Live Cell Imaging of CEACAM1 Dynamics and Self-Association during Bacterial Binding

by

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A thesis submitted in conformity with the requirements for the degree of Master of Applied Science
Institute of Biomaterials and Biomedical Engineering
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Abstract

The carcinoembryonic antigen-related cellular adhesion molecule 1 (CEACAM1) is a human receptor that facilitates adhesion with neighbouring cells, as well as with certain pathogens. CEACAM1 at the cell surface exists as a mixture of monomers and dimers in a heterogeneous distribution that is thought to regulate the balance of its functions, including those associated with pathogen binding. We used live cell fluorescence and homogeneous Förster resonance energy transfer (homo-FRET) microscopy on a combined total internal reflection fluorescence polarization (TIRFPM) confocal microscopy platform to investigate the distribution, dynamics, and monomer-dimer equilibrium of CEACAM1-4L-EYFP on live cells that were parachuted onto surfaces coated with CEACAM1-binding Neisseria gonorrhoea. Both CEACAM1-4L-EYFP and a monomeric mutant form of the receptor are rapidly recruited to bacteria and lead to downstream effector recruitment. Homo-FRET data indicate that wild-type CEACAM1-4L-EYFP was predominantly monomeric at bacterial contact sites. Preferential monomeric binding during bacterial adhesion controls the infection process.
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List of Abbreviations

CEA  carcinoembryonic antigen
CEACAM  CEA-related cell adhesion molecule
CEACAM1-L  CEACAM1-long cytoplasmic tail
CEACAM1-S  CEACAM1-short cytoplasmic tail
CFP  cyan fluorescent protein
d  depth of evanescent field penetration
DiO-C16  3,3'-dihexadecyloxacarbocyanine perchlorate
EYFP  enhanced yellow fluorescent protein
FCS  fluorescence correlation spectroscopy
FITC  fluorescein isothiocyanate
FLIM  fluorescence lifetime imaging microscopy
FOV  field of view
FRAP  fluorescence recovery after photo-bleaching
FRET  Förster resonance energy transfer
$F_{x,y,z}$  fluorescence intensity polarized along x-, y- or z- axis, respectively
$F_{||}$  fluorescence intensity oriented parallel to excitation polarization
$F_{\perp}$  fluorescence intensity oriented perpendicular to excitation polarization
G  G factor
GFP  green fluorescent protein
GPI  glycosylphosphatidylinositol
Ig-like  immunoglobulin-like
IgC-like  Ig-like constant domain
IgV-like  Ig-like variable domain
ITIM  immunoreceptor tyrosine-based inhibitory motifs
$K_a, K_b, K_c$  high NA-correction factors
n  refractive index
N  number of cells
NA  numerical aperture
NF-κB  nuclear factor κB
N&B  number and brightness analysis
Opa  opacity-associated
PALM  photoactivated localization microscopy
PI3K  phosphatidylinositol 3-kinase
PI(3,4,5)  phosphatidylinositol-3,4,5 phosphate
PSG  pregnancy-specific glycoprotein
Q  fluorescence quantum yield
r  fluorescence anisotropy
$r_c$  high NA-corrected anisotropy  
$R$  intermolecular distance  
$R_0$  Förster radius, distance at which FRET efficiency is 50%  
ROI  region of interest  
SE  standard error  
SHP  Src homology region 2 domain-containing phosphatase  
STI  sexually transmitted infection  
STORM  stochastic optical reconstruction microscopy  
TCR  T cell receptor  
TIRFPM  total internal reflection fluorescence polarization microscopy  
tr-FAIM  time-resolved fluorescence anisotropy imaging  
$a_i$  incident angle  
$a_c$  critical angle  
$\lambda$  wavelength  
$\varepsilon$  extinction coefficient  
$\sigma$  half-cone angle of objective  
$\tau$  fluorescence lifetime  
$(\lambda)$  spectral overlap integral for two fluorophores  
$\kappa^2$  dipole orientation factor
CHAPTER 1

1 Introduction

Membrane proteins are important moderators of cellular behaviour, allowing cells to identify and interact with the surrounding environment, relaying signals between extracellular and intracellular compartments, and acting as key players in the pathogenesis of infectious disease. The carcinoembryonic antigen-related cellular adhesion molecule 1 (CEACAM1) is a membrane glycoprotein expressed on the surface of human epithelial, endothelial, and immune cells. It participates in both homotypic and heterotypic binding interactions that can induce stimulatory or inhibitory effects on cell signaling to regulate many normal processes, including cell proliferation, differentiation, and immune responses [1]. However, CEACAM1 also participates in interactions that facilitate the uptake of pathogenic bacteria, and its inappropriate expression is associated with cellular transformation and tumorigenesis [2-4]. A better understanding of how this receptor’s many functions are normally balanced and controlled would help to clarify its role in disease.

CEACAM1 participates in trans-homotypic (between cell) and cis-homotypic (on the same cell) interactions, in addition to interacting with other proteins, such as the Neisseria gonorrhoeae colony opacity-associated (Opa) integral membrane protein. These Opa proteins offer a selective advantage to bacteria during invasion, aiding in establishment of the sexually transmitted infection (STI) gonorrhea, one of the most commonly acquired STIs worldwide [5]. Though gonorrhea is a treatable infection, the development of N. gonorrhoeae antimicrobial resistance is becoming a severe public health concern, motivating exploration into alternative therapeutic options [6].

Advances in microscopy have enabled the study of membrane diffusion, cell adhesion and signal transduction at the molecular level. Live-cell fluorescence microscopy has revealed a great deal about the dynamic and heterogeneous nature of CEACAM1 in healthy cells, though many of the molecular details of its role in both normal and pathogenic processes are not yet
well understood. Characterizing and clarifying CEACAM1 organization and responses with advanced microscopy techniques, which allow for a real-time imaging of receptor interactions and dynamics in living cells during the initial stages of bacterial adhesion, is fundamental to generating a complete understanding of the CEACAM1 function and regulation, and ultimately exploiting its function for therapeutic purposes.

This chapter presents an overview of what is known about CEACAM1 and its role in healthy and detrimental processes, as well as a review of fluorescence imaging techniques that can be used to investigate what is not yet known.

1.1 CEACAM1

1.1.1 Structure and Function

Within the carcinoembryonic antigen (CEA) family, CEA-related cellular adhesion molecules (CEACAMs) make up a subfamily of membrane glycoproteins involved in binding interactions that influence cell growth and differentiation in normal and cancerous mammalian cells (Figure 1.1 A). These receptors are primarily surface-anchored and have structures that typically include glycosylated immunoglobulin-like (Ig-like) extracellular domains and either a transmembrane domain or a glycosylphosphatidylinositol (GPI) anchor [4].

Human CEACAM1, a broadly expressed member of this subfamily with homologues in other mammals, has a number of different splice isoforms that vary in cytoplasmic tail length and in Ig-like extracellular domain number (Figure 1.1 B) [1]. The CEACAM1-4L isoform contains one extracellular N-terminal Ig-like variable (IgV-like) domain followed by three extracellular Ig-like constant (IgC-like) domains, a transmembrane domain, and a long 71-amino acid cytoplasmic tail. CEACAM1-4S is similar in structure but has only a short 10-amino acid tail. CEACAM1 is expressed by epithelial cells, endothelial cells, and lymphocytes, and is involved in many pathways related to the immune response to cancer and infection [1]. The expression of this receptor is down-regulated in some forms of cancer and elevated in others, emphasizing the
complexity of its regulation and the balance of its functions [7]. CEACAM1 also plays a role in other processes, such as angiogenesis and the regulation of insulin levels [8, 9].

Figure 1.1 Human CEA Family and CEACAM1 Isoforms
(A) Basic structure and tissue expression of major CEA family members, including CEACAMs and the pregnancy-specific glycoproteins (PSGs). The N-terminal IgV-like domain is often referred to as the N domain. (B) CEACAM1 naturally occurs as a combination of a number of different isoforms that vary in their number of glycosylated Ig-like extracellular domains. Isoforms with a transmembrane domain also vary in cytoplasmic tail length. CEACAM1-4L has four Ig-like domains and a long (L) cytoplasmic tail. Figure reproduced from reference [1] with permission.
CEACAM1 participates in many protein-protein interactions via both extracellular and cytoplasmic domains. It can interact in \textit{trans}- with itself or the related family member CEACAM5 on neighbouring cells to facilitate adhesion and immunological responses [10], as well as engaging a number of pathogen adhesins to facilitate attachment and invasion. These \textit{trans}-interactions occur via a non-glycosylated β-sheet on the N-terminal IgV-like domain [11]. CEACAM1 localizes to cell-cell contacts to facilitate adhesion, and Rho-like GTPases that act on the CEACAM1 transmembrane domain, as well as the actin cytoskeleton, appear to be important for this localization to occur [12, 13]. In addition to these \textit{trans}-homotypic and \textit{trans}-heterotypic interactions, CEACAM1 can also interact with itself in \textit{cis} to form non-covalently linked dimers. Certain signalling molecules may bind to dimers more readily than monomers, and vice versa, implicating a role for the CEACAM1 monomer-dimer distribution in signal transduction [14, 15].

Cytoplasmically, the CEACAM1-4L isoform contains two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) with tyrosine phosphorylation sites that mediate primarily inhibitory signalling pathways [16]. This motif allows for the binding of proteins such as the Src family protein tyrosine kinases and Src homology region 2 domain-containing phosphatases (SHP -1 and -2) [14, 17]. Though the CEACAM1-4S isoform lacks ITIM motifs, it and CEACAM1-4L can interact intracellularly with other proteins such as calmodulin and actin [12, 18]. Calmodulin binds to CEACAM1 in a calcium-dependant manner, causing a down-regulation of CEACAM1 oligomerization [18, 19]. Some studies have shown that CEACAM1-4L can interact with G-actin and proteins associated with the cytoskeleton, such as tropomyosin and paxillin, though other studies have shown that its interaction with F-actin cytoskeleton is not direct [12, 20, 21].

\subsection*{1.1.2 Molecular Organization at the Cell Surface}

Total internal reflection fluorescence microscopy (TIRFM) of enhanced yellow fluorescent protein (EYPF)-tagged human CEACAM1-4L transiently expressed in HeLa cells has shown that the receptor is heterogeneously distributed at the cell surface, forming bright dense clusters at ezrin-rich regions that are surrounded by regions of more diffuse CEACAM1-4L-EYFP [22].
Time-lapse TIRFM revealed the dynamic nature of these bright clusters, which can move and change shape and size over the time-course of several seconds to minutes [22].

Biochemical studies have revealed that human CEACAM1-4L exists as a mixture of monomers and dimers at the surface of epithelial cells [23]. Steady-state Förster resonance energy transfer (FRET) experiments confirmed that the cell surface contains both monomeric and dimeric CEACAM1, and showed that the bright clustered regions contain primarily monomeric CEACAM1, whereas more diffuse CEACAM1 is largely dimeric [24]. Investigations into this receptor often unearth intricate and even conflicting functions for CEACAM1, likely due in part to its involvement in both stimulatory and inhibitory processes, even within the same cell type [15, 25]. It has been suggested that the monomeric and dimeric forms of the receptor may initiate opposing signalling pathways [15].

Computational models predict that kinase effectors can bind both the monomeric and dimeric form of the receptor, whereas downstream phosphatase effectors can bind only monomeric CEACAM1 due to steric hindrance from the dimeric form [15]. As such, the predominance of either the dimeric or monomeric receptor could regulate transduction of either stimulatory activity facilitated by kinases or inhibitory activity facilitated by phosphatases. Dimerization is controlled by the cell through the binding of Ca^{2+}-loaded calmodulin, which binds to a membrane-proximal site in the cytoplasmic domain of CEACAM1 [18, 19, 26]. Additionally, co-expressing CEACAM1-4L with the CEACAM1-4S isoform, with which it is capable of forming a heterodimer, also leads to fewer CEACAM1-4L homodimers [14]. The C-terminal cytoplasmic domain is not required for dimerization, and though both the N-terminal and transmembrane domains play a role, a GXXG sequence in the transmembrane domain of both –L and –S CEACAM1 isoforms was shown to be critical for dimerization [14, 27]. Correspondingly, a mutation in this motif, generating G432,436L-CEACAM1, results in a completely monomeric form of the receptor that is still capable of participating in trans-homotypic interactions [24, 27].

The CEACAM1 transmembrane and N-terminal domains are important for other aspects of cell surface receptor organization, as they are required for CEACAM1 microdomain organization in cholesterol-rich lipid rafts [28-31]. Lipid rafts are involved in many processes, such as the
trafficking of membrane components and associated proteins, as well as the grouping of cell signalling machinery [32, 33], and therefore may play a key role in CEACAM1-related functions. However, there is still much to be learned and confirmed with respect to the formation of these microdomains and their significance. The CEACAM1 cytoplasmic tail also governs CEACAM1 organization more generally across the surface of certain cell types. Polarized epithelial cells show differences in the distribution of CEACAM1-L, which localizes to both apical and lateral surfaces, and the distribution of CEACAM1-S, which predominantly localizes to apical surfaces [34]. This organization is regulated by cell signalling cascades, which modulate the surface organization and surface expression level of each isoform, confirming that, at least in certain cell types, CEACAM1 organization is of significance and is under systematic control.

1.1.3 CEACAM1 as a Pathogen Receptor

CEACAMs can act as host receptors for a number of different pathogens to mediate invasion and the cellular response to infection. The pathogenic Neisseria species Neisseria gonorrhoeae, which causes gonorrhea, and Neisseria meningitidis, a common cause of bacterial meningitis, are Gram-negative diplococci that exclusively infect humans. Both of these bacteria can express colony opacity-associated (Opa) proteins that act as adhesins and invasins during infection. These are integral membrane proteins that interact with host receptors via surface exposed loops [35]. Neisserial strains have a number of different Opa alleles, with high sequence variation in regions encoding the surface exposed loops, which are differentially expressed in a phase-variable manner to allow for functional and antigenic diversity and immune evasion [36]. These proteins are thought to play an important role in N. gonorrhoeae pathogenesis, as almost all bacteria retrieved from infected humans are Opa+, even when those humans were initially infected with Opa- strains [37-39]. N. gonorrhoeae infections are typically acquired by sexual contact, which introduces the bacteria to mucous membranes in the human urogenital tract, though the infection may spread to or originate in a number of tissues.

Most Opa variants bind to human CEACAMs, allowing bacteria to form a tight association with epithelial, endothelial, and immune cells, which normally takes place after an initial pili-
mediated stage of attachment [40, 41]. This interaction occurs between the Opa surface-exposed loops and the non-glycosylated β-sheet on the CEACAM N-terminal IgV-like domain [42-45]. The interaction allows for bacterial uptake in many cell types, such as mucosal epithelial cells that allow for the transcytosis of *N. gonorrhoeae* and consequent breaching of the epithelial barrier, leading to invasive disease.

Certain Opa variants can bind to CEACAM1 on host cells [46-51]. Though bacterial uptake mediated by the related family member CEACAM3, expressed on neutrophils, occurs through a well-characterized actin microfilament-dependent mechanism akin to traditional phagocytosis [46, 52-54], the mechanism of CEACAM1-mediated uptake is atypical and not well understood. It is not prevented by actin polymerization inhibitors or tyrosine kinase inhibitors, but has instead been suggested to occur through a “zipper-like” mechanism as bacterial Opa proteins sequentially become engaged by cellular CEACAM1 until bacteria are enveloped [31, 46, 49]. Transmission electron microscopy of the cell membrane morphology at sites of CEACAM1-mediated *N. gonorrhoeae* invasion of HeLa cells revealed that small pseudopods enclose bacteria, whereas sites of CEACAM3-mediated invasion are riddled with long filopodial protrusions that extend around bacteria [46]. Though the integrity of the actin cytoskeleton is required for the transcytosis of *N. gonorrhoeae* across epithelial cell barriers, and so it is important for the movement of bacteria once they are inside certain host cells [55], the CEACAM1-mediated uptake process results in very little F-actin structure formation at uptake sites [46].

Surprisingly, the entire CEACAM1-4L cytoplasmic domain is unnecessary for the uptake process, as a truncated CEACAM1 mutant lacking this domain allows for the unhindered internalization of a number of different pathogens, including *N. gonorrhoeae* [31]. In contrast, it appears that the transmembrane domain, which is responsible for localizing CEACAM1 to cholesterol and sphingolipid-rich membrane microdomains, is important for *N. gonorrhoeae* internalization [29, 31]. The addition of cholesterol depleting agents that disrupt membrane microdomains inhibits bacterial uptake, suggesting a lipid raft–dependent mechanism for CEACAM1-mediated bacterial invasion [31]. Clustered lipid rafts have been shown to act as an entryway for a number of different pathogens [56]. The CEACAM1-mediated internalization of
other pathogens with similar or diverse bacterial adhesins, such as *N. meningitidis* and *Haemophilus influenzae*, is similarly influenced by inhibitors and CEACAM1 mutations, and therefore likely occurs by a mechanism that is the same or related to *N. gonorrhoeae* internalization [31].

Investigations into the cellular signalling response to the CEACAM1-Opa binding interaction have shown that there is a rapid increase in phosphatidylinositol-3,4,5 phosphate PI(3,4,5)P at sites of CEACAM1-mediated bacterial attachment [57]. Phosphatidylinositol 3-kinase (PI3K) activity is involved, and the inhibition of this kinase, as well as the overexpression of the PI(3,4,5)P phosphatase SHIP, reduces levels of bacterial uptake. These effects appear to involve the CEACAM1 IgC-like extracellular domains, as PI3K inhibitor sensitivity requires these domains to be present [57]. Though tyrosine phosphorylation of the CEACAM1 ITIMs does not appear to be required for bacterial uptake, it does play an important role in other signals initiated by the Opa-CEACAM1 interaction. Phosphorylation of the ITIM can mediate inhibitory effects through the recruitment of cellular phosphatases such as SHP-1 and SHP-2. Opa binding to CEACAM1 has been shown to have an immunosuppressive effect, for example causing the reduced activation and proliferation of T cells, reduced antibody production by B cells, and a reduced toll-like receptor 2 (TLR-2)-directed inflammatory response in pulmonary epithelial cells [1, 58-60]. This may contribute to the ability of pathogenic *Neisseria* to evade the adaptive immune response. The binding of pathogens to CEACAM1 on mucosal epithelial cells has also been shown to trigger the expression of CD105, which promotes cellular adhesion to the extracellular matrix and prevents cell detachment in response to infection, countering the exfoliation response that infected epithelial cells often employ as a defense mechanism [61].

Similar to other CEACAM1 binding processes, molecular re-organization of CEACAM1 during these Opa interactions and subsequent signal transduction processes has not been well characterized, and it is not yet known whether the bacterial Opa proteins bind to monomers, dimers, or both monomers and dimers (Figure 1.2). We know that monomers and dimers have the propensity to initiate different signalling events, and so clarification regarding the dynamic organization and oligomerization status of CEACAM1 during Opa binding may help develop our understanding of bacterial attachment and uptake, a fundamental stage of pathogenesis.
Figure 1.2 Models for CEACAM1 Attachment to Opa⁺ Neisseria

Bacterial attachment may be mediated primarily by CEACAM1 monomers (A) or could be mediated by CEACAM1 dimers (B). Though it has been suggested that each form of the receptor may function differently, it is also possible that both monomers and dimers participate in Opa binding.

1.2 Fluorescence Microscopy Tools and Techniques

1.2.1 Techniques for Investigating Membrane Protein Organization and Dynamics

In living cells, the mobile and changeable nature of membrane proteins gives rise to their ability to regulate signal transduction pathways and many other critical processes. Classical biochemistry techniques have provided many insights into protein structure and function, but biochemical and fixed-cell microscopy studies do not typically provide spatial and temporal information at the single cell and single molecule level. Sample preparation can also damage or interfere with cellular components as they are removed from normal physiological conditions when cells are lysed, fixed, or otherwise processed.

In contrast, live cell fluorescence imaging techniques permit the visualization of individual events as they occur over time, often allowing for a better understanding the dynamics of the processes involved. In recent years, many fluorescence microscopy techniques for visualizing and quantifying aspects of biological processes have developed. Fluorescently-tagged membrane proteins can be subjected to a number of procedures to gain information about their
cellular localization and organization, transport routes, diffusion, oligomerization, and association with other proteins or structures, in addition to many other details, all in living cells.

Super-resolution microscopy techniques such as photoactivated localization microscopy (PALM) break the diffraction limit of optical microscopy and allow for a dramatic resolution of protein organization at the cell membrane [62-65], while time-lapse imaging on a variety of available microscope configurations can reveal dynamic processes. Though the fast time-scales of many dynamic events make them a challenge to detect and interpret, many techniques for investigating these dynamics have developed (summarized in Figure 1.3). Methods such as fluorescence recovery after photobleaching (FRAP) provide diffusion information, while methods like single particle or cluster tracking providing information about the trajectory of protein movement [66-68]. Protein interactions at the cell surface are also of critical importance, as they often control protein function and signal transduction. Fluorescence correlation spectroscopy (FCS) and related techniques involve the interpretation of fluctuations in fluorescence intensity measured within a very small (sub-femtoliter) sample volume to draw conclusions regarding diffusion and the number of particles of a species in a complex [69-71]. Meanwhile, direct protein interactions can be probed with techniques such as FRET, discussed later in more detail, which detects interacting species in very close proximity [72, 73]. Often many of these techniques can be used in combination, and the strengths and limitations of each must be carefully compared when trying to address a specific research question.

The investigation of protein dynamics by these methods has led to a number of discoveries regarding the behaviour and function of membrane proteins. Studies of receptor dynamics have helped to clarify processes such as receptor trafficking, notably for a number of G-protein coupled receptors (GPCRs) [74-77]. Fluorescence microscopy has also revealed information about pathogenic processes like microbial infection, and about how these processes cause or are facilitated by changes in cellular constituents. The high-resolution imaging of pathogen association over time can help to describe host cell factor dynamics during infection, as has been shown by Ehsani et al. with time-lapse and super-resolution microscopy of Shigella flexneri uptake by host cells [78]. A medley of fluorescence microscopy techniques for studying membrane dynamics has also revealed the mechanism of entry of a pathogen into plant cells.
Dynamic imaging can reveal the details of cellular morphology and supramolecular changes during physiologically relevant perturbations.

**Figure 1.3** Fluorescence Techniques for Monitoring Membrane Protein Dynamics

Fluorescence correlation spectroscopy (FCS) relies on the movement of fluorophores in and out of a small detection volume (A, B), which is measured as intensity fluctuation over time (C). These intensity traces are autocorrelated (D), and the autocorrelation function provides information about protein diffusion and concentration. In fluorescence recovery after photobleaching (FRAP), a detection volume is selectively photo-bleached with a laser (E, F). The return of fluorescence signal, due to the diffusion of un-bleached fluorophores into the detection volume (G), is monitored over time to determine protein diffusion rates (H). Single particle tracking (SPT) involves high-speed image acquisition of a region of the sample (I, J). The movement of single fluorescent particles is tracked over these image sequences to plot particle positions over time (K) and detect protein trajectories and mean square displacement (MSD; L). Figure reproduced from reference [80] with permission.
1.2.2 Fluorescence Microscopy Imaging Modalities

Evolving imaging modalities that employ methods of optical sectioning, which reduce or eliminate background fluorescence outside of the focus plane, have enhanced the capacity of fluorescence microscopy to clarify protein organization, lateral movement, and trafficking to and from the membrane (Figure 1.4). Confocal laser scanning microscopy allows for optical sectioning of the sample by incorporating a spatial pinhole that filters out fluorescence outside of the focal plane. Two-photon microscopy, often used in conjunction with laser scanning microscopy (TPLSM), further minimizes light scattering, background fluorescence, and photobleaching by exciting fluorophores with an infrared light source, from which two photons must be absorbed for excitation. This allows for the imaging of cellular features, including membrane components, with high resolution and even at high depth in living tissues [81-83]. In light sheet fluorescence microscopy (LSFM), the excitation laser source is tapered into a thin light sheet that is directed perpendicular to the observation plane, so that only the region under observation is illuminated. The specimen is then exposed to only a narrow sheet of light, which reduces photobleaching and phototoxicity and allows for imaging over long time periods in living samples [84, 85]. Though some level of physiological relevance must often be forfeit in order to observe the molecular details of a biological system, emerging fluorescence microscopy techniques such as these are making the high-resolution imaging of living cells, tissues and even organisms more feasible.

Total internal reflection fluorescence microscopy (TIRFM) is a powerful tool for imaging membrane proteins, as it restricts the illumination of a sample, such as a cell resting on a glass dish, to a very thin region just at the sample surface. This greatly reduces background out-of-focus fluorescence in comparison to standard epifluorescence (Figure 1.4), and provides an excellent means of visualizing the basal surface of adherent cells [86-88].
Figure 1.4 Fluorescence Imaging Modalities

Epifluorescence microscopy (A) illuminates fluorophores throughout the sample, resulting in a high degree of background fluorescence outside of the focal plane. Developing imaging modalities, which employ optical sectioning to block or prevent background fluorescence, enhance contrast and clarify the structure and distribution of fluorescent features in a sample. In confocal laser scanning microscopy (B) a laser is scanned across the sample as images are acquired point by point. A confocal pinhole prevents background fluorescence outside of the focus plane from reaching the detector. In total internal reflection fluorescence microscopy (TIRFM; C), a reflected laser beam generates an evanescent field that excites fluorophores only at the very bottom surface of the sample. In two-photon laser-scanning microscopy (TPLSM; D), an infrared laser is once again scanned as in confocal microscopy. Two photons are required to excite fluorophores, so there is a lower probability that fluorophores outside of the plane of focus will become excited. Figure reproduced from reference [89] with permission.
This restricted TIRF illumination is achieved by exciting the sample with a laser beam that is angled such that the incident beam hits the glass-sample interface at or beyond the critical angle ($\alpha_c$) for total internal reflection. The critical angle is the angle of incidence ($\alpha_i$), for a light ray passing through a medium of one refractive index ($n_1$) and striking a boundary between that medium and one of a lower refractive index ($n_2$), at or beyond which none of the light ray refracts into the low index medium. The light completely back reflects into the medium of high refractive index, often a glass slide or dish ($n = 1.52$) in TIRFM (Figure 1.5). An evanescent electromagnetic field is generated at the glass-sample boundary, penetrating into the sample and decaying, but exciting fluorophores within 300nm of the surface [86, 90]. The precise depth of penetration of the evanescent field ($d$) depends on the wavelength and is modelled by:

$$d = \lambda_o/4\pi \cdot (n_1^2 \sin^2 \alpha_i - n_2^2)^{-1/2}$$

where $\lambda_o$ is the wavelength of the incident light in a vacuum.

**Figure 1.5 Critical Angle for Total Internal Reflection**

When a light ray strikes the boundary between a medium of high refractive index and a medium of lower refractive at an angle of incidence ($\alpha_i$) that is below the critical angle ($\alpha_c$) (red ray, $\alpha_i < \alpha_c$), then some of that light refracts into the lower index medium. Beyond this critical angle (blue ray, $\alpha_i > \alpha_c$) no refraction occurs, resulting in total internal reflection.
One of two approaches is typically used for achieving total internal reflection at the glass-sample interface. In prism-based TIRFM, a prism is used to adjust the excitation laser beam to the critical angle for total internal reflection. More commonly, in objective-based TIRFM the excitation source is focused to the back focal plane of the microscope imaging objective and directed to the periphery of the aperture cone, so that the angle of incidence is at the critical angle for total internal reflection when it reaches the optical surface. The numerical aperture (NA) of an objective is:

$$NA = n \sin \sigma$$

(2)

Where $n$ is the refractive index of the medium, and $\sigma$ is the half-cone angle of the high numerical objective used. Samples such as living cell typically have a refractive index of approximately 1.33 to 1.37, and an objective with a numerical aperture (NA) of 1.4 or greater is required for the excitation source to be totally reflected at the glass sample-interface.

An excitation laser beam with a Gaussian transverse intensity distribution is commonly used for TIRFM. When the laser is focused by the objective into total internal reflection the evanescent illumination generated at the optical plane has an elliptical Gaussian shape. This means that regions of the field of view (FOV) receive different illumination intensity depending on how far away from the center of illumination they are. This is in contrast to raster scanning modalities such as confocal laser scanning microscopy, where each region in the FOV is sequentially illuminated with the same intensity. Also in contrast to scanning methods of image acquisition, TIRFM is a wide-field technique in which all pixels in the image are captured simultaneously by the pixel array of a camera. The development of improved camera technologies, such as electron-multiplying charged coupled device (EMCCD) digital cameras, has allowed for image acquisition at high speeds and with low readout noise. As with other fluorescence microscopy modalities, multiple laser lines and filter sets allow for the imaging of several probes within the same sample, allowing for relationships between labeled molecules or structures to be established.
1.2.3 Homo-Förster Resonance Energy Transfer

The chemical structure of a fluorophore has a dipole moment. When a fluorophore is optimally oriented such that its dipole moment is aligned parallel to the electric field of an excitation light source, it has a high probability of becoming excited. This fluorophore can then relax to the ground state in a number of ways, such as by emitting a photon, which results in fluorescence that is polarized parallel to the fluorophore dipole.

A fluorophore has a characteristic average lifetime ($\tau$) between excitation and photon emission. Specifically, $\tau$ refers to the time taken for a population of excited fluorophores to decay to $1/e$ of its original size. Fluorophore rotation during the excited phase changes the direction of its dipole, typically leading to a greater disparity between excitation and fluorescence light polarizations. However, a large fluorophore such as a fluorescent protein undergoes very little rotation during its fluorescence lifetime, and so its emission dipole remains relatively unchanged. When fluorescence occurs from an optimally oriented fluorophore, it has approximately the same polarization as the excitation source.

If a sample of fluorophores is illuminated with a polarized light source, then optimally oriented fluorophores will be selectively excited. They will emit fluorescence that is highly polarized with respect to the excitation source, assuming that fluorophores are isolated and undergo negligible rotational diffusion during the fluorescence lifetime, as is the case for fluorescent proteins (Figure 1.6 A). However, if fluorophores with overlap between their absorption and emission spectra are not isolated and are capable of associating closely with one another, then optimally oriented “donor” fluorophores can become excited and subsequently relax instead by non-radiatively transferring energy to other nearby fluorophores via Förster resonance energy transfer (FRET). These other “acceptor” fluorophores, which are typically free to move and adopt many different orientations, will unlikely have their dipole moments aligned in that same orientation, and will therefore emit fluorescence that is not oriented with the polarization of the excitation source (Figure 1.6 B).
Figure 1.6 Homo-FRET Between Identical Fluorophores

The excited isolated fluorescent protein in (A) emits fluorescence of the same polarization as that which it absorbed. The fluorescent protein in (B) is excited and then transfers energy to a second fluorescent protein in close proximity but with a different orientation. This second fluorescent protein then emits fluorescence that is “depolarized” with respect to the excitation light. Small arrows indicate fluorophore dipole moments.

If FRET occurs to a spectrally distinct red-shifted fluorophore, for example from cyan fluorescent protein (CFP) to green fluorescent protein (GFP), then this heterogeneous (hetero)-FRET is typically detected as the quenching of donor fluorescence and enhanced fluorescence emission from the acceptor fluorophore. Conversely, if FRET occurs between fluorophores of the same type with a relatively small Stokes shift (a small shift between emission and absorption maxima) then homogeneous (homo)-FRET between identical fluorophores is detected by the depolarization of fluorescence emission. In addition to having rotation times that are much longer than their fluorescence lifetime, GFP and its variants have a small Stokes shift (10-45 nm), making them ideal for homo-FRET experiments [91].

The occurrence of FRET is dependent on the distance between fluorophores, such that the distance at which energy transfer is at 50% efficiency (the Förster radius, $R_0$) is approximately 3-6 nm for most fluorophore pairs [92]. This Förster radius depends on properties of the two fluorophores involved, including their spectral overlap integral ($\langle \lambda \rangle$), the orientation of their dipole moments ($\kappa^2$; dipole orientation factor, assumed to be 2/3), the donor fluorophore quantum yield ($Q_D$) and the acceptor fluorophore extinction coefficient ($\varepsilon_A$). It can be calculated for a fluorophore pair with the following equation:

$$R_0 = [2.8 \times 10^{17} \cdot Q_D \cdot \kappa^2 \cdot \varepsilon_A \cdot \langle \lambda \rangle]^{1/6} \text{ nm}$$ (3)
The extent of FRET strongly depends on the proximity of fluorophores, which has a sixth power relationship to the FRET efficiency \(E_{\text{FRET}}\), calculated by:

\[
E_{\text{FRET}} = \frac{1}{1 + (R/R_0)^6}
\]

where \(R\) is the intermolecular distance. FRET between fluorescent proteins that are attached to proteins of interest is used as an indicator of protein interactions, which can bring the fluorophores in close enough proximity for FRET, as well as protein conformation states, when the fluorophores are attached to different parts of the same protein and are only close enough for FRET in certain conformations. Homo-FRET is well suited to probing protein self-association, as there is no need to correct for issues that arise with hetero-FRET, such as unequal donor and acceptor fluorescent construct expression levels or channel bleed-through. Fluorophores tagged onto a protein of interest may come into close enough proximity for FRET if the protein forms dimers or oligomers. Thus, a low degree of fluorescence polarization is expected for tagged proteins that form oligomers, whereas a high degree of polarization is expected for isolated proteins with fluorophores that do not allow for FRET. This degree of polarization is measured as the fluorescence polarization anisotropy \(r\), which is corrected with a G factor to account for differences in the ability of a specific optical configuration to transmit light of different polarizations:

\[
r = \frac{F_\parallel - GF_\perp}{F_\parallel + 2GF_\perp}
\]

where \(F_\parallel\) is fluorescence intensity collected through a polarizer that is parallel with respect to the excitation source (polarized fluorescence) and \(F_\perp\) is fluorescence intensity collected through a polarizer that is perpendicular to the excitation source (“depolarized” fluorescence). Low fluorescence anisotropy is therefore characteristic of labeled protein dimers or higher order oligomers that engage in FRET.

During the fluorescence lifetime, exponential fluorescence decay occurs over fast (nanosecond) timescales as a population of excited fluorophores return to the ground state in the absence of further excitation. This rate of decay in the fluorescence signal can be measured in the time
domain by exposing the sample to a short excitation pulse and then measuring photon emission over picosecond timescales by time-correlated single photon counting (TCSPC). Alternatively, fluorescence intensity can be measured in the frequency domain by exciting the sample with a modulated excitation light source. In this case the phase lag and demodulation of the fluorescence emission are used to calculate fluorescence lifetime [93]. Both time and frequency domain methods are used for fluorescence lifetime imaging microscopy (FLIM). If total fluorescence emission is divided into $F_{\parallel}$ and $F_{\perp}$ components, then the decay of each separate component of fluorescence emission can also be measured. The decay of each component can in turn be used to calculate the decay in fluorescence anisotropy. Anisotropy decay occurs as fluorophores rotate, and so it will decay more slowly for fluorescent species with a greater molecular mass, though it will decay more quickly if FRET is taking place [94, 95]. Thus, the rate of anisotropy decay is used as an indicator of homo-FRET in time-resolved fluorescence anisotropy imaging (tr-FAIM) [91, 96]. This method of homo-FRET detection has some degree of sensitivity in differentiating between protein dimers and higher-order oligomers. However, it requires a pulsed illumination source and instrumentation for detecting photon emission over the very fast timescales of fluorescence decay, which may limit its ability to provide spatial information. In contrast, steady-state homo-FRET detection is relatively simple to implement. In this method, microscope cameras capture each component of the fluorescence intensity over an imaging exposure time. Through ratiometric image analysis, this method can be used to generate a spatial distribution of anisotropy values across a sample, though it is difficult to differentiate between dimers and higher order oligomers from these steady-state anisotropy values.

### 1.2.4 Steady-State Homo-FRET by TIRF Polarization Microscopy

TIRF polarization microscopy (TIRFPM) can be used to collect $F_{\parallel}$ and $F_{\perp}$ image pairs for the detection of homo-FRET between probes at or near the basal cell membrane. The evanescent wave generated in TIRF will be polarized in an orientation that depends on the polarization of the incident laser beam [97]. When the beam is polarized along the plane of incidence (the plane formed by incident and reflected rays, defined here as the x-z plane of the microscope) it is deemed “p-polarized” and will generate an evanescent wave that is elliptically polarized and
cart-wheels in the plane of incidence (Figure 1.7). When the beam is polarized normal to the plane of incidence it is deemed “s-polarized” and will generate an evanescent wave that is also polarized normal to the plane of incidence (the y-direction) (Figure 1.7). A laser beam with s-polarization is used for fluorophore illumination in homo-FRET experiments, and so fluorophores with dipole moments aligned in the y direction during illumination have a high probability of becoming excited.

In objective-based TIRFM, the polarized excitation beam can become slightly depolarized as it is concentrated by the high NA objective lens, resulting in light from different axes escaping into the $F_{\parallel}$ and $F_{\perp}$ channels. To correct for this, a three dimensional interpretation of fluorescence must be made, so that intensity of the polarization components along each imaging axis are considered [98-100]. $F_{\parallel}$ and $F_{\perp}$ become:

$$F_{\parallel} = K_a F_z + K_b F_x + K_c F_y$$ (6)

$$G F_{\perp} = K_a F_z + K_c F_x + K_b F_y$$ (7)

Where the correction factors $K_a$, $K_b$, and $K_c$ are:

$$K_a = \frac{1}{6} \left( 2 - 3 \cos \sigma + \cos^3 \sigma \right) (1 - \cos \sigma)^{-1}$$ (8)

$$K_b = \frac{1}{24} \left( 1 - 3 \cos \sigma + \cos^2 \sigma - \cos^3 \sigma \right) (1 - \cos \sigma)^{-1}$$ (9)

$$K_c = \frac{1}{8} \left( 5 - 3 \cos \sigma - \cos^2 \sigma - \cos^3 \sigma \right) (1 - \cos \sigma)^{-1}$$ (10)

These correction factors depend on the NA of the objective lens (Equation 2). The corrected fluorescence anisotropy equation then becomes:

$$r = \frac{(K_a + K_b + K_c)(F - G F_{\perp})}{(K_a - 2K_b + K_c)F + (-K_a - K_b + 2K_c)GF_{\perp}}$$ (11)
Figure 1.7 TIRFPM Evanescent Field Polarization

Incident light that is \( p \)-polarized will generate an evanescent field that is elliptically polarized, whereas incident light that is \( s \)-polarized will generate a field that is linearly polarized normal to the plane of incidence. For homo-FRET experiments \( s \)-polarized light is used to excite fluorophores. Figure reproduced from reference [3] with permission.

1.3 Thesis Objectives

As discussed earlier in section 1.1.3, despite the importance of the CEACAM1-Opa interaction during \textit{Neisseria} pathogenesis, many uncertainties regarding the nature of this interaction remain. Cellular signalling effectors associated with the CEACAM1-Opa interaction have been suggested to preferentially bind the monomeric form of CEACAM1, and so it seems likely that monomeric CEACAM1 plays a role in bacterial adhesion. The binding of bacterial Opa proteins to cellular CEACAM1 may alter the organization of CEACAM1-4L on the surface and cause a shift in its monomer-dimer equilibrium, in order to facilitate bacterial attachment and cell signal transduction.

To address some of the uncertainties associated with this process, the objective of this thesis is to describe receptor dynamics in live cells that have been transfected to express EYFP-tagged
CEACAM1-4L as the cells contact Opa⁺ bacteria. Advanced fluorescence microscopy techniques can be used to study the CEACAM1-4L membrane distribution and oligomerization state and investigate the co-localization of signalling molecules that have been suggested to preferentially bind to one form of the receptor.

Chapter 2 of this thesis focuses on characterizing the recruitment of CEACAM1-4L-EYFP to strains of *N. gonorrhoeae* using a combination of confocal and TIRF microscopy. These methods allow for live cell imaging of the dynamic process of CEACAM1-4L reorganization during infection. These basic imaging modalities can be used to implement more advanced fluorescence techniques for studying protein dynamics and interactions.

In Chapter 3, a closer investigation is made into the involvement of CEACAM1-4L monomers and dimers in bacterial binding and uptake, as well as the signalling effects that result from the Opa interaction. Live cell homo-FRET by TIRFPM can be used to visualize the spatial distribution of protein oligomerization states at the cell membrane over time, and is therefore well-suited to studying the reorganization of CEACAM1-4L-EYFP monomers and dimers during pathogenic processes such as infection.

This thesis provides insight into the functional role of the CEACAM1-4L membrane distribution and oligomerization state during bacterial adhesion, generating a more complete understanding of the receptor dynamics involved in infection.
CHAPTER 2

2 Characterization of CEACAM1-4L Recruitment to *Neisseria gonorrhoeae*

2.1 Chapter Summary

Combinatorial confocal and TIRF microscopy was used to characterize recruitment of the fluorescently labeled CEACAM1-4L receptor to sites of bacterial adhesion. HeLa cells parachuted onto immobilised *Neisseria gonorrhoeae* were capable of recruiting CEACAM1-4L-EYFP to Opa⁺ bacterial contact sites within 20 minutes of coming into contact with bacteria. CEACAM1-4L-EYFP recruitment occurred as a result of receptor re-organization at the cell surface, in addition to possibly recruitment from internal stores, as clusters of the receptor spread over the bacteria until they formed a shell-like structure or completely enveloped them. Receptor reorganization and recruitment led to the localization of labeled tyrosine phosphatases SHP-1 and SHP-2 to bacterial contact sites when they were co-expressed with labeled CEACAM1-4L, implying that these signalling molecules bound to the phosphorylated CEACAM1-4L ITIM.

2.2 Introduction

Host cell binding and entry, a key step in microbial pathogenesis, is a dynamic process that involves host receptor trafficking and re-organization at the cell surface, morphological changes in the membrane, and signal transduction within the cell. Fluorescence microscopy, which can reveal spatial and temporal information about such processes as they occur in living cells, was therefore applied to the study of CEACAM1 during its association with a pathogen.
CEACAM1 binds to a number of pathogen adhesins, including the Opa proteins expressed on *Neisseria gonorrhoeae*. This helps to facilitate bacterial attachment and uptake in many cell types, and also influences cellular signalling pathways primarily through the inhibitory activity of the CEACAM1 ITIM. CEACAM1 surface localization in T cells has been shown to increase upon exposure to bacteria, suggesting that it must be recruited from internal stores in these cells [23, 101]. However, a well-defined mechanism for receptor recruitment and recycling has not yet been established. Over longer (2 hour) timescales, *N. gonorrhoeae* lipopolysaccharide (LPS)-induced cytokine production leads to an up-regulation of CEACAM1 expression in epithelial cells, by a mechanism involving activation of the transcription factor nuclear factor κB (NF-κB), though bacterial uptake can occur over shorter (less than 30 minute) timescales [102].

When transfected into HeLa cells, which do not normally express CEACAM1, the fluorescently-tagged CEACAM1-4L isoform forms high intensity clusters on cell projections. Time-lapse TIRFM has revealed that even in resting adherent cells these clusters are very dynamic, moving and changing shape over second-long timescales. Biochemical and fixed cell microscopy studies have shown that HeLa cells expressing CEACAM1 effectively bind to and engulf *N. gonorrhoeae* [47-50]. TIRFM permits the time-lapse imaging of membrane proteins with high spatial and temporal resolution in living cells, and thus could reveal information about the dynamic CEACAM1 response to bacterial binding. However, it allows for visualization of only the basal portion of a sample, and so a protocol had to be implemented for coordinating cell-bacteria interactions so that they were visible by TIRFM. Cell and ligand receptor interactions have been effectively orchestrated by parachuting cells onto substrates such as lipid bilayers or micro-patterned surfaces [89, 103-105]. In addition to being viewable by TIRFM, this ensures that the plane of the cell-substrate interaction (and therefore the desired imaging plane) is predictable and remains consistent, making time-course imaging studies more feasible, especially beginning from time-points early on in the interaction.

By parachuting cells onto surfaces decorated with bacteria, the organization of fluorescently-labeled CEACAM1, as well as other cellular components such as signalling molecules that associate with CEACAM1, during bacterial binding could be investigated by combinatorial confocal and TIRF microscopy. Here, confocal microscopy was used to visualize the whole-cell
3-dimensional CEACAM1-4L distribution in relation to the positions of fluorescently-labeled immobilised *N. gonorrhoeae*, whereas TIRFM provided a high-resolution 2-dimensional view of the CEACAM1-4L basal membrane distribution.

Fluorescence microscopy studies have shown that the tyrosine phosphatases SHP-1 and SHP-2 colocalize with CEACAM1 in cells infected with *Neisseria*, though these have been restricted to fixed and pervanadate-treated cells [23]. This likely supports the inhibitory signalling effects of the CEACAM1-Opa interaction, which lead to outcomes such as immunosuppression in a cell-type-dependent manner. Investigations into the relative association of these phosphatases with monomeric and dimeric CEACAM1-4L will help to ultimately relate molecular organization of CEACAM1, including its oligomerization state as discussed later in Chapter 3, to functional outcomes within the cell.

### 2.3 Materials and Methods

#### 2.3.1 Molecular Cloning

Members of the Gray-Owen group (University of Toronto) generated labeled CEACAM1 constructs by attaching enhanced yellow fluorescent protein (EYFP) or mCherry to the C-terminal end of the CEACAM1-4L cytoplasmic tail, creating CEACAM1-4L-EYFP and CEACAM1-4L-mCherry. For a more detailed description of how these constructs were generated, see H. Lee’s thesis [2]. Lifeact-RFP, provided by S. Grinstein (University of Toronto), was used to label F-actin.

#### 2.3.2 Cell Culture and Transfections

HeLa cells were maintained in RMPI-1640 media (Life Technologies, ON, Canada) supplemented with 10% fetal bovine serum and 1% GlutaMAX (Life Technologies) at 37°C and 5% CO₂. For transfection, cells were trypsinized and either plated onto 35mm WillCo-dish Glass Bottom dishes (Series GWSt-3512, WillCo Wells, Amsterdam, Netherlands) for TIRFM,
or onto 35mm UpCell dishes (Model 174903, Nunc, Thermo Scientific, Waltham, MA). UpCell temperature-sensitive dishes are hydrophobic at 37°C, allowing for cell adhesion, but become hydrophilic at temperatures below 32°C, causing cells to detach without the need for trypsinization. Plated cells were left to incubate overnight, and then transfected in OptiMEM media (Life Technologies) with 1μg of each plasmid DNA using XtremeGENE HP transfection reagent (Roche, PQ, Canada), and imaged 18-24 hours later.

### 2.3.3 Bacteria Preparation

*Neisseria gonorrhoeae* strains N313 (expressing CEACAM1-binding Opa57) and N302 (Opa−), derived from a *N. gonorrhoeae* MS11 mutant that lacks pili, were grown on GC agar plates (Difco, Oakville, Ontario) supplemented with 1% IsoVitaleX Enrichment (BBL, Becton Dickinson Microbiology Systems, Cockeysville, MD) at 37°C and 5% CO₂. Bacteria were harvested into phosphate-buffered saline (PBS), heat inactivated for 30 minutes at 56°C, and fluorescently labeled chemically with AlexaFluor 647 Succinimidyl Ester (Life Technologies, Carlsbad, CA). Meanwhile, WillCo dishes were coated with poly-L-lysine (PLL) to improve adhere

### 2.3.4 Live Cell Manipulation

Cells plated onto WillCo dishes were imaged directly in the dishes, whereas cells plated into UpCell dishes were allowed to detach so that cell suspensions could be transferred by pipetting into other prepared WillCo dishes for imaging. In some cases, a micromanipulator (Patchman NP2 and CellTram Oil Microinjector, Eppendorf, New York, NY) was used to transfer individual cells into prepared dishes for imaging. All images were acquired 10-40 minutes after
cell suspensions were added to prepared imaging dishes, unless otherwise indicated in time-course experiments.

### 2.3.5 Confocal Microscopy

Laser scanning confocal microscopy was carried out on a Fluoview 500 microscope (Olympus Canada, Ontario, CAN). EYFP and GFP labeled constructs were imaged with a 488nm laser beam using a 505-525nm band-pass filter. CEACAM1-4L-mCherry was imaged with a 543nm laser using a 560-600 nm band-pass filter. AlexaFluor 647-labeled bacteria were imaged with a 633nm laser and 660nm long-pass filter. With the same 60X 1.45 NA objective as used for TIRFM, stacks of 512 x 512 pixel images were acquired at a slow scan speed with a 250nm z-direction step size, using Fluoview software (version 4.3).

### 2.3.6 TIRFM

Fluorescence microscopy was carried out on a home-built TIRFM system, configured on a Fluoview 500 confocal microscope (Olympus Canada, Olympus, CAN), with a 60X 1.45 NA oil-immersion objective (Olympus Canada) (Figure 2.1), as described in J. Lo’s thesis [24]. EYFP and GFP labeled proteins were excited with a 473nm laser beam, and fluorescence emission was passed through a 505nm cut-on wavelength dichroic mirror and a 500-550nm band-pass interference filter. CEACAM1-4L-mCherry was excited with a 532nm laser beam, and fluorescence emission was passed through a 564nm cut-on wavelength dichroic mirror and a 565-605nm band-pass interference filter. Images were captured with water-cooled, Excelon-equipped Evolve 512 EMCCD cameras (Photometrics, AZ, USA) at a gain of 35, using μManager software (version 1.4, [http://www.micro-manager.org](http://www.micro-manager.org)). One of the cameras was provided by J. Audet (University of Toronto).
Figure 2.1 The TIRFM system

(1) 532nm, 473nm, and 405nm lasers were used to excite fluorescent protein samples. (2) The excitation laser beam polarization was refined with a Glan-Taylor linear polarizer and was then passed through a half-wave plate to generate an s-polarized excitation source, important for TIRFPM experiments discussed in Chapter 3. (3) With a translatable mirror the laser beam was directed to the periphery of a 60X 1.45NA objective to achieve total internal reflection. (4) Here, the z-axis is defined as the optical axis, the y-axis as the axis is parallel to the s-polarized excitation beam, and the x-axis as the axis perpendicular to the excitation polarization. (5) Fluorescence emission was directed through the appropriate emission filter and detected by an Evolve EMCCD camera. On the two-camera set-up for homo-FRET detection, fluorescence emission was passed through a polarizing beam-splitter, positioned before the two cameras, which sends light of one polarization ($F_\parallel$) to one camera, and light of the opposite polarization ($F_\perp$) to a second camera. Adapted from original figure, courtesy of Gillian Vanderlee.
2.3.7 Data Analysis

All image analysis was carried out with ImageJ software (version 1.47, [http://rsbweb.nih.gov/ij/](http://rsbweb.nih.gov/ij/)). Pearson’s colocalization coefficients were determined using the “JaCoP” plugin ([http://rsbweb.nih.gov/ij/plugins/track/jacop.html](http://rsbweb.nih.gov/ij/plugins/track/jacop.html) version 2.1.1) [106], and CEACAM1-4L-EYFP clusters were manually tracked using the “MtrackJ” plugin (version 1.5.0) [107].

2.4 Results

2.4.1 CEACAM1-4L-EYFP in Uninfected HeLa Cells

CEACAM1-4L-EYFP in HeLa cells is located primarily at the cell surface, where it forms bright clustered regions, which are predominantly located around actin rich membrane protrusions, over the entire surface of the cell (Figure 2.2). CEACAM1-4L-EYFP has been shown to colocalize with both labeled ezrin (part of the actin end-cap that localizes to membrane protrusions) and the membrane label 3,3'-dihexadecyloxycarbocyanine perchlorate (DiO-C<sub>16</sub>) [22]. CEACAM1-4L-mCherry shares this same clustered morphology [24]. Cells that are parachuted from suspension onto bacteria-free glass surfaces take on the same clustered CEACAM1-4L-EYFP appearance as they adhere to the surface (Figure 2.3).

2.4.2 Confocal Imaging of Cellular CEACAM1-4L-EYFP in Contact with *Neisseria gonorrhoeae*

Typically in bacterial adhesion and uptake assays, bacteria are added to dishes already containing adherent cells. However, this would make imaging by TIRFM impractical, and so a protocol had to be developed for coordinating the cell-bacteria interaction so that it could be imaged from below the cell. *N. gonorrhoeae* were immobilised onto the bottom surface of the
dish, and suspensions of cells were then added to the bacteria-containing dishes. The cells were therefore not initially adherent, but would eventually parachute down and adhere to the bottom surface, coming into contact with the bacteria. To ensure that CEACAM1-4L-EYFP would be recruited to sites of bacterial contact and permit bacterial uptake, and to characterize the CEACAM1-4L-EYFP distribution in a cell that is contacting bacteria in this fashion, cells resting on bacteria were imaged by confocal microscopy, which allows for the visualization of all planes throughout the cell.

Figure 2.2 Summary of Cellular CEACAM1-4L-EYFP Organization

(A) When expressed in HeLa cells, confocal microscopy images indicate that CEACAM1-4L-EYFP is found predominantly at the cell surface, where it forms bright clustered regions on both apical and basolateral surfaces of the cell. The xz slice shown was taken at the white line indicated in the basal slice image. (B) TIRFM images of the bottom surface of a HeLa cell expressing CEACAM1-4L-EYFP and Lifeact-RFP, which labels F-actin. The CEACAM1-4L-EYFP clusters tend to form at actin-rich membrane protrusions.
Figure 2.3 CEACAM1-4L-EYFP Membrane Organization on a Parachuted Cell

A HeLa cell expressing CEACAM1-4L-EYFP and Lifeact-RFP was parachuted onto a bacteria-free glass surface and imaged by TIRFM. Within 30 minutes after contact with the surface the cell has begun to adhere and CEACAM1-4L-EYFP clusters are apparent. Clusters are located at actin rich protrusions and are otherwise similar in morphology to those found on resting adherent HeLa cells.

Membrane receptor binding tends to cause more receptor recruitment, which was visible as an increase in CEACAM1-4L-EYFP fluorescence intensity at sites of cell contact with bacteria, confirming its ability to bind Opa proteins expressed on the bacteria. Within 20 minutes of dropping the cells onto Opa\(^+\) N313 bacteria, CEACAM1-4L-EYFP recruitment to sites of contact is visible (Figure 2.4). In a majority of cases, the bacteria simply continued to rest underneath the cells as CEACAM1-4L-EYFP formed a shell-like structure over them (Figure 2.4 D), though they occasionally became fully enclosed within the cell.

As a negative control to ensure that CEACAM1-4L-EYFP was being recruited to bacteria due to the Opa proteins expressed on their surface, the same procedure was repeated with \textit{N. gonorrhoeae} strain N302, which does not express Opa proteins. CEACAM1-4L-EYFP recruitment was not observed when cells contacted these bacteria (Figure 2.5). These experiments confirmed CEACAM1-4L-EYFP recruitment and binding to Opa-expressing bacteria by parachuted HeLa cells.
Figure 2.4 CEACAM1-4L-EYFP Recruitment to Opa⁺ Bacteria

(A) Fluorescently labeled *N. gonorrhoeae* strain N313, expressing the CEACAM1-binding Opa57. (B) Whole cell xy projection of CEACAM1-4L-EYFP, which was recruited to the positions of bacteria on the basal surface of the cell. (C) Overlay of bacteria and CEACAM1-4L-EYFP fluorescence images. The xz slice shown was taken at the white dashed line indicated. The selected inset region in the xz projection image is 3.5 x 3.5 μm. CEACAM1-4L-EYFP is mainly recruited to apical and lateral surfaces of the immobilised bacterium to form a shell-like structure over-top of it (D), though occasionally bacteria are completely enveloped.
Fluorescently labeled *N. gonorrhoeae* strain N302 does not express Opa proteins. CEACAM1-4L-EYFP recruitment to these bacteria was not observed, as seen in the overlay of bacteria and CEACAM1-4L-EYFP fluorescence.

**2.4.3 Imaging CEACAM1-4L-EYFP Recruitment to *Neisseria gonorrhoeae* Contact Sites by Time-Lapse TIRFM**

Confocal microscopy images were captured after receptor recruitment to bacterial contact sites had occurred, and therefore provided no information about dynamic changes in CEACAM1-4L-EYFP organization in response to cellular contact with *N. gonorrhoeae*, or about the mechanism of receptor recruitment. Time-lapse image sequences of CEACAM1-4L-EYFP were captured by TIRFM as cells were parachuted onto Opa⁺ bacteria, in order to visualize CEACAM1 reorganization over time and possibly clarify the mechanism of receptor recruitment. From the perspective offered by TIRFM, which allowed for visualization of only the bottom surface of the cell, initial receptor recruitment appeared to occur from a cluster of CEACAM1 moving towards a bacterial contact site (Figure 2.6). This cluster then moved upwards (in the z direction above the imaging plane, presumably to the top of the bacterium) and briefly out of focus, before spreading down over the bacterium. As shown previously from confocal microscopy images, CEACAM1 forms a shell-like structure over top of each bacterium. However, because TIRFM only allows for a view of the cell’s basal surface, and because the height of the bacterium (0.6-0.8μm in diameter) extends above the TIRF evanescent field, this structure appears as a ring-shaped feature in CEACAM1-4L-EYFP TIRFM images.
Figure 2.6 Time-lapse TIRFM of CEACAM1-4L-EYFP Recruitment to Bacteria

Videos of receptor recruitment were generated by capturing images every 5 seconds for 20 minutes as a cell expressing CEACAM1-4L-EYFP came into contact with a surface coated with *N. gonorrhoeae* strain N313. The image sequence above shows representative images from 0 to 10 minutes after image acquisition began, shortly after cell contact with bacteria, as a CEACAM1-4L-EYFP cluster at the position denoted by the arrow was recruited to a bacterium. The confocal overlay identifies the positions of bacteria relative to the cell.

After the initial stage of receptor recruitment, it appeared that additional CEACAM1 was recruited from internal stores in the form of small clusters, which move into the TIRF focal plane and towards the bacterial contact site. Particles were tracked using the “MTrackJ” plugin for ImageJ [107], and occasionally multiple particles were observed following the same route, suggesting that they may be trafficked along cytoskeletal tracks (Figure 2.7 A). The lateral
movement of these particles near the cell membrane could be tracked by TIRFM, though no information about particle movement and speed in the z-direction was obtainable by this method. The lateral speed of the tracked particles was discontinuous, but had an average value of $0.098 \pm 0.031 \, \mu\text{m/sec}$, which is comparable to the discontinuous movement and speed of vesicles trafficked along microtubules [108, 109].

**Figure 2.7 CEACAM1-4L-EYFP Particle Movement Towards a Bacterial Contact Site**

Time-lapse TIRFM revealed that small CEACAM1-4L-EYFP particles move to sites of cell contact with *N. gonorrhoeae*. (A) TIRFM image of a cell showing CEACAM1-4L-EYFP recruitment at a bacterial contact site denoted by the arrow. The tracking of small particles at or near the cell surface revealed that these features move towards the sites of bacteria along similar paths. The tracks of two particles are shown in the enlarged image. Small circles denote the position of each cluster over successive frames, with 2 second intervals between frames. (B) A plot of the lateral speeds of particles between frames, where frame 1 for each particle is the first frame after that particle first appeared in the image sequence. Particle speed fluctuates between frames, as particles dwell and go through periods of fast movement. Though the average speed of particle 1 (yellow track, 0.13\mu m/sec) was faster than the average speed of particle 2 (red track, 0.08\mu m/sec), each particle went through similar periods of fast and slow movement.
Figure 2.8 CEACAM1-4L-EYFP Clusters on Cells in Contact with Bacteria

Though clusters were present on cells contacting *N. gonorrhoea* (A), there were fewer clusters on cells that had a high density of bacterial contact sites (B).

The dynamic CEACAM1-4L-EYFP clusters observed on resting HeLa cells remained present on cells contacting bacteria. However, there were fewer clusters on cells with a high density of bacterial contact sites across their surface, suggesting that all or most became recruited to bacterial contact sites when there were enough contact sites available (Figure 2.8).

### 2.4.4 Live Cell CEACAM1-4L Effector Colocalization

To begin to relate these changes in CEACAM1-4L-EYFP organisation at the cell surface, and ultimately changes the CEACAM1 monomer and dimer distribution that will be discussed later, to functional outcomes within the cell, preliminary investigations into the concurrent recruitment of downstream CEACAM1 effectors were conducted by TIRFM. Colocalization between CEACAM1-4L-mCherry and the labeled tyrosine phosphatases SHP-1-GFP and SHP-2-GFP was quantified by determining the Pearson’s colocalization coefficient using the “JaCoP” plugin for ImageJ [3]. The Pearson’s coefficient is a measure of linear correlation between the intensity values in each image channel, and has a value of 1 for perfectly colocalized channels, a value of 0 if there is no colocalization, and a value of -1 for inversely correlated channels. Bleed-through between the mCherry channel and the GFP channel did not occur at the exposure times used for imaging these constructs. Due to differences in the beam size of the 532 and 473 lasers used to excite mCherry and GFP, the area illuminated by the 532 laser was larger than
that of the 473 laser, and so regions of interest (ROIs) at the center of the aligned illumination fields were selected for Pearson’s coefficient calculations. It is also important to note that, at the same incident angle for total internal reflection, the penetration depth of the evanescent wave generated by the 532nm laser was slightly greater than that of the 473nm laser due to its longer wavelength (Equation 1).

Resting HeLa cells expressing SHP-1-GFP and CEACAM1-4L-mCherry showed no colocalization, as did resting cells expressing SHP-2-GFP and CEACAM1-4L-mCherry (Pearson’s coefficients of 0.093 and 0.003 respectively, for 3x3μm ROIs at the center of each cell). In cells that have landed on Opa⁺ bacteria (approximately 15 minutes after contact), both SHP-1-GFP and SHP-2-GFP showed colocalization with CEACAM1-4L-mCherry, with Pearson’s coefficients of 0.638 and 0.865 at selected 3x3μm bacterial contact sites (Figure 2.9). SHP-1-GFP and SHP-2-GFP were not visibly recruited to bacteria when expressed alone without CEACAM1 (Appendix 1).

Figure 2.9 SHP-1 and SHP-2 Colocalize with CEACAM1-4L at Bacterial Contact Sites
(A) SHP-1-GFP and CEACAM1-4L-mCherry, when co-expressed in a cell that is contacting Opa⁺ bacteria, are nominally co-localized with a Pearson’s coefficient of 0.638 at the highlighted bacterial contact site (inset). (B) In another cell, co-expressed SHP-2-GFP and CEACAM1-mCherry show more obvious co-localization at the highlighted bacterial contact site (Pearson’s coefficient of 0.865). Inset images of bacterial contact sites are 3x3 μm.
2.5 Discussion

Confocal microscopy offered 3-dimensional perspective of CEACAM1-4L-EYFP organization at sites of cell contact with immobilised *N. gonorrhoeae*, whereas TIRFM allowed for the high-resolution imaging of only the bottom surface of the sample, and could be used for capturing homo-FRET image pairs, as discussed in Chapter 3. Bacteria were occasionally completely enveloped by the cell, in a manner which may depend on the CEACAM1 expression level, as well as how securely each bacterium is immobilized onto the dish. However, for TIRFM imaging purposes, CEACAM1-4L-EYFP maintained at the surface was ideal for imaging.

The combinatorial microscopy approach assisted with the interpretation of TIRFM time-lapse images of CEACAM1-4L-EYFP receptor recruitment to Opa⁺ bacterial contact sites, which suggested that at least some recruited CEACAM1 originated as a pre-formed cluster at the cell surface that spread over and encircled bacteria (Figure 2.10). Though the high-intensity clusters, independent of high-intensity features at sites of bacterial adhesion, were still present on cells that are contacting bacteria, they appeared reduced in number, especially around regions with many bacterial contact sites. This supports the model of cluster recruitment to adhering *N. gonorrhoeae*.

However, the tracking of additional small CEACAM1-4L-EYFP particles that appeared to come from within the cell and moved towards bacterial attachment sites indicated that the mechanism of CEACAM1 receptor recruitment may be a combination of the movement of pre-formed clusters already at the cell surface as well as recruitment or replenishment from internal stores via small transport vesicles. The apparent directed trafficking of CEACAM1 clusters suggests that cytoskeletal tracks may play a role in cluster formation and dynamics, though studies have shown that actin destabilization has only a minor effect on bacterial attachment and uptake [49]. It may be that the additional receptor trafficking occurs but it is not required for bacterial attachment and uptake.
Figure 2.10 Model for CEACAM1-4L-EYFP Recruitment to Immobilised *N. gonorrhoeae*
A CEACAM1-4L cluster moves towards the site of an immobilised bacterium and spreads around it.

The entire cytoplasmic domain is not required for CEACAM1-mediated bacterial invasion, and so presumably direct interactions between CEACAM1 and the cytoskeletal molecules are not required for the process. However, cytoskeletal networks often play a role in the maintenance and trafficking of lipid rafts, which CEACAM1 is believed to associate with, and so it is possible that the cytoskeleton indirectly modulates the movement of CEACAM1 cytoplasmic domain mutants. Regardless, it appears that substantial cytoskeletal involvement is not absolutely required during the process of bacterial uptake, it is has been shown still to occur in the presence of actin remodelling inhibitors [31, 49].

In addition to the methods of receptor recruitment observed, CEACAM1 may be recruited from diffuse stores at the cell surface, which would be difficult to identify by diffraction-limited TIRFM, which is incapable of identifying individual features within the diffuse CEACAM1 pool. Though this type of congregation would appear as a gradual increase in fluorescence intensity at the bacterial contact site, it is undetectable above the large scale recruitment of CEACAM1 clusters.

Though these time-lapse studies show how the CEACAM1-4L-EYFP organisation changes upon bacterial contact, time-lapse experiments that include other labels, such as a membrane label for visualizing the position of the bottom surface of the cell, or an actin label for associating CEACAM1 dynamics with any cytoskeletal changes, might provide further insights into the cellular and molecular mechanism of CEACAM1 receptor trafficking and recruitment. In preliminary investigations involving the fluorescent labeling of other cellular components,
CEACAM1-4L and effector colocalization studies confirmed that CEACAM1-4L recruitment lead to SHP-1-GFP and SHP-2-GFP localization at bacterial contact sites in living cells.

Finally, the parachuting of cells onto bacteria-coated tissue culture dishes was shown to be an effective means of coordinating the CEACAM1-Opa interaction, so that multiple sites of bacterial adhesion could be monitored on a single plane by TIRFM, beginning from early time-points in the interaction. This controlled method for visualising the cell-bacteria interaction could be extended to the study of CEACAM1 recruitment to other pathogens, or even to other host receptor and pathogen interactions.
CHAPTER 3

3 Homo-FRET Imaging of CEACAM1-4L-EYFP during Bacterial Adhesion

3.1 Chapter Summary

Homo-FRET experiments, carried out on a two-camera TIRFPM system, were capable of differentiating between the fluorescence anisotropy of monomeric and dimeric Venus protein controls in living cells, and also depicting the heterogeneous CEACAM1-4L-EYFP oligomerization state at the cell surface. This technique was used to monitor CEACAM1-4L-EYFP spatial and temporal anisotropy distributions in cells that came into contact with *Neisseria gonorrhoeae*, to draw inferences about the receptor’s structural state and function during pathogen binding. On cells contacting the Opa⁺ bacteria, regions of recruited CEACAM1-4L-EYFP had a higher anisotropy than the rest of the cell throughout the time-course of receptor recruitment, and these higher anisotropy values were comparable to the average anisotropy of monomeric G432,436L-CEACAM1-4L-EYFP, indicating that there is a greater proportion of CEACAM1-4L monomers at sites of bacterial engagement. Monomeric G432,436L-CEACAM1-4L-EYFP was efficiently recruited to bacterial contact sites and allowed for SHP-1-GFP and SHP-2-GFP recruitment. Together these results suggest that the monomeric CEACAM1-4L receptor state is predominantly involved in processes relating to Opa binding and pathogenesis by *Neisseria gonorrhoeae*.

3.2 Introduction

Investigations into CEACAM1 function have led to many complex and even conflicting interpretations of its role in human cells, likely due in part to its capacity to influence both
stimulatory and inhibitory pathways under different circumstances. CEACAM1 is capable of undergoing cis-interactions to form dimers, and also possibly higher-order oligomers [19]. It has been suggested that the different structural states of the receptor are responsible for transmitting alternate signals, offering a potential mechanism by which complex CEACAM1 signalling is regulated.

Monomers and dimers both exist at the cell surface, and the predominance of each is regulated by the cell through the binding of Ca\(^{2+}\)-loaded calmodulin. Calmodulin binding, which occurs at a membrane-proximal region of the CEACAM1 cytoplasmic domain of both long and short (-L and -S) receptor isoforms, leads to the dissociation of dimers [18, 19, 26]. Treating cells with the Ca\(^{2+}\) ionophore ionomycin therefore leads to a shift towards more monomeric CEACAM1 [23]. Serine and threonine residues located within the calmodulin binding site of the receptor can become phosphorylated, and so the activity of serine/threonine kinases may also regulate the receptor oligomerization state, though this has yet to be confirmed [110]. Additionally, co-expressing CEACAM1-4L with the CEACAM1-4S isoform, with which it can form a heterodimer, also leads to fewer CEACAM1-4L homodimers [14].

As a further indication of the significance of the CEACAM1 oligomerization state, there is an apparent difference in the capacity of monomers and dimers to bind various downstream effectors. Computational modeling of the CEACAM1 and SHP-1 association has suggested that steric hindrance would prevent the tyrosine phosphatase from binding to CEACAM1 dimers, and so binding to the monomeric form would be preferred [15]. In support of this analysis, biochemical studies have shown that monomeric but not dimeric CEACAM1 co-immunoprecipitates with SHP-1[23]. In contrast, the kinase c-Src, another downstream effector thought to participate mainly in CEACAM1 stimulatory pathways, was predicted to be capable of binding both monomeric and dimeric CEACAM-1[15]. Phosphatases and other inhibitory effectors are the signalling molecules responsible for CEACAM1’s immunosuppressive influences during infection.

Though CEACAM1 mutant studies have shown that the integrity of the N-terminal IgV-like domain is important for CEACAM1 cis-interactions, the region of the receptor critical for dimerization has been mapped to a GXXG motif in the transmembrane domain [14, 27]. A
mutation in this region generated the monomeric G432,436L-CEACAM1-4L, which is incapable of undergoing cis-homotypic interactions but remains capable of trans-homotypic interactions and is capable of mediating the adhesion and uptake of *N. gonorrhoeae* expressing appropriate Opa proteins (Gray-Owen group, personal communication). When visualized by TIRFM, the labeled monomeric mutant receptor forms high intensity clustered regions and has a morphology that is otherwise indistinguishable from that of wild-type CEACAM1-4L-EYFP [24].

Human CEACAM1-4L has been shown by biochemical studies to exist as a mixture of monomers and dimers when expressed in HeLa cells [23]. Through homo-FRET imaging, in which fluorescence anisotropy is measured to differentiate between monomeric and oligomeric species, previous Yip lab researchers have shown that fluorescently labeled CEACAM1-4L monomers and dimers are heterogeneously distributed at the HeLa cell surface. High intensity CEACAM1-4L-EYFP clusters are predominantly monomeric, whereas diffuse CEACAM1-4L-EYFP is more dimeric, though both populations also appear to have a heterogeneous distribution of receptor states [22, 24]. Perturbations in this distribution could be detected after the addition of ionomycin, as well as polyclonal α-CEA antibody, which both caused increases in anisotropy suggestive of a shift towards more monomeric CEACAM1, in agreement with results previously reported [14, 23]. Homo-FRET by TIRFPM could effectively detect relative changes in anisotropy across the same cell, though anisotropy values between cells would be less consistent and predictable. As such, this technique is best suited to studying the relative proportion of monomeric and dimeric species spatially or temporally within the same cell [24].

As with changes in the total CEACAM1 distribution upon cellular contact with *N. gonorrhoeae*, described previously in Chapter 2, changes in the distribution of CEACAM1 monomers and dimers may provide important information, such as functional roles for the different receptor states. It is not known which form of the receptor binds to the pathogen Opa receptors, or which form transmits the cellular signals stimulated by the CEACAM1-Opa interaction. As such, homo-FRET imaging was carried out by TIRFPM on cells expressing CEACAM1-4L-EYFP in contact with these Opa⁺ bacteria, to investigate any shifts in the heterogeneous receptor oligomerization state at the cell surface.
3.3 Materials and Methods

3.3.1 Molecular Cloning

Through a collaboration with the Rocheleau group (University of Toronto) a Venus fluorescent protein tandem dimer construct was generated from two monomeric (A206K mutant) Venus constructs and a 10 amino acid linker, as described by Rizzo et al. [24, 111]. In addition to the fluorescent CEACAM1-4L constructs described in section 2.2.1, members of the Gray-Owen group (University of Toronto) generated labeled monomeric mutant G432,436L-CEACAM1 constructs by attaching enhanced yellow fluorescent protein (EYFP) or cyan fluorescent protein (CFP) to the C-terminal end of the G432,436L-CEACAM1-4L cytoplasmic tail, creating G432,436L-CEACAM1-4L-EYFP and G432,436L-CEACAM1-4L-CFP.

3.3.2 Cell Culture, Transfections, and Bacteria Preparation

Cells were maintained and transfected as described in section 2.2.2. Bacteria were grown and immobilized onto tissue culture dishes as described in section 2.2.3.

3.3.3 Microscopy

Cells parachuted onto surfaces were imaged 10-40 minutes after coming into contact with the bottom surface of the tissue culture dish, except where indicated in time-course experiments. For each cell, TIRFPM homo-FRET images of labeled CEACAM1-4L were captured first, before confocal stacks of both CEACAM1-4L and the fluorescently-labeled bacteria were captured as described in section 2.2.5. TIRFPM CEACAM1-4L-EYFP images were overlaid with confocal bacteria images to illustrate the positions of bacteria relative to the cells, though the time taken to switch between imaging modalities allowed for some bacteria and receptor movement between captures. Images of G432,436L-CEACAM1-CFP were captured by TIRFM, for which samples were excited with a 405nm laser, and fluorescence emission was passed
through a 400nm cut-on dichroic mirror and a 460-490nm band-pass interference filter. Confocal and TIRFPM images of other constructs were captured with the lasers and filter sets described in sections 2.2.5 and 2.2.6 respectively. All images shown and analysed are representative of two or more experiments.

3.3.4 Steady-State Homo-FRET by TIRFPM

Homo-FRET image pairs were captured on the TIRFPM system. The 473nm laser beam used to excite both CEACAM1-EYFP and Venus constructs was s-polarized, such that it selectively excited fluorophores oriented with the y-axis as defined in Figure 2.1. Previously with a one-camera system, $F_{||}$ and $F_{\perp}$ images were captured in sequence through emission polarizing filters mounted in a rotating filter turret. The $F_{||}$ image was first collected through a polarizing filter aligned parallel to the excitation polarization, after which the filter turret was manually rotated so that the $F_{\perp}$ image could be collected through a polarizing filter aligned perpendicular to the excitation polarization. In order to allow for the acquisition of both images simultaneously, a two-camera imaging system was developed (Figure 3.1). In this new system, fluorescence emission was directed through a polarizing beam splitter (PBS251, ThorLabs, Newton, NJ) which synchronously sends light of one polarization sideways to one camera and light of a perpendicular polarization upwards to a second camera. Both cameras could then acquire images at the same time, allowing for the simultaneous capture of $F_{||}$ and $F_{\perp}$ images for homo-FRET detection. All anisotropy images shown in this section were captured using the two-camera system. A 1500ms exposure time was used to excite EYFP constructs, and a 500ms exposure time was used to excite Venus constructs, which would consistently result in images with intensities that spanned the dynamic range of camera pixel values and avoid pixel saturation. Pixel-wise fluorescence anisotropy values were determined using a lab-written ImageJ macro that carried out the corrected anisotropy calculation (Equation 11) on background-subtracted $F_{||}$ and $F_{\perp}$ images to generate anisotropy images depicting fluorescence anisotropy across the cell surface (Figure 3.2) [24]. Pixels at high CEACAM1-4L-EYFP intensity regions were isolated using ImageJ’s “MaxEntropy” and “Triangle” dark image background auto-threshold routines (http://pacific.mpi-cbg.de/wiki/index.php/Auto_Threshold).
Two EMCCD cameras, mounted off a top port on the microscope above the eyepiece, were used to collect fluorescence emission through a polarizing beamsplitter that sends fluorescence of one polarization \( F_{\parallel} \), aligned parallel to the excitation polarization, to one camera, and fluorescence of the perpendicular polarization \( F_{\perp} \), perpendicular to the excitation polarization, to a second camera.

Anisotropy calculations are applied to the intensity values in \( F_{\parallel} \) and \( F_{\perp} \) images on a pixel-by-pixel basis, using a home-written macro for ImageJ. This macro generates corrected anisotropy \( r_c \) images such as the one shown above, in which each pixel is coloured according to its calculated anisotropy, where more red values indicate a high anisotropy and more blue values indicate a lower anisotropy. For more detail regarding the macro for anisotropy image generation, see J. Lo’s thesis [24].
3.3.5 Two-Camera Homo-FRET Detection System Set-up and Calibration

Due to very small differences in the optical path for both polarizations, the field of view (FOV) detected by each EMCCD chip on the two camera system was slightly different, resulting in image pairs that were translated and rotated with respect to one another. To register the cameras so that both camera EMCCD arrays captured the same FOV on the sample, one camera was set up on a multi-axis translation stage so that its position could be adjusted until the FOV that it detected matched that of the other camera. Registration of captured image pairs was fine-tuned using the “StackReg” plugin freely available for ImageJ (http://bigwww.epfl.ch/thevenaz/stackreg/)[112].

Furthermore, due to the fact that homo-FRET image pairs were collected on two different EMCCDs, and that these EMCCDs both had slightly different sensitivities (or photo-electron to pixel unit conversion factors), approaches to accounting for this difference were investigated. To correct for the difference in the two EMCCD sensitivities, the gain on one camera was increased while leaving the second camera gain at 35 until the captured intensity of an isotropic fluorescein isothiocyanate (FITC) solution, detected with a 10x objective in epifluorescence mode, was the same on both cameras. It was determined that when the first camera (collecting $F_\perp$) gain was increased to 40, the collected intensities of a dye solution matched those captured on the second camera (collecting $F_{\parallel}$) with a gain of 35, over a range of exposure times and dye concentrations that resulted in intensity values comparable to those normally obtained with fluorescent live-cell samples (Appendix 2). Enabling the Evolve 512 EMCCD “Quant-view” feature, which back-calculates from pixel unit intensity values to give each pixel the value of its theoretical photo-electron count, was also investigated as a promising method of correcting for the differences in camera sensitivities, though all images shown here were obtained with the gain correction method of sensitivity correction.

With camera gains adjusted, the calculated G-factor, a correction factor to account for polarization bias in the microscope optical path, was determined to be 1 for images of an isotropic solution of FITC on the two-camera system containing a polarizing beam splitter.
G-factors were calculated with the following equation:

\[ G = \frac{F_{yx} \times F_{xy}}{F_{yx} \times F_{xx}} \]  

where \( F \) is the intensity and first letter in the subscript after \( F \) indicates excitation polarization oriented along either the \( x \)-axis or \( y \)-axis of the imaging plane, and the second letter indicates the direction of the emission polarization [98]. The G factor was routinely monitored over a range of FITC concentrations and exposure times.

### 3.3.6 Data Analysis

All image analysis was once again carried out with ImageJ. Two-tailed, unpaired Student’s T-tests were used to determine any significant differences using Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA).

### 3.4 Results

#### 3.4.1 Two-Camera TIRFPM Differentiates Monomeric and Dimeric Venus Anisotropy in Live Cells

Monomeric Venus and the tandem dimeric Venus described in section 3.2.1 have been used in the past to validate homo-FRET measurements on our TIRFPM system [4]. As such, these constructs were once again used for live cell imaging to validate the new two-camera system.

Cells expressing monomeric Venus had an average fluorescence anisotropy of 0.198 ± 0.002, whereas cells expressing dimeric Venus had an average anisotropy of 0.144 ± 0.003 (Table 3.1). These values were comparable to those captured on the old one-camera system, and the anisotropy of the monomeric fluorophore was significantly higher than that of the dimeric fluorophore (\( p<0.0001 \)). Representative anisotropy images (Figure 3.3) show that anisotropy
values were fairly homogenous across the cell surface. At cell edges, where detected intensities drop off to very low values due to the decay of illumination intensity at regions further away from the center of the illumination field, anisotropy values became less consistent. This is likely due to these pixel intensity values residing outside of the range of intensity values that are properly corrected for by the gain correction method of camera sensitivity adjustment.

Though the monomeric and dimeric Venus controls are only isolated fluorescent proteins and thus their average anisotropy values do not represent what would be expected for completely monomeric and completely dimeric CEACAM1-4L-EYFP populations, they could be used to confirm that the two-camera homo-FRET detection system was capable of differentiating between monomeric and dimeric species. The Venus controls were run regularly and any time after a change to our microscope configuration was made, and plots of anisotropy versus total intensity throughout the sample were routinely monitored to ensure that fluctuations in intensity did not correlate with fluctuations in anisotropy.

![Figure 3.3 Monomeric and Dimeric Venus Anisotropy Controls](image)

Representative corrected anisotropy images of cells expressing monomeric Venus (A) and the tandem dimeric Venus (B). Anisotropy histograms (right) display the distribution of anisotropy values calculated for each pixel. Both fluorescent proteins have a homogenous anisotropy distribution.
3.4.2 Homo-FRET Imaging of CEACAM1-4L-EYFP on Parachuted Cells

To determine the CEACAM1-4L-EYFP anisotropy distribution in the absence of bacterial adhesion, suspensions of cells were parachuted onto dishes that were treated as described in section 3.2.2 without the addition of bacteria. CEACAM1-4L-EYFP-expressing HeLa cells had an average anisotropy of 0.145 ± 0.004 (Table 3.1). In contrast to the Venus controls, which showed a homogenous anisotropy distribution, CEACAM1-4L-EYFP had a more heterogeneous anisotropy distribution, in agreement with CEACAM1-4L existing as a mixture of monomers and dimers at the cell surface (Figure 3.4 A). Cells expressing the monomeric mutant G432,436L-CEACAM1-4L-EYFP that were parachuted onto bacteria-free surfaces had a higher average anisotropy of 0.169 ± 0.004 (Table 3.1). Though this receptor population is expected to be completely monomeric, the anisotropy distribution appeared less homogenous than that of the monomeric and dimeric Venus controls (Figure 3.4 B).

<table>
<thead>
<tr>
<th>Construct</th>
<th>Average $r_c$ ± SE</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomeric Venus</td>
<td>0.198 ± 0.002</td>
<td>37</td>
</tr>
<tr>
<td>Dimeric Venus</td>
<td>0.144 ± 0.003</td>
<td>35</td>
</tr>
<tr>
<td>CEACAM1-4L-EYFP</td>
<td>0.145 ± 0.004</td>
<td>34</td>
</tr>
<tr>
<td>G432,436L-CEACAM1-4L-EYFP</td>
<td>0.169 ± 0.004</td>
<td>33</td>
</tr>
</tbody>
</table>

The average corrected anisotropy ± standard error (SE) for N cells expressing the construct indicated. Monomeric and dimeric Venus were imaged in adherent HeLa cells and CEACAM1 constructs were imaged in cells parachuted onto bacteria-free surfaces. Monomeric Venus had a significantly higher (p<0.0001) anisotropy than dimeric Venus.
Figure 3.4 CEACAM1-4L-EYFP and G432,436L-CEACAM1-4L-EYFP Anisotropy

Representative fluorescence and anisotropy images of cells expressing CEACAM1-4L-EYFP (A) and G432,436L-CEACAM1-4L-EYFP (B) that have been parachuted onto bacteria-free surfaces. Anisotropy images and histograms indicate that the CEACAM1-4L-EYFP distribution is heterogeneous, while the G432,436L-CEACAM1-4L-EYFP distribution also shows some heterogeneity.

3.4.3 Anisotropy of G432,436L-CEACAM1-4L-EYFP on Bacteria-Engaging Cells

HeLa cells expressing the monomeric G432,436L-CEACAM1-4L-EYFP that were parachuted onto Opa$^{57}^{+}$ N. gonorrhoeae showed marked receptor recruitment to bacterial contact sites. This was visible by TIRFM as bright fluorescent rings that corresponded to the bottom edges of shell-like structures that the recruited receptor formed over immobilised bacteria, confirming that this monomeric receptor allowed for N. gonorrhoeae adhesion. These features therefore presumably contained Opa-engaging CEACAM1. The whole cell average anisotropy for the cell shown in Figure 3.5 was 0.174 ± 0.073, which is comparable to the average G432,436L-CEACAM1-4L-EYFP anisotropy in uninfected parachuted cells (0.169).
Figure 3.5 G432,435L-CEACAM1-4L-EYFP at Bacterial Contact Sites

Labeled bacteria (A) led to cellular recruitment of G432,435L-CEACAM1-4L-EYFP to sites of *N. gonorrhoeae* adhesion (B,C). The cell average anisotropy was $0.174 \pm 0.073$ (D).

3.4.4 CEACAM1-4L-EYFP Anisotropy during the Time-course of Bacterial Attachment

Early anisotropy experiments on the one-camera TIRFPM system detected a greater average CEACAM1-4L-EYFP fluorescence anisotropy for cells parachuted onto a high density of unlabeled Opa$^+$ bacteria, in comparison with cells parachuted onto Opa$^-$ bacteria or bacteria-free surfaces (Appendix 4 and 5), suggesting that CEACAM1-4L-EYFP was more monomeric on these cells. These global experiments revealed little information about relative changes in anisotropy and its distribution across a single cell, and so time-course experiments were conducted on the two-camera TIRFPM system using bacteria that were fluorescently labeled in order to determine their positions relative to cells. Cells were monitored as they came into contact with Opa$_{57}^+$ *N. gonorrhoeae* and as receptor recruitment occurred, to provide insight into CEACAM1-4L-EYFP monomer and dimer distributions beginning from early time points during bacterial attachment. Homo-FRET image pairs were captured every five minutes to minimize photobleaching of the sample, which can interfere with the accuracy of homo-FRET measurements. Images show that CEACAM1-4L-EYFP intensity once again increased over a period of 20 minutes as the receptor was recruited to bacterial contact sites (Figure 3.6). Using an intensity thresholding tool during image analysis, high-intensity pixels corresponding to recruited CEACAM1-4L-EYFP at bacterial attachment sites could be isolated for anisotropy.
measurements. This allowed for the determination of anisotropy values specifically for recruited CEACAM1-4L-EYFP, which could then be compared to anisotropy values across the rest of the cell, as well as other cells, for a more quantitative analysis (Figure 3.7).

The average anisotropy at the isolated high intensity bacterial contact regions was 0.175 ± 0.037 at the 20 minute time-point, which is comparable to the average anisotropy of the resting monomeric G432,436L-CEACAM1-4L-EYFP (0.169) as well as the average G432,436L-CEACAM1-4L-EYFP anisotropy for a cell resting on bacteria (0.174). Furthermore, CEACAM1-4L-EYFP at regions contacting bacteria had a higher average anisotropy than non-contacting regions for each region at each time point, indicating that there was a greater proportion of CEACAM1-4L-EYFP monomers at the high-intensity sites throughout the time-course of bacterial attachment. Anisotropy histograms for values at each bacteria contact region (Figure 3.7 C) are up-shifted with respect to histograms of values at bacteria-free regions, though they remain within the range of these values.

![Figure 3.6](image)

**Figure 3.6** Time-course of CEACAM1-4L-EYFP Recruitment to Bacterial Contact Sites

(A) CEACAM1-4L-EYFP recruitment to *N. gonorrhoeae*, beginning from a time point immediately after cell contact with the bacteria-coated surface (time 0 min). (B) Positions of labeled N313 bacteria. (C) Overlay of bacteria and CEACAM1-4L-EYFP 15 minute time point images, depicting the relative positions of the bacteria and cell.
Figure 3.7. Time-course CEACAM1-4L-EYFP Anisotropy during Bacterial Adhesion

(A) Enlarged image of the cell shown in Figure 3.6 at the 15 minute time point, with three bacterial contact sites highlighted (white boxes) and one bacteria-free region highlighted (red box). (B) CEACAM1-4L-EYFP fluorescence recruitment over the time-course of bacterial attachment is shown for each highlighted region in (A) (upper rows). Anisotropy ($r_e$) maps for high-intensity pixels in these bacterial contact regions were calculated (lower rows). (C) CEACAM1-4L-EYFP fluorescence anisotropy histograms for each selected region at the indicated time points. For each bacterial contact region (regions 1-3), the average anisotropy calculated from high-intensity pixels was greater than the average anisotropy at a non-contacting region of the cell (region 4) at each time point, suggesting that CEACAM1-4L-EYFP was proportionally more monomeric at these sites.
3.4.5 Anisotropy Fluctuations at Bacterial Contact Sites

When homo-FRET images were taken rapidly in sequence at two separate time points, though the average anisotropy values for high intensity CEACAM1-4L-EYFP at bacterial contact sites remained consistent (0.173 ± 0.0008 for eight images), fluctuations in the pixel anisotropy values were observed (Figure 3.8). Isolated fluorescent protein tags on CEACAM1 monomers would be expected to consistently emit highly anisotropic fluorescence, whereas the fluorescence emitted from tags on dimers, which may or may not be engaging in FRET, and for which the acceptor fluorophore can be in different orientations, would be of a lower, less consistent anisotropy. The fluctuations observed may therefore mean that these bacterial contact sites, though predominantly monomeric, contain some dimeric species. Alternatively, the fluctuations may be due to levels of crowding-induced depolarization. There is a high density of the fluorescently-labeled receptor at bacterial contact sites, and though very small distances are required between fluorophores for FRET to take place, it is possible that the monomeric receptors occasionally move close enough for it to occur without dimerization.

Figure 3.8 Pixel Anisotropy Value Fluctuations

After CEACAM1-4L-EYFP recruitment to a bacterial contact site had occurred, four homo-FRET image pairs were collected in succession at two time points, (A) time 1 and (B) time 2, five minutes later. Surface plots show the spatial distribution of anisotropy values at a 2x2μm (15x15 pixel) bacterial contact site. Though the average anisotropy for high intensity CEACAM1-4L-EYFP at the selected bacterial contact site remained consistent for all images (0.173 ± 0.0008), small fluctuations in the pixel anisotropy values occur.
3.4.6 G432,436L-CEACAM1-4L and Effector Recruitment to Bacterial Contact Sites

To investigate whether both a completely monomeric CEACAM1-4L construct and the SHPs localized to *N. gonorrhoeae*, the monomeric G432,436L-CEACAM1-4L-CFP and SHP-GFPs were co-expressed in cells that were parachuted onto bacteria. At high exposure times (>1000ms), for cells singly transfected with G432,436L-CEACAM1-4L-CFP, there was a moderate amount of CFP fluorescence bleed-through into the GFP channel used for imaging SHP-1-GFP and SHP-2-GFP. However, these GFP constructs were very bright and required only a 300ms exposure time for imaging. At this exposure time, a negligible amount of G432,436L-CEACAM1-4L-CFP bleed-through occurred. Though G432,436L-CEACAM1-4L-CFP required exposure times of greater than 1000ms in the CFP channel, no GFP bleed-through occurred into this channel for cells singly transfected with SHP-1-GFP or SHP-2-GFP.

Resting uninfected HeLa cells co-expressing G432,436L-CEACAM1-4L-CFP and SHP-1-GFP had a colocalization coefficient of 0.416 for 3x3μm ROIs selected at the center of the cell. This modest relationship may reflect a basal level of association between the proteins, or may be an artifact due to the low level of channel bleed-through. Similarly, cells co-expressing G432,436L-CEACAM1-4L-CFP and SHP-2-GFP had a Pearson’s colocalization coefficient of 0.364.

After coming into contact with the Opa⁺ bacteria, G432,436L-CEACAM1-4L-CFP was efficiently recruited to bacterial contact sites. It also allowed for SHP-1 and SHP-2 recruitment, with Pearson’s coefficients of 0.844 and 0.862 respectively for 3x3μm ROIs at bacterial contact sites (Figure 3.9).
3.5 Discussion

3.5.1 Two-camera TIRFPM Set-up and Live Cell Controls

The two-camera homo-FRET detection system offered many advantages, as it reduced the time required for homo-FRET image acquisition and reduced the effects of photo-bleaching as image acquisition required only one exposure time, in contrast to image acquisition with the old set-up where two exposure times were required to collect $F_{\parallel}$ and $F_{\perp}$ images separately. This reduction in photobleaching made time-lapse anisotropy imaging more feasible. Furthermore, on the old set-up it was shown that even acquisition of the first $F_{\parallel}$ homo-FRET image was sufficient to
photobleach Venus and CEACAM1-EYFP slightly, and so the $F_\perp$ image would be collected from a dimmer photobleached sample [24], causing potential anisotropy calculation inaccuracies that were avoided with the new set-up.

EMCCD cameras produce images by generating electrons when incident photons hit the CCD array. These electrons are converted into analog-to-digital units (ADUs) that determine what greyscale value will be given to each pixel in the array. Each EMCCD chip will have a different sensitivity, and therefore a slightly different electron-to-ADU conversion factor. When an isotropic solution of FITC, which should send approximately equal fluorescence intensity to both cameras from the polarizing beam-splitter in the two-camera set-up, was imaged over a range of concentrations and exposure times, the intensities captured on each camera ($F_{\parallel}$ and $F_\perp$) were quite different and could not be matched with a corrective $G$ factor. Though adjusting the gain of one camera was sufficient to correct for this, enabling the “Quant-view” feature for the Evolve 512 EMCCD cameras was another potential correction method. This feature gives each pixel the theoretical value of its photo-electron count instead of a somewhat arbitrary ADU, allowing for more quantitative imaging, especially when comparing intensities from two different cameras. Its potential use in homo-FRET experiments was investigated, and when images of an isotropic fluorescein solution were collected over a range of exposure times on both cameras with the Quant-view feature enabled, the intensities of $F_{\parallel}$ and $F_\perp$ images increased in unison, with $F_\perp$ values being slightly lower than $F_{\parallel}$. When a corrective $G$-factor of 1.2 (determined by Equation 12) was applied to the $F_\perp$ image, intensity values acquired by each camera matched (Appendix 2). Thus, this feature could potentially be used for homo-FRET experiments, as it provided an accurate way of comparing the intensities of the two cameras. However, in low intensity regions of cells the photo-electron counts became very low or had values of zero, making the imaging and anisotropy calculations of dimmer samples difficult.

Though live cell Venus control experiments using this feature detected a significant difference between monomeric and dimeric Venus anisotropy, pixel intensity and anisotropy values obtained by the gain correction method were comparable to results obtained on the one-camera system, and so this method was used for consistency (Appendix 3). However, the Quant-view method may be useful to explore for future homo-FRET imaging applications.
3.5.2 CEACAM1-4L-EYFP Anisotropy at Bacterial Contact Sites

Homo-FRET detection by TIRFPM was previously shown to be capable of detecting relative anisotropy changes on a cell during a shift in the CEACAM1-4L-EYFP monomer-dimer distribution, induced by the addition of the calcium ionophore ionomycin or α-CEA antibody [24]. The homo-FRET technique was therefore applied to study any possible perturbations in CEACAM1-4L-EYFP fluorescence anisotropy distribution in response to *N. gonorrhoeae* binding. Relative to the rest of the cell, the higher anisotropy values for regions of recruited CEACAM1-4L-EYFP at Opa⁺ bacterial contact sites, which were comparable to the average anisotropy of cells expressing the monomeric G432,436L-CEACAM1-4L-EYFP, suggest that these regions contained a greater proportion of CEACAM1-4L-EYFP monomers.

The higher proportion of monomers at the bacterial contact sites could mean that Opa binding specifically required the monomeric receptor, and/or that signalling events within or on the cell caused the recruitment of CEACAM1-4L monomers. In section 2.2.3, CEACAM1-4L-EYFP recruitment to bacteria was shown to occur, at least in some cases, as a result of the recruitment of a pre-formed cluster at the cell surface. These clusters have been shown to contain a high proportion of CEACAM1-4L monomers, and so it seems that a proportion of CEACAM1-4L is readily recruited in the monomeric state.

The fluctuations in pixel anisotropy values at bacterial contact sites over short interval times suggest that there may be some level of either dimerization or crowding-induced FRET that decreases the calculated anisotropy at these sites. It is reasonable to believe that some level of receptor crowding would occur, as there was a high density of CEACAM1-4L-EYFP in those regions. It is not known whether each Opa protein binds to one relatively isolated CEACAM1 receptor, or if many receptors crowd around each Opa protein, or if a combination of both scenarios can occur depending on the availability of host receptors.
3.5.3 Implications for CEACAM1-4L Monomer Function

Bright CEACAM1-4L-EYFP clusters on resting adherent HeLa cells have been shown to contain a high proportion of CEACAM1 monomers, by both hetero- and homo-FRET experiments [22, 24]. These dynamic clusters were present on parachuted cells, and were observed to bind to Opa+ *N. gonorrhoeae*. The clusters are present on all surfaces of the cell, and so it may be that they serve as high concentration monomeric receptor sites on epithelial cells that pathogens can initially “stick” to before envelopment begins. In the method used here, the bacteria were fixed in place, and so this was difficult to investigate. It appeared that the clusters could also be recruited to the bacteria by the cell shortly after contact. However, it is possible that the cluster movement observed was not directed recruitment, but was instead the normal random movement of clusters that simply “caught” the bacteria as they came close. Like the bright CEACAM1-4L-EYFP clusters, it appeared that CEACAM1-4L-EYFP was monomeric as it was engaging the Opa proteins expressed on the bacteria.

The labeled monomeric G432,436L-CEACAM1-4L was also recruited to bacterial contact sites and colocalized with SHP-1-GFP and SHP-2-GFP during bacterial adhesion. Without a dimeric CEACAM1-4L control, it cannot be confirmed that the dimeric form of the receptor is incapable of performing these functions. However, CEACAM1-4L-EYFP anisotropy data suggest that it is the monomeric form of CEACAM1-4L that is predominantly recruited to the bacteria and subsequently recruits these signalling molecules (Figure 3.10).

Though it seems that there are lower proportions of dimers at contact sites, it is possible that the dimeric form is still involved in Opa interactions, and may transduce separate effects, though anisotropy data indicated that the monomeric CEACAM1-4L state is principally involved.
Figure 3.10 CEACAM1-4L Monomers Function in Opa Binding and Signaling

CEACAM1-4L monomers, possibly originating as a pre-formed monomeric cluster at the cell surface, are recruited to sites of cell contact with *N. gonorrhoeae*, where they bind to Opa proteins. This results in the phosphorylation of the CEACAM1-4L ITIM by cellular kinases, which allows for tyrosine phosphatase (SHP-1) binding to the monomeric receptor. CEACAM1-4L dimers are present elsewhere on the cell surface.
CHAPTER 4

4 Conclusions and Future Directions

4.1 Chapter Summary

Conclusions and future directions regarding the study of CEACAM1 dynamics by advanced fluorescence microscopy techniques are discussed, both in the context of its function as a pathogen receptor, as well as its other roles in the human body. In general, though the methodology presented in this research could be extended to the study of other cellular receptor and pathogen interactions, further insights into CEACAM1 behaviour and function could be obtained by a number of different approaches.

4.2 CEACAM1-4L Organization and Dynamics in Relation to other Cellular Components

Steady-state multi-colour fluorescence experiments have revealed that CEACAM1-4L-EYFP clusters are located at actin-rich membrane protrusions and colocalize with ezrin. Time-lapse imaging has revealed that the clusters are very dynamic even in resting cells, and that the movement of some clusters becomes more directed toward bacterial contact sites during bacterial adhesion. In a number of cases, small round CEACAM1-4L-EYFP particles were tracked as they moved towards the bacterial contact sites, and occasionally multiple particles were observed following the same path. This suggests that they may be small CEACAM1 transport vesicles being delivered from internal stores, possibly along filamentous actin tracks. TIRFM can be used to track vesicle trafficking and fusion with the cell membrane, and additional labels on other cellular structures could aid with our understanding of the mechanisms of receptor transport in both resting and infected cells. Though disrupting the actin cytoskeleton does not prevent CEACAM1-mediated bacterial uptake, and bacterial binding does
not induce substantial actin remodeling, CEACAM1 is capable of binding to actin and associated proteins like tropomyosin [12, 20, 49]. It is possible that cytoskeletal involvement does normally occur during infection in unperturbed cells, and further actin labeling studies would help to clarify this. Also, a membrane label on the parachuted cells would clarify if CEACAM1 is recruited *after* or *as* the cell basal surface reaches the bacterium, though this might be difficult to determine by TIRFM and would likely require the inclusion of confocal time-lapse imaging.

Likewise, a more thorough investigation of signalling pathways, through effector colocalization, tyrosine phosphorylation, and inhibitor studies, would reveal more information about the functional outcomes of CEACAM1 reorganization at the cell surface, as well as the kinetics of these outcomes. However, there were limitations associated with the time-lapse TIRFM imaging of effector recruitment kinetics in a parachuted cell, as the early stages of effector recruitment correlated with early stages of the cell settling on the bottom surface, and so the intensity of these cytoplasmic effectors would of course increase as a larger volume of the cell entered the TIRF field. As these interactions are often transient in live cells, imaging by other modalities, which would allow for the visualization of bacterial contact on adherent cells, might make time-course and colocalization studies more feasible. One research group has successfully used time-lapse multi-colour imaging of cellular host factors involved in *Shigella flexneri* invasion to reveal information about the distribution and kinetics of host effector recruitment [78]. It may be of use to track the dynamics of other factors involved in CEACAM1-mediated pathogen invasion, though this group also showed that the kinetics of effector recruitment varied from cell to cell, and occurred over fast timescales, which would likely making imaging by the method used in this research difficult.

Furthermore, it is important to note that there are differences in the cellular machinery of different cell types, and cell-type specific differences in CEACAM1 organization and functions have already been noted. HeLa cells do not normally endogenously express the CEACAM1 receptor, and so cells transfected to express the fluorescently labeled receptor provided a simple model for studying the receptor organization and interactions by homo-FRET, in which it could be assumed that all CEACAM1 was labeled with a fluorescent protein. However, carrying out
experiments in more relevant cell type might be more appropriate for CEACAM1 effector investigations.

In additional to the role of other cellular components during bacterial infection, there are many features and processes related to CEACAM1 organization that are not well understood even in uninfected resting cells. A closer investigation into the composition of high-intensity CEACAM1 clusters observed by TIRFM could be made with certain pharmacological substances. For example, CEACAM1 has been shown to associate with lipid rafts, and so it is possible that clusters correspond to patches of these cholesterol-rich microdomains. In agreement with this, Yip lab researchers have shown that a tagged receptor mutant, RQ43,44SL-CEACAM1-4L-EYFP, with a mutation in the N-terminal domain, had a reduced number of high intensity clusters and a reduced difference in intensity between clusters versus diffuse regions [24]. The N-terminal domain has been shown to be important for the organization of microdomains [28]. The application of cholesterol depleting substances could also potentially perturb the clusters, on resting or infected cells. Alternatively, receptor organization could also be compared to CEACAM3, which does not appear to associate with lipid rafts. As with the study of these events in infected cells, a careful selection of appropriate cell types should be made.

Flow-through experiments with a fluorescent dye showed that there is some space between the high intensity clustered regions and the glass surface [24]. However, the concurrent labeling of other structures, such as integrins, may clarify how or if the positions of these clusters are related to focal adhesions. CEACAM1 has been shown to influence integrin functions and signalling [29, 113], and so there is evidence that a relationship may exist.

It may also be of interest to incorporate other CEACAM1 isoforms, such as CEACAM1-4S, or mutant constructs into these experiments. The ratio of -S and -L isoforms has been shown to influence homotypic CEACAM1-4L dimerization levels, and the isoforms are expressed at a different ratio in proliferating versus quiescent cells [7, 14]. Experiments which incorporate the CEACAM1-4S isoform, expressed singly or in combination with CEACAM1-4L using a different fluorescent label, may reveal new insights.
4.3 CEACAM1 during Cell-Substrate Interactions

HeLa cells parachuted onto prepared surfaces were capable of adhering to the surface and interacting with *N. gonorrhoeae* bacteria that had been immobilised below them. This controlled cell-bacteria interaction made the time-lapse imaging of CEACAM1-4L-EYFP receptor reorganization by TIRFM, beginning from early time-points in the adhesion process, more feasible. In this protocol the bacteria were randomly dispersed onto the bottom surface of tissue culture dishes, though the stamping down of bacteria into predictable patterns was briefly investigated. Using poly(dimethylsiloxane) (PDMS) stamps that form a consistent rectangle array pattern, it was possible to immobilise the bacteria in regular patterns (Figure 4.1). In the future, this technique could be used to control the cellular engagement of sequential bacteria, as a cell adhering to the bottom surface would progressively spread over-top each bacterium. This might make tracking the recruitment of CEACAM1 and associated molecules more feasible, and make it easier to differentiate between bacteria-interacting and non-interacting cell regions.

In addition to micropatterning the full bacteria, laying down bacterial outer membrane vesicles (OMVs) would be another method of coordinating the CEACAM1-Opa interaction in a way that could offer advantages. *Nesseria* OMVs naturally produced by the bacteria during growth contain Opa proteins which are capable of binding CEACAM1 [114]. The full intact bacteria are wider than the TIRF evanescent field, and so when cells were parachuted on to these bacteria and imaged by TIRFM, the fluorescently tagged CEACAM1 recruited to the most apical surfaces of the bacteria was above the TIRF field. Though CEACAM1 also spread around the edges of bacteria, forming the characteristic fluorescent ring structures visible by TIRFM, the small size of OMVs, which are typically less than 100nm, would mean the all or most of the CEACAM1 engaging these OMVs would be within the TIRF zone. These OMVs could also be micropatterned onto the bottom surface, so that cells parachuted onto them would contact a regular and predictable array of Opa-covered and Opa-free regions, making it easier to compare CEACAM1 properties at each region. In addition to the micopatterning of Opa-containing substrates, PDMS stamps can be used for microcontact printing of protein monolayers onto surfaces [105]. Cells can then be parachuted onto these surfaces to observe their interactions with the protein patterns [104].
Figure 4.1 Micropatterning of CEACAM1-Interacting Substrates

(A) PDMS stamps can be “inked” with substances and stamped onto tissue culture dishes, where cells can be parachuted onto the micropatterns and imaged during cell-substrate contact. Proteins such as α-CCM antibody can be micropatterned and then visualized using a fluorescently-labeled secondary antibody (B), or the stamps can be used to lay down full bacteria into regular rows (C).

CEACAM1 has many functions outside of pathogen binding. Live cell fluorescence microscopy studies on other aspects of CEACAM1 function may reveal novel information. CEACAM1 can interact with CEACAM1 and CEACAM5 on neighbouring cells to facilitate intercellular adhesion. As with bacterial adhesion, there is still much to be determined with respect to its behaviour and the significance of its different oligomerization states during intercellular homotypic and heterotypic interactions. Also similar to cell-bacteria interactions, the mobile and irregular nature of the cell-cell interaction interfaces makes it difficult to predict and monitor over time by fluorescence microscopy. Interactions at cell junctions have been successfully simulated through cell-bilayer interactions. Supported lipid bilayers—which can be prepared to include membrane proteins of interest—act to model one cell, while membrane proteins or other molecules in a whole living cell resting on top of that bilayer are studied by TIRFM. This has been done extensively to simulate the formation of an immune synapse at the cell junction.
between T cells and antigen presenting cells (APCs). The APC is replaced by a lipid bilayer that contains relevant APC surface components, such as the peptide-loaded major histocompatibility complex (MHC). Live T cells are then parachuted onto these bilayers and observed by fluorescence imaging modalities such as TIRFM as the spatial reorganization of membrane receptors occurs to form the immune synapse [89]. The tracking of TCR microcluster formation and movement at cell junctions has also been carried out by this method [115]. If model lipid bilayers containing a CEACAM1-engaging receptor, such as the GPI-anchored CEACAM5 or a mutant GPI-anchored CEACAM1, could be prepared, then cells expressing CEACAM1-4L-EYFP could be parachuted onto them. Similar to the experiments conducted on cell-bacteria interactions, TIRFM could then be used to monitor CEACAM1 surface organization and movement at cell junctions.

All methods discussed could be used not only to study changes in the clustered organization of CEACAM1, but also to observe CEACAM1 self-association dynamics through homo-FRET experiments by TIRFPM.

Finally, live cell fluorescence microscopy investigation of cellular CEACAM1 organization is not limited to TIRFM. Previous Yip lab researchers have demonstrated that homo-FRET experiments conducted on our confocal microscope can differentiate between monomeric and dimeric species. Though the interaction interface would be less uniform and predictable, making time-lapse studies more difficult, homo-FRET imaging of cell-bacteria and cell-cell contacts at regions other than the basal surface of the cell could be carried out. Through single cell manipulation, two non-interacting cells can be brought into contact to allow for adhesion between them, so that the interaction interface between two living cells can be monitored by fluorescence microscopy and accompanying techniques.

### 4.4 Additional Fluorescence Microscopy Techniques

Many fluorescence microscopy techniques for studying membrane proteins have emerged. Though TIRFM has allowed for the imaging of CEACAM1-4L-EYFP cell surface organization with reasonably high resolution, it remains a diffraction-limited approach, making it difficult to
resolve the finer details of features such as the CEACAM1 clusters or CEACAM1 recruited to bacterial contact sites.

Recently, a number of “super-resolution” techniques that enhance the resolution of fluorescence microscopy beyond the diffraction limit have been developed. These include localization microscopy techniques such as photoactivated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM), which localize photo-convertible or photo-activatable probes with nanometer resolution [63, 116]. These probes undergo an intramolecular reaction upon activation by ultraviolet light, changing their fluorescent properties. After a short pulse of exposure to ultraviolet light, a small subset of photo-activatable (PA) probes is converted from a dark state to a bright fluorescent state. Fluorescence emission from this small subset is then captured, and the precise location of each fluorophore can be estimated, due to the reduced overlap of single molecule fluorescence signals. This process is carried out repeatedly, and each subset of localized fluorophores is combined into one super-resolved fluorescence image (Figure 4.2). A PA-GFP probe could replace EYFP on CEACAM1-4L to carry out PALM on the receptor. Alternatively, other techniques like super-resolution optical fluctuation imaging (SOFI), rely on a statistical analysis of the sporadic fluorescence emission from standard fluorescent proteins like EYFP [117].

**Figure 4.2 Super-Resolution Localization Microscopy**

In diffraction limited microscopy, individual single molecule fluorescent features cannot be resolved. In super-resolution localization microscopy, subsets of photo-switchable probes are activated and imaged in sequence, so that the fluorescence emission from a single fluorophore can be precisely localized. These subsets of single molecule locations are combined in to one super-resolved image, which depicts molecular locations with single molecule resolution.
Each of these methods requires that many frames be captured rapidly in sequence, so that the sparse subset of fluorescent molecules activated in each frame can be combined into one complete super-resolution image. These techniques are well-suited to the study of membrane proteins, as they can be carried out by TIRFM [62]. Though they are more commonly used on fixed cells, high-speed imaging and the post-acquisition statistical analysis of super-resolved images has extended their application to include live cell samples. Live cell PALM images have been used to visualize membrane protein clustering and domain formation with nanometer-level precision [62].

Biochemical and diffraction-limited fluorescence microscopy studies have already shown that the CEACAM1 surface distribution is multifaceted and involves receptor organization into monomers and dimers, and possibly higher-order oligomers, which in turn localize to lipid rafts, which then are presumably localized to larger clusters and/or diffuse regions at the cell surface. Super-resolution images that clarify this organization may help to relate it to CEACAM1 function. Live-cell super-resolution microscopy has already revealed the intricacies of receptor organization in greater detail. For example, high-speed live cell PALM has revealed secondary domains within T cell receptor microclusters, as well as their dynamics, and how these relate to the functions of the receptor [118, 119]. PALM has also been implemented for the study of cellular membrane proteins interacting with pathogen, for example revealing the highly organized arrangement and oligomerization state of cellular tetherin at sites of human immunodeficiency virus (HIV) budding from the plasma membrane [120]. Another super-resolution technique has shown how the non-random nanoscale spatial organization of a dendritic cell receptor optimizes it for pathogen binding [121]. In a similar manner, super-resolution techniques could also be used to clarify the CEACAM1 distribution during pathogen binding and at sites of bacterial adhesion.

Homo-FRET image pairs are collected by diffraction-limited techniques, and so generated anisotropy images are therefore also diffraction limited. Nevertheless, the possibility remains that super-resolution methods of interpreting fluorescence signals could be applied to anisotropy imaging. The CEACAM1 monomer and dimer organization appears to be very heterogeneous, and so diffraction-limited pixels in the anisotropy images likely correspond to a mixture of
monomer and dimer states in many cases. Histograms of anisotropy values for cellular CEACAM1-4L-EYFP have a broad distribution, as they include anisotropy values that would be expected for both the monomeric, dimeric, and also possibly even oligomeric species. It is possible that, through peak-fitting methods, these histograms could be separated into two peaks, corresponding to monomeric anisotropy and dimeric anisotropy distributions individually. If this could be done, anisotropy images could be separated into separate fluorescence images, likewise corresponding to monomeric and dimeric fluorescence intensities.

Successive frames of these separated images could also be subjected to super-resolution methods of interpreting fluorescence signals, such as SOFI, to generate a super-resolved image of monomeric and dimeric species, clarifying heterogeneity in the oligomerization state. However, this would likely require that a dimeric CEACAM1-4L mutant be generated, so that the anisotropy distribution of a dimer could be determined.

In addition to super-resolution microscopy, there are many other fluorescence microscopy techniques for studying membrane protein dynamics, as discussed in section 1.2.1. The plethora of FCS-based techniques, which rely on an interpretation of spatio-temporal fluorescence intensity fluctuations within a very small detection volume to determine qualities relating to protein dynamics and oligomerization states [122], would offer an ideal complement to homo-FRET studies, in addition to possibly overcoming some of its limitations.

Homo-FRET is a sensitive method, as it requires very close association (less than 10nm) between fluorophores to be detected as a decrease in anisotropy. We assumed that EYFP on the CEACAM1-4L cytoplasmic domain was flexible and allowed for detectable FRET between protein dimers, as CEACAM1-EYFP mutants with shorter, less flexible cytoplasmic tails have previously been shown by TIRFPM to have higher average fluorescence anisotropy than the long CEACAM1-4L-EYFP [24]. However, future monomer and dimer equilibrium perturbation studies may benefit from the incorporation of these different techniques, which may identify the presence or disappearance of dimers in a more apparent way. Conversely, concerns about crowding induced FRET, which can reduce the observed anisotropy in the absence of protein interactions, are also avoided by other methods. Finally, in our case we simply obtained information regarding the relative proportion of monomers and dimers, whereas other
approaches can determine values such as the number and brightness (N&B) of protein complexes [123, 124]. Similar to maps of anisotropy values, this technique can be used to generate maps of molecular brightness, a value that corresponds to the number of fluorescently-tagged molecules per complex, providing more definitive information about protein oligomerization states.

A similar combinatorial approach, in which time-resolved homo-FRET studies were united with fluorescence fluctuation and brightness analysis methods to reveal detailed in vitro information regarding the molecular organization of Venus-tagged subunits in the calcium-calmodulin dependent protein kinase-II (CaMKIIα) holoenzyme, was recently carried out by Nguyen et al. [125]. The homo-FRET component, as a very sensitive method that requires very close association between fluorophore pairs to be detected, was used to confirm the association of certain subunit pairs within the larger complex, whereas the fluctuation analysis component could be used to infer the number of subunits in the whole complex, regardless of subunit proximities. Combinatorial biophysical fluorescence techniques, including FRET, N&B, and FRAP, have also recently been used by Crosby et al. to provide a through description of annexin A4 membrane distribution and self-association dynamics, providing details that no one technique alone could supply [126].

At a larger scale, CEACAM1-4L particles and clusters at the cell surface were shown to be quite dynamic, and so methods for tracking the gross movement of these proteins should be employed. Single particle tracking and cluster tracking techniques can be used to describe membrane protein mobility and to infer the mechanism of that mobility, which could be due to influences such as guidance by the underlying cytoskeletal meshwork or confinement in membrane microdomains [127]. Efforts are already underway to analyse these dynamics by quantifying the appearance, disappearance, speed, and changes in shape of these clusters over time to better characterize them and understanding their importance. There is still much to be learned with respect to the functional significance of CEACAM1 organization and dynamics at the plasma membrane under different physiological circumstances.
References


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Appendix 1. No SHP Recruitment to Bacterial Contact Sites

Neither SHP-1-GFP (A) or SHP-2-GFP (B) visibly localize to sites of cellular contact with *N. gonorrhoeae* when expressed alone in the absence of CEACAM1-4L.
Appendix 2. Methods of Camera Sensitivity Correction

(A) When an isotropic solution of FITC was imaged at a range of exposure times with the gain of one camera (detecting $F_\perp$) increased to better match the second camera (detecting $F_{ll}$), the intensity detected by each camera matched well over a large range of exposure times and fluorescein solutions. (B) When an isotropic solution of FITC was imaged at a range of exposure times with the “Quant-view” feature enabled, the intensity detected by each camera matched when a corrective G-factor of 1.2, to account for polarization bias in the microscope optical path, was applied to the intensities collected from the $F_\perp$ images. When intensity values were very low, such as those collected from a dilute 0.05mg/ml fluorescein solution at short exposure times, the two camera intensities were not as well matched.
Appendix 3. Average Monomeric and Dimeric Venus $r_c$ by Different Imaging Methods

<table>
<thead>
<tr>
<th>System</th>
<th>Construct</th>
<th>Average $r_c$ ±SE</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>One Camera</td>
<td>Monomeric Venus</td>
<td>0.190 ± 0.010</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Dimeric Venus</td>
<td>0.110 ± 0.006</td>
<td>13</td>
</tr>
<tr>
<td>Two-Camera with Gain Correction</td>
<td>Monomeric Venus</td>
<td>0.198 ± 0.002</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>Dimeric Venus</td>
<td>0.144 ± 0.003</td>
<td>35</td>
</tr>
<tr>
<td>Two-Camera with Quant-view</td>
<td>Monomeric Venus</td>
<td>0.277 ± 0.008</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Dimeric Venus</td>
<td>0.195 ± 0.003</td>
<td>10</td>
</tr>
</tbody>
</table>

The average corrected anisotropy ± SE for N cells expressing the construct indicated calculated from images captured on the TIRFPM system indicated. One camera images were captured sequentially through polarizing filters, as described in 3.3.4. Two-camera images were captured simultaneously with a polarizing beam-splitter, using different methods of camera sensitivity correction (gain correction versus Quant-view) as discussed in sections 3.3.4 and 3.5.1.

Appendix 4. Average Whole-Cell $r_c$ of Cells Resting on Bacteria

<table>
<thead>
<tr>
<th>Strain</th>
<th>Average $r_c$ ±SE</th>
<th>N</th>
</tr>
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<tr>
<td>Opa$_{57}^+$ N313</td>
<td>0.198 ± 0.008</td>
<td>12</td>
</tr>
<tr>
<td>Opa$^-$ N302</td>
<td>0.151 ± 0.005</td>
<td>12</td>
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</table>

Average CEACAM1-4L-EYFP corrected anisotropy ± SE for N cells parachuted onto a high density of unlabeled immobilised *N. gonorrhoeae* of the strain indicated. Homo-FRET images for calculating anisotropy values were captured on the one-camera TIRFPM system described in section 3.3.4. Images were taken 10-40 minutes after cell suspensions had been added to bacteria-containing dishes. The average anisotropy of cells contacting N313 bacteria was significantly higher (p<0.0001) than the average anisotropy of cells contacting N302 bacteria.
Appendix 5. One-Camera TIRFPM of CEACAM1-4L-EYFP Resting on Bacteria

Representative images of cells expressing CEACAM1-4L-EYFP that were parachuted onto glass surfaces patterned with (A,B) Opa<sup>+</sup> N. gonorrhoeae; (C,D) Opa<sup>-</sup> N. gonorrhoeae; (E,F) bacteria-free glass, and imaged by TIRFPM on the one-camera system as they made contact. Anisotropy images (B,D,F) depict the CEACAM1 monomer-dimer distribution.
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