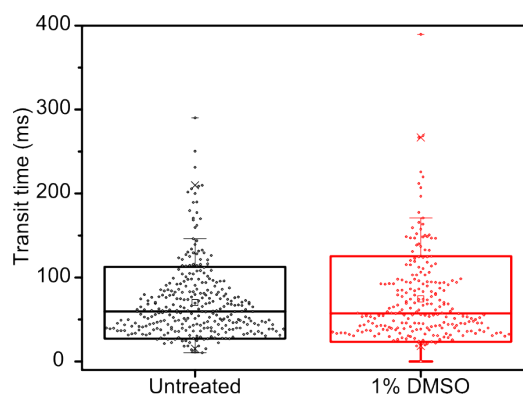


Figure 5.10: Effect of DMSO on hLYVE-1 GFP mobility on HDLECs. sFCS box charts showing the effect of DMSO on the transit times of hLYVE-1 FL GFP.

As most of the actin depolymerisation drugs were dissolved in DMSO, the effect of DMSO on the diffusion of LYVE-1 was tested in the hLYVE-1 FL GFP transfected HDLECs. As shown in the results, the values of transit times of LYVE-1 GFP in untreated and 1% DMSO treated cells was almost identical (Fig. 5.10), thus it can be concluded that DMSO did not affect the diffusion of LYVE-1.

5.5 Identifying the diffusion mode of LYVE-1 in HDLECs

The diffusion studies using drug treatment in the previous results have shown evidence of LYVE-1 lateral mobility being hindered by the cortical actin cytoskeleton. This hindrance could be due to confinement within the actin meshwork (Model 1) or transient direct interactions (Model 2) or both, all resulting in different diffusion

modes. For example, Model 1 would result in compartmentalised or hop diffusion, where LYVE-1 would diffuse freely within a meshwork compartments but crossing to the next compartment would result in a hindrance, while Model 2 would result in a trapped diffusion, where freely diffusing LYVE-1 is transiently held at a place due to interaction with actin. In order to determine such diffusion modes, with a minimally invasive technique and no drug treatments, STED-FCS measurements on live primary HDLECs were performed. By measuring the diffusion coefficient (D), using STED-FCS, in observation spot sizes between 100 nm to 200 nm in diameter (d), one can highlight possible hindered diffusion modes, such as transient trapping, where the D values are expected to decrease towards smaller d , or hop diffusion where the D values would increase towards a smaller d [71, 100, 101, 113, 140]. In contrast, this relationship ($D(d)$) is expected to be constant for free diffusion. The various observation spot sizes (as tuned by the STED laser power) were calibrated with a fluorescent lipid analog (DHPE-KK114) in a fluid DOPC lipid bilayer as described in the methods before carrying out the measurement on the cells.

STED-FCS measurements were carried out on primary HDLECs at varying STED powers and thus observation spot diameters. Figure 5.11 shows the determined D values of LYVE-1 against the observation spot diameter (d). The $D(d)$ dependencies obtained from individual cells and cell positions showed huge variations, as expected from primary cells. Both, free (constant $D(d)$ dependency) as well as slight to strong transient confinement (decrease of D with small d) was observed (Fig. 5.11 A (i-iii)).

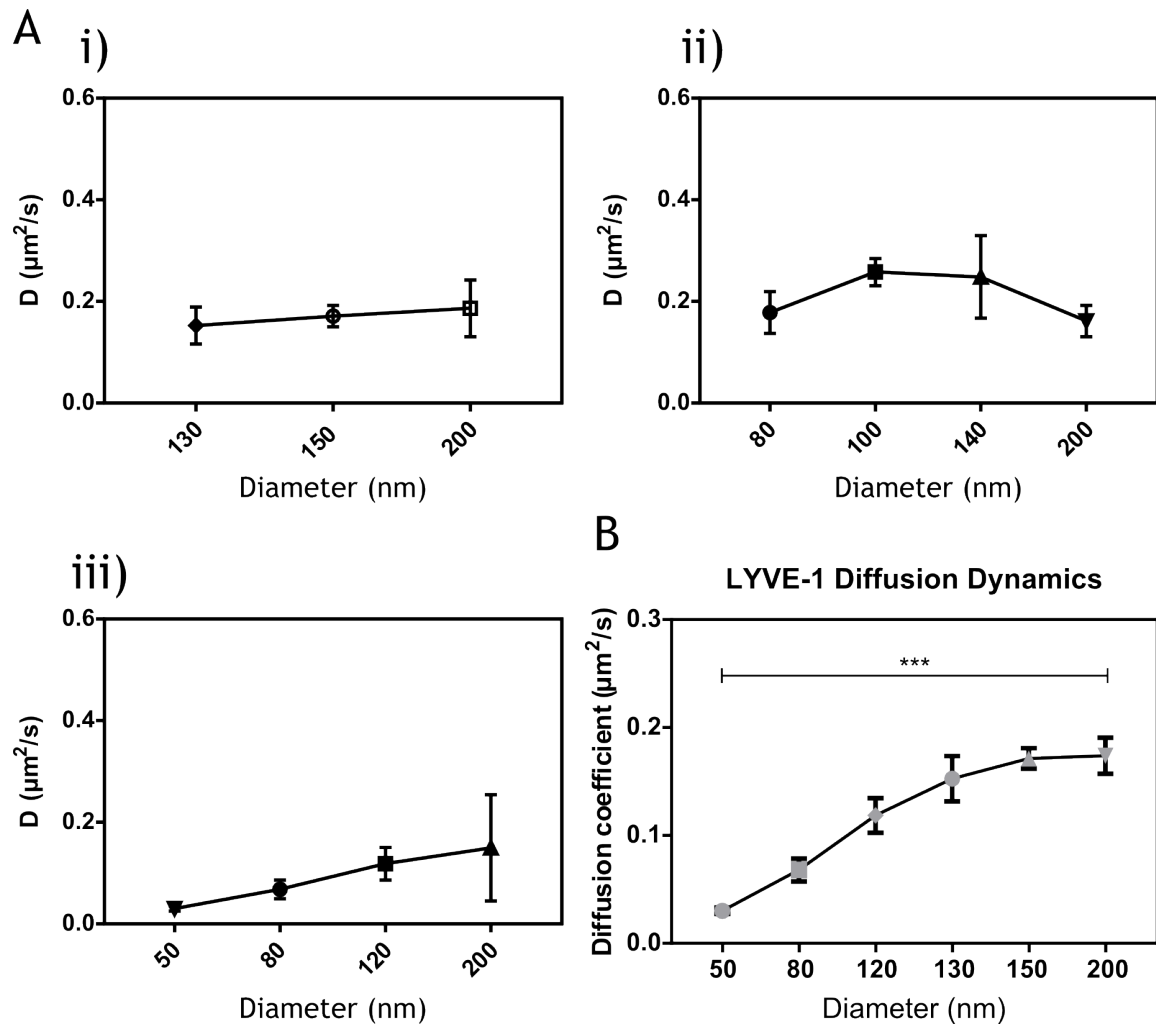


Figure 5.11: Observation of LYVE-1 trapped diffusion with varying STED spot diameter. A) Determined diffusion coefficients of LYVE-1 in primary HDLECs, labelled with fluorescent fab KK114 showing a variability with each replicate (i-iii). B) Pooled data set of the 3 replicates showing LYVE-1 diffusion at 200 nm spot size $0.17 \pm 0.06 \mu\text{m}^2/\text{s}$ decreasing to $0.03 \pm 0.002 \mu\text{m}^2/\text{s}$ at smaller spot size of 50 nm indicating trapped diffusion of LYVE-1.

However, when the data sets were combined, a clear decrease of D towards smaller d indicating trapped diffusion was detected. Diffusion at $d = 200$ nm was found to decrease from $0.17 \pm 0.06 \mu\text{m}^2/\text{s}$ to $0.03 \pm 0.002 \mu\text{m}^2/\text{s}$ at $d = 50$ nm (One way ANOVA with Tukey's multiple comparison; $P = 0.0006$) (Fig. 5.11 B). Combining the results of the previous experiments, this provided further evidence that LYVE-1 predominantly interacts with actin structures.

5.6 Discussion

In this chapter, it was established that the lateral mobility of LYVE-1 is orchestrated by the cortical actin cytoskeleton. Furthermore LYVE-1 was found to be confined

by the cortical actin meshwork, and also transiently bound to it. This transient binding and confinement restricts LYVE-1 in its native or steady state from interacting with its ligand HA.

A number of intrinsic and extrinsic factors can effect of the cells behaviour, and it was critically important to control the effect of this on the experimental results. Here, it was established that culture length after confluency did not affect LYVE-1 mobility on the surface despite the fact that LYVE-1 surface density increased (3 fold) over 3 days, and depending on the labelling method and techniques used, the diffusion coefficient of LYVE-1 varied (Table 2 and 3), suggesting to observe relative trends rather than absolute values between different observation and labelling techniques.

Transmembrane receptors such as TR, epidermal growth factor receptor (EGFR), MHC-I, E-cadherin, CD22 have been shown to alter their lateral mobility upon actin turnover [10, 102, 103, 129, 131, 179]. Edidin et al have shown that MHC I molecules were able to travel longer length scales on the membrane surface of cells, when the cytoskeletal barriers were removed. This was shown to be due to the surface receptors often found confined or bound by the cortical actin cytoskeleton [102, 103]. Similarly, diffusion of LYVE-1 was identified to be confined by the cortical actin meshwork when studied by FRAP in combination with drug treatments such as CK-666.

LYVE-1 was also found to interact with the actin filaments in primary HDLECs. Cells treated with Cyto D and Lat B resulted in an increase in lateral mobility (a 1 fold increase) with sFCS showing evidence for interaction with the cortical actin. Whether this interaction is indirect by the aid of adaptor proteins or direct is a question that still remains unexplored. However, it was established, by co-ip pull down of LYVE-1:actin complexes, that the interaction is transient, as often only 2 % of LYVE-1 is interacting with actin at a time. In order confirm the interaction of the cytoplasmic tail with actin, LYVE-1 cytoplasmic truncation mutants hLYVE-1 Δ 263 and FL hLYVE-1 with and without a GFP tag were generated in HDLECs.

sFCS data show that hLYVE-1 Δ 263, with only five amino acids in the tail region, has faster mobility on the surface of HDLECs compared to its full length version ($0.12 \pm 0.03 \mu\text{m}^2/\text{s}$ to $0.14 \pm 0.01 \mu\text{m}^2/\text{s}$; Unpaired student t-test, $P = 0.0020$). Even if the mobility was found to be increased, indicating lower or no interaction with actin, the co-ip experiments showed that the truncated mutants still interacted with actin. Contrary to this, sFCS results of hLYVE-1 Δ 263 GFP with actin depolymerisation showed no difference (One way ANOVA $P = 0.6721$ and 0.3645 respectively) in LYVE-1 mobility on the membrane surface after actin depolymerisation. The shorter tail of LYVE-1 cytoplasmic domain may have allowed a faster mobility on the cell surface but it may still interact with actin when encountering a much dense cortical compartment. Similar interactions using SPT have been studied for truncated MHC-I molecules and E-cadherin [103]. Both these truncated receptors were capable of travelling longer distance on the membrane before being hindered again by the cortical actin. Even though, an interaction between hLYVE-1 Δ 263 and actin was observed in the co-ip experiments, it may be that the transient binding and unbinding occurs at a fast time frame that cannot be picked up in sFCS method. Therefore, much more temporally sensitive techniques like SPT and also STED-FCS may need to be employed to study this interaction.

As previously introduced, sequence homology studies of LYVE-1 cytoplasmic tail revealed a ERM-like domain similar to CD44 (Wang, YJ. et al, unpublished). In the truncation mutant hLYVE-1 Δ 263, the ERM consensus sequence (about 59 amino acids) was deleted, only leaving the potential tyrosine phosphorylation site (Fig. 5.6) and a few basic residues. Therefore, the reason the interaction was still present in the co-ip may be due to the remaining tyrosine site on the truncated tail region which could interact with the actin cytoskeleton *via* and unknown method.

Finally to conclude, the confinement or binding mechanism of LYVE-1 in primary HDLECs was confirmed by the non-invasive super-resolution STED-FCS measurements. Employing this technique, LYVE-1 diffusion at varying STED spot size was determined which clearly exhibited a trapped mode of diffusion of LYVE-1 on the

cell surface ($0.17 \pm 0.06 \mu\text{m}^2/\text{s}$ at 200nm to $0.03 \pm 0.002 \mu\text{m}^2/\text{s}$ at 50 nm). This along with functional data can provide a clear indication for the regulation of LYVE-1 function by the cortical actin.

Chapter 6

Conclusion

In this thesis, I set out to answer three main questions. What is the nanoscale organisation of LYVE-1 that potentiates HA binding? Is there an influence of the actin cytoskeleton on LYVE-1 organisation and HA binding function? Could the lateral mobility of LYVE-1 on the membrane surface be a mechanism of LYVE-1 modulating receptor functions? The results from this thesis help answer those questions. LYVE-1 clusters that potentiate HA binding were organised as 100 nm clusters on the surface of LECs but the clusters were not dependent on the cortical actin cytoskeleton. Nevertheless, it was found that removing the actin cytoskeleton does induce an increase in LYVE-1 binding to HA. Finally, a direct and transient interaction of LYVE-1 with actin was identified along with evidence to LYVE-1 exhibiting a confined mode of diffusion on the surface of primary HDLECs.

Working with primary cells was challenging as batch to batch variation in the cells were affecting the results. Thus every experimental variable was optimised to the best possible degree in this thesis. Using transfected hLYVE-1 HDLECs, the fixation studies were optimised and further on, the primary cell culture length and labelling strategies were also optimised for studying lateral dynamics of LYVE-1.

At the first instance, I observed the native distribution of LYVE-1 in primary human dermal lymphatic endothelial cells (HDLECs) supertransfected with LYVE-1. To study the native organisation, LYVE-1 was labelled with a fluorescent fab conjugate of LYVE-1 (8C) mAb at the N-terminus. This and live cell labelling *via* the C-terminus using Halo ligand showed that surface LYVE-1 is fairly homogeneous in distribution with no visible clustering. The process of switching from native to cross-linked state was not as straight forward as expected for labelling. Labelling with primary LYVE-1 mAb and secondary fluorescent conjugate altered the distribution of the clusters when compared to direct immunolabelling with bivalent LYVE-1 mAb.

It turned out that the process of immunolabelling, using PFA alone for fixation, was prone to artefacts that interfered with studying LYVE-1 clustering. As often, these artefacts are due to improper fixation techniques prior to immunolabelling. Fortunately, advances in imaging techniques provided better labelling strategies which have now been imposed for confocal and super-resolution imaging [115, 162, 164]. In this study, I combined fixation methods of 1% PFA with a second fixative, GA at 0.2%, to completely immobilise the LYVE-1 receptors. Using this fixation technique, I imaged the cross-linked state of LYVE-1 by super-resolution STED microscopy and analysed the clusters formed on the cell surface. The analysis revealed that cross-linked LYVE-1 organised into 100 - 150 nm clusters on the surface of HDLECs. LYVE-1 clustering induced by physiologically mAb cross-linked HA, Group A Streptococcus (GAS) generated LYVE-1 clustering, or those caused by DC trans migratory cups were not investigated in this thesis but are planned for future work.

Clustering of membrane proteins that contributes to functional changes is often mediated by the membrane lipid layer and the cortical actin cytoskeleton [8, 105, 119, 189]. Hitherto, there has been no evidence which links the function of LYVE-1 or its surface clustering to the cortical actin cytoskeleton. In the LYVE-1 homologue CD44, the receptor has been shown to interact with the cytoskeleton through the Ezrin/Radixin/Moesin (ERM) and ankyrin adaptor proteins. When this interaction was destabilised it altered the membrane organisation of CD44 to smaller or loose clusters. CD44 facilitated rolling function of neutrophils on E-selectin expressing endothelial cells was also found to be impaired when the cytoplasmic ERM/Ankyrin region was deleted revealing a crucial role of actin in mediating CD44 clustering and function [8]. Hence, I was curious to know if the clustering of LYVE-1 induced by cross-linking was also mediated by the actin cytoskeleton. Strangely, clustering of mAb cross-linked LYVE-1 on primary HDLECs did not alter significantly when the actin cytoskeleton was depolymerised. Although this was disconcerting, similar conclusions were observed for the transmembrane receptor CD22 [179]. Looking at the reduced interaction in the later co-ip experiments, it provides some explanation

why there was little change in receptor clustering. The co-ip results showed that at any given time, there was only a small percentage of LYVE-1 molecules interacting with actin. Therefore, it may be that at one fixed time point and method, the mAB cross-linked LYVE-1 clusters on the cell surface are all the same whether or not the actin was depolymerised.

Proteins can associate with lipid rich environments on the plasma membrane as seen for CD44 in fibroblasts and epithelial cells. Antibody clustering of CD44 was found to co-cluster with annexin II membrane proteins. This co-clustering was found to be in actin and cholesterol rich sites [105]. A similar mechanism may hold true for LYVE-1, with LYVE-1 interacting with other membrane proteins in lipid rich areas. Baluk et al, have shown that CD31 was located in a similar position to LYVE-1 at the tips of overlapping flaps in the cells of the initial lymphatics [22]. In platelet cells, CD31 was found to be held in lipid rich sites while interacting with the FcR region on the membrane glycoprotein VI (GPVI-FC γ) and once the cholesterol was removed, it resulted in enhanced clumping of platelet cells [190]. Hence, it is possible that LYVE-1 could also be interacting with CD31 in lipid rich sites, that prevent changes in receptor clusters when actin alone is depleted. Also, cholesterol removal from T cells caused the dispersal of CD44 from cholesterol rich areas and also increased adhesion and rolling of these cells on endothelium *via* HA binding to CD44 on the T cells [191]. Preliminary experiments of LYVE-1 binding to cross-linked HA was found to be increased in cholesterol depleted primary cells (Appendix Fig. 6.3). Therefore, clustering might vary if both actin and cholesterol were removed at the same time. Also, the clustering of LYVE-1 here was studied by fixing the cells after adding the cross-linking primary antibody, and chemically fixing the cells. This may not be the ideal way to study receptor clustering because this mechanism is a highly dynamic process.

The interaction of CD44 to the cytoskeleton occurs *via* ERM protein associations [8, 93, 105]. As introduced earlier, sequence homology of LYVE-1 cytoplasmic tail with CD44 showed evidence of ERM-like protein sites on the cytoplasmic tail

(Wang, YJ., et al. unpublished) suggesting that ERM proteins may link LYVE-1 to the actin cytoskeleton. However, blotting for endogenous levels of ERM proteins in the preliminary co-ip assays did not show evidence of ERM proteins in pulled down with LYVE-1:actin complex (data not shown). Imaging of CD44 clusters along with annexin II [105] indicated that actin bundle rearrangements cause the clusters to form in actin rich areas. Unfortunately, direct visualisation of actin and LYVE-1 using confocal imaging indicated no evidence for LYVE-1 clusters present in actin rich areas (Appendix Fig. 6.1). This may fall in line with the co-ip and sFCS experiments where I exhibited evidence for the interaction of LYVE-1 to be transient, with only a small percentage of LYVE-1:actin complexes identified to be interacting at a given time or only a 1 fold increase in LYVE-1 mobility upon actin disassembly. Hence, interactions to the actin cytoskeleton could occur *via* other binding partners of LYVE-1, or it links to phosphorylated forms of ERM proteins, or LYVE-1 link directly to the actin in a transient fashion without the aid of actin binding proteins.

In CD22, the organisation of the receptors did not change but the lateral mobility was increased when the actin cytoskeleton was depleted leading to an increase in BCR signalling function [179]. This promoted the question of whether the lateral dynamics of LYVE-1 receptors in primary HDLECs contribute to the avidity induced increase in functions upon actin depolymerisation. To assess this, primary HDLECs were tested for HA binding after the actin cytoskeleton was depolymerised. This indeed contributed to an increase in LYVE-1 binding to complexed HA and even with uncomplexed linear HA polymers (Appendix Fig. 6.2). I can only speculate that the increase in HA binding, without the aid of cross-linking, was due to the cross-linked HA molecules seizing enough receptors to provide the necessary avidity required for HA interaction.

Another possible reason for the increase in HA binding could be due to associations with proteins such as galectins. Galectins are carbohydrate binding proteins virtually present in all living organisms. They bind to N- and O- glycans present on glycoproteins. Galectin-8 has been shown to have high-affinity binding to integrins and to

a variant of CD44 in the extracellular matrix. This binding to integrins ($\alpha 3\beta 1$ and $\alpha 6\beta 1$) regulates their adhesive and signalling behaviour [192]. Galectin-1, is known to bind to several transmembrane receptors such as CD3, CD7, CD43 and CD45 thereby cross-linking the receptors and forming a lattice, which aids in organising receptors on the membrane and increase ligand interaction [193]. Similar mechanisms could be involved with LYVE-1:HA binding in the LECs, once LYVE-1 is released after actin depolymerisation, as galectins (galectin-8) are also expressed by LECs [194].

The lateral mobility of transmembrane receptors are regulated by the membrane and its actin cytoskeleton. The dynamics of these receptors often command the functional properties of the receptors. In order to study lateral mobility, three hypothetical models of LYVE-1 interaction with the actin cytoskeleton were proposed. Receptors such as EGFR, MHC-I, E-cadherin, CD14, CD22 and TR were found to be either linked to the actin cytoskeleton [129, 131, 179] or to the lipid rich environments like that of TLR receptors [132] and for other receptors, for example, like CD44 are linked to lipids rich domain and the actin cytoskeleton [8, 191]. For studying actin related dynamics, it is important to understand that the close proximity of the cortical actin to the plasma membrane generates cortical compartments to which transmembrane receptors can be confined [9, 10, 102, 103]. Hence, membrane receptors and proteins are often directly bound to the actin cytoskeleton or they are confined with the dense cortical meshwork.

When the LYVE-1 mobility was measured by sFCS, before and after depolymerisation of actin using Lat B and Cyto D, it indicated that LYVE-1 mobility increased upon actin depolymerisation. As these drugs act on the disassembly of F-actin by preventing filament extension it creates an environment where LYVE-1 cannot bind or interact with the filaments. Thus, this proved the hypothesis that LYVE-1 is bound to the cortical actin and once released shows faster mobility. Similarly, LYVE-1 mobility was also found to be confined within the cortical actin compartments as treatment with CK-666 increased the mobility of LYVE-1 when measured using FRAP. This drug as introduced earlier targets Arp 2/3 complex and prevents the formation of

filament branching, thus creating larger cortical mesh sizes. Cytoplasmic truncated versions of hLYVE-1, Δ 263 (with 5 amino acids residues in the cytoplasmic tail), also showed faster mobility when compared to full length versions of LYVE-1 indicating that the lateral diffusion of LYVE-1 is hindered by its cytoplasmic tail interacting with the cortical actin. This has also been evidenced in the movement of MHC I molecules on the membrane of hepatoma cells (HEPA-OVA), where truncated MHC I molecules (with 4 amino acid tail residues) and E-cadherin (with 21 amino acid residues in the cytoplasmic tail) travelled longer length scales on the membrane surface compared to their full length versions. Eventually, the movement of truncated MHC I was hindered indicating that it is still confined within actin cytoskeleton compartments [102, 103]. Although, truncated version of hLYVE-1, Δ 263, did not show any confinement or binding to the actin cytoskeleton in the dynamic studies using sFCS, co-ip results indicated that hLYVE-1 Δ 263:actin complex was still present highlighting there is some residual interaction to the cortical actin by truncated LYVE-1. This may be due to the potential tyrosine phosphorylation site (as shown in Chapter 5, Fig. 5.6) in the 5 remaining amino acid residues of the cytoplasmic tail.

Finally, using advanced super-resolution STED-FCS, the diffusion mode of LYVE-1 on the surface of primary HDLECs was identified. The $D(d)$ dependency of LYVE-1 diffusion indicated that LYVE-1 mobility exhibits confinement on the membrane of primary HDLECs. As studied using sFCS, FRAP and co-ip, the diffusion may be hindered due to the interaction of LYVE-1 with the actin cytoskeleton. The confinement may also be due to interaction with several other molecular players that could contribute to the confinement of LYVE-1 such as membrane lipids like cholesterol or interaction with membrane proteins and receptors like galectins and CD31 [8, 22, 192].

As highlighted from the results from Chapter 4 and 5, the role of actin in the HA binding of LYVE-1 provides new insights on the regulation of the receptor. Thus, opening up several areas of exploration for LYVE-1 such as signalling and endocytosis mediated by the cytoplasmic tail of LYVE-1.

A Biological Role

The results from this thesis provide a link between LYVE-1 receptor diffusion and its function with respect to binding HA modulated by the actin cytoskeleton. But the question does arise: how does this mechanism prove to be physiologically relevant?

In normal physiological conditions, the actin cytoskeleton stabilise the junctions of endothelial cells *via* VE-cadherin thereby providing barrier and structural integrity to the endothelium. It is now well known that the junctional VE-cadherin/ catenin complex interacts with the actin cytoskeleton. The junctional integrity of all cells is mostly maintained by the Arp2/3 complex of the actin cytoskeleton [76]. There is surmounting evidence of actin involvement in endothelial junctional remodulation. These events are largely studied in blood endothelial cells but similar mechanism could be emulated by LECs as they are also known to have VE-cadherin rich junctions [22, 76]. In skin epidermal layer, CD44 was found to regulate tight junction assembly, where the absence of CD44 causes a disturbance in the junctional integrity [195]. Similarly, VE-cadherin and LYVE-1 rich junctions were altered when mouse LECs were treated with LYVE-1 mAbs or low molecular weight HA (Wang, YJ., et al. unpublished). LYVE-1 ligands (PDGF) induce disruption of VE-cadherin mediated intercellular adhesion and opening of the intercellular junction in LEC monolayers [53]. This junctional remodelling, often modulated by Arp2/3 in endothelial cells [76], causes changes in actin turnover, which in turn could alter the mobility of LYVE-1 and hence, its interaction with HA. Hence the changes in lateral diffusion and function mediated by treatment with CK-666 could be an imitation of the physiological setting, as described above, leading to endothelial barrier disfunction and hence increased adhesion of, for example, HA coated DC and migration *via* the lymphatics [3] as described below.

The cell membrane is in constant communication with its surroundings at normal and pathological conditions. This allows it to effectively respond to signals received from extracellular environment. These signals are transduced to the inside of the cell

to initiate effector functions (such as signalling, adhesion, cell migration or changes in barrier integrity). The cortical actin and the plasma membrane are well known to orchestrate receptor signalling events. This can be evidenced in CD44 receptors, where an inflammatory environment triggers the expression of CD44 on lymphocytes and its ability to bind HA increases [60]. Also, this expression on leukocytes facilitates binding to E-selectin on endothelial cells and permits the rolling of the cells on the endothelium [8] for extravasation. Similarly, the CD44 lymphocytes coated in HA could foster the binding or adhesion to LYVE-1 expressed on LECs [196] prior to entering or exiting the lymphatics [23, 41]. This mechanism has been imitated by HA coated macrophages that engage LYVE-1 on LECs, facilitating their adhesion and transit across lymphatic endothelium [6]. Therefore, it is plausible for other lymphocytes mimicking this mechanism of cell trafficking.

Bacterial toxins have shown to hijack the actin controlling machinery (Rho-GTPases) or directly target actin (by AADP ribosylation) to alter the cytoskeleton. Remodelling of the actin cytoskeleton is then mediated by the assembly and disassembly of the actin filaments which depend on the ratio of G-actin monomers available and on the actin binding and regulating proteins [76]. Thus, the actin disassembly mediated by the Cyto D and Lat B drugs that increase HA binding and lateral mobility of receptors can re-enact this mechanism by which GAS hijack the lymphatic endothelial cells to bind to HA and disseminate *via* the lymphatics [4].

Another unexplored function that could be mediated by the actin cytoskeleton and lateral dynamics is the ectodomain shedding of LYVE-1. Shedding of LYVE-1 has been shown to occur in the presence of VEGF-A [197] and in the presence of inflammatory cytokine, TNF- α [1]. It could be possible that during an inflammatory response, endothelial remodelling occurs due to actin turnover, which may contribute to shedding of LYVE-1 as a result of complete release of LYVE-1 from the cortical actin. It can be speculated that the release of LYVE-1 from the actin network could either prevent or enhance receptor shedding which in turn either mediates or blocks the turnover of LYVE-1 [1, 197].

As evidenced in this thesis, I have only studied the interactions of LYVE-1 with HA during actin turnover. There are several other membrane associated proteins and receptors that could contribute to LYVE-1 clustering, lateral diffusion and functional binding to HA. A few examples include interaction of LYVE-1 with CD31 present on similar sites, integrins and ICAM and VCAM, or galectins that could mediate adhesion and transmigration of immune cells during inflammatory response [1, 17, 22, 192, 198].

Future Work

An immediate exploration would be in identifying the diffusion modes exhibited by the truncations of LYVE-1, including hLYVE-1 Δ 263, and of tail-less LYVE-1 mutants using STED-FCS. Alternatively, to confirm this, the confined mode of diffusion will also be tested in primary cells in the presence of actin depolymerisation drugs. It would be curious to know if the HA binding ability of the tail-less and truncated mutants of LYVE-1 improves compared to the full-length versions when transfected into HDLECs as seen by actin depolymerised HDLECs. The HA binding can also be tested by using of physiologically relevant HA ligands such as TSG6:HA [199], GAS [4], DC with endogenous HA [3], to test binding to HA upon in primary cells after actin depolymerisation.

The transmigration of DCs or tumour cells through a monolayer of HDLECs that have been transfected with the different truncations, and tail-less versions of LYVE-1 can be compared to the full length version and native wild type cells. This could accurately depict physiological relevance of the cytoplasmic tail interaction with the actin cytoskeleton on the migration of cell through LECs.

Other interacting partners of LYVE-1, such as cholesterol that could contribute to receptor clustering and lateral diffusion will be further investigated using sFCS and STED-FCS. The actin components that interact with actin can be delineated by pre-forming co-ip of LYVE-1 or actin and detecting the presence of actin binding proteins that facilitate this interaction or move to an open ended approach *via* proteomics.

The membrane topology and curvature could also have an effect on the dynamics

and function of the LYVE-1 [200, 201]. This can be modulated by other actin cytoskeletal structure such as the microtubules [202] and intermediate filaments [74] which is a potential extension of this project.

D.Phil publications

- **Tess A. Stanly**, Marco Fritzsche, Suneale Banerji, Esther García, Jorge Bernardino de la Serna, David G. Jackson, Christian Eggeling: *Critical importance of appropriate fixation conditions for faithful imaging of receptor microclusters*. Biology Open 07/2016; 5(9):bio.019943., DOI:10.1242/bio.019943
- Erdinc Sezgin, Fatma Betul Can, Falk Schneider, Mathias P Clausen, Silvia Galiani, **Tess A Stanly**, Dominic Waithe, Alexandria Colaco, Alf Honigmann, Daniel Wuestner, Frances Platt, Christian Eggeling: *A comparative study on fluorescent cholesterol analogs as versatile cellular reporters*. Journal of Lipid Research 12/2015; 57(2)., DOI:10.1194/jlr.M065326
- **Tess A. Stanly**, Marco Fritzsche, Suneale Banerji, Dilip Shrestha, Christoffer Largerholm, David G. Jackson, Christian Eggeling: *LYVE-1 lateral dynamics and function is modulated by the actin cytoskeleton*. Manuscript in preparation.

Appendix

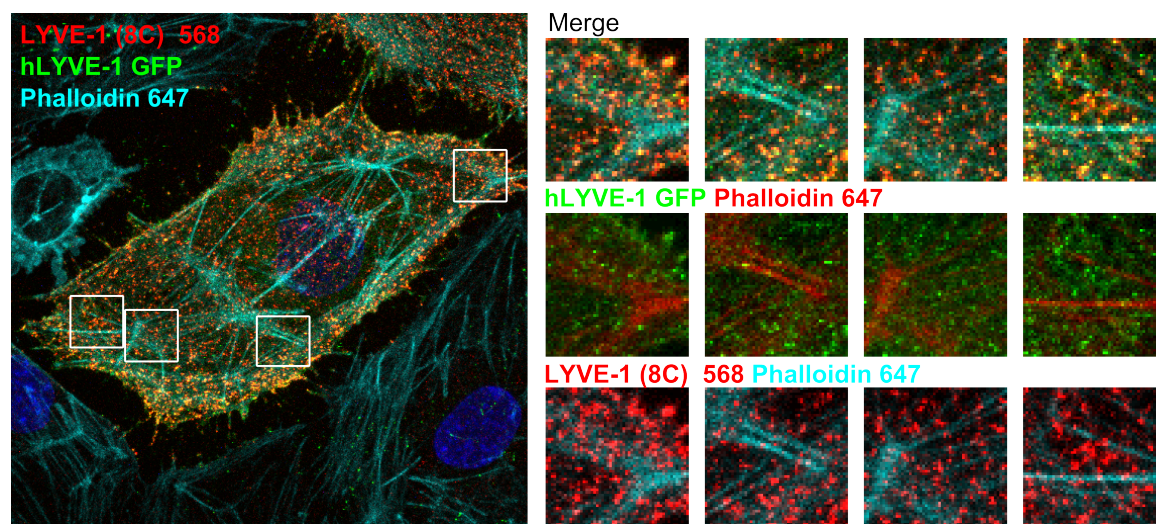


Figure 6.1: Confocal images of LYVE-1 and F-actin. Confocal images of hLYVE-1 GFP HDLECs labelled with Phalloidin 647 (cyan), hLYVE-1 GFP (green), LYVE-1 (8C) red, nucleus (blue). The magnified regions show actin rich areas with minimal to no LYVE-1 interaction. The F-actin colour was changed to red from cyan in the middle panel for better visualisation of LYVE-1 clusters and the F-actin.

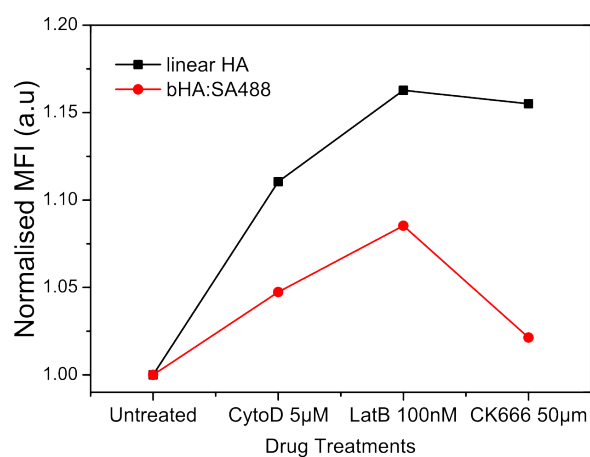


Figure 6.2: Linear and complexed HA binding to LYVE-1 HDLECs after actin depolymerisation. Primary HDLECs were treated with Cyto D, Lat B and CK-666 and tested for the ability to bind linear or bHA:SA488 complexed form of HA by FACS. The results show that the actin depolymerisation lead to an increase in HA binding to LYVE-1 even when using linear forms of HA.

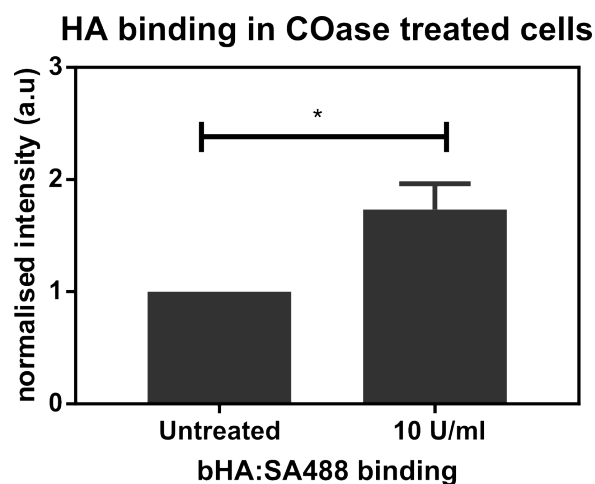


Figure 6.3: bHA:SA488 binding to LYVE-1 after cholesterol depletion determined by FACS. Primary HDLECs were depleted of the membrane cholesterol using cholesterol oxidase (COase) lead to an increase in bHA:SA488 binding the the cells. $n=3$, error bar indicate SEM, Unpaired Student t-test, $P = 0.0331$.

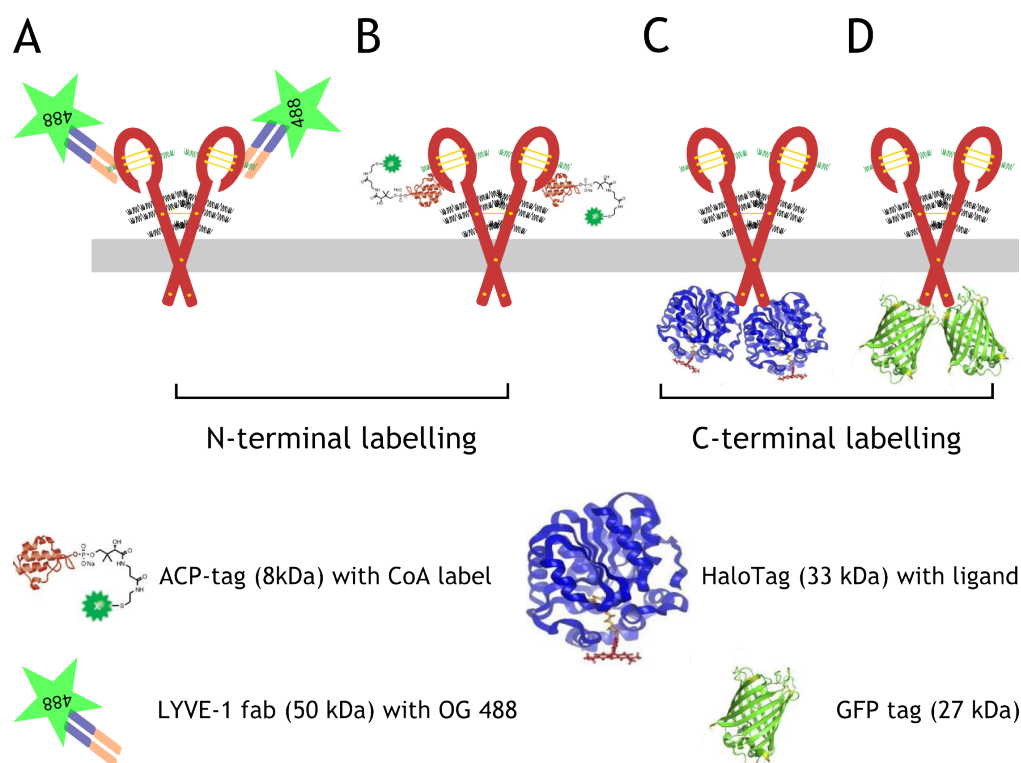


Figure 6.4: Labelling techniques for LYVE-1. LYVE-1 was labelled using different techniques A, B) Labelling of LYVE-1 *via* the N-terminus using a fluorescent monovalent fab Oregon Green[®] 488 conjugates (A) or with Co-enzyme A to label the hLYVE-1 transfectants carrying an N-terminus ACP-tag (B). C, D) C-terminus labelling using HaloTag[®] membrane permeable ligand Halo OG 488 (C) or GFP tag (D) expression at the C-terminus of hLYVE-1 cytoplasmic tail.

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