

Macromolecular crowding in *Rhodobacter sphaeroides*



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Statement of COVID-19 impact on DPhil research

I was in the fourth year of my DPhil when the university went into full lockdown due to the COVID-19 pandemic. As a result of the department closure in addition to the unexpected changes of my DPhil sponsorship (lack of Year 4 funding), I was unable to continue some of the lab work I had in mind. Therefore, I was encouraged to stop practical lab work and focus on writing up my thesis.

The initial plan of this project was to track different-sized proteins in *Rhodobacter sphaeroides* grown under aerobic and photoheterotrophic conditions. Using photoactivatable fluorescent protein PAmCherry, I measured the diffusion of medium-sized proteins (27 - 41 kDa) and obtained preliminary data for the diffusion of large ribosomal subunit L1 from an inducible plasmid. However, I was unable to proceed with the construction of a fully functional L1-PAmCherry fusion at the native *R. sphaeroides* chromosome, even though significant time and effort were spent performing homologous recombination and plasmid construction.

I was also unable to complete the electroporation project where the aim was to track protein smaller than the typical 27 kDa fluorescent protein. Before the pandemic, I was at a promising stage where the target protein CheY₆ was successfully labelled with an organic dye and internalized into *E. coli* and aerobic *R. sphaeroides*. However, due to time and funding constraints, I could not proceed with the tracking experiment in photoheterotrophic cells, including screening for new dye candidates that are compatible with the autofluorescence of photoheterotrophic cells.

Unfortunately, the pandemic has led to the disruptions of a few experiments that could provide a comparison regarding the diffusion of a small 14 kDa protein, a typical 27 kDa fluorescent protein, and large ribosomes, measured using different techniques.

Abstract

Macromolecular crowding is thought to have an important role in protein dynamics and molecular diffusion in bacteria. However, the dynamic and heterogeneous nature of the cytoplasm makes it challenging to study and test the significance of crowding in live cells. Previous studies have used diffusion coefficient of protein to estimate excluded volume in osmotically stressed or energy depleted cells, but the effects of crowding in unperturbed cells are unclear. The alphaproteobacterium *Rhodobacter sphaeroides* is a good candidate for studying crowding *in vivo* as it generates different number of intracytoplasmic membrane (ICM) vesicles occupying different amounts of cytoplasmic space under different light intensities. When grown heterotrophically under low light illumination, *R. sphaeroides* cells generate 3 to 4 times more vesicles than cells grown under high light, resulting in a dramatic change in the cellular architecture and increased crowding.

Through expression of a genetically encoded fluorescence resonance energy transfer (FRET) probe *in vivo*, this thesis shows that low light grown *R. sphaeroides* cells have higher crowding than high light grown cells, reflected by an increase in FRET signal. The probe senses a difference in excluded volume occupied by ICM vesicles by adopting a more condensed conformation under crowded condition.

Single molecule tracking also shows that the diffusion of different-sized protein molecules (> 27 kDa) is affected by crowding effects of ICM vesicles in *R. sphaeroides*. A slow-diffusing population of PAmCherry and PAmCherry-CheY₆ was observed in photoheterotrophically grown cells but was absent in aerobic cells. As the cells transitioned from aerobic to low light growth, the diffusion of protein was found to decrease by around an order of magnitude. The apparent diffusion coefficient of PAmCherry and PAmCherry-CheY₆ changed from 3.62 ± 0.23 and $3.23 \pm 0.18 \mu\text{m}^2\text{s}^{-1}$ respectively, to around $0.3 \mu\text{m}^2\text{s}^{-1}$. Preliminary data also shows that the larger ribosomal L1 subunit fused to PAmCherry has decreased mobility in photoheterotrophic cells.

To track proteins < 27 kDa, the 14 kDa response regulator CheY₆ was labelled with an organic dye, iFluor633 and electroporated into *E. coli* and *R. sphaeroides*. The labelling, purification, and electroporation protocols developed here allow the efficient internalization of fluorescently labelled protein molecules into live cells, and the data show promise for rapid single molecule imaging of small protein *in vivo*.

In general, this thesis proposes that under normal growth conditions, cellular components such as ICM vesicles may contribute to crowding and can decrease protein mobility. Macromolecular crowding may constrain segregation of low copy number large proteins and affect signalling rates. Therefore, excluded volume effects should be considered in *in vivo* studies investigating protein dynamics and molecular diffusion.

Declaration

The work in this thesis was performed in the Department of Biochemistry and the Clarendon Laboratory, Department of Physics, University of Oxford. Work was performed from October 2016 until September 2020 under the supervision of Prof. J. P. Armitage and Prof. R. Berry. All the work in this thesis is my own unless otherwise stated and has not been submitted for a degree at this or any other university.

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Contents

Abstract	ii
Abbreviations	ix
1 Introduction	1
1.1 Macromolecular crowding	1
1.1.1 Background and definition	1
1.2 Macromolecular crowding effects <i>in vitro</i>	7
1.3 Macromolecular crowding in the bacterial cytoplasm	14
1.3.1 Studying crowding and molecular diffusion <i>via</i> fluorescence mi- croscopy	15
1.3.2 Understanding macromolecular crowding effects on diffusion <i>via</i> perturbations	23
1.3.3 Bacteria may maintain homeocrowding	27
1.4 <i>Rhodobacter sphaeroides</i>	30
1.4.1 Background	30
1.4.2 Crowding in photoheterotrophic <i>R. sphaeroides</i>	32
1.4.3 Light dependent crowding in photoheterotrophic <i>R. sphaeroides</i> .	41
1.5 Chemotaxis in <i>Rhodobacter sphaeroides</i>	45
1.5.1 Chemosensory clusters	45
1.5.2 Response regulator CheY ₆	47
1.6 Single molecule fluorescence microscopy	48
1.6.1 Photoactivated Localization Microscopy (PALM)	49
1.6.2 Electroporation technique using organic dyes	50
1.7 Aims of project	51
2 Materials and methods	53
2.1 Strains, plasmids and primers	53

2.2	Growth conditions	56
2.3	Antibiotics	56
2.4	BChl extraction using acetone / methanol	56
2.5	Swim plates	57
2.6	Genetics techniques	57
2.6.1	Plasmid extraction	57
2.6.2	Polymerase Chain Reaction (PCR)	57
2.6.3	Restriction digest	58
2.6.4	Agarose gel electrophoresis	59
2.6.5	Purification of DNA fragments	59
2.6.6	DNA ligation	59
2.6.7	Plasmid DNA transformation	60
2.6.8	Site directed mutagenesis	60
2.6.9	Preparing electrocompetent <i>E. coli</i>	60
2.6.10	Preparing electrocompetent <i>R. sphaeroides</i>	60
2.6.11	Conjugations	61
2.6.12	DNA Sequencing	61
2.7	Protein expression and purifications	62
2.7.1	Overexpression of CheY ₆	62
2.7.2	SDS-PAGE	62
2.8	Protein labelling and analysis	63
2.8.1	Dye storage	63
2.8.2	Protein-dye conjugation	63
2.8.3	Free dye removal	63
2.8.4	Size exclusion chromatograph	64
2.8.5	Labelling yield	64
2.9	Electroporation	65
2.10	Circular dichroism	65

2.11	Agarose pads preparation	66
2.12	Tunnel slides	66
2.13	Fluorescence microscopy	67
2.13.1	FRET	67
2.13.2	PALM	67
2.13.3	Data analysis using Stormtracker	68
2.13.4	Electroporation	68
3	Quantifying crowding levels in photoheterotrophic <i>R. sphaeroides</i>	70
3.1	Quantification of the number of cells in liquid culture at OD ₇₀₀	70
3.2	Quantification of ICM vesicles <i>via</i> BChl extraction	71
3.3	FRET sensor for quantifying crowding <i>in vivo</i>	75
3.4	Discussion	83
4	Studying crowding effects on protein diffusion <i>via</i> Photoactivated Localization Microscopy (PALM)	88
4.1	Functionality of CheY ₆ fusions	88
4.2	Choosing the right photoactivatable fluorescent protein for PALM	91
4.3	Autofluorescence	94
4.4	Cell viability and phototoxicity	97
4.5	Diffusion of PAmCherry in <i>E. coli</i> and aerobic <i>R. sphaeroides</i>	99
4.5.1	Experimental setup for PALM	99
4.5.2	Data analysis using Stormtracker	100
4.6	Diffusion of PAmCherry in high light and low light <i>R. sphaeroides</i>	104
4.7	Diffusion of PAmCherry-Y ₆ in <i>E. coli</i> and <i>R. sphaeroides</i>	108
4.8	L1-PAmCherry fusions	112
4.9	Diffusion of L1-PAmCherry in <i>R. sphaeroides</i>	114
4.10	Discussion	117

5	Electroporation of maleimide dye labelled CheY₆	124
5.1	Generation of cysteine-modified CheY ₆ mutants for enhanced labelling	124
5.2	Dye candidates	129
5.3	Optimizations to standard labelling and purification protocols	132
5.4	Optimized electroporation of protein into <i>E. coli</i> and <i>R. sphaeroides</i>	135
5.5	Discussion	143
6	Summary and implications	146
6.1	FRET sensor to study macromolecular crowding	148
6.2	Single molecule tracking for studying crowding and diffusion <i>in vivo</i>	149
6.2.1	Electroporation of small, fluorescently labelled protein	150
6.2.2	Tracking of different-sized protein using PALM	152
6.3	Future work	154
6.4	Implications	155
	References	157
	Appendix	178

Abbreviations

3D	three dimensional
ATP	adenosine triphosphate
BChl	bacteriochlorophyll
CD	circular dichroism
D	diffusion coefficient
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DoL	degree of labelling
DTT	dithiothreitol
dNTP	deoxynucleoside-5'-phosphate
EP	electroporated
FOV	field of view
FP	fluorescent protein
FRAP	fluorescence recovery after photobleaching
FRET	Förster resonance energy transfer
GFP	green fluorescent protein
ICM	intracytoplasmic membrane
IPTG	Isopropyl β -D-thiogalactoside
kDa	kilo Dalton
LB	Lysogeny broth
LH	light harvesting
MB	motility buffer
NEP	non-electroporated
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PALM	photoactivated Localization Microscopy

PAmCherry	photoactivatable mCherry
PCR	polymerase chain reaction
Nal	nalidixic acid
Ni-NTA	nickel-nitrilotriacetic acid
WT	wild type
SDS	sodium dodecyl sulphate
SEC	size exclusion chromatography
SUX	succinate medium
TCEP	tris(2-carboxyethyl)phosphine
YFP	yellow fluorescent protein

1 Introduction

1.1 Macromolecular crowding

1.1.1 Background and definition

Prokaryotic and eukaryotic cells are two major structural classes of cells. Animals, plants, and fungi are some examples of eukaryotes; whereas bacteria and archaea are grouped under prokaryotes. All cells have a cytoplasmic membrane that separates the cytoplasm from the outer environment. However, eukaryotic cells tend to be significantly larger than prokaryotic cells and they contain specialized, membrane-bound organelles such as the nucleus, mitochondria, and chloroplasts that are important for cellular activities and survival. The internal structures of prokaryotic cells are less complex; both bacteria and archaea lack membrane-enclosed organelles and the cytoplasm is filled with essential components such as proteins, DNA, metabolites, and ribosomes that may self-organize or localize to different regions through diffusion (Figure 1). A typical rod-shaped bacterium, such as *Escherichia coli*, has a small size of about 2 to 3 μm by 1 μm but it is able to perform multiple functions such as signal sensing, locomotion, and toxin secretion. How these single-celled, microscopic organisms carry out diverse activities and behaviours remains an interesting subject and information can be gained by dissecting the structure and properties of the bacterial cytoplasm.

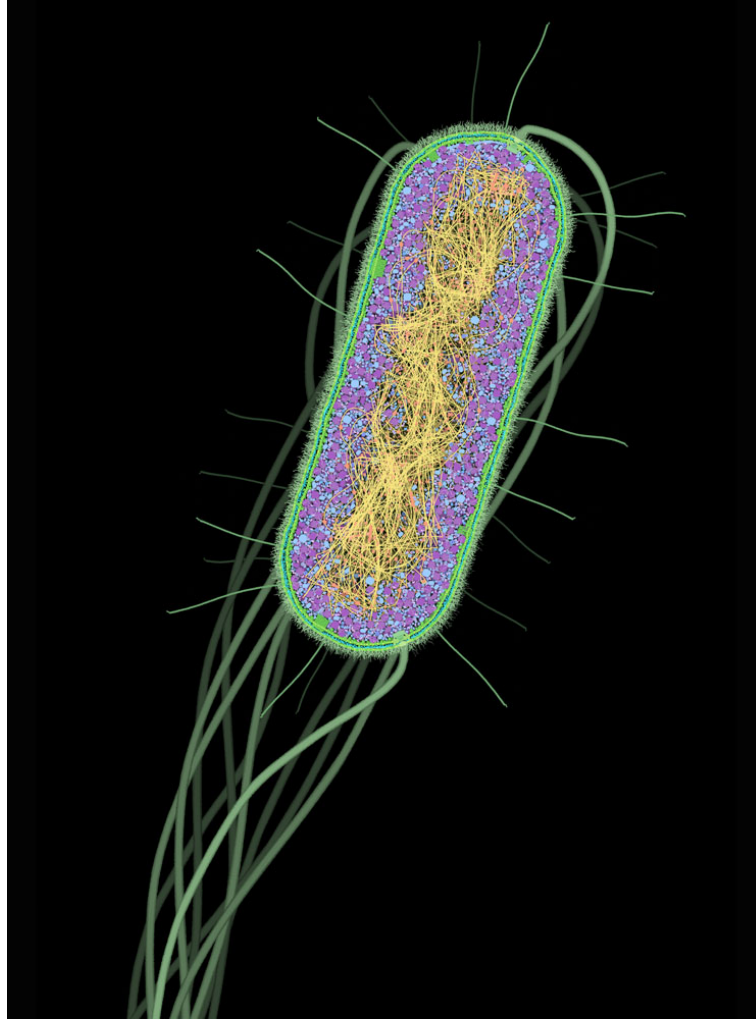


Figure 1: The cross-section of the bacterium *E. coli* shows its highly crowded cytoplasm that is packed with macromolecules. Instead of membrane-bound organelles that can be found in eukaryotic cells, the bacterial cytoplasmic space (coloured blue and purple) contains ribosomes, proteins, ions and metabolites that appear to be relatively unstructured. An important feature of the *E. coli* cell architecture is the nucleoid (coloured yellow and orange) that occupies the centre of the cell. The nucleoid refers to the highly compact 4.6 Mbp circular genome in *E. coli* and it contains high density of DNA and DNA binding proteins. Figure taken from David S. Goodsell [47].

Bacterial cells are more crowded than eukaryotic cells as they have higher protein and DNA concentrations confined within a small compartment [81]. Studies have shown that protein displays higher mobility in eukaryotic cytoplasm than in bacterial cytoplasm [81]. It is known that Green Fluorescent Protein (GFP) [158][19] diffuses around 3 times slower in the cytoplasm of *E. coli* (around $7 \mu m^2 s^{-1}$ [39]) as compared to the cytoplasm of fibroblasts ($27 \mu m^2 s^{-1}$ [137]) and slime molds ($24 \mu m^2 s^{-1}$ [103]). The slower GFP diffusion in bacterial cell may reflect higher intracellular viscosity or a higher probability of encountering diffusive barriers. The bacterial cytoplasm is a highly dynamic environment that is packed with different-sized macromolecules, ranging from large chromosomes (the entire length of the circular *E. coli* chromosome is around $4 \mu m$ long [157]), plasmids (>100 nm), molecular machines (diameter of flagellar motor is 50 nm [127]), ribosomes (20 nm), proteins (2 to 10 nm [41]), to small metabolites and ions (<1 nm) (Figure 2). Typically, macromolecules such as proteins, RNA, and DNA take up around 20 to 30% of the total cellular volume [38]. In *E. coli*, the total concentration of protein and RNA in the cytoplasm is around 300 mg / mL [167] and they occupy the most cytoplasmic space. In addition, the 4.6 Mbp DNA of *E. coli* forms a compact nucleoid that spans across almost 75% of the cytoplasmic space [42] and it also contributes to crowding.

Despite the high concentration of molecules, the bacterial cytoplasm remains a fluid and aqueous environment. Proteins, nucleic acids, and metabolites are surrounded by water molecules, forming a hydration shell that could affect molecular mobility and interactions with other molecules. Water molecules bind to charged surfaces and ions through Coulombic interactions (attraction force between negative and positive charges) and hydrogen bonding. This form of solute-solvent interaction or hydration shell is sometimes referred as "bound" water and it takes up around 10 - 15% of intracellular water [76]. Molecular dynamics simulations also predicted that metabolites and proteins would form non-covalently interacting structures with each other in the cytosol [25]. These properties of the bacterial cytoplasm make it a very distinctive environment compared to pure water or an ideal dilute solution.

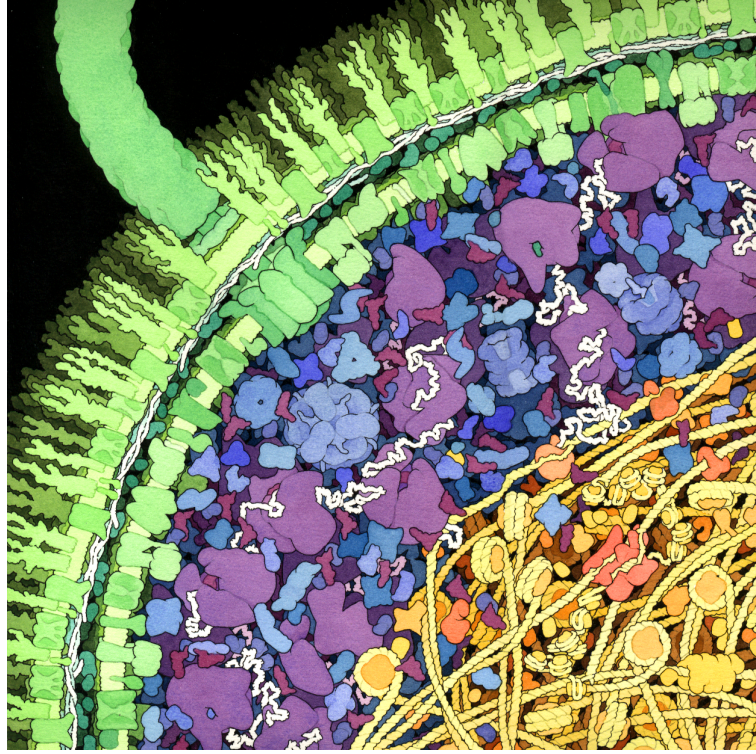


Figure 2: By taking a closer look at the cross-section of an *E. coli* cell, larger molecules such as ribosomes (large purple molecules), proteins or enzymes (blue), DNA (yellow), and strands of mRNA (white) could be observed. A large flagellar motor (green) that is embedded in the cell membrane can also be seen. Water, amino acids, metal ions, and other smaller molecules fill the spaces in between those macromolecules but they are too small to be visualized at this magnification. Figure taken from David S. Goodsell (<http://mgl.scripps.edu/people/goodsell/illustration/public>).

High density of macromolecules such as proteins, DNA, and ribosomes in the cytoplasm leads to the phenomenon called macromolecular crowding and it is an important characteristic of all living cells. These macromolecules are confined within the cytoplasm and they cannot overlap with each other, resulting in a reduction of accessible space for all macromolecules to move around the cell. Therefore, macromolecular crowding is most commonly defined as the excluded volume effect as a result of the steric repulsion between the molecules [143][37][38]. This nonspecific steric repulsion is universal and it occurs between all macromolecules that are confined within a crowded environment, regardless of whether any other attractive or repulsive interactions between the molecules are present.

Under the excluded volume model, all molecules in a solvent that occupy a certain volume and can contribute to molecular crowding through steric repulsion are defined

as crowders. Crowders come in different shapes and components but they were often modelled as hard-spheres in crowding studies. A tracer molecule is often introduced to the pool of crowders to study its interactions with the crowders. When there is a high concentration of crowders in a solution, a tracer molecule will experience a high excluded volume effect by bumping into neighbouring crowders.

However, the magnitude of crowding is strongly affected by the size of the crowders and tracer molecule [38][143][84]. In a compartment filled with a single species of crowders, a small tracer molecule can access most of the space unoccupied by the crowders; whereas a large tracer molecule with a size comparable to the size of the crowder species experiences a much higher volume exclusion (Figure 3). This suggests that the relationship between crowding and the size of molecules is highly non linear. When crowding increases (the number or size of the molecules in the compartment increases), all molecules become more confined, with decreased ability to diffuse around freely. As a result, the entropy of the molecules decreases (molecules become less randomly distributed), and the total free energy of the system increases [164]. This entropy penalty will then enhance binding and association of molecules to increase the available volume in the system. Studies have found that crowding encourages protein folding [139], formation of oligomeric structures, and condensation of DNA [28]. Another effect of crowding is the reduction of molecular diffusion rates by creating impenetrable barriers to mobile molecules [167]. Molecules have been shown to diffuse 2 to 10 times slower in a cell than in water [38]. Crowding could also lead to confinement where macromolecules are trapped within microcompartments. This suggests that crowding may affect signalling pathways, gene transcription, translation, and other cellular processes that are regulated by diffusion.

In summary, macromolecular crowding has great consequences on molecular interactions and diffusion rates. Therefore, it is important to understand how biomolecules function in the native, crowded state of the bacterial cytoplasm and how crowding affects bacterial physiology.

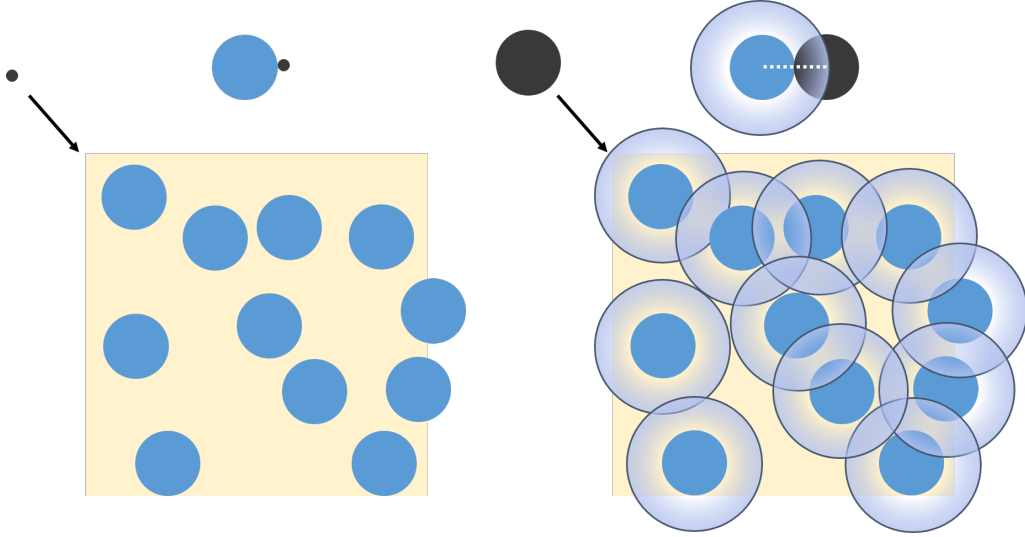


Figure 3: The magnitude of crowding or excluded volume effect by crowders (blue) is dependent on the size of the tracer molecule (black). Assuming the position of each molecule is specified only by the position of its centre, the yellow square shows the volume available to the centre of the tracer molecule. The rings around the crowders have a radius that is equal to the sum of radii of both the tracer molecule and crowder (indicated by the white dashed line). The rings resembled the excluded volume that is inaccessible by the centre of the tracer molecule. In this case, a tracer molecule that is as big as the crowders is excluded from most of the space. As the concentration of crowders increases, the space available to a tracer molecule decreases significantly. However, a tracer molecule that is relatively smaller than the crowders can freely explore most of the space that is unoccupied. This shows that crowding effect is significantly stronger between large molecules. Figure adapted from [84]

1.2 Macromolecular crowding effects *in vitro*

The study of crowding has traditionally been conducted in ideal systems using purified macromolecules in solutions. Natural or synthetic crowding agents such as cell lysates, Ficoll, and polyethylene glycol (PEG) are added to dilute solutions to induce the effects of crowding in the cytoplasmic environment. The effect of the crowder on the reaction equilibrium, kinetics, and interactions is then studied in combination with theoretical approaches. Even though these synthetic systems cannot fully mimic the complex intracellular environment such as the bacterial cytoplasm, they serve as a good starting point for understanding the excluded volume effects on molecular behaviours and reactions. Having a simple system also allows a more precise control on the experimental variables and straightforward data interpretation, and eventually additional complexity may be added to the system until it resembles the native environment of the biomolecules.

Ficoll, Dextran, and PEG are some of the most commonly used synthetic crowders to study crowding. These crowding agents are often used in experiments and simulations as ideal representations of hard spheres that do not interact with other macromolecules or with themselves to provide direct measurements of excluded volume effects. In general, high concentration of synthetic crowders is thought to limit protein conformational flexibility (the ability of a protein to undergo changes in conformation or structure) and encourage protein molecules to adapt a more compact conformation *via* excluded volume effects. The compactness of a protein molecule has been described as its accessible surface area over the surface area of an ideal sphere of the same volume [74]. Compared to a protein's unfolded or loose packing state, a compact protein conformation has a smaller radius of gyration, R_g (the distance from the centre of mass of a polymer in solution about an axis through its centre of mass [109]) and is entropically favoured under the effects of crowding as it occupies less space in solution [164]. The addition of synthetic crowding agents *in vitro* has also been shown to enhance protein folding [3][139][83], stability [161][131][112], aggregation [51], and self-association [15][106][125][166].

However, there are limitations with using synthetic crowders and describing them under the generalized excluded volume model. Firstly, not all synthetic crowders behave as ideal hard-spheres or hard-rods as expected. Synthetic crowders can rearrange themselves differently in solution and develop different radius of gyration (R_g) depending on their concentrations [62]. Under dilute conditions, each synthetic crowder can be treated as an isolated hard sphere with a certain R_g . However, as concentration increases to a semi-dilute level, the polymer chains of synthetic crowders would start to overlap and interpenetrate to form a compact polymer meshwork [62] (Figure 4). An example of this would be Dextran. Dextrans are linear, flexible polysaccharide molecules that can be compressed at high concentration due to self-crowding [94]. In a few theoretical studies that used Dextran as a crowding agent, an inconsistent radius was assumed in calculations and therefore causing the results to be quantitatively incomparable between the studies [37].

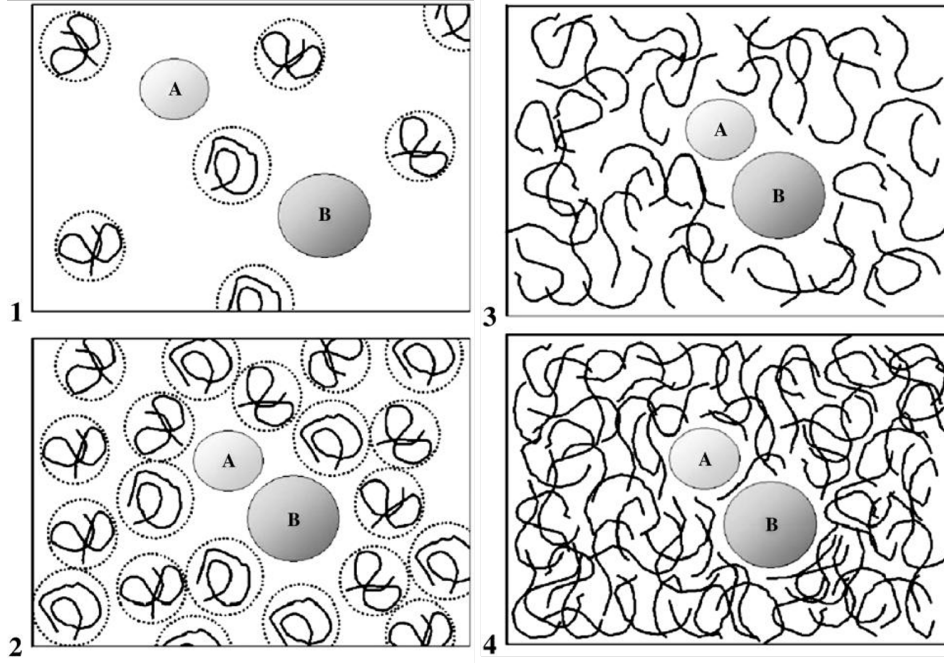


Figure 4: The changes in conformation and arrangement of synthetic crowders in solution under different concentrations (dilute, critical / crossover, semi-dilute, and concentrated). The synthetic crowders are shown as lines whereas A and B are proteins (tracer molecules). Under dilute condition (panel 1), each synthetic crowder behaves as isolated hard spheres. In panel 2, the crowders reach a critical / crossover concentration where their polymer chains start to overlap each other while the crowders can still maintain their spherical structures. When the crowders reach the semi-dilute range (panel 3), the crowders interpenetrate each other and a dense polymer meshwork is formed. In the concentrated regime (panel 4), the polymer meshwork becomes highly compact and the proteins (tracer molecules) are caged within a small amount of solvent. Figure taken from [62]

The changes in crowder concentrations and possibly the crowder conformation in solution have been shown to have different effects on the stability of a random coil domain of the activator of thyroid and retinoid receptor (ACTR) [110]. ACTR is an intrinsically disordered protein (IDP) that has several transiently helical regions [110]. Using Ficoll as synthetic crowders, the authors found that 300 g / L Ficoll destabilized the ACTR transiently helical regions but 400 g / L Ficoll had a stabilizing effect. The dependency of ACTR stability on Ficoll concentrations is possibly due to the different arrangement and structure of Ficoll molecules (Figure 5) [110]. Even though Ficoll has been described as having a spherical shape, its branched structure could be compressed and deformed at higher concentrations [147]. At 300 g / L, Ficoll molecules may reach a critical concentration where their polymer chains make contacts with each other, trapping the ACTR segments within the Ficoll spheres *via* hydrogen bonding and destabilizing the transient helical domains [110]. When Ficoll molecules reach the semi-dilute range (400 g / L), their polymer chains will be compressed to form a nonpenetrable spheroidal meshwork that forces ACTR into a more compact configuration within the solvent area through excluded volume effect [110].

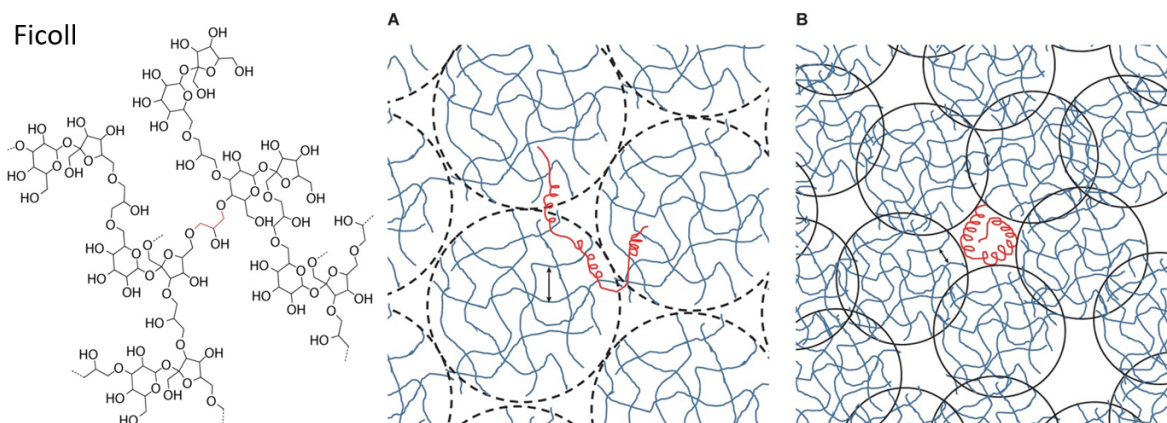


Figure 5: Ficoll is a highly cross-linked, polysaccharide synthetic crowder that has a spherical shape. The structure on the left shows a Ficoll segment with a number of hydroxyl groups that can engage in hydrogen bonding with proteins or crowding agents. (A) and (B) show structure and arrangement of Ficoll molecules (blue lines) changing under different concentrations and how Ficoll interacts with ACTR (red lines). Under critical concentration (A), the polymer chains of Ficoll start to overlap each other while maintaining their spherical shape. ACTR helical regions are able to penetrate into the Ficoll spheres and form hydrogen bondings with the Ficoll hydroxyl groups, thus destabilizing the helices. In semi-dilute regime (B), Ficoll spheres are highly compressed to form a nonpenetrable polymer meshwork. ACTR segments are excluded from the interior of the Ficoll spheres and forced to adapt a compact configuration due to excluded volume effect. Figure taken from [110].

Secondly, some crowders may have unspecific attractive interactions with the tracer molecule / protein and this interaction would counteract the volume exclusion effect. Depending on the chemical properties and concentration of the synthetic crowders, non-specific interactions such as hydrogen bonds, electrostatic forces, and hydrophobic interactions may stabilize or destabilize the conformations of protein. For instance, PEG has been shown to interact with hydrophobic surface of proteins in solutions and thus it is not an inert crowder [27][140][164]. These results show the importance of using well-characterized crowding agents in experiments to understand the effects of excluded volume at a quantitative level.

However, the excluded volume effect is not the sole factor affecting macromolecular interactions in live cells. Crowding effects in live cells can be a lot more complex and are dependent on other environmental variables. For example, proteins such as dihydrofolate reductase, enolase, and GFP could not refold properly in a solution crowded with Ficoll 70 as their folding is dependent on the GroEL/GroES chaperonin system in the presence of ATP hydrolysis [80]. Viscosity, pH, nonspecific attractive and repulsive interactions, chaperons and other variables have strong impacts on molecular behaviours and a single crowder species cannot reproduce the complexity of the cytoplasm. Indeed, there are studies that set out to investigate the effects of mixed crowders on the behaviour of the tracer molecule [163][33]. For example, Du *et al.* found that having mixed crowders can enhance protein folding compared to using a single crowder species at the same concentration [33]. However, *in vitro* studies performed in dilute solutions are nowhere near the complexity and inhomogenous environment of the cytoplasm. Natural crowding agents such as cell lysates and proteins are also used because they are more biologically relevant and can better represent the intracellular environment. Wang *et al.* showed that a tracer protein portrayed different diffusive behaviours when it was added with natural or synthetic crowders respectively [152]. This suggests that synthetic crowders could not mimic the non specific interactions between natural crowders and proteins in live cells.

Despite some variations regarding the magnitude of crowding effects on protein be-

haviours, the general consensus is that crowding stabilizes protein, promotes association and polymerization of molecules, and encourages protein folding. Even though synthetic and natural crowders provide useful insights regarding the role of crowding in biological systems, synthetic systems cannot address the complexities of crowding by real macromolecules in an actual living cell. Scientists are now encouraged to switch from *in vitro* studies to study protein behaviours in their native environments [46]. A review on the *in vivo* studies of crowding and its challenges will be presented in the following sections.

1.3 Macromolecular crowding in the bacterial cytoplasm

The dynamically structured bacterial cytoplasm could not be reproduced by *in vitro* experiments added with homogeneous and well-mixed crowders. Proteome analyses are used to calculate the estimated intracellular macromolecular volume fraction but they do not provide information on the actual protein behaviours and motions within the cytoplasm. As mentioned in subsection 1.1.1, the bacterial cytoplasm is a highly crowded environment that is very different to dilute solutions or pure water. One of the major environments of the cytoplasm is the cytosol and it is defined as the more dilute or aqueous part of the cell that contains less biomolecules [128]. The complexity of the cytosol is strongly affected by its high concentration of metabolites, ions, proteins, and the physicochemical interactions between these molecules. Atomistic simulations of the *E. coli* cellular cytosol revealed the formation of non-covalently interacting metabolite structures (NIMS) mostly through hydrogen bonds, pi stacking, and ionic interactions [25]. Since most cytosolic metabolites and macromolecules are negatively-charged, the anionic surface charges would cause molecules to repel each other through screened electrostatic forces. Anions such as potassium ions and magnesium ions play important roles in maintaining electro-neutrality and serving as osmolyte protectants in the cytoplasm [129].

The cytoplasm can be up to 10 times more viscous than water [104], meaning that molecules experience greater friction in fluids, and its structural heterogeneity is affected by cellular processes such as folding of polypeptide chains into functional proteins, formation of oligomeric structures, protein degradation, and DNA condensation. In live cells, assembly of protein complexes and clustering of nucleic acids lead to spatially organized subcellular structures that are essential for cellular activities and metabolism. Examples of these structures include nucleoid, filaments, metabolons, and intracellular bodies and they may increase the local crowding of the cytoplasm (Figure 6). Furthermore, macromolecules such as soluble proteins and RNAs can undergo phase separations to form aggregate-like assemblies with increased local concentration [153]. As a result of

this spatial organization, there are microcompartments in the cytoplasm that are supercrowded and regions that are dilute [143][130]. Previous modeling work has shown that heterogeneous crowding would lead to a wide range of diffusive behaviors that deviate from normal diffusion [124]. Therefore, depending on its size and location within the cell, a molecule will face varying degree of crowding and this can be reflected by the mobility of the molecule.

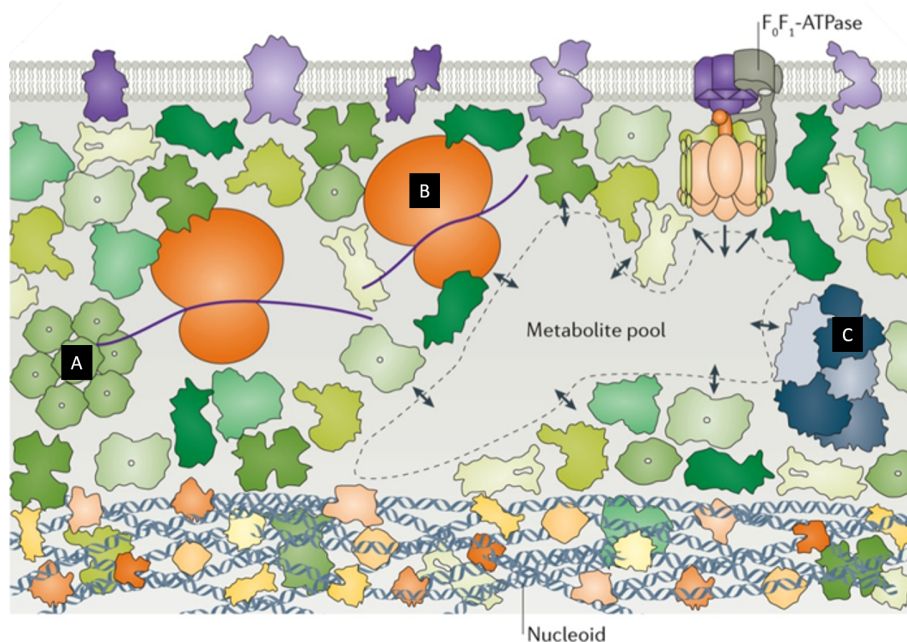


Figure 6: The bacterial cytoplasm is highly organized with microcompartments of unequal crowding. Intracellular bodies (A) and metabolons (C) are some of the biomolecules that contribute to the local crowdedness of the cytoplasm. (B) shows ribosomes that are excluded from the nucleoid. These subcellular structures lead to regions that are supercrowded and dilute solutions of metabolites. The arrows show that the metabolite pool is highly dynamic with small molecules and ions rapidly diffusing in and out. Figure taken from [143]

1.3.1 Studying crowding and molecular diffusion *via* fluorescence microscopy

The complex architecture of the bacterial cytoplasm makes it experimentally challenging to study crowding *in vivo* and its heterogeneity can complicate interpretation of the results. The study of crowding in live cells is usually through the introduction of fluorescently or isotopically labelled molecules into the cellular interior. Fluorescence

microscopy or NMR spectroscopy is then used to study the properties and motions of the molecules, which provide insights on the structure and macromolecular crowding in the cell. This section focuses on the role of fluorescence microscopy as a powerful tool to study crowding *in vivo* and what it can tell us about the physical nature of the bacterial cytoplasm.

Fluorescence microscopy allows researchers to directly visualize motions of tracer molecules in cells and study the structure of the cytoplasm. Previous studies have shown the importance of fluorescence imaging in understanding molecular dynamics and interactions in bacteria [45]. A tracer molecule is usually labelled with a fluorescent protein or an organic dye and introduced into live cells through recombinant expression or electroporation. The diffusion of the tracer molecules in the available cytoplasmic volume and how they interact with cellular components provide a measure of crowding and physical environment in cell. Some of the earliest experiments that provided a glimpse on the interior of filamentous *E. coli* cells through tracking of fluorescent proteins are the work by Elowitz *et al.* [39] and Mullineaux *et al.* [87]. By using fluorescence recovery after photobleaching (FRAP) microscopy, the studies showed that green fluorescent protein (GFP) molecules have lateral diffusion coefficients of $7.7 \pm 2.5 \mu\text{m}^2\text{s}^{-1}$ [39] and $9.0 \pm 2.1 \mu\text{m}^2\text{s}^{-1}$ [87] in the cytoplasm, which is about 11 times slower than in water [138]. These measurements reflect not only the crowding effects on protein diffusion but also the potential influence of viscosity, binding, and confinement effects within the cytoplasmic space. To understand how crowding and confinement affect protein mobility in live cells, the diffusion coefficient of GFP in the cytoplasm of osmotic stressed *E. coli* was measured using FRAP microscopy [60]. In *E. coli* cells that underwent osmotic upshift, an increase in macromolecular volume fraction occurs and macromolecules can take up to 40% of the total cellular volume (400 mg / mL) [18]. A significant decrease in GFP diffusion coefficient (down to $0.01 \mu\text{m}^2\text{s}^{-1}$) was observed following the formation of visible plasmolysis spaces in osmotically stressed cells [60]. This reduction in GFP mobility reflects the increase in macromolecular crowding in *E. coli*.

However, conventional FRAP experiments can only measure bulk diffusion of fluorophores and have difficulties in distinguishing the heterogeneities of molecular motion. FRAP measurements provide an average signal of thousands of fluorescent molecules and they cannot reflect the different diffusion modes of individual molecules in the cytoplasm. Furthermore, if the fluorescent proteins are distributed into several microcompartments within the cell, it will be challenging to differentiate between the mixed populations or there may be a bias towards the dominant population. To overcome the problem, a modified FRAP-based technique, pulsed-FRAP, was developed to detect heterogeneities of protein diffusion in osmotic stressed *E. coli* [144][82]. At high osmolarities, van den Bogaart *et al.* observed compartmentalization of GFP molecules into discrete pools of slow-diffusing or immobile subpopulations in the cytoplasm. This non-uniform distribution of GFP reflects the presence of cytoplasmic diffusion barriers and confinement of molecules within microcompartments of the cell.

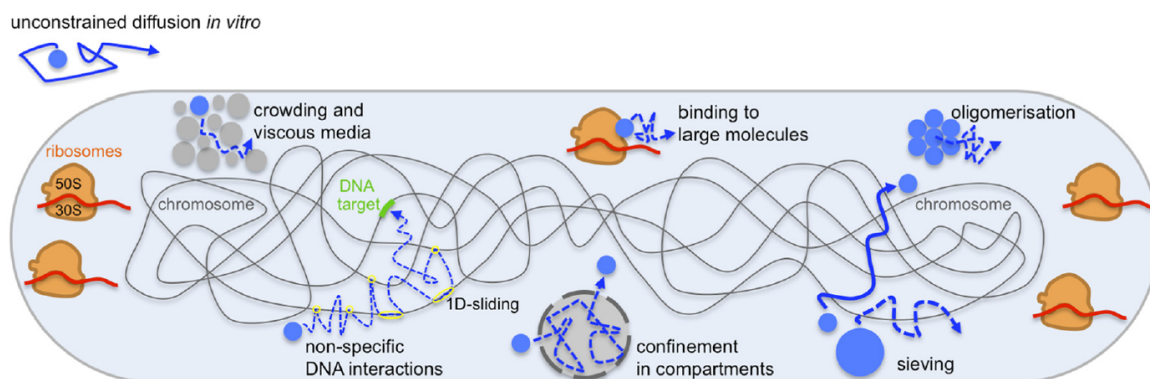


Figure 7: Biomolecules exhibit different diffusive behaviours through interactions with native cellular components at various sites of the cytoplasm; whereas molecules in water or dilute solutions will display unconstrained (free) diffusion. Mobilities of molecules in the cytoplasm decrease through specific or nonspecific binding to structures such as DNA and ribosomes. Tracer molecules could also be trapped within cytoplasmic microcompartments or be excluded from crowded environments such as the nucleoid. Single molecule tracking is used to visualize motions of tracer molecules in different cellular environment. Figure taken from [55]

More recently, the development of single molecule fluorescence microscopy has enabled the study of molecular motion *in vivo* under the effects of crowding at the single molecule level. This is described in detail in subsection 1.6. Single molecule tracking has been

used to directly visualize the diffusion modes of protein molecules in different cellular environments (Figure 7). For example, Lasker *et al.* used single molecule tracking and superresolution imaging to study the diffusion of the CtrA pathway signalling molecules in and out of the PopZ microdomain in *Caulobacter crescentus* (Figure 8).

The membraneless PopZ microdomain is formed by the cell pole organizing protein PopZ and it creates a ribosome and DNA free zone in the cytoplasm [16][35]. There is evidence suggesting that PopZ microdomain interacts with phosphosignalling proteins CckA and ChpT and transcription factor CtrA to form a gradient of phosphorylated CtrA in the cytoplasm [65]. By following the motion of the three proteins using 3D single molecule tracking, Lasker *et al.* were able to capture both the entry and exit of single ChpT and CtrA molecules from the microdomain in real time. The diffusion coefficients of ChpT and CtrA in the cytoplasm decreased from $1.8 \mu m^2 s^{-1}$ by more than an order of magnitude when the molecules entered the PopZ microdomain (to around $0.1 \mu m^2 s^{-1}$ and $0.01 \mu m^2 s^{-1}$) [65]. The increased dwell time and higher concentration of signalling molecules within the PopZ microdomain is important for determining the differential cell fate of *C. crescentus* daughter cells [65]. This study shows that the cytoplasm can be spatially organized and can create microcompartments by recruiting and concentrating the protein PopZ at the poles. Other proteins can exhibit complex diffusive behaviours within these microcompartments.

Another spatially organized structure that can affect protein mobility is the nucleoid. The tracking of single ribosomes labelled with eYFP in *E. coli* showed ribosomes being confined to the cell endcaps, away from the compact nucleoid [9] as they were too large to diffuse through the DNA meshwork. This nucleoid-ribosome segregation is probably caused by excluded volume and entropic effects of the condensed chromosomal DNA in *E. coli* [85]. However, in *C. crescentus*, where the chromosome occupies the entire cytoplasm, weak clusters of ribosomes were observed throughout the cell instead of localizing to the cell periphery [11], illustrating major differences between species. In addition to ribosomes, plasmids [105] and protein aggregates [24] have also been shown to localize to

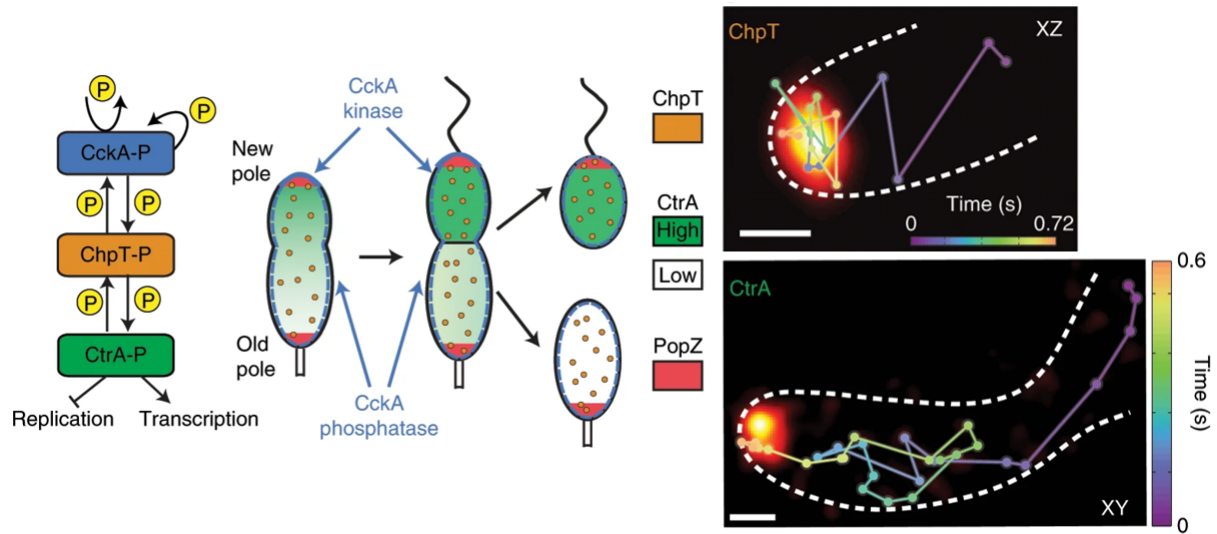


Figure 8: Single molecule tracking was used to study the diffusion of CtrA pathway phosphosignalling molecules in *C. crescentus*. Right: Figure shows the phosphotransfer pathway of CckA/ChpT/CtrA with respect to PopZ microdomain to establish a gradient of CtrA-P in the cytoplasm. To phosphorylate the transcription factor CtrA, phosphotransferase ChpT must enter the PopZ microdomain to interact with the transmembrane kinase CckA. Right: 3D single molecule tracking allows the direct visualization of protein motions at real time. Single ChpT and CtrA molecules labeled with eYFP were shown to diffuse slower when they entered the PopZ microdomain coloured in yellow-orange. In the microdomain, ChpT and CtrA had diffusion coefficients of $0.1 \mu\text{m}^2\text{s}^{-1}$ and $0.01 \mu\text{m}^2\text{s}^{-1}$ respectively, but their mobilities increased to $1.8 \mu\text{m}^2\text{s}^{-1}$ once they returned to the rest of the cytoplasm. Scale bar 200 nm. Figure taken from [65]

the *E. coli* cell poles *via* excluded volume effects of the nucleoid. Reyes-Lamothe *et al.* showed that single plasmid molecules were able to diffuse freely in the nucleoid free regions with occasional movement across the dense nucleoid. This shows that macromolecules such as the nucleoid can act as crowders or diffusion barriers to restrict diffusion and lead to confinement of molecules.

The effects of macromolecular crowding and confinement by physical barriers on molecular diffusion are stronger for molecules with high molecular weights. Large molecules are slowed more than small molecules [143] and they are more likely to be trapped within supercrowded microcompartments of the cell. For instance, free ribosomal subunits have been shown to diffuse freely in the *E. coli* nucleoid whereas fully assembled ribosomes are confined to the nucleoid free zones [111] (Figure 9). The tracking of a 26 kDa fluorescent

protein mEos2 *via* stroboscopic illumination also showed that mEos2 could explore the entire cytoplasm freely without any sign of confinement [40]; whereas a homotetradimer fluorescent protein Kaede (4 times the size of a GFP) was not excluded from the nucleoid but exhibited slower diffusion in the ribosome-rich areas of *E. coli* cells possibly due to crowding [7]. Since cytoplasmic protein diffusion depends heavily on size, the cytoplasm has also been described as a molecular sieve [82][63].

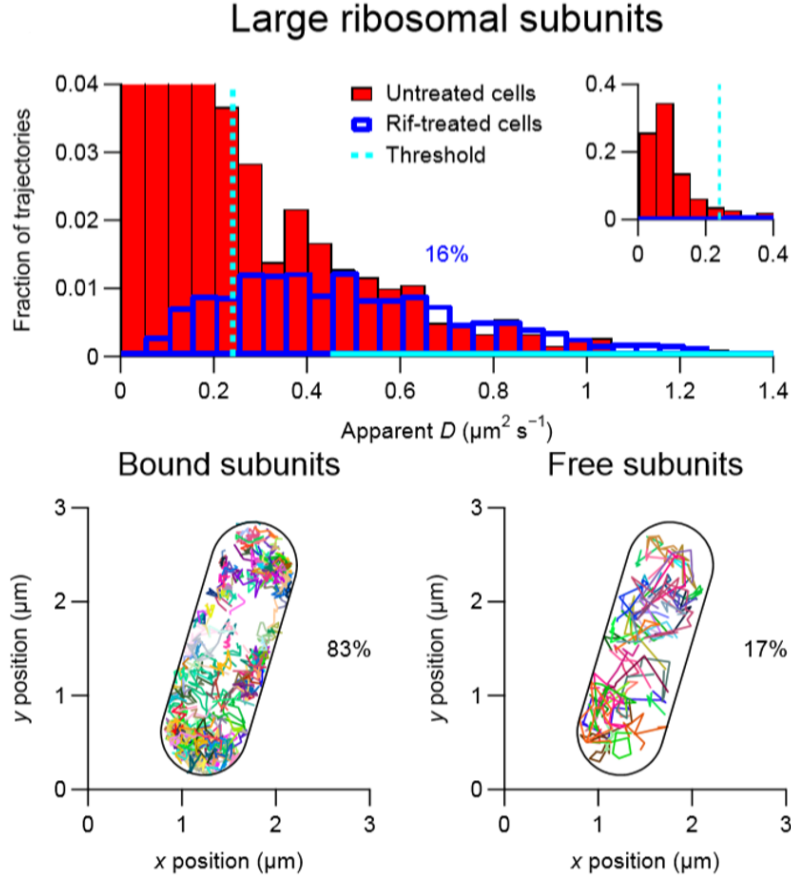


Figure 9: The apparent diffusion coefficients and tracks of bound and free 50s ribosomal protein L1 in *E. coli*. L1 protein was fused to photoconvertible fluorescent protein mEos2 and its diffusion was measured by single molecule tracking. As a control, cells were also treated with rifampicin to deplete mRNA and show the diffusion of free ribosomal subunits. A threshold was established to separate the apparent diffusion coefficients of bound and free ribosomal subunits. The trajectories showed the spatial distributions of bound and free subunits: (left) fully assembled, translating ribosomes were excluded from the nucleoid; (right) free 50s subunits could access the entire cell including the nucleoid. Figure taken from [111]

The relationship between crowding and diffusion of different sized molecules has also

been studied in yeasts *via* the use of genetically encoded multimeric nanoparticles (GEMs) as tracer molecules [29]. GEMs consist of genetically encoded scaffold proteins that are tagged with fluorescent proteins and they are able to self-assemble to form 20 nm and 40 nm particles when expressed in cells [29] (Figure 10). Delarue *et al.* demonstrated that crowding by the 30 nm ribosomes (regulated by the mTORC1 pathway) affected the diffusion of 20 nm GEMs and other larger tracer molecules in yeast cells but not smaller GFP dimers (radius = 5 nm). Since GEMs are made up of archaeal and bacterial scaffold proteins, the decreased diffusion coefficients were likely due to excluded volume effects by ribosomes rather than specific interactions with the native cellular contents of yeasts [29]. Even though crowding conditions between the bacterial and eukaryotic cytoplasm are quite distinctive (for example, the bacterial cytoplasm was described as more crowded [81] and prokaryotic ribosomes are smaller [12]), this study agrees with previous experiments that described the size dependence of diffusion and excluded volume effect. It also introduces GEMs as a useful, nonperturbative tool for studying macromolecular crowding in the cytoplasm *via* tracking of different sized tracer molecules [132]

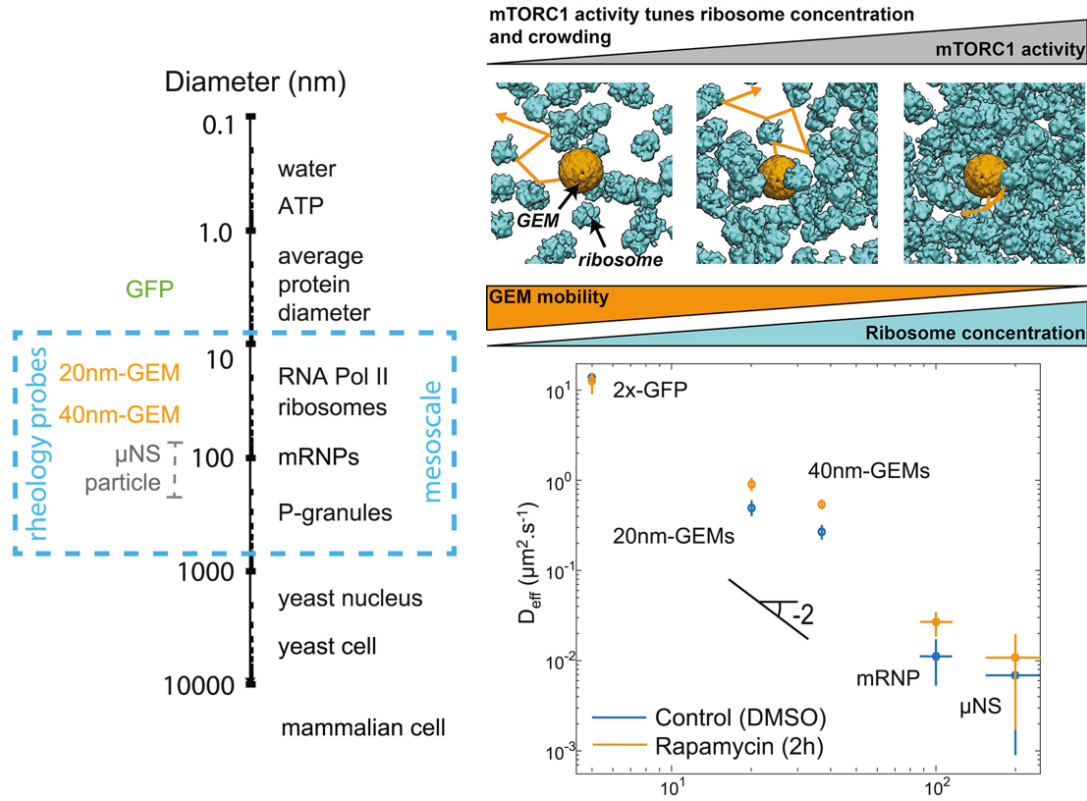


Figure 10: The mTORC1 signalling pathway affect diffusion of GEMs and other molecules by controlling the concentration of ribosomes in yeast. Rapamycin inhibited the mTORC1 pathway and reduced the concentration of ribosomes by almost two-fold. The decrease in crowding by ribosomes led to the increase in effective diffusion of 20 nm or larger tracer molecules by more than two-fold. However, the diffusion of smaller GFP dimer with 5 nm radius was unaffected. The figure on the left shows the diameters of GEMs and how they compare to molecules such as GFP and ribosomes. GEMs are of a similar size to large complexes such as the eukaryotic ribosome. Figure taken from [29]

In summary, fluorescence microscopy improves our knowledge of the structure of the cytoplasm and the effects of macromolecular crowding on molecular diffusion. Tracking tracer molecules within the cells reveals the complexity and heterogeneity of molecular diffusion. However, it remains challenging to fully understand how molecular diffusion is affected by the effects of excluded volume alone. This is because the mobility of molecules *in vivo* are also affected by other factors such as viscosity, binding to other cellular structures, or unspecific interactions with other native components in addition to crowding effects.

1.3.2 Understanding macromolecular crowding effects on diffusion *via* perturbations

There has been an increasing consensus among the scientific community on the heterogeneity of the cytoplasm and how it affects diffusion of molecules. However, there are currently limited studies on crowding effects in live cells and the results of these studies are not directly comparable due to the differences in techniques, organisms, and tracer molecules that were used. Furthermore, interpretation of data is difficult and complex because the cytoplasm of a live cell is dynamic and subject to constant changes. To better understand the relationship between crowding and diffusion, a series of studies were conducted to induce cellular changes that promote or reduce crowding and formation of cytoplasmic barriers that can restrict molecular diffusion. Most of these studies involved the use of stressed conditions such as osmotic upshift, antibiotic treatments, and starvation in the experiments so a comparison between the perturbed and the native state within the same bacterium can be obtained.

The work of Konopka *et al.* on osmotic stressed *E. coli* is one of the earliest studies looking at the effects of macromolecular crowding on protein diffusion. When *E. coli* cells were incubated in hyperosmotic buffer lacking the osmoprotectant K^+ , the cells underwent plasmolysis (the cytoplasm suffered a loss in water) and the mean biopolymer volume fraction increased, causing an increase in crowding, binding and confinement effects [60]. At high osmolarity (osmotic upshift values of ≥ 0.28 osmolal), the inner membrane detached from the cell wall due to the high surface / volume ratio and formation of visible plasmolysis spaces (VPS) could be observed [60]. A general decrease in GFP mobility was observed in these stressed cells despite some spread in diffusion coefficient values due to cell-to-cell variation and formation of VPS [60].

The same dramatic reduction in GFP mobility has also been observed in other osmotic upshift studies [144][82]. These studies were able to amplify crowding, confinement, and sieving effects by using osmotic stress to change the cytoplasmic state of *E. coli*. It was shown that GFP molecules that would otherwise diffuse freely in the cytoplasm with

homogeneous fluorescence became confined in discrete pools probably due to the formation of cytoplasmic diffusion barriers [144].

The relationship between crowding and diffusion of different molecular weight molecules was also investigated using osmotically stressed *E. coli*. Under extreme osmotic upshift conditions, diffusion barriers started to form and the diffusion coefficients of three different-sized fluorescent tracers: β -gal-gfp oligomer (four subunits of GFP-tagged β -galactosidase), GFP, and NBD-glucose decreased significantly as an effect of crowding [82]. However, the diffusion barriers only prevented the mobility of GFP (26 kDa) and β -gal-gfp oligomer (582 kDa) but not small NBD-glucose molecules [82]. The photobleaching of compartmentalized GFP on one end of the cell resulted in local loss of fluorescence but the fluorescence signal from the other end (separated by the nucleoid) was unaffected (Figure 11) [82]. Therefore, Mika *et al.* proposed that the nucleoid, with contribution by high cytoplasmic crowding, became the physical barrier that inhibited GFP diffusion. This observation supported the view of the cytoplasm as a molecular sieve for larger molecules [81]. Together, these studies suggested that high osmolarities can further introduce dramatic heterogeneity (formation of diffusion obstacles), crowding, confinement, and sieving effects within the cytoplasm.

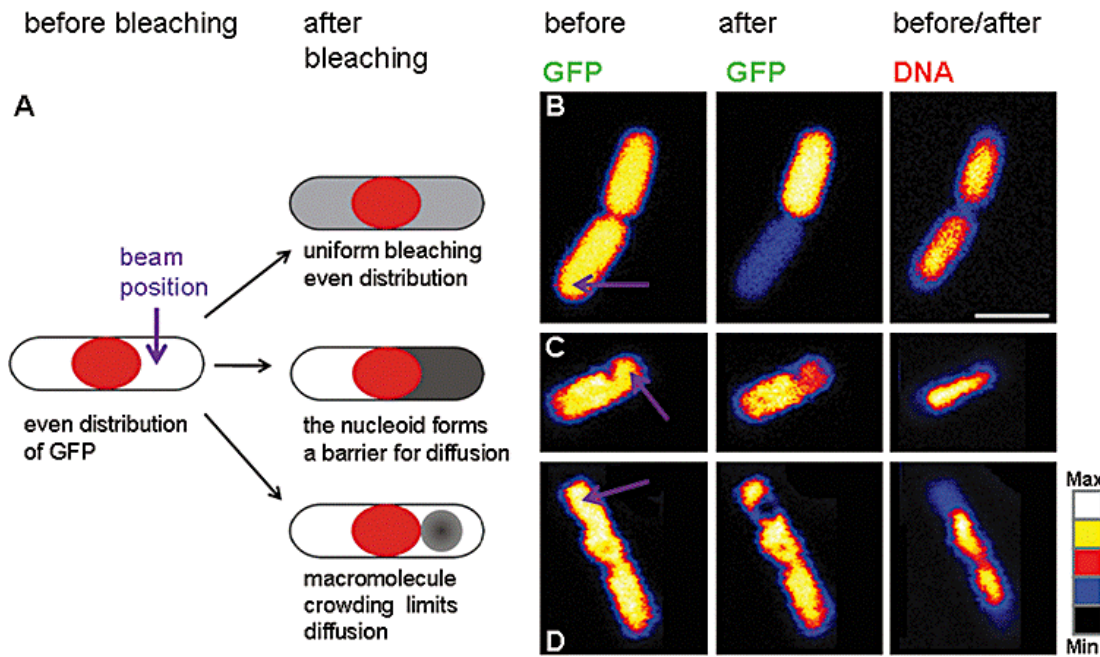


Figure 11: Formation of diffusion barriers could be observed in osmotically stressed *E. coli* cells that were expressing GFP molecules. (A) Demonstrates the three phenotypes that could be observed in the osmotic upshift experiment (mixed phenotypes could be observed in 30% of the population). (B) In the remaining population, 20% of the osmotically stressed *E. coli* cells did not exhibit diffusive barriers within the cytoplasm. Photobleaching by pulsed-FRAP showed homogeneous loss of GFP fluorescence. (C), (D) The phenotypes where the nucleoid (20%) and high macromolecular crowding (60%) act as physical obstacles to inhibit GFP diffusion respectively. Chromosomal DNA of the same cells was also co-stained with Draq-5 to show the spatial distribution of the nucleoid. Scale bar 2 μm . Figure taken from [82]

As mentioned previously, the nucleoid in *E. coli* has been shown to be a crowder that affects diffusion of certain large molecules such as ribosomes and plasmids. In addition to osmotic upshift, the excluded volume effect of the nucleoid can also be amplified or reduced through the use of antibiotics such as chloramphenicol and rifampicin. For example, rifampicin has been shown to cause nucleoid expansion by inhibiting transcription [134] and increased mobility of DNA loci and GFP- μNS particles in the cytoplasm [154]. Another study by Neeli-Venkata *et al.* also showed that changes in the nucleoid size, generated by antibiotic stresses and changes in medium richness, affected the positioning of protein aggregates that would usually be excluded from midcell by the nucleoid [89].

Another study by Parry *et al.* also demonstrated a change in the fluid state of the *C. crescentus* and *E. coli* cytoplasm to 'glassy-state' where the motions of large tracer

molecules became confined within cellular compartments [98]. The study showed that in energy-depleted or carbon-starved *C. crescentus* and *E. coli*, large molecules such as crescentin-GFP structures and mini-RK2 plasmids lost their mobilities in the cytoplasm; whereas in metabolically active cells, the same molecules could freely explore the cytoplasm [98] (Figure 12). The authors proposed that acidification of the cytoplasm occurred when the cellular metabolism was shut down, causing large particles to be trapped within the cytoplasm. The diffusion of molecules in these energy-depleted / starved cells was also dependent on the molecular weight of the tracer molecules. By tracking different-sized GFP- μ NS particles, Parry *et al.* found that molecules smaller than 30 to 40 nm were able to diffuse freely within the cell despite the "solid-like" state of the cytoplasm.

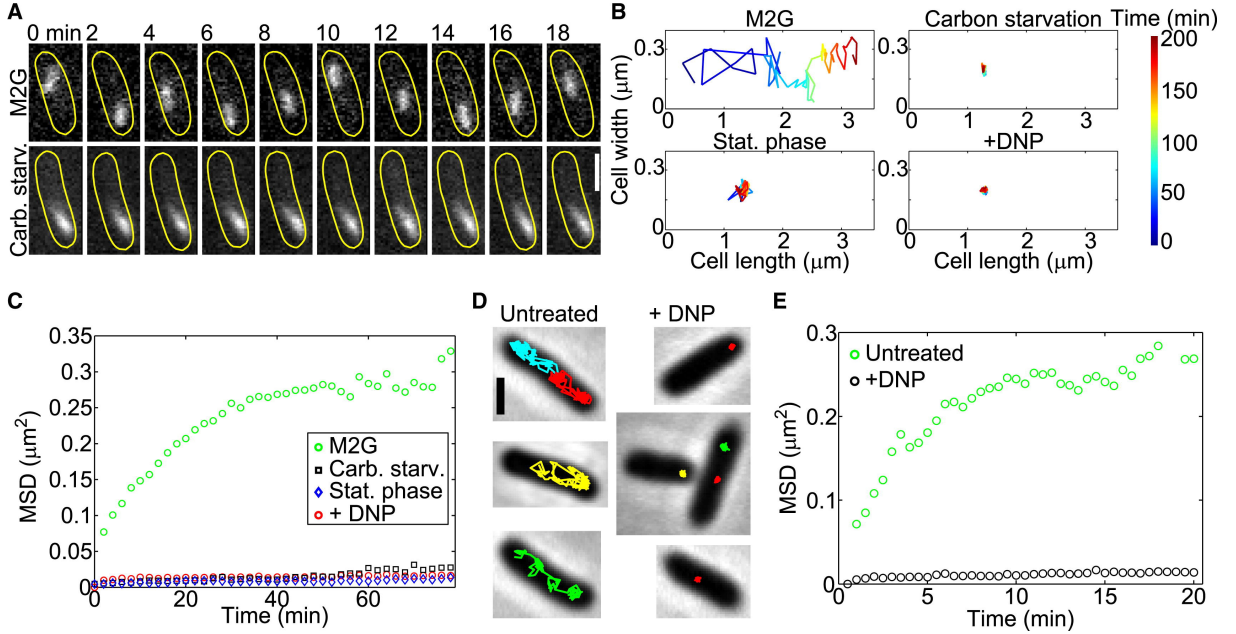


Figure 12: The diffusion of crescentin-GFP structures and mini-RK2 plasmids in energy-depleted *C. crescentus* and *E. coli* cells. (A) Time-lapse images show motions of crescentin-GFP structures in the *C. crescentus* cytoplasm under normal growth (top) and carbon starvation conditions (bottom). (B) Trajectories of crescentin-GFP structures in actively growing *C. crescentus* cells (M2G) and energy-depleted / stationary cells. 2,4-dinitrophenol (DNP) treatment led to ATP and GTP depletion in cells. (C) MSD of crescentin-GFP structures in conditions described in (B). (D) 2D trajectories of crescentin-GFP structures and mini-RK2 plasmids in metabolically active and DNP-treated *E. coli* cells. (E) MSD of mini-RK2 plasmids in metabolically active and DNP-treated cells. Scale bar 1 μm . Figure taken from [98]

Mechanical perturbations have also been used to induce changes in the cytoplasmic

volume and structure. *E. coli* cells were compressed and flattened using microfabricated / microfluidics valve and the mobilities of fluorescent proteins, cytoplasmic particles and chromosomal loci were investigated [95][160]. The compression was believed to cause a loss in water from the cell and an increase in macromolecular crowding [95]. It was found that the diffusion coefficient of an RFP decreased from $14 \pm 4 \mu m^2 s^{-1}$ to below $1 \mu m^2 s^{-1}$ following the compression [95]. This observation was also supported by the work of Yu *et al.*, who recorded a dramatic reduction in diffusion of GFP- μ NS particles in compressed cells. However, the slowing of chromosomal loci in these flattened cells was less significant and this difference between the motions of cytoplasmic molecules and DNA loci was still unclear [160].

In summary, the tracking of tracer molecules under perturbed and stressed conditions is important because it helps us to better understand the relationship between macromolecular crowding and mobility of molecules. However, the cellular responses of bacteria upon external perturbations are complex and it is unclear how this layer of complexity can affect molecular diffusion and data interpretation. More experiments that enable the study of crowding under normal growth conditions are needed to improve our knowledge on the field.

1.3.3 Bacteria may maintain homeocrowding

It has been proposed that bacterial cells maintain optimal levels of macromolecular crowding where biochemical reaction rates are maximized without limiting the lateral diffusion of biomolecules within the cytoplasm. The term 'homeocrowding' was introduced to describe this ability of the cell to control the concentration of macromolecules within a narrow range [143]. By studying the response of *E. coli* to changes in nutrient availability and osmotic stress, van den Berg *et. al.* proposed two mechanisms in which cells could maintain optimal macromolecular densities, and both mechanisms involve regulation of cell size and volume to some extent.

Homeocrowding in *E. coli* may be achieved through the carbon catabolite repression

(CCR) pathway and regulation of cell size in response to increased growth rate [143][165] (Figure 13A). The CCR pathway is activated in cells with rapid growth rates to promote selective substrate uptake and maintain an optimal cytoplasmic density [165]. By growing *E. coli* cells in media supplied with mixed carbon sources, Zhou *et. al.* observed both an increased utilization of glucose and a higher production of acetate in cells with high growth rates following the activation of CCR. This suggests that in rapidly growing cells, oxidative phosphorylation is down-regulated to allow a switch to glycolysis / fermentation. This metabolic switch is important because the synthesis of oxidative phosphorylation enzymes would take up 2 times more cytoplasmic space than in the case of fermentation, resulting in overcrowding of the cytoplasm [146] [143]. In addition to activated CCR, cells that grew faster also had increased cellular volume to cope with the high biomass synthesis rate, therefore crowding / cytoplasmic density remained constant despite the differences in growth rates [165].

Osmolyte transport also plays an important role in controlling the cellular volume to maintain optimal crowding when the cell is placed under osmotic stress conditions. At high osmolarities, the cell volume shrank following plasmolysis and the mobility of GFP in the cytoplasm decreased in *E. coli* cells [61]. However, cells were able to adapt to high osmolarities *via* accumulation of osmoregulatory solutes such as K^+ [155]. The increase in osmolyte concentration would allow water to flow into the cytoplasm to expand the cell volume, restoring the diffusion of GFP to its previous level before the osmotic upshift [143] (Figure 13B). When cells underwent osmotic downshift, osmolytes would be transported out of the cell to prevent excessive gain in cell volume and lysis. These examples demonstrate the potential functions of osmoregulatory transporters and mechanosensitive channels in regulating the concentration of macromolecules by controlling the cellular volume [143].

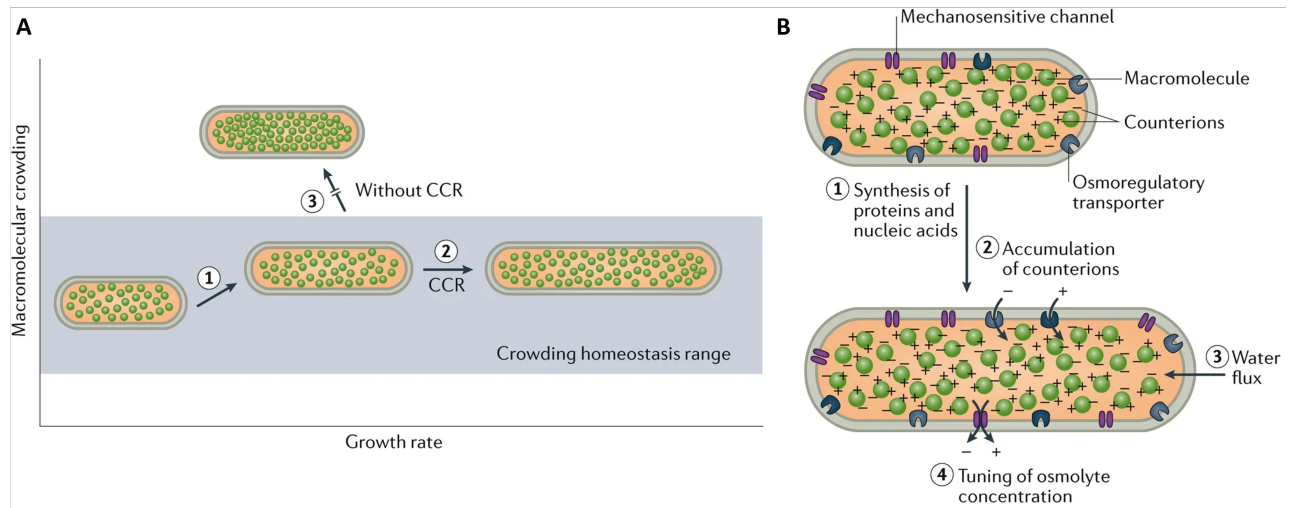


Figure 13: Schematic shows the two possible mechanisms that are involved in maintaining homeocrowding in bacterial cells. (A) Carbon catabolite repression (CCR) pathway is activated in cells with increased growth rate. (B) Osmolyte transport regulate crowding in cells by controlling the cell volume. Figure taken from [143]

The precise mechanisms for achieving homeocrowding are not known, but it is likely that regulation of cellular volume and cell size plays an important role. There are still many unresolved questions in the field of crowding: How do bacteria sense the crowdedness of the cytoplasm? How do bacterial cells control the spatial heterogeneity of different cellular components when there is a sudden change in crowding? How different is cytoplasmic crowding level in bacteria other than *E. coli*?

1.4 *Rhodobacter sphaeroides*

1.4.1 Background

Rhodobacter sphaeroides is a purple nonsulfur α -proteobacterium and a model organism for studying photosynthesis and complex signalling pathways such as chemotaxis [101]. It is metabolically versatile as it can grow photosynthetically or use aerobic or anaerobic respiration depending on the the availability of light, oxygen, and carbon sources. Like most purple nonsulfur bacteria, *R. sphaeroides* grows especially well as photoheterotroph (use light as energy source but cannot use carbon dioxide as the sole carbon source) under illuminated, anoxic conditions containing organic compounds [78]. It is widespread in the environment and can be isolated from mud, lake water, or sewage samples [145][77][120].

Interestingly, aerobically and photoheterotrophically grown *R. sphaeroides* cells have distinctive cell architecture. When *R. sphaeroides* is grown aerobically in the dark, it has a cytoplasmic morphology that is more similar to a typical *E. coli* cell; however, when it is grown under photosynthetic condition, the cytoplasm develops extensive membrane invaginations containing photosynthetic apparatus to maximize light absorption. Like most purple bacteria, the development of intracytoplasmic membrane (ICM) in *R. sphaeroides* depends on the oxygen level and light intensity available in the environment. High oxygen tension has been shown to inhibit the synthesis of photosynthetic complexes in *R. sphaeroides* [58] while its cellular ICM levels are inversely correlated to light intensity [1][23]. Figure 14 shows *R. sphaeroides* undergoing significant changes in cell architecture by generating an extensive network of ICM vesicles following a decrease in oxygen level [58][91]. At 0 h, the cells had just been transferred from aerobic to low oxygen condition (3% oxygen) and the cytoplasmic membrane was positioned near the outer membrane. At 6 and 12 h, the invagination of ICM occurred and the cells were slowly filled with ICM vesicles until they reached a steady state at 24 h.

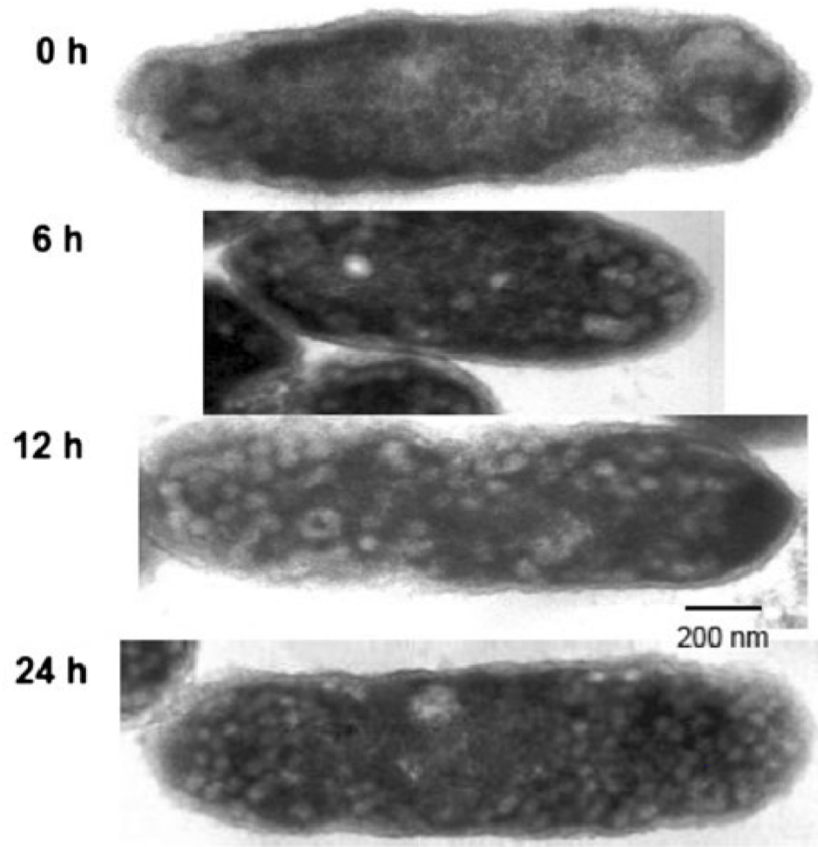


Figure 14: Transmission electron micrographs of *Rhodobacter sphaeroides* undergoing sequential induction and invaginations of intracytoplasmic membrane (ICM) when the cells were exposed to low oxygen level (3% oxygen). At 0 h, the cells resembled the architecture of aerobic cells where the cytoplasmic membrane was positioned near the outer membrane. At 6 and 12 h, budding of ICM began to occur and a dense network of ICM vesicles started to occupy the cytoplasmic space until it reached a steady state at 24 h. Figure taken from [58][91].

This dramatic morphological change in the cell architecture of *R. sphaeroides* from aerobic to photosynthetic growth may contribute to crowding of the cytoplasm. A discussion on the structure and organization of the ICM vesicles in *R. sphaeroides* is presented in subsection 1.4.2.

1.4.2 Crowding in photoheterotrophic *R. sphaeroides*

When *R. sphaeroides* is grown photoheterotrophically, it generates invaginations of intracytoplasmic membrane (ICM) that bud off to form discrete ICM vesicles or chromatophores in the cytoplasm (Figure 15). These vesicles contain bacteriochlorophyll (BChl), carotenoids, and other photosynthetic apparatus and they serve to increase the surface area for light absorption and energy production. Depending on the light intensity levels, *R. sphaeroides* cells are filled with varying number of chromatophores [2] that may contribute to macromolecular crowding within the cytoplasm.

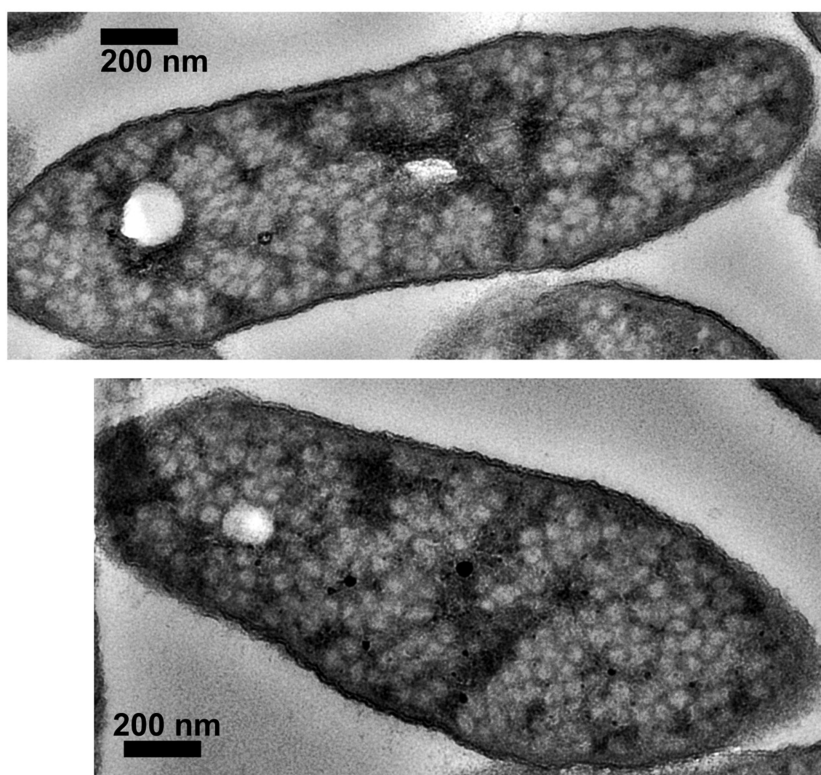


Figure 15: The architecture of the ICM vesicles in photoheterotrophic *Rhodobacter sphaeroides* grown at low light shown by transmission electron microscopy (TEM). Figure taken from [2]

Photosynthesis in *R. sphaeroides* occurs on its invaginations of ICM where there is an abundance of BChl-protein complexes. These BChl-protein complexes, also known as light harvesting (LH) complexes, capture and transfer photons to a reaction center (RC), where charge separation occurs across the membrane to generate a proton gradient for

ATP synthesis. The *R. sphaeroides* LH complexes consist of light absorbing pigments, BChl and carotenoids, and integral membrane proteins that increase the efficiency of light capture [91].

There are two types of LH complexes on the ICM surface of *R. sphaeroides*; the LH1 or the core complex that is closely associated with the RC and the LH2 antenna complex. A few techniques were used to study the membrane dynamics and organization of chromatophores [86]. Atomic force microscopy (AFM), a powerful tool for obtaining topographs of membrane proteins at subnanometer resolution, revealed that the ICM of *R. sphaeroides* grown under high or moderate light consisted of linear arrays of interconnected RC-LH1 core complexes and peripheral LH2 antenna (Figure 16) [6]. The RC-LH1 complexes are about 12 nm in diameter and they form rows of up to six dimers, while the 7 nm diameter LH2 complexes occupy the spaces around rows of RC-LH1 dimers so that excitations can be transferred to LH1 and the RC [6]. The organization of these light harvesting complexes are important for efficient capture and transmission of photons in high and moderate illuminated *R. sphaeroides*. Under low light condition, the ICM surface consists of mainly LH2 complexes [6][2]. The higher ratio of LH2 to RC-LH1 complexes in low light ICM is probably due to the need to enhance the surface area for light capturing *via* the increase in LH2 complexes. Using the available data on the organization of the light harvesting complexes, a three-dimensional reconstruction of a chromatophore at the atomic level has been made (Figure 17) [118].

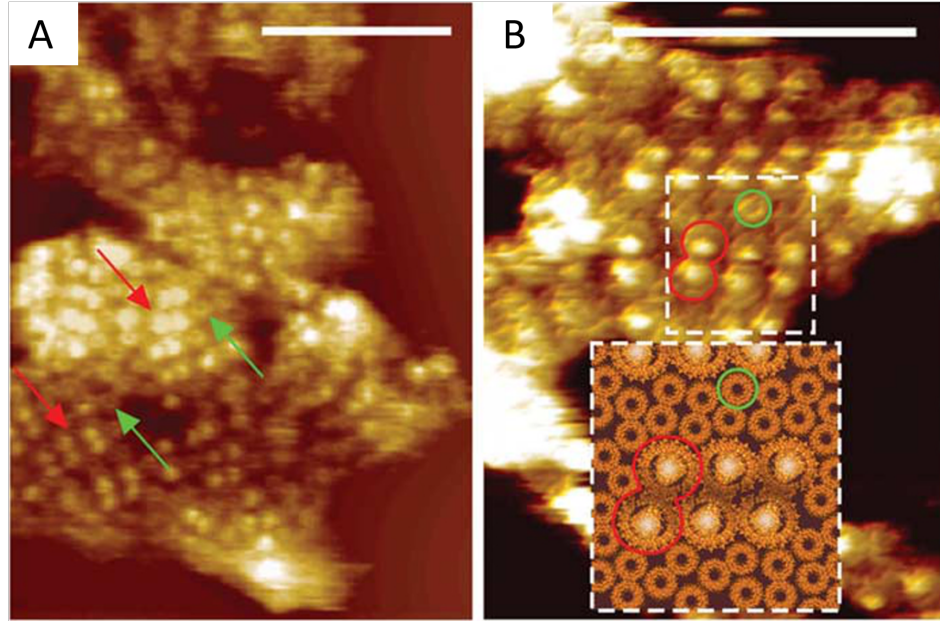


Figure 16: AFM of purified *R. sphaeroides* ICM vesicle membranes revealed the organization of photosynthetic complexes RC-LH1 and LH2 antenna complex grown under high or moderate light intensities. (A) shows the RC-LH1 core complex (red arrows) and the associated LH2 complex (green arrows). The bottom inset of (B) is a reconstruction of the upper dashed box using known structures of RC, LH2, and RC-LH1 from previous studies. The large circular complexes with a central RC complex are the RC-LH1 complexes (red) and they form rows of up to six dimers. The smaller rings of LH2 antenna complexes (green) occupy the spaces between the rows of RC-LH1 dimers. Scale bar 100 nm. Figure taken from [6]

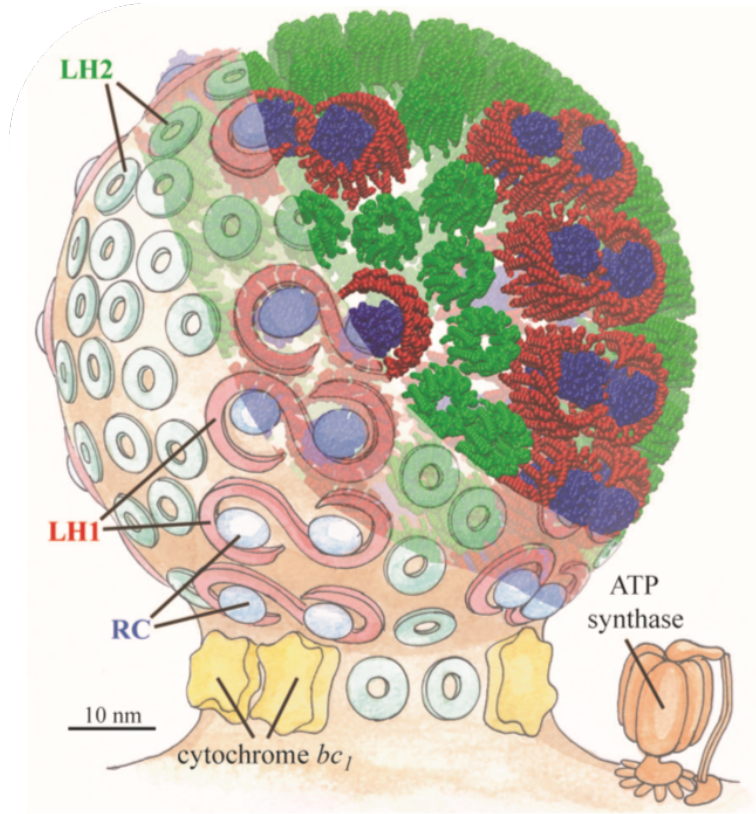


Figure 17: Three dimensional reconstruction of a spherical *R. sphaeroides* ICM vesicle grown under moderate light using data from AFM and linear dichroism (LD) studies. The chromatophore has a diameter of around 70 nm and consists of LH1 (red) and LH2 (green) complexes that capture photons and transfer the electronic excitations to a RC (blue). The RC will then initiate transmembrane electron transfer to establish a proton gradient and generate ATP. This vesicle contains 18 dimeric RC-LH1 complexes, 101 LH2 complexes, and 3879 BChl molecules. The ratio of surface area occupied by RC-LH1 core complexes and LH2 complexes in this moderate / high light vesicle is 1:1.31. Scale bar 10 nm. Figure taken from [118].

The development and connectivity of the ICM vesicles in *R. sphaeroides* have been studied through the isolation of the ICM vesicles initiation sites, upper pigmented band (UPB), which sediments more slowly than single ICM vesicles in rate zone sucrose density gradients (Figure 18)[92][141]. Cells are first disrupted using a French press and the bands are separated through rate-zone ultracentrifugation on sucrose gradients. This gives rise to membrane fractions that retain most of their morphologies and physical shapes as confirmed by transmission electron microscopy (TEM) and AFM [141]. UPB, the precursor of ICM vesicles, contains hollow, hemispherical membrane structures while

closed, spherical structures are found in the ICM fraction. Since UPB band is the link between the cytoplasmic membrane and the ICM vesicles, it contains respiratory cytoplasmic membrane markers (electron transport and transfer proteins) and high levels of BChl synthase [92]. ICM vesicles, on the other hand, are enriched in LH2 polypeptides, ATP synthase, and cytochrome bc_1 complex [91]. By using pulse-chase radiolabelling and running the isolated UPB and ICM vesicle fractions on SDS-PAGE gels, Niederman *et al.* found that clustering and sequential assembly of RC-LH1 complexes and then the LH2 complexes lead to the initiation of membrane curvatures and budding to form ICM vesicles [92].

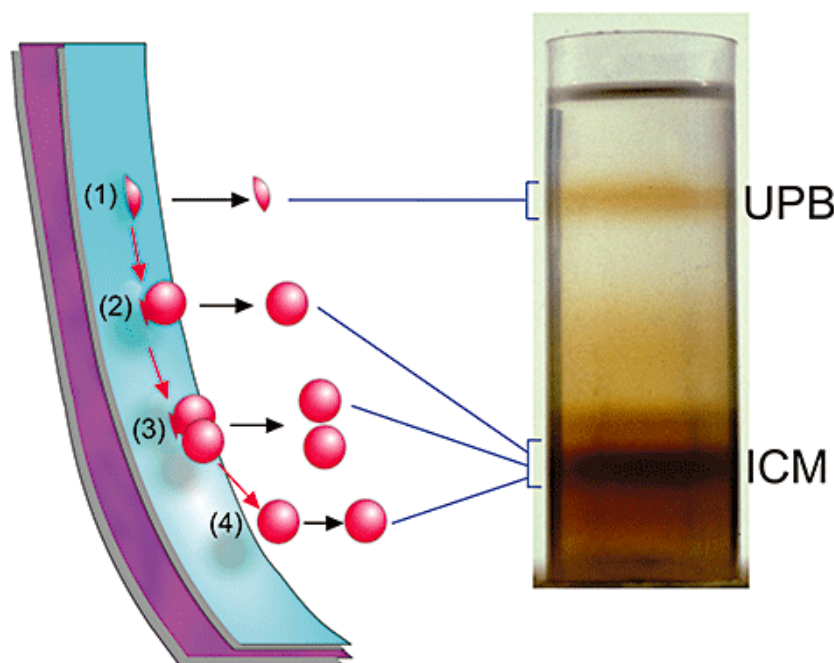


Figure 18: The diagram shows the bands of upper pigmented band (UPB) and ICM fractions in the 5 - 35% w/w sucrose density gradients and their respective position in the cell. Cells were first disrupted using a French press and passed through rate-zone centrifugation in sucrose gradients to give rise to two membrane fractions. (1) The UPB fraction contains the initial invagination sites of ICM vesicles and it sediments more slowly than the ICM fraction. (2), (3), (4) are all part of the ICM fraction that have been released (may or may not due to cell disruption) from the cytoplasmic membrane into the cytoplasm following further invagination from (1). (3) Secondary budding event could also initiate from an existing vesicle, forming a double spherical structure that breaks off into two isolated structure. Figure taken from [141]

Electron microscopy tomography and cryo-electron tomography (cryo-ET) have also

been used to study the overall spatial organization of ICM vesicles within *R. sphaeroides* cells. There are controversies regarding the connectivity of the ICM network. Previously, it was unclear whether the ICM vesicles in *R. sphaeroides* exist as isolated chromatophores or as a continuous reticulum that links all the vesicles to each other and to the cytoplasmic membrane. For example, work by Scheuring *et al.* suggested that the connectivity of vesicles to each other is a general feature of *R. sphaeroides* and the interconnected network may be important for material transport and coordinated response to internal and external cues of the system (Figure 19)[115]. On the other hand, Tucker *et al.* believed that ICM vesicles are discrete, independent structures that occupy the cytoplasm [141].

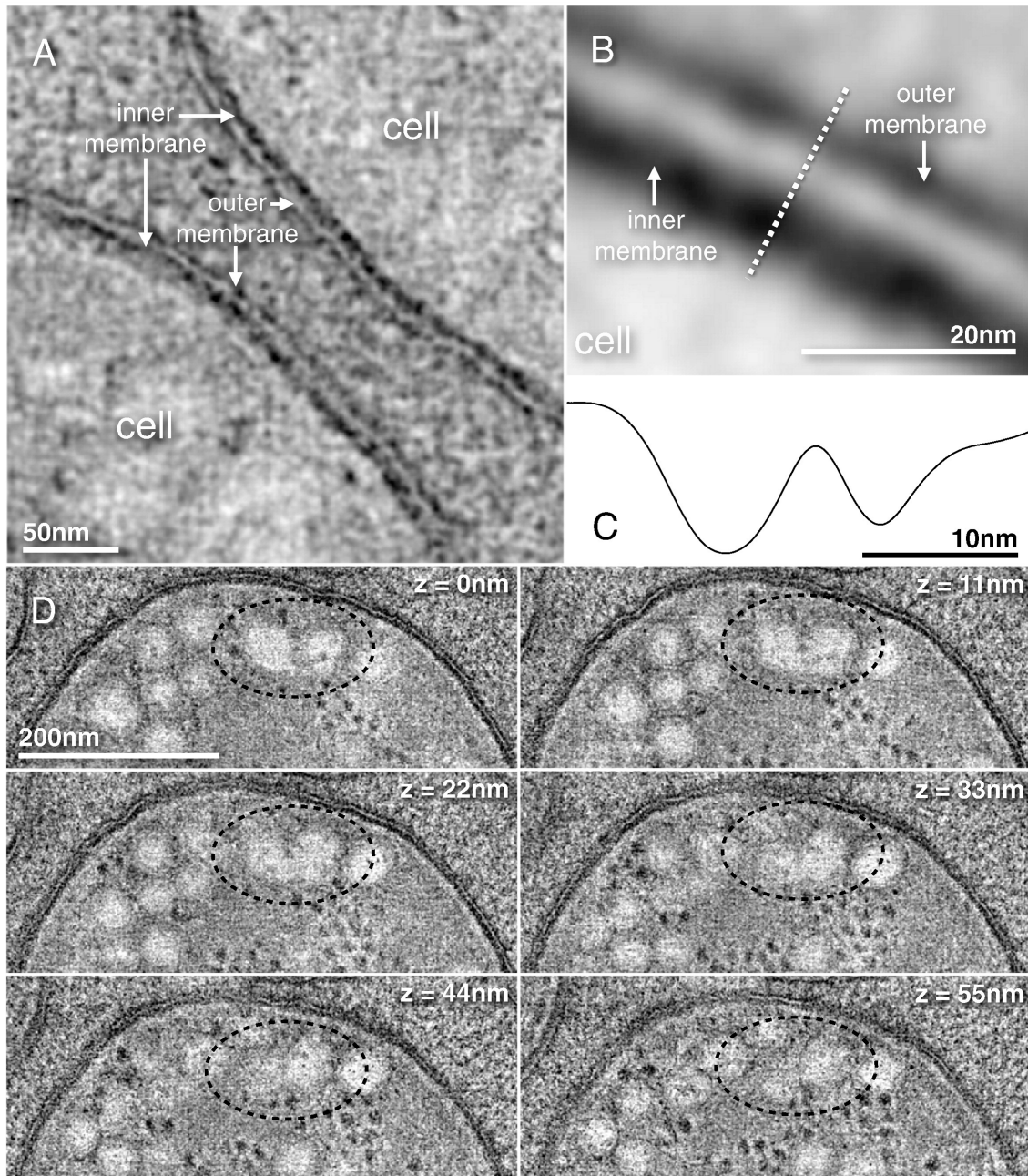


Figure 19: Electron microscope tomography slices showing that chromatophores in photoheterotrophic *R. sphaeroides* exist as connected network that occupy the cytoplasmic space. (A) and (B) The inner and outer membranes are indicated by the white arrows. (D) shows tomographic slices of ICM vesicles that are fused to each other to form structures of varying diameter (dashed lines). Figure adapted from [115]

More recently, cryo-ET performed on 300 nm thick whole *R. sphaeroides* cell slices produced by cryo-focused ion beam (cryo-FIB) milling showed that the generation of ICM vesicles occurs in multiple developmental stages (Figure 20) [93]. During the earliest stage, initiation of ICM vesicles starts to occur and indentations in the cytoplasmic membrane can be observed. The vesicle remains attached to the cytoplasmic membrane through an extended neck-like structure as it develops into a spherical shape during early and late budding stage. As the vesicle reaches the pre-detachment phase, it will form a full sphere with a minimal contact point linking it to the cytoplasmic membrane. Once the vesicle is released into the cytoplasm, it will remain close to the cytoplasmic membrane as isolated chromatophore while the older vesicles will occupy the centermost region [93]. These isolated vesicles are often known as mature vesicles.

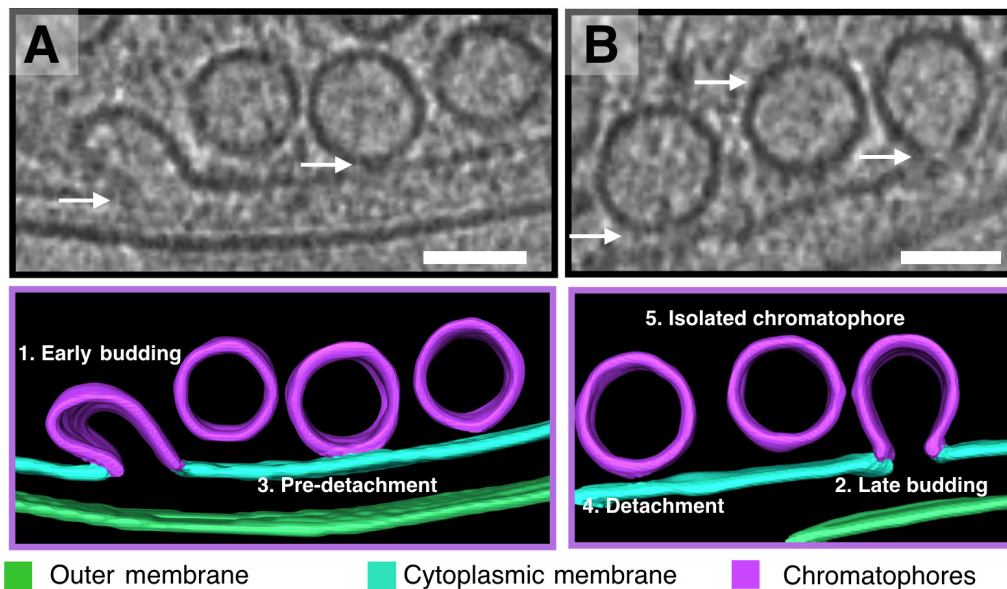


Figure 20: Cryo-ET on thin *R. sphaeroides* cell slices shows the different stages that lead to the formation of mature ICM vesicles (top row). The bottom row is the corresponding 3D segmentations that is colour-coded. During early budding (1) and late budding (2) phase, vesicles start to develop a spherical shape while they form a neck-like structure that extends into the cytoplasmic space while connecting to the inner membrane. During the pre-detachment phase (3), the vesicle has a minimal point of contact with the cytoplasmic membrane. After detachment (4), the isolated chromatophore will remain close to the cell periphery. Scale bar 50 nm. Figure taken from [93]

In contrast to the isolated chromatophores near the cell membrane, Noble *et al.* also showed that the older ICM vesicles located at the cell centre are often linked to neighboring vesicles *via* 8 - 10 nm long linkers and occasionally, have secondary budding events [93]. Previously, it had been difficult to access the ICM architecture at the center of the cell due to the thickness of *R. sphaeroides*. Noble *et al.* showed that these linked vesicles take up the majority of the chromatophores in *R. sphaeroides* (62.8%) while isolated vesicles consist of 30.8% of the chromatophores. The function of these linkers are unclear and they do not have access to the interior of the vesicles [93]. Despite the linker, each vesicle is likely to be a closed and discrete compartment.

Overall, previous studies suggested that the ICM network in *R. sphaeroides* exists as both discrete chromatophores and as a connected network that occupy the cytoplasmic space [141][93][115][2]. The connectivity of the ICM vesicles with each other and the inner cell membrane depends heavily on the developmental stage of the chromatophores. Currently, it is unclear how crowding caused by these ICM vesicles can have an effect on protein diffusion, localization of protein complexes, and spatial heterogeneity of the cytoplasm. Understanding the distribution of these 70 nm vesicles within the cytoplasm could provide useful insights for the field of macromolecular crowding.

1.4.3 Light dependent crowding in photoheterotrophic *R. sphaeroides*

Gene expression and protein synthesis in *R. sphaeroides* can be affected by light dependent regulatory pathways, mediated by photoreceptors that are able to absorb different wavelengths of light and undergo reversible structural changes [91][57]. This includes the levels of photosynthetic pigments and apparatus such as BChl, LH, and RC complexes in photoheterotrophic *R. sphaeroides*. In response to downshifts in light intensity, *R. sphaeroides* cells develop dense network of ICM vesicles to increase the surface area for capturing photons. Earlier work showed that the overall BChl concentration and chromatophores per cell increased following a transition from high light to low light.

The adaptation of *R. sphaeroides* cells to changes in light intensity has been studied using different techniques, such as AFM, transmission electron microscopy (TEM), and pigment extraction. The ICM contents of high light cells grown at $1100 \mu\text{mol photons } m^{-2}s^{-1}$ versus low light cells grown at $20 \mu\text{mol photons } m^{-2}s^{-1}$ were compared using TEM [2]. Low light and high light *R. sphaeroides* cells from freshly grown cultures irradiated with compact fluorescent lamps were fixed and embedded in resin before cutting into thin, 60 - 90 nm slices. The cell sections were then imaged by shining a high energy beam of electrons that was transmitted through the specimen and focused onto a camera to form an image. TEM micrographs in Figure Figure 21 showed that the cytoplasm of low light cells were packed with ICM vesicles, with an estimate of 1500 vesicles per cell ($195 \text{ vesicles} / \mu m^2 \text{ section}$), which is around four times more than the 369 vesicles / cell in high light cells ($48 \text{ vesicles} / \mu m^2 \text{ section}$) [2].

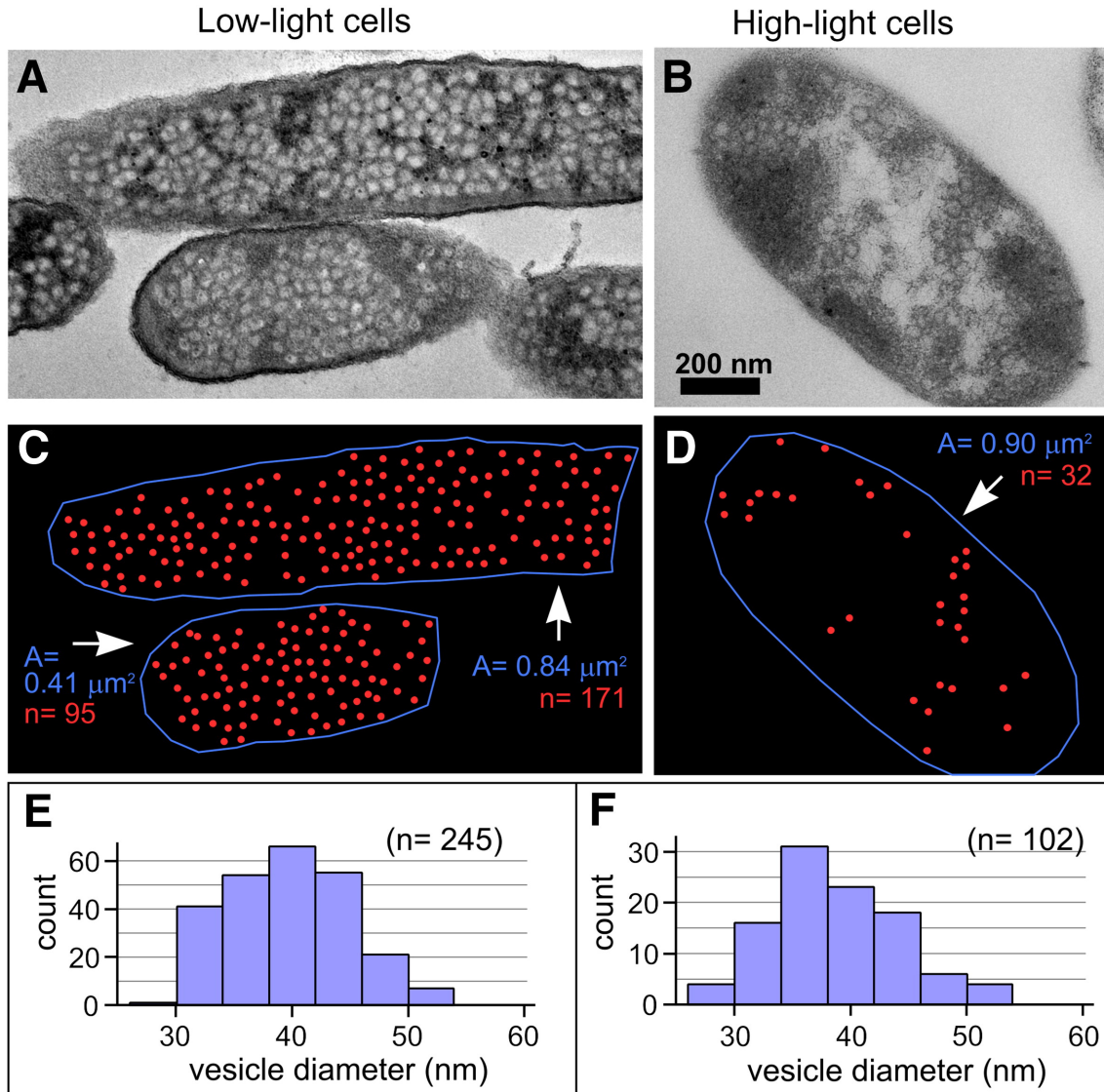


Figure 21: Photoheterotrophic *Rhodospirillum rubrum* cells are filled with varying amount of ICM vesicles depending on the light intensities. (A) TEM showed the cytoplasm of low light cells ($20 \mu\text{mol photons m}^{-2}\text{s}^{-1}$) filled with 30 - 50 nm vesicles. (B) High light cells ($1100 \mu\text{mol photons m}^{-2}\text{s}^{-1}$) had less vesicles and empty regions within the cytoplasm. The red dots in (C) and (D) resembled each vesicle that was identified and the area of the cytoplasm (μm^2) is coloured in blue. Low light cells contained an estimate of 195 vesicles / μm^2 section while high light cells had an average of 48 vesicles / μm^2 section. (E) and (F) showed the distribution of the diameter of low light and high light cells, centered around 40 nm. Scale bar 1 μm . Figure taken from [2].

The TEM data in Figure 21 (E) and (F) also suggested that both the high light and low light ICM vesicles were around 40 nm in diameter [2]. However, this data probably underestimated the actual *in vivo* diameter of a vesicle due to the artefacts of the TEM specimen preparation step [2]. Before imaging, whole *R. sphaeroides* cells were fixed in chemicals, dehydrated, washed, and embedded in resins. These procedures may have a damaging effect on the size and structure of the actual ICM vesicles. The cell sections may also contain incomplete sections of the vesicles since the slices did not cut through the centre of every vesicle. Additional data provided by TEM and AFM on purified ICM vesicles estimated that low light and high light vesicles are similar in size with an outer diameter of 60 - 65 nm [2]. This agrees well with values reported previously [118][141][93].

The higher ICM content in low light cells is also reflected by its higher concentration of BChl molecules extracted using 7:2 acetone / methanol. The molar concentration of BChl molecules can be determined by measuring the absorbance of extracted pigment at 770 nm and calculated using the extinction coefficient of $0.076 \mu\text{M}^{-1} \text{cm}^{-1}$ [20]. Pigment extraction showed that low light cells had 6.5×10^6 BChl molecules per cell, which was around four times the BChl molecules compared to high light cells (1.6×10^6 molecules per cell) [2]. This corresponds to around 1468 vesicles per cell for low light and 274 vesicles per cell for high light [2], calculated using the modelling data of 4000 BChl molecules per vesicle [118].

The levels of RC, LH1, and LH2 complexes in low light cells had also been shown to increase on the surface of these ICM vesicles during downshifts in light. AFM analysis on low light and high light vesicle membrane revealed lower density of RC-LH1 core complexes in low light cells than high light cells [2][141]. Low light vesicles were found to have higher levels of LH2 complexes relative to LH1 core complexes, even though both high light and low light vesicles remain a similar size. This is because low light vesicles have to increase the surface area for capturing and absorbing light energy by creating more LH2 antenna complexes.

A comparison of the UPB and ICM fractions of low light and high light was done by

rate-zone centrifugation on 20 - 45% sucrose density gradients [2]. UPB fraction contained the precursors and the development sites where ICM invaginations first originated. Even though low light cells had a significantly larger ICM fraction, the authors estimated that there was a similar number of membrane development sites in high and low light cells (704 and 829 UPB development sites respectively) [2]. This suggests that fewer ICM invaginations in high light cells form mature vesicles due to the fast growth rate.

Adams *et al.* also found that the sizes of low and high light cells were within the range of 1.49 - 1.58 μm , suggesting that both high and low light cells have similar cell size. However, low light cells have slower doubling time than high light cells. Under light limited conditions, the cells probably have a slower rate of generating ATP from light for growth. In order to maximize light harvesting, *R. sphaeroides* cells grown under low light generate approximately $16 \times 10^6 \text{ nm}^2$ of additional photosynthetic membrane to increase the surface area [2]. It is important to understand whether the dramatic increase in intracytoplasmic membrane surface area would impact the motions and dynamics of other biomolecules within the cell.

As shown in TEM images, ICM vesicles occupy a large portion of the cytoplasmic space in photoheterotrophic *R. sphaeroides*. These vesicles may increase macromolecular crowding in the cell and affect spatial organization of large complexes such as the chemosensory cluster or positioning of chromosomal DNA. In addition, the different compositions of low light and high light ICM membrane showed by AFM could also affect protein diffusion. It is possible that a tracer molecule diffusing in the cell will have different nonspecific interactions to LH2 rich regions or RC-LH1 core complexes due to the differences in chemical properties.

1.5 Chemotaxis in *Rhodobacter sphaeroides*

R. sphaeroides is able to perform chemotaxis, which means it can migrate towards favourable environments by swimming in the direction of attractants such as oxygen, light, and carbon sources. Chemotaxis functions through complex intracellular signalling. Swimming in *R. sphaeroides* depends on the rotation of the flagellar motor and multiple chemotaxis proteins that are localized to various parts of the cell. Some examples include chemoreceptor proteins that form the chemosensory clusters and response regulators that controls the stopping of the flagellar motor.

Bacterial chemotaxis is a classic example of a two-component signalling pathway. Changes in environmental stimuli result in a signal that is transduced by the chemoreceptors across the membrane to a histidine kinase through autophosphorylation. The phosphoryl group is then transferred to a response regulator, where it binds to the flagellar motor and cause the cell to tumble.

1.5.1 Chemosensory clusters

The main difference between the *R. sphaeroides* and *E. coli* chemotaxis pathways is the presence of two chemosensory clusters in *R. sphaeroides* that are essential for chemotaxis. Under laboratory conditions, two chemotaxis operons, *cheOp2* and *cheOp3*, are expressed, leading to the formation of a transmembrane and a cytoplasmic cluster [101] (Figure 22). *In vivo* imaging of fluorescent protein fusions and immuno-gold electron microscopy have been used to study the localizations of chemosensory clusters [150]. Both the chemosensory clusters consist of methyl-accepting chemotaxis proteins (MCP) that form trimers of dimers to sense ligand concentrations or stimuli from the environment and cytoplasm [17].

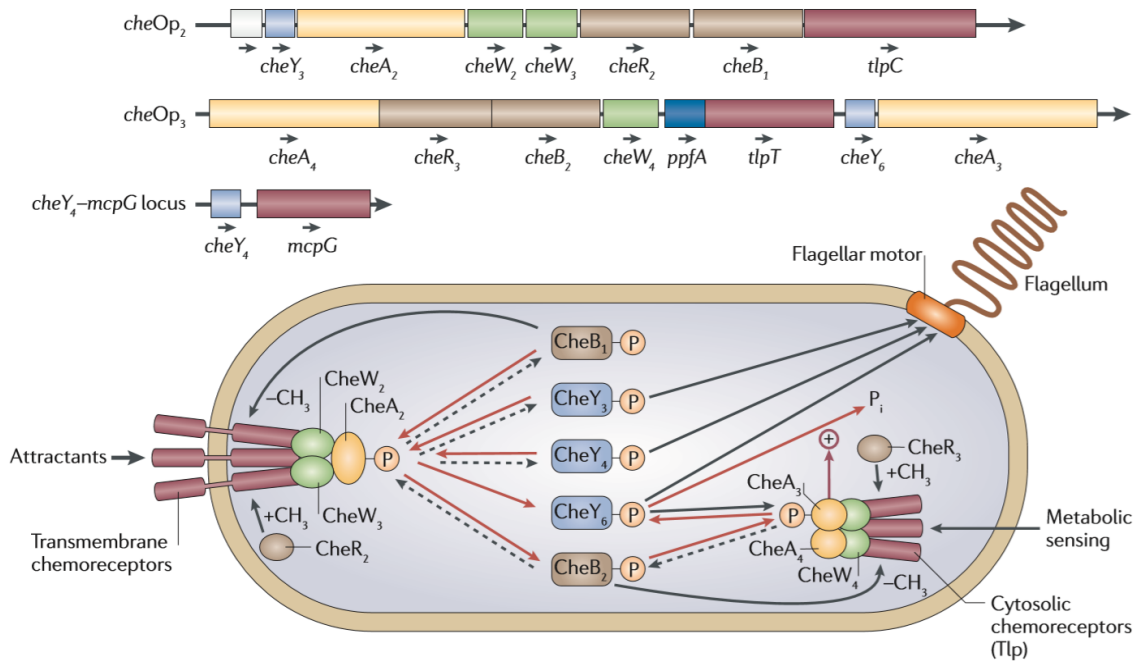


Figure 22: Chemotaxis in *R. sphaeroides*. Two chemotaxis operons, *cheOp2* and *cheOp3*, are expressed to form a transmembrane and a cytoplasmic chemosensory cluster. The polar transmembrane cluster is important for sensing periplasmic ligands concentration; whereas the soluble cytoplasmic cluster is thought to detect the metabolic state of the cell. Response regulators such as CheY₆ are phosphorylated by the histidine kinase CheAs in the clusters to stop rotation of the flagellar motor. The red arrows show the role of CheY₆ as a phosphate sink for the other response regulators during phosphorylation. The dashed arrows show the CheB-mediated phosphorylation reactions that connect the two chemosensory clusters. Figure taken from [101].

It is still unclear how the cell regulate localization of the chemosensory clusters. The mechanisms that control the spatial organization of the receptor clusters and motor within a cell are poorly understood. The localization of clusters to different sites of the cell may be important for cluster segregation during cell division and for preventing direct cross-talk between different chemotaxis pathways. It is also possible that chemosensory cluster is being positioned near the flagellar motor so the rate of signal transduction from the receptor cluster to the flagellar motor is not limited by diffusion.

1.5.2 Response regulator CheY₆

The response regulator CheY₆ is probably one of the best studied proteins in the chemotaxis pathway. In *R. sphaeroides*, CheY₆ and either CheY₃ or CheY₄ are required for chemotaxis [102]. *In vivo* imaging of fluorescent protein fusions and immuno-gold electron microscopy found that CheY₆ is associated with the cytoplasmic cluster and is randomly distributed throughout the cytoplasm [102]. Once it is phosphorylated by the CheA proteins, CheY₆ binds to FliM of the flagellar motor to stop its rotation. The diffusion of CheY₆ can be especially important in *R. sphaeroides* since the response regulators connect the two chemosensory clusters and the flagellar motor that are localized to different regions of the cell.

1.6 Single molecule fluorescence microscopy

Fluorescence-based microscopy techniques have been used to study the diffusion, localization, and molecular dynamics in live cells. This includes macromolecular crowding experiments described in subsection 1.3.1. In a typical experiment, the target protein is labelled with a fluorescent tag such as a fluorescent protein or an organic dye. Genetically encoded fluorescent proteins such as GFP and its derivatives have traditionally been used to tag protein molecules because they do not interact with cellular components.

One of the most commonly used fluorescence techniques is Fluorescence Recovery After Photobleaching (FRAP) microscopy [99][5]. FRAP measures the bulk diffusion of fluorophores by photobleaching a small region of the cell and observing the recovery of fluorescence signal to the bleached region. In this case, rapid fluorescence recovery suggests fast molecular diffusion; whereas slow recovery may be due to slower diffusion due to presence of diffusive barriers, confinement, and / or bound molecules. However, ensemble techniques such as FRAP rely on the average signal from hundreds to thousands of fluorescent proteins and they cannot efficiently differentiate the different diffusion profiles of the fluorophores within the cell. For example, biomolecules are known to exhibit Brownian motion (free or normal diffusion) or non-Brownian (anomalous or confined) motion in the cell. Even though ensemble techniques provide a good signal-to-noise ratio (the intensity of fluorescence signal compared to the background intensity of the same area), it does not allow direct visualization of the complex diffusion modes in the cell.

More recently, single molecule fluorescence microscopy or single molecule tracking has been developed to overcome the limitations of previous ensemble techniques. The single molecule approach is restricted to low densities of fluorophores during imaging to prevent overlap of signals. The technique tracks the location of a single fluorophore over time over the background noise using high precision localization or filtering. The localizations from the consecutive time frames are then linked to form a trajectory if they appear within a tracking window of a certain distance within a cell. The mean-squared displacement (MSD) of the trajectory can be calculated to determine the diffusion modes and apparent

diffusion coefficient, D of the molecule [55][142].

Single molecule tracking allows the study of molecular behaviours in cells but the technique suffers from low signal-to-noise ratio and low photons counts. Cellular autofluorescence and other freely-diffusing fluorophores will contribute to a noisy background beneath the fluorescence signal from a single fluorophore. For instance, photosynthetic bacteria are known to exhibit high BChl autofluorescence [64] which can be problematic for single molecule experiments. To minimize autofluorescence, a prebleaching step in Photoactivated Localization Microscopy (PALM) experiments can be applied before imaging. Brighter fluorescent dyes that can be conjugated to proteins and DNA templates are also created as an alternative of fluorescent proteins.

1.6.1 Photoactivated Localization Microscopy (PALM)

Photoactivated Localization Microscopy (PALM) [52][13] is one of the methods used to acquire single molecule images. PALM relies on photoactivatable fluorescent proteins (PA-FPs) such as PA-mCherry that can be activated from a dark state to a fluorescent state through a short or weak pulse of irradiation. The fluorescence of the activated PA-FPs is then observed using a second, longer wavelength excitation laser. PALM provides the advantage of activating only a small fraction of fluorophores each time (one to two molecules) by adjusting the power of the activation laser. It also ensures homogenous and complete labelling of the target protein in the cell. At the end of the imaging session, a large number of single molecule tracks can be generated within a single cell. By analyzing the trajectories and calculating the diffusion coefficients, the presence of different subpopulations or molecular behaviours can be observed.

However, there are also issues to consider with PALM experiments. First, PA-FPs are bulky molecules and it could affect the diffusion and / or function of the tagged protein. In addition, PA-FPs are less photostable and they photobleach relatively faster than synthetic dye molecules. Several photoactivatable fluorescent dyes have been developed to overcome these limitations.

1.6.2 Electroporation technique using organic dyes

Small organic dyes have been created to replace the bulky fluorescent protein tags for *in vivo* imaging. Organic dyes are more photostable and have a longer fluorescent lifetime [30][119], meaning that they emit more photons before photobleaching. These characteristics are important for single molecule tracking, especially imaging of rapidly diffusing, motion blurred molecules, because fluorescent dyes can produce good signal-to-noise images and longer tracks. However, fluorescent dyes often require extensive *in vitro* labelling and purification procedures.

A novel technique of internalizing organic dye labelled protein molecules *via* electroporation has been developed recently [26]. The target protein with a cysteine residue is overexpressed, purified, and labelled with a maleimide dye. The excess dye is then removed using size exclusion chromatography and the protein-dye conjugate is incubated with electrocompetent cells. An electric field is applied to disrupt the lipid bilayer of the membrane and to internalize the protein into live cells. The electroporated cells are recovered in rich medium and the non-internalized protein was removed by washing before imaging.

Previously, the small *E. coli* CheY (14 kDa) had been labelled with the Atto647 maleimide dye (1 kDa) and electroporated into *E. coli* cells [31]. This shows that the electroporation technique allows the tracking of small fluorescently labelled protein molecules without the bulky fluorescent protein tag. However, electroporation also has its limitations: it is a harsh technique that kills a large percentage of cells. By lowering the voltage of the electric field, the cell viability increases but the internalization efficiency decreases [26]. In addition, blinking of dyes and simultaneous bleaching events may lead to ambiguity during data analysis.

1.7 Aims of project

Crowding studies to date have used stress conditions to change the cytoplasmic state or induce crowding effects, making data interpretation difficult. Therefore, the aims of this study are to investigate whether the packing of intracytoplasmic membrane (ICM) vesicles in unperturbed, photoheterotrophic *Rhodobacter sphaeroides* lead to macromolecular crowding effects that can be detected *in vivo* by a genetically encoded FRET sensor and reflected through the reduction of protein diffusion rates.

First, a comparison of the macromolecular crowding levels in high light and low light photosynthetically grown *R. sphaeroides* were made by:

- *In vitro* bacteriochlorophyll (BChl) extraction to estimate the number of ICM vesicles per cell
- Expression of a FRET probe *in vivo* and calculation of FRET ratios

Second, the diffusion of different sized proteins were measured in *R. sphaeroides* under three different crowding levels by single molecule tracking (Figure 23). When *R. sphaeroides* cells were grown aerobically, it has a 'normal' cytoplasmic structure that is similar to *E. coli*'s; whereas when the cells were grown photosynthetically, the cytoplasm is filled with varying amount ICM vesicles depending on the light intensities. Specifically, I would like to:

- Measure the diffusion of 30 kDa PAmCherry
- Measuring the diffusion of 45 kDa CheY₆ labelled with PAmCherry
- Measuring the diffusion of ribosomes by labelling ribosomal protein L1 with PAmCherry

in aerobic, high light, and low light *R. sphaeroides* using single molecule microscopy. A comparison of the apparent diffusion coefficients was made.

Third, the diffusion of a small CheY₆ (15 kDa) molecule labelled with an organic dye was measured in *E. coli* and aerobic *R. sphaeroides* through the electroporation technique. A modified protocol for the protein purification and electroporation technique has been generated using new cysteine-modified CheY₆ mutants.

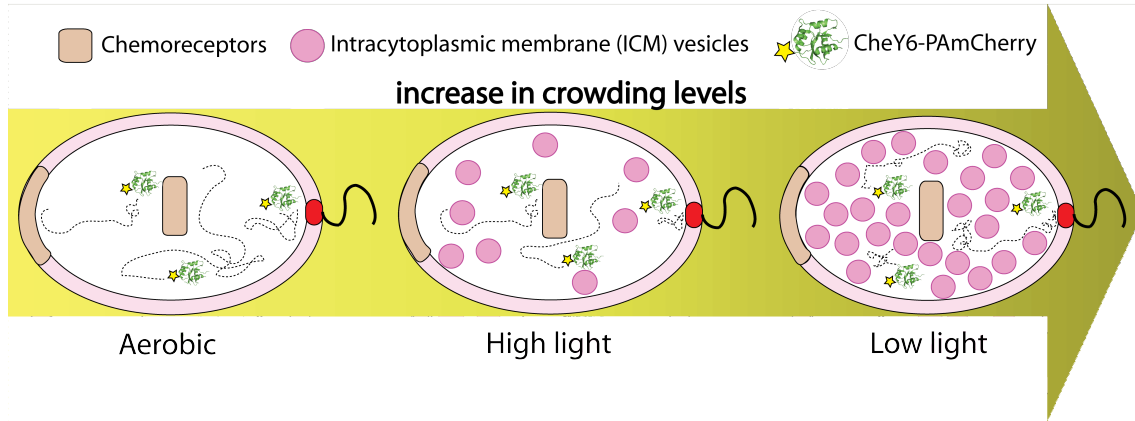


Figure 23: Macromolecular crowding level in *R. sphaeroides* increases following the decrease in light intensity and induction of ICM vesicles.

2 Materials and methods

2.1 Strains, plasmids and primers

The strains, plasmids, and primers used in this study are shown in Table 1, Table 2 and Table 3. Late exponential or stationary phase *E. coli* and *R. sphaeroides* cultures were stored as 20% glycerol stocks at - 80°C.

Table 1: Bacterial strains used in this study.

Strains	Description	Source
<i>E. coli</i>		
RP437	Wildtype for chemotaxis	J S Parkinson
BL21(DE3)	For expression of recombinant protein	Life Technologies
DH5 α	For cloning and storage of plasmids. Nal ^R	(Ind <i>et al.</i> , 2009)
S17	Donor strain for introducing plasmids into <i>R. sphaeroides</i> <i>via</i> conjugation. Sm ^R	(Penfold and Pemberton, 1992)
<i>R. sphaeroides</i>		
WS8N	Spontaneous Nal ^R mutant of wildtype WS8	(Sackett <i>et al.</i> , 1990)
JPA1213	CheY ₆ (D56N) derivative of WS8N	(Porter <i>et al.</i> , 2006)

Table 2: Plasmids used in this study.

Plasmids	Description	Source
pIND4	Inducible expression plasmid for <i>Rhodobacter sphaeroides</i> . Km ^R	(Ind <i>et al.</i> , 2009)
pIND4-PAGFP-Y ₆	Plasmid encoding YFP-CheY ₆ fusion. Km ^R	This study
pIND4-PAmCherry	Plasmid encoding free PAmCherry. Km ^R	This study
pIND4-PAmCherry-Y ₆	Plasmid encoding YFP-CheY ₆ fusion. Km ^R	This study
pIND4-crTC2.1	FRET sensor (mTurquoise2.1-mCitrine) cloned into pIND4	This study
pIND4-YFP-Y ₆	Plasmid encoding YFP-CheY ₆ fusion. Km ^R	This study
pK18 <i>mobsacB</i>	Suicide vector for allelic exchange. Km ^R	(Schafer <i>et al.</i> , 1994)
pQE30	Expression vector. Amp ^R	Qiagen
pQE80-CheY ₆ (G3C)	CheY ₆ with 6xHis-tag at N terminus, Cys inserted	This study
pQE80-CheY ₆ (S4C)	CheY ₆ with 6xHis-tag at N terminus, Cys inserted	This study
pQE80-Y ₆ (A134C)	CheY ₆ with A134C point mutation	M. W. Smith
pFLOAT-YNC-His	CheY with Cys at N terminus, 6xHis-tag at C terminus	D. Di Paolo
pRSET A-crTC2	Plasmid encoding FRET sensor (mTurquoise2-mCitrine) for crowding quantification	(Liu <i>et al.</i> , 2018)
pIND4-L1-PAmCherry	Plasmid encoding L1-PAmCherry fusion	This study

Table 3: Primers used in this study.

Primers	Sequence (5' to 3')	Description	Restriction site
JK001-F	CGCGCCATGGTGAGCAAGGGCG AGGAGC	PAGFP/YFP	NcoI
JK002-R	CGCGAAGCTTTTACTTGTACAGC TCGTCCATGCCG	PAGFP	HindIII
SG001-F	TGTACCATGGTGAGCAAGGGCG AGGAG	PAmCherry	NcoI
SG002-R	ACGTAAGCTTACTTGTACAGCT CGTCCATGCCG	PAmCherry	HindIII
SG003-F	AATAGGGGTTCCGCGCACATTT CC	Colony PCR in pIND4	-
SG004-R	GGCGGGCAAGAATGTGAATAAA GGCC	Colony PCR in pIND4	-
JK005	AGGAGAAATTAACCTATGAGATGC TCGCATCACCATCACCAT	CheY ₆ cys insertion (G3C)	-
JK011	ATGGTGATGGTGATGCGAGCATC TCATAGTTAATTTCTCCT	CheY ₆ cys insertion (G3C)	-
JK0013-R	AATACGGATCCTCAGGCGGCCAT CAGCGTC	YFP-CheY ₆	BamHI
JK0016-F	GAGGAGGCGCGCGCGCATGCCCT ACAATGTCATGATC	CheY ₆	BseRI
JK0031-F	CGCGTCATGATGCATCATCACCA TCAT	FRET probe	BspHI
JK0032-R	GGATCAAGCTTTTATTTGTACAG CTC	FRET probe	HindIII
JK0033-F	GCGGGATCCGCGGCGTCGTCAC CCTGCCG	Upstream of L1	BamHI
JK0034-R	GCGTCTAGAGTGGCTGGTGGCG GAGGTCAGGTC	Upstream of L1	XbaI
JK0035-F	GCGGTCGACTGAGGCGGGGGCT TTGAGAATTC	Downstream of L1	SalI
JK0036-R	GCGGCATGCGCAACCTTCGCCG CAGCGACG	Downstream of L1	SphI
JK0037-F	GCGTCTAGAGTGAGCAAGGGCG AGGAGGATAAC	PAmCherry -L1	XbaI
JK0038-R	GCGGTCGACTTACTTGTACAGCT CGTCCATGCCGCCGGTGGAG	PAmCherry -L1	SalI
JK0039-F	GCGTCTAGAGTGAGCAAGGGCG AGGAGCTG	YFP for L1 labelling	XbaI
JK0039-R	GCGGTCGACTTACTTGTACAGC TCGTCCATG	YFP for L1 labelling	SalI

2.2 Growth conditions

R. sphaeroides cells were grown in succinate medium (SUX) [121] at 30 °C, either aerobically with 225 rpm shaking, or photoheterotrophically in sealed eppendorfs or medical flats under constant illumination. A tungsten lamp (Crompton Lamps, 120 W / 2600 K warm white, PAR38) and an LED lamp (Luceco Guardian Slimline, 22 W - 300 W / 5000 K day light) were used to provide the light source in the light cabinet. The light intensity was measured by a light meter (Skype, SKP 200) and the temperature in the light cabinet was kept at around 30 °C. Photoheterotrophic cells were grown at either low light ($15 \mu\text{mol photons } m^{-2}s^{-1}$) or high light ($800 \mu\text{mol photons } m^{-2}s^{-1}$).

E. coli cells were grown at 37 °C in Luria-Bertani (LB) medium with 225 rpm shaking.

2.3 Antibiotics

Table 4: Antibiotic concentrations used in this work.

Antibiotic	Concentration for <i>E. coli</i> ($\mu\text{g} / \text{mL}$)	Concentration for <i>R. sphaeroides</i> ($\mu\text{g} / \text{mL}$)
Nalidixic Acid	-	20
Kanamycin	25	25
Ampicillin	100	100
Chloramphenicol	30	30
Rifampicin	50	50
Cephalexin	10	10

2.4 BChl extraction using acetone / methanol

1 mL of *R. sphaeroides* culture grown to the appropriate OD was harvested by 13,300 xg for 2 minutes. The pellet was resuspended in 1 mL of acetone / methanol (7:2 by volume) and centrifuged at 13,300 xg for 3 minutes. The supernatant containing the extracted pigments was collected and absorbance at 770 nm was measured. The BChl content was

determined by using the extinction coefficient of $0.076 \mu\text{M}^{-1} \text{ cm}^{-1}$ (refer to the FRET chapter).

2.5 Swim plates

R. sphaeroides strains carrying the appropriate plasmids were grown photoheterotrophically in the light cabinet to stationary phase. 0.25% agar swim plates were made in M22 minimal media with 100 μM attractants (propionate in MiliQ water, 0.22 μm filtered) and IPTG at 0, 10, 100, 1000, or 10000 μM . The plates were inoculated with 5 μL of stationary phase culture as a single drop and incubated at 30°C. Each set was carried out in triplicate and the swarm diameters were measured after 48 hours.

2.6 Genetics techniques

2.6.1 Plasmid extraction

E. coli cultures containing the appropriate plasmids were grown in LB with antibiotics at 37°C until stationary stage. Plasmid DNA was extracted using Miniprep Kit (Qiagen) and stored at -20 °C.

2.6.2 Polymerase Chain Reaction (PCR)

PCR reactions for amplification of DNA were carried out using Phusion High-Fidelity DNA Polymerase (New England Biolabs) and the appropriate primers according to the protocols in Table 5.

Table 5: Components of PCR reaction mixture

Component	Quantity (μ L)
template DNA (< 250 ng)	-
dNTPs (10 mM)	5
DMSO	2.5
Forward primer (10 μ M)	2.5
Reverse primer (10 μ M)	2.5
5 $\times$ Phusion HF buffer	10
Phusion High-Fidelity DNA polymerase	0.5
MiliQ	to 50

PCR reaction mixtures were then run in a thermocycler according to the conditions stated in Table 6. For colony PCR, template DNA was obtained by boiling an isolated bacterial colony in water at 100°C for 10 minutes and collecting the supernatant.

Table 6: Thermocycling conditions

Step	Temperature ($^{\circ}$ C)	Time
Initial denaturation	98	30 seconds
(30 cycles)		
Denaturation	98	10 seconds
Annealing	72	15 - 30 seconds / kb
Extension	72	1 minute
Final extension	72	5 minutes
Hold	4	indefinite

2.6.3 Restriction digest

PCR products were digested with restriction enzymes and buffers from New England Biolabs. Restriction digest were performed using two restriction enzymes simultaneously (double digest) at 37 $^{\circ}$ C for 1 hour followed by a heat inactivation step. The final concentration for glycerol in the reaction mixture should be less than 5% to minimize star activity.

Table 7: Composition of restriction digest mixtures for plasmids and DNA inserts.

Component	Vector Quantity (μL)	DNA insert Quantity (μL)
DNA	5	20
Buffer	2	5
Enzyme	1	1
Enzyme	1	1
Water	to 20	to 50

2.6.4 Agarose gel electrophoresis

DNA fragments were analyzed and separated using agarose gel electrophoresis. 1% agarose gel was made using $0.5\times$ TBE buffer and stained with SyBr Safe. DNA samples added with DNA loading dye and 1kb plus DNA ladder (Invitrogen) were loaded on the agarose gel and at 120 V for 45 minutes.

2.6.5 Purification of DNA fragments

DNA fragments were purified using PCR Cleanup Kit (Qiagen) or were extracted from agarose gels using the Gel Extraction Kit (Qiagen).

2.6.6 DNA ligation

Ligation reactions were performed in a final volume of 50 μL using T4 DNA ligase (New England Biolabs). The volume of vector DNA and DNA insert are adjusted according to their concentrations to fit the molar ratio of 3:1.

Table 8: Ligation

Component	Quantity (μL)
Vector DNA	-
DNA insert	-
T4 ligase	3
$5\times$ buffer	3
MiliQ	to 30

2.6.7 Plasmid DNA transformation

Plasmid DNA was added to 50 μ L chemically competent *E. coli* cells and incubated on ice for 30 minutes. Cells were heat shocked at 42 °C for 2 minutes and incubated on ice for 5 minutes. 950 μ L SOC was added and the cells were incubated at 37 °C for 1 hour with constant 225 rpm shaking. The recovered cells (100 μ L) were plated on LB agar plates with the appropriate antibiotics and incubated at 37 °C overnight.

2.6.8 Site directed mutagenesis

Site directed mutagenesis was performed with the appropriate primers using the Q5 Site Directed Mutagenesis kit (New England Biolabs) according to its protocol.

2.6.9 Preparing electrocompetent *E. coli*

4 mL of stationary *E. coli* culture was added to 500 mL of Tryptone Broth (TB) and grown to OD₆₀₀ 0.35 - 0.4. The culture was cooled to 4 °C for 15 - 30 minutes and centrifuged at 3000 rpm (1000 xg) for 15 minutes. The pellet was resuspended in 500 mL ice cold water and centrifuged at 3000 rpm for 20 minutes. The pellet was then resuspended in 250 mL and 10 mL 10% glycerol and harvested by 3000 rpm for 20 minutes each time. Finally, the pellet was resuspended in 1 mL ice cold GYT medium and flash frozen in liquid nitrogen.

2.6.10 Preparing electrocompetent *R. sphaeroides*

Stationary *R. sphaeroides* was added to 500 mL SUX medium in a ratio of 1:4,000 and incubated at 30 °C overnight with 225 rpm shaking. When the OD₇₀₀ reached 0.4, the culture was cooled to 4 °C for 15 minutes. The cells were harvested by 1,000 xg at 4 °C for 10 minutes. The pellet was washed with 500 mL, 250 mL, and 125 mL ice cold water and centrifuged at 1,000 xg for 10 minutes each time. The pellet was then resuspended in 10 mL 10% glycerol and harvested by 1,000 xg for 10 minutes. Finally, the pellet was resuspended in 1 mL 10% glycerol and flash froze in liquid nitrogen.

2.6.11 Conjugations

Plasmids were introduced into *R. sphaeroides* via conjugation with *E. coli* S17 donor strain. S17 cells with targeted plasmid were grown to early exponential phase and incubated with stationary *R. sphaeroides* in a ratio of 1:10. The mixture was placed onto a nitrocellulose filter on LB plate and incubated at 30 °C overnight. The filter was then vortexed vigorously in 800 μ L LB to wash off the cells. 100 μ L culture was spread onto LB plates containing antibiotics to select for *R. sphaeroides* with the plasmid.

2.6.12 DNA Sequencing

DNA sequencing was performed using the Snager sequencing service from Source Bioscience and SnapGene was used for analysis.

2.7 Protein expression and purifications

2.7.1 Overexpression of CheY₆

E. coli BL21(ED3) cells containing the plasmid pQE80-CheY₆ were grown in 2X TY rich medium at 37 °C with 225 rpm shaking until mid exponential phase. IPTG was added to a final concentration of 0.5 mM and the cells were incubated at 18 °C with 225 rpm shaking overnight. After induction, the cells were harvested by 7000 xg centrifugation at 4 °C for 20 minutes. Cell pellet was resuspended in 40 mL lysis buffer with protease inhibitor and 1 mM DTT. The cells were then lysed by sonication (60% amplitude, 4 minutes sonication time, 5 seconds on, 15 seconds off). Lysate was centrifuged at 35,000 xg at 4 °C for 45 minutes and filtered to remove insoluble cell debris. As the targeted protein contained a His-tag, cleared lysate was applied to a Nickel-NTA column and the protein was eluted using elution buffer. When necessary, eluted protein samples were concentrated using protein concentrators.

2.7.2 SDS-PAGE

20 μ L protein samples were added with 6 μ L 4 $\times$ LDS loading buffer and 4 μ L of β -mercaptoethanol. The samples were boiled at 100 °C for 10 minutes and loaded on Expedeon RunBlueTM Bis-Tris protein gels. The gels were ran at constant voltage of 180 V for 45 minutes and stained with coomassie blue.

2.8 Protein labelling and analysis

2.8.1 Dye storage

Maleimide dyes were dissolved in DMSO and stored as 20 mM aliquots at -80 °C, away from light and moisture.

2.8.2 Protein-dye conjugation

Before labelling, pure protein aliquot was buffer exchanged into labelling buffer containing 0.1 mM TCEP and concentrated to a final concentration of 200 μ M. Labelling buffer has a pH that is optimal for maleimide dye labelling (around pH 7.5) and TCEP is a reducing agent that prevents protein aggregation by cysteine residues. The addition of TCEP will not inhibit the labelling process.

200 μ L of 200 μ M protein sample aliquot was diluted with labelling buffer to a reaction volume of 2 mL and a final concentration of 20 μ M. 10 μ L of 20 μ M dye resuspended in DMSO was added to the reaction sample drop by drop with gentle mixing. For optimal labelling, the concentration of dye should be about 5 - 10 $\times$ molar excess of the protein sample. The percentage of DMSO in the reaction sample should always be kept under 4 % vol / vol. The conjugation reaction was allowed to proceed in the dark at room temperature for 1 hour before adding 20 mM DTT to quench the reaction.

2.8.3 Free dye removal

Nickel column and size exclusion chromatograph were used to remove the excess free dye from the labelled protein.

A nickel affinity chromatography column was prepared by pouring 200 μ L of Ni-NTA agarose slurry (brand) into a drip column. The column was allowed to settle for 10 minutes and was equilibrated with 10 mL labelling buffer containing 1 mM DTT. The protein-dye conjugate was applied to the Ni-NTA column and wash with 50 mL buffer containing 1 mM DTT (at least 30 column volume). The labelled protein was eluted with

around 600 μL of elution buffer containing 1 mM DTT.

2.8.4 Size exclusion chromatograph

The sample was loaded onto a HiLoad 16/600 Superdex 75 pg column (GE Healthcare) through a 1 mL sample loop. The column was attached to an AKTA system and a UV detector with the correct excitation and emission wavelengths. When iFluor633 maleimide was used, the excitation was 638 nm and emission was 655 nm. The sensitivity and gain settings were adjusted to 2. The system was supplied with electroporation buffer added with TCEP at a constant flow rate of 0.8 mL / minute. The eluted protein fraction was buffer exchanged from elution buffer into electroporation buffer. The purified elutions were collected using a 96-well sample collector and concentrated to a final volume of 500 μL . The protein fractions were stored in small aliquots at $-80\text{ }^{\circ}\text{C}$, away from light and moisture.

2.8.5 Labelling yield

The purified protein-dye conjugate was loaded on Expedeon RunBlueTM Bis-Tris protein gels with a transparent loading buffer. The gels were ran at constant voltage of 180 V for 45 minutes and scanned for fluorescence using a UV-Vis Pharos Molecular Imager (BioRad). The fluorescence of labelled protein versus free dye was analyzed using ImageJ.

Labelling yield was also quantified by measuring the absorption of protein and dye using a NanoDrop. The molar concentration of labelled protein was calculated using the degree of labelling (DoL) formula (refer to Electroporation chapter).

2.9 Electroporation

Labelled protein samples were added to 20 μL of electrocompetent cells in a pre-chilled 1.0 mm gap electroporation cuvette. The cuvette was placed in a Bio-Rad Electroporator and an electric field pulse ranges from 1.2 kV / cm to 1.8 kV / cm was applied. 500 μL of SUX medium or SOC medium was immediately added to *R. sphaeroides* or *E. coli* cells respectively. Electroporated cells were recovered at 30 °C for 10 minutes or longer with 350 rpm shaking.

After recovery, the cells were collected by centrifugation at 7,000 rpm for 3 minutes and washed with SUX medium for *R. sphaeroides* or MB for *E. coli* to remove non-internalized proteins. The pellets were then resuspended in 500 μL buffer. A 1:5 dilution was made by taking 100 μL of resuspended cells and adding with 400 μL buffer to reach an OD₆₀₀ of 0.5. The resuspended cells were then washed 3 times with 500 μL buffer using 0.22 μm Ultrafree-MC Centrifugal Filter (Merck) with hydrophilic PVDF and 0.5 mL volume. A final spin for 1 minute was used to remove any residual buffer. The washed cells were resuspended in 50 μL buffer and placed on 1% agarose pads with a burnt coverslip for imaging.

2.10 Circular dichroism

Protein sample was first dialyzed in 600mL CD buffer for 4 hours at 4 °C and then in fresh 600mL buffer overnight. CD was performed using a Jasco J-815 Spectropolarimeter and a 1mm path length quartz cuvette. The system was purged with 5 L/min nitrogen for 5 minutes before measurement. A spectrum of 190 – 250 nm was taken at a scanning speed of 100 nm/min for a total of 9 scans. The final spectrum was corrected by subtracting the baseline (blank). Circular dichroism traces of the protein mutants were compared to the wild type spectrum. The presence of alpha-helices in these proteins showed that the proteins were folded.

2.11 Agarose pads preparation

1% agarose pads were made in SUX medium for *R. sphaeroides* and motility buffer (MB) for *E. coli*. Around 50 μL of agarose was pipetted within a 1×1 cm Gene Frame (Thermo Scientific) adhered to a microscope slide. The Gene Frames have two adhesive sides that can stick to the slide and coverslip to hold the sample in place. A clean coverslip was placed on top to flatten the agarose layer and the agarose was left to dry for around 10 minutes. 2 μL of cells were pipetted onto the pad and a burnt coverslip was placed on top of the cells onto the adhesive end of the Gene Frame. Coverslips used for fluorescence microscopy imaging were burned in furnace to remove fluorescence impurities or dirt.

2.12 Tunnel slides

Tunnel slides were prepared for organic dyes fluorescence studies. Two strips of Scotch double-sided tape were adhered to a microscope slide and a burnt coverslip was placed on top of the tape, forming a narrow tunnel. A pipette tip was used to gently tap and stick down the coverslip (avoiding the tunnel area) and excess tape was removed. Buffer solutions were flushed through the tunnel *via* capillary action by pipetting and using Kimwipes (Kimtech Science) at the other end of the tunnel. Diluted dye sample was applied to the tunnel after the washing steps and the open ends of the tunnel were sealed using nail varnish to prevent evaporation.

2.13 Fluorescence microscopy

2.13.1 FRET

R. sphaeroides cultures containing the FRET sensor on pIND4 were grown photoheterotrophically under high and low light as described above in the presence of 1 mM IPTG. A negative control was included with each FRET experiment, using a strain containing an empty pIND4 vector. Chloramphenicol was added at $30 \mu\text{g mL}^{-1}$ to inhibit protein synthesis and the culture was incubated at 30°C with rigorous shaking for 30 minutes to allow proper folding and maturation of the remaining immature fluorescent protein.

The cells were washed with SUX medium and placed on agarose pads and imaged using a laser scanning confocal microscope (Zeiss 780). A 405 nm laser was used to excite the donor protein. The emission of the donor and acceptor proteins were split into two channels: 449 - 506 nm and 508 - 600 nm and the fluorescence in both channels were analyzed using ImageJ. Exposure time was 30 ms.

2.13.2 PALM

R. sphaeroides cultures containing the appropriate PAmCherry fusions on pIND4 were grown photoheterotrophically under high and low light as described above in the presence of 1 mM IPTG. A negative control was included with each PALM experiment, using a strain containing an empty pIND4 vector. The cells were placed on agarose pads and imaged on a custom-built PALM/TIRF microscope at Micron Advanced Imaging Consortium, University of Oxford. The microscope was equipped with an Andor iXon 897 ultra electron multiplying CCD (EMCCD) camera and a Toptica Multi Laser Engine with 405 nm, 488 nm, 561 nm, and 640 nm lasers.

Bright field images of the targeted cells from a 64×64 pixels field of view (FOV) were recorded before imaging. The 564 nm laser was used to bleach the targeted cells until the autofluorescence was minimized. After the prebleach step, the cells were imaged with 405 nm ($336 \mu\text{W}$) laser for photoactivation and 70% (9.5 mW) 564 nm laser for excitation of

PAmCherry. The intensity of the 405 nm laser was increased gradually as the PAmCherry molecules in the cells started to photobleach over time. Around 6 movies (800 frames each) were recorded for each FOV. Exposure time was 7 ms and a cycle time was 7.75 ms. PAmCherry filter sets ET600/50 (575 - 625 nm) was used.

2.13.3 Data analysis using Stormtracker

Single molecule analysis for the PALM experiment was performed using the automated tracking analysis software Stormtracker [142] (modified by Dr. Matthew Stracy and Dr. Helen Miller). Fluorescent spots were localized through a self-defined threshold of 20 - 40 depending on the signal-to-noise ratio. The localization threshold indicates the minimum intensity of the detected pixel divided by the standard deviation of the background [142]. A tracking window of 8 pixels (pixel size 96 nm) was selected to link 5 localizations together to produce 4-step trajectories. The final apparent diffusion coefficient was calculated from the mean squared displacement (MSD) formula:

$$MSD = \frac{1}{N-1} \sum_{i=1}^{N-1} [(x_{i+1} - x_i)^2 + (y_{i+1} - y_i)^2]$$

where N is the number of localizations [142] and $\Delta t = 7.75$ ms. If autofluorescence molecules were observed in the negative control, all of the imaging data for the same session was discarded.

2.13.4 Electroporation

The microscopy samples were prepared at the same time and imaged immediately in the order of electroporated (EP), non-electroporated (NEP), and EMPTY on a custom-built fluorescence microscope from the Berry Group, University of Oxford. The microscope was equipped with multiple illumination modalities and a high sensitivity and high speed camera; EMCCD Andor iXon 888, and had a pixel size of 51.5 nm. If autofluorescence molecules were observed in the negative control (EMPTY), all of the imaging data for

the same session was discarded.

3 Quantifying crowding levels in photoheterotrophic

R. sphaeroides

R. sphaeroides develops invaginations of intracytoplasmic membrane (ICM) vesicles in the cytoplasm when the bacterium is grown photoheterotrophically. Crowding induced by varying number of ICM vesicles in *R. sphaeroides* grown under high and low light was measured using bacteriochlorophyll (BChl) extraction and a FRET sensor. Acetone/methanol extraction was performed on *R. sphaeroides* grown under low and high light intensities to estimate the BChl concentration and number of vesicles in each cell, assuming that the amount of BChl per vesicle remained constant under different light intensities.

A genetically encoded FRET sensor was also used to quantify crowding in *R. sphaeroides* *in vivo*. IPTG inducible plasmid pIND4 containing the FRET sensor was introduced into *R. sphaeroides* and the cells were grown photoheterotrophically with IPTG induction. The crowding level in single cells expressing the FRET probe was measured by calculating the ratiometric FRET ratio. A higher FRET ratio was observed in low light cells that contained approximately 3 times more vesicles as compared to high light cells.

3.1 Quantification of the number of cells in liquid culture at

OD₇₀₀

Wild type *R. sphaeroides* (WS8N) was grown anaerobically under high light (800 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$) and low light (15 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$) using tungsten and LED lamps in SUX medium (see Materials and methods). The number of photoheterotrophic *R. sphaeroides* cells in SUX culture was determined by counting the colony forming units (CFUs) from cell plating experiments. In this study, the optical density of *R. sphaeroides* cells was measured at 700 nm, to avoid artefacts caused by absorbance peaks of photosynthetic pigments in photoheterotrophic *R. sphaeroides* [88][23]. Serial dilutions

from fresh growing *R. sphaeroides* cultures were made with M22 at different OD₇₀₀ units and plated on LB agar. Measurements from at least four independent cultures were plotted in Figure 24 and fitted linearly using least-squares regression.

The number of cells per OD₇₀₀ unit for both high light and low light *R. sphaeroides* were similar: one OD₇₀₀ unit contained approximately 8.0×10^8 high light cells ($R^2=0.8442$) and 1.0×10^9 low light cells ($R^2=0.883$) respectively. It is possible that the higher turbidity of high light cells is caused by the larger size of high light cells compared to low light cells.

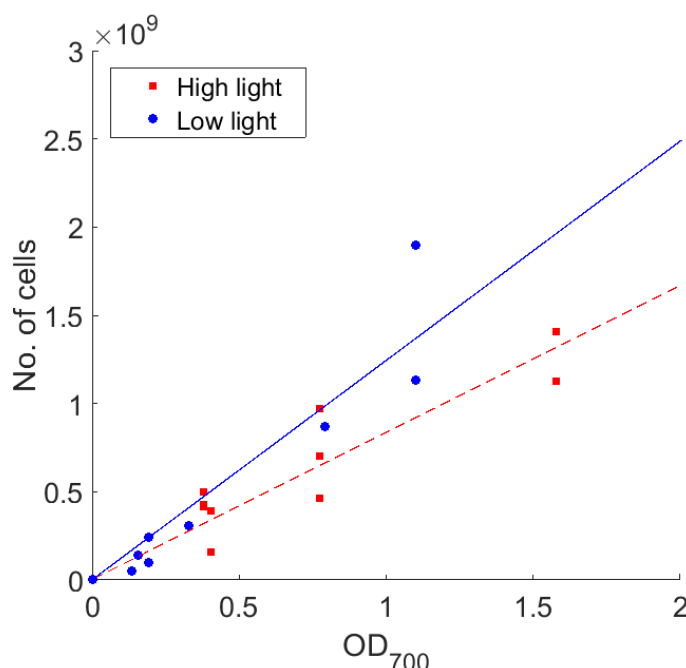


Figure 24: Number of photoheterotrophic *R. sphaeroides* cells per OD₇₀₀ unit. CFUs per mL at different OD₇₀₀ measurements of high light and low light cells were fitted with linear best-fit lines using Microsoft Excel. Low light cells gave a conversion factor of 1.0×10^9 cells / OD_{700nm} unit, $R^2=0.883$, while high light cells gave a conversion factor of 8.0×10^8 cells / OD₇₀₀ unit, $R^2=0.8442$.

3.2 Quantification of ICM vesicles *via* BChl extraction

The number of vesicles in photoheterotrophic *R. sphaeroides* cells was calculated using acetone/methanol extraction (see Materials and methods) and number of cells per OD₇₀₀ unit conversion factors calculated from section 3.1. The absorbance of extracted pig-

ments was measured at 770 nm and the BChl concentration was determined by using the extinction coefficient of $0.076 \mu\text{M}^{-1} \text{ cm}^{-1}$ [20]. BChl concentration provides a good estimate to the number of vesicles because most of the BChl molecules are incorporated into the intracytoplasmic membrane with only a small unbound, cytoplasmic fraction [156]. Previous models estimated that each ICM vesicle contained around 3879 to 4464 bacteriochlorophyll molecules [118]. In this study, the number of vesicles per cell was determined by assuming each vesicle has an average of 4000 BChl molecules. Table 9 provides a detailed example on vesicles quantification.

Table 9: Quantification of ICM vesicles per cell. Table 9 demonstrates an example of estimating the number of vesicles and BChl content in low light and high light cells.

	Low light	High light	Descriptions
OD ₇₀₀	0.67	0.514	Absorbance of <i>R. sphaeroides</i> cells at 700 nm
No. of cells / mL	6.70×10^8	4.11×10^8	No. of cells calculated using conversion factors from cell plating experiments in section 3.1
BChl extract at OD ₇₇₀	0.4	0.102	Absorbance of extracted BChl at at OD ₇₇₀ using 7:2 acetone/methanol
BChl ($\mu\text{mol} / \text{L}$)	5.26	1.34	Calculated using extinction coefficient of $0.076 \mu\text{M}^{-1} \text{ cm}^{-1}$ [20]
BChl (molecules / mL)	3.17×10^{15}	8.08×10^{14}	Calculated using Avogadro constant of $6.022 \times 10^{23} \text{ mol}^{-1}$
BChl (molecules / cell)	4.73×10^6	1.97×10^6	Calculated by dividing the no. of BChl molecules / mL by the no. of cells / mL
No. of vesicles / cell	1183	491	No. of vesicles per cell could be estimated by assuming each ICM vesicle contains about 4000 BChl molecules [118]

Development of ICM vesicles in *R. sphaeroides* is inversely correlated to light intensity [1][23]. Previous study using transmission electron microscopy (TEM) and acetone/methanol extraction found that low light *R. sphaeroides* grown at $20 \mu\text{mol photons } m^{-2}s^{-1}$ had almost five times as many vesicles as high light cells grown at $1100 \mu\text{mol photons } m^{-2}s^{-1}$.

tons $m^{-2}s^{-1}$ [2]. Therefore, the number of vesicles in photoheterotrophic *R. sphaeroides* cells may also increase at high OD₇₀₀ units due to self-shading effects [122][23]. BChl extraction performed on cells taken from fresh growing *R. sphaeroides* cultures at different OD₇₀₀ units showed that low light cells had a higher number of vesicles when grown to high OD₇₀₀ units (Figure 25). However, the number of vesicles in high light cells remained constant over increasing OD₇₀₀ units. This observation could be explained by the use of extremely high light intensity in this study ($800 \mu\text{mol photons } m^{-2}s^{-1}$) in comparison to the bacterium's natural habitat where light is limited [97]. If light intensity reaches a saturating level, it is possible that the motile bacterium is able to capture sufficient light for growth even at high OD₇₀₀ by generating a minimum number of vesicles. This also agrees with previous study that found the effects of self-shading to be less significant when the BChl concentration is lower than $5 \mu\text{g} / \text{mL}$ [122] [156]. The BChl concentration of high light *R. sphaeroides* in this study was in the range of 0.4 to $3.4 \mu\text{g} / \text{mL}$ (molecular weight of BChl is $911.5 \text{ g} / \text{mol}$).

On the other hand, the low light condition in this study is closer to the natural environment of photosynthetic bacteria such as *R. sphaeroides*. *R. sphaeroides* and many other purple non-sulfur bacteria can be isolated from stagnant ponds, lake water, mud, or sewage [145][77][120]. Light penetration in lakes is low in general due to the colour of lake water and the presence of particulate material [97]. It is likely that *R. sphaeroides* made adaptations to survive at low light intensity (less than $15 \mu\text{mol photons } m^{-2}s^{-1}$) by developing large number of vesicles and BChl content to maximize light absorption.

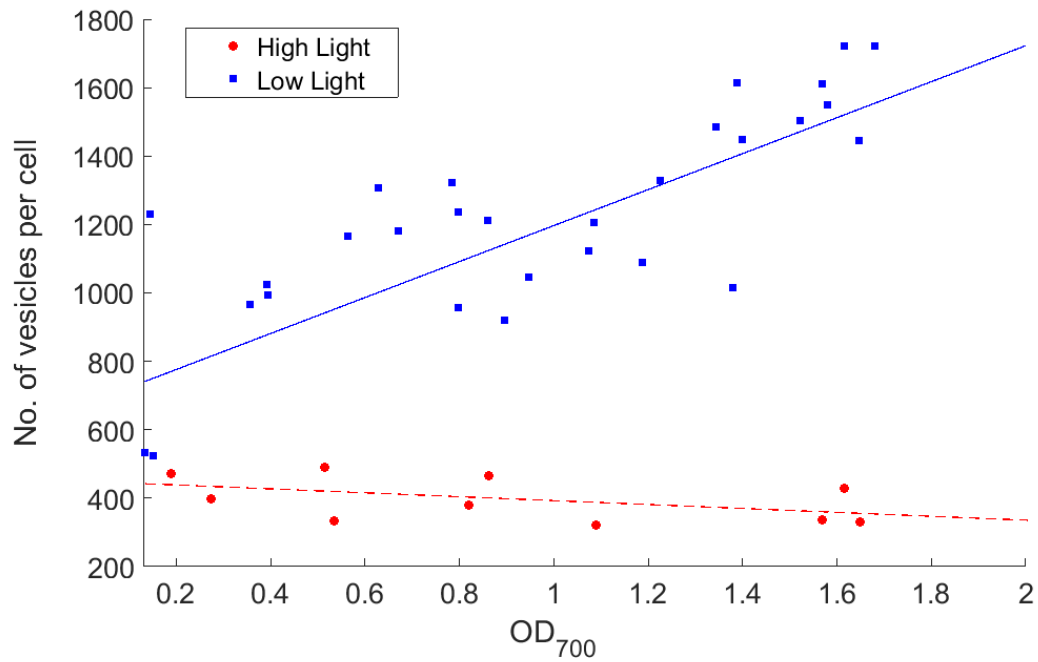


Figure 25: Number of vesicles in photoheterotrophic *R. sphaeroides* decreased at increased light intensity.

Overall, low light cells had approximately three to four times more vesicles than high light cells (Figure 25). The values calculated in this study were comparable to that of previous study [2] where the cells were grown at similar ‘low’ and ‘high’ light intensities. Slight variations could be attributed to the differences in experimental setup such as light intensities, sources of light, spectrophotometer model, and growth stage.

3.3 FRET sensor for quantifying crowding *in vivo*

Boersma *et al.* developed a genetically encoded fluorescence resonance energy transfer (FRET) probe to investigate effects of macromolecular crowding in osmotically stressed *E. coli* cells [14][72]. The original sensor crGE consists of a CFP - YFP (mCerulean3 - mCitrine) pair connected by a flexible linker with repeating regions of (EAAAK)₆ helices and (GSG)₆ coils (Figure 26) [14][71]. When the probe was placed in crowded *in vitro* and *in vivo* environments, it underwent compression and adapted a more condensed conformation. The full protein sequence of the linker is shown below:

$$(CFP)(GSG)_6A(EAAAK)_6A(GSG)_6A(EAAAK)_6A(GSG)_6(YFP)$$

The crGE probe was expressed in bacterial and mammalian cells to detect changes in excluded volume as an effect of osmotic upshift [14]. The probe also responded to compression *in vitro* by various macromolecular crowders such as Ficoll, PEG, and BSA [14][71]. In this study, an improved version of the FRET probe (crTC2, obtained from Prof. B. Poolman) consisting of a donor and an acceptor protein, mTurquoise2 and mCitrine [73] was used in all experiments.

To determine the effects of crowding induced by vesicles, the FRET probe was cloned onto inducible pIND4 plasmid and expressed in photoheterotrophic *R. sphaeroides*. *R. sphaeroides* cells were grown anaerobically under high light and low light light condition with 1 mM IPTG induction. When the cells reached exponential phase, chloramphenicol was added at 30 $\mu\text{g mL}^{-1}$ to inhibit protein synthesis and the culture was incubated at 30°C with rigorous shaking for 30 minutes to allow proper folding and maturation of the remaining immature fluorescent protein. Fluorescence microscopy showed that fluorescence signal was evenly distributed in the cytoplasm and the FRET probe was functional in living *R. sphaeroides* cells (Figure 26).

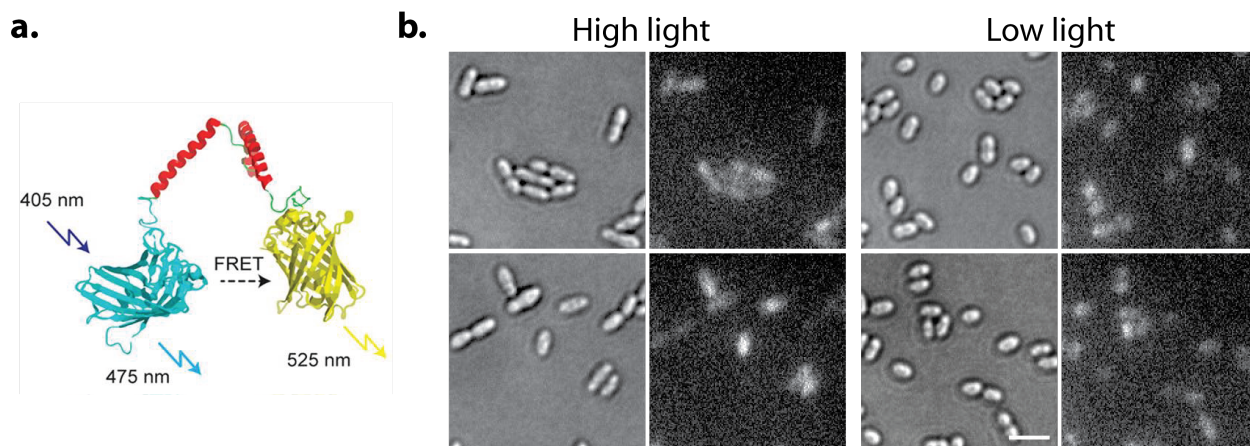


Figure 26: **a.** FRET probe (crGE, image adapted from [73]). **b.** Examples of the homogeneous distribution of FRET signal from the acceptor (500 - 550 nm) in *R. sphaeroides* when the FRET probe was excited using 405 nm laser. Exposure time, 30 ms. Scale bar, 3 μ m

Using a laser scanning confocal microscope (Zeiss 780), the emission of acceptor protein (508 - 600 nm) and donor protein (449 - 506 nm) in live cells were recorded upon excitation of mTurquoise2 using a 405 nm laser (Figure 27). The fluorescence intensity for each cell in both channels was determined using ImageJ. In high light cells ($n = 633$), the fluorescence signal was 1286 ± 27 a.u. (mean $\pm$ standard error of the mean (SEM)) in the donor channel and 678 ± 9 a.u. in the acceptor channel; whereas in low light cells ($n = 768$), the mean $\pm$ SEM was 875 ± 19 a.u. in the donor channel and 530 ± 8 a.u. in the acceptor channel (Table 10). The higher donor and acceptor fluorescence in high light cells suggested that the FRET probe was being expressed at a higher level under high light condition even though both conditions were induced with equal concentration of IPTG. This is not too surprising because gene expression in *R. sphaeroides* is affected by light intensity[107].

Since photosynthetic bacteria were known to emit high level of autofluorescence [4][64], wild type *R. sphaeroides* cells with an empty pIND4 plasmid were also imaged under the same condition. In this study, the autofluorescence of negative control cells was much lower than the signals of cells containing FRET probe (Figure 27). High light negative control cells ($n = 336$) gave 335 ± 3 a.u. (mean $\pm$ SEM) in the donor channel and 309

± 2 a.u. in the acceptor channel (Table 10). Low light negative control cells ($n = 366$) gave 333 ± 3 a.u. (mean $\pm$ SEM) in the donor channel and 305 ± 2 a.u. in the acceptor channel (Table 10). The fluorescence intensity of the probe-containing cells was usually around 2 - 3 times higher than the background autofluorescence of negative control in both channels. This confirms that the observed signal was mainly due to the FRET probe rather than native autofluorescence of photoheterotrophic *R. sphaeroides*.

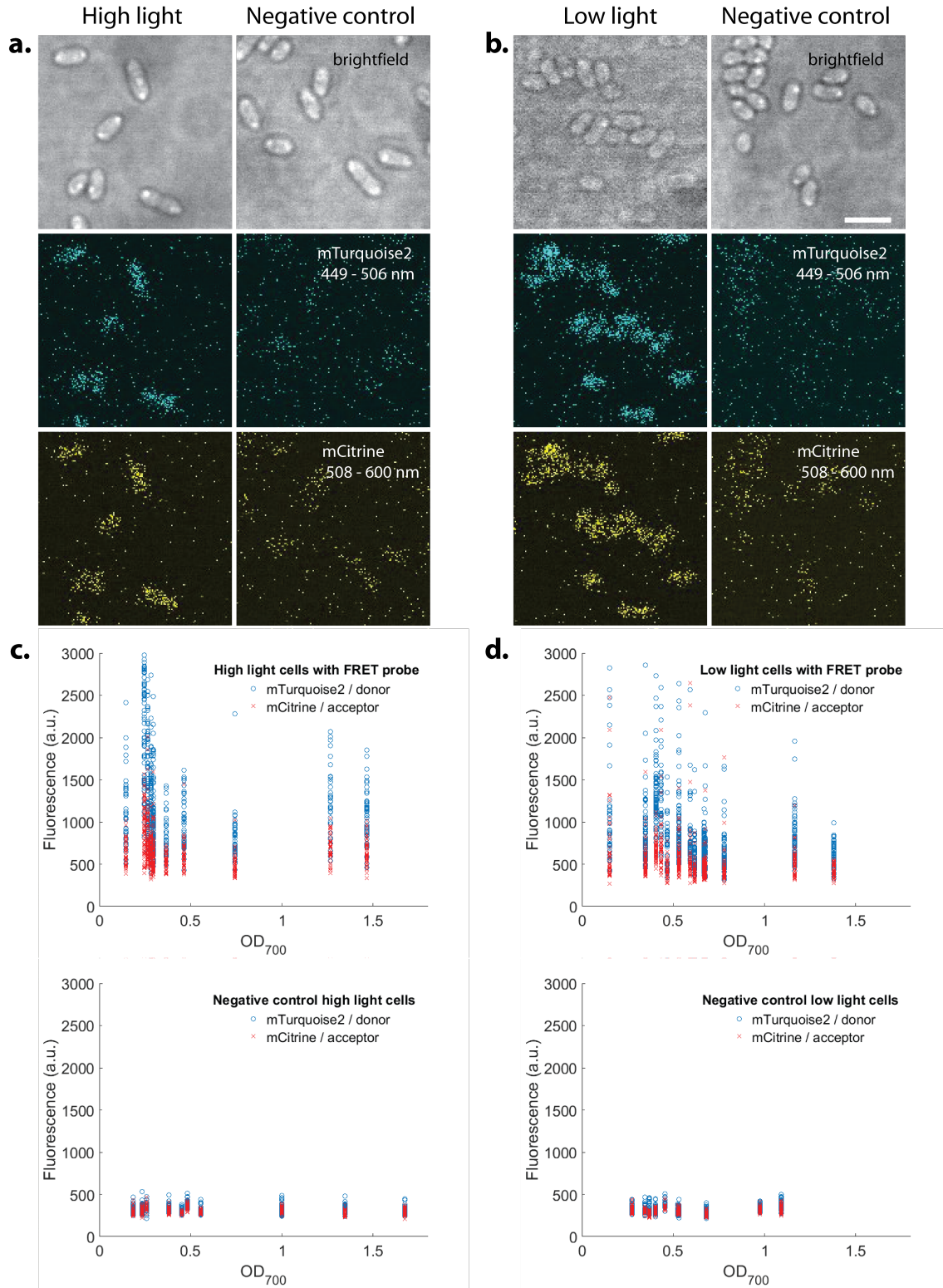


Figure 27: FRET probe for measuring crowding in photoheterotrophic *R. sphaeroides*. **a.** and **b.** show images of high light and low light *R. sphaeroides* cells expressing the FRET probe on an inducible pIND4 plasmid upon 405 nm laser excitation. Negative control cells contained an empty pIND4 plasmid without the FRET probe. Scale bar, 3 μ m. **c.** and **d.** shows the fluorescence distributions of FRET donor mTurquoise2 and acceptor mCitrine emitted by single *R. sphaeroides* cells at different OD₇₀₀.

Table 10: Fluorescence (a. u.) of low light (LL) and high light (HL) *R. sphaeroides* in the acceptor and donor channel. The mean $\pm$ standard error of the mean (SEM) was determined using fluorescence intensities taken from Figure 27.

		Channel 1: 449 - 506 nm	Channel 2: 508 - 600 nm
	No. of cells (n)	Mean $\pm$ SEM (a. u.)	Mean $\pm$ SEM (a. u.)
High light	633	1286 $\pm$ 27	678 $\pm$ 9
HL negative control	336	335 $\pm$ 3	309 $\pm$ 2
Low light	768	875 $\pm$ 19	530 $\pm$ 8
LL negative control	366	333 $\pm$ 3	305 $\pm$ 2

The single cell ratiometric FRET ratio was calculated by subtracting the background fluorescence of negative control and dividing the fluorescence signal of acceptor (508 - 600 nm) by the signal of donor (449 - 506 nm) from Figure 27 **c.** and **d.**. The calculated FRET ratios for all the individual low light and high light cells were plotted onto two histograms and fitted with a Gaussian (Figure 28). The mean FRET ratio was calculated by averaging all the individual cells FRET ratios. A high mCitrine / mTurquoise2 ratio represents compression of the FRET probe by macromolecular crowding. High light cells were found to have a FRET ratio of 0.3907 ± 0.003 (mean $\pm$ SEM, $n = 633$); while low light cells had a mean FRET ratio of 0.4028 ± 0.005 ($n = 768$), p -value = 0.000227. The higher mCitrine / mTurquoise2 ratio in low light cells is in agreement with the BChl extraction results and it shows that the FRET probe is sensitive to differences in crowding induced by ICM vesicles.

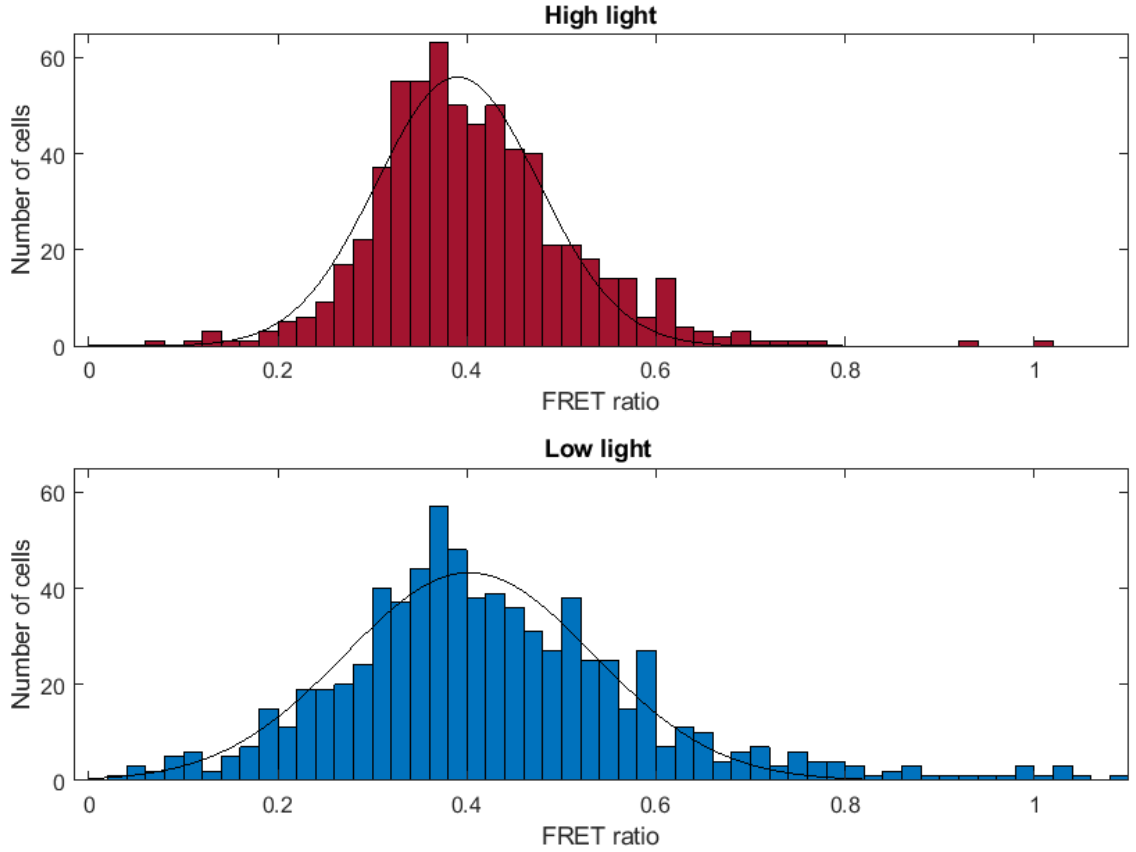


Figure 28: The distributions of ratiometric FRET probe for high light and low light *R. sphaeroides* cells. Data fitted with Gaussian using MATLAB. The mean FRET ratio was 0.3907 ($n = 633$) for high light cells and 0.4028 ($n = 768$) for low light cells. Two tailed t-test showed that $p\text{-value} = 0.000227$.

To better understand the dependence of crowding on OD_{700} , single cell FRET ratios over different OD_{700} units were also calculated and plotted on Figure 29. The data were fitted with linear least squares regression using MATLAB. Despite cell-to-cell variations, a trend of increasing FRET ratios over increasing OD_{700} could be observed in low light cells. Both high and low light cultures had an initial FRET ratio of around 0.4 calculated by extrapolation. As OD_{700} increased, the FRET ratio in low light cells increased steadily from around 0.4 to 0.5; whereas the ratio in high light cells remained constant at approximately 0.4. These observations are in line with findings shown in Figure 25. In general, low light cells were found to have higher FRET ratio compared to high light cells. This contrast in FRET ratio is especially obvious during mid to late exponential

phase of the growth curve ($\text{OD}_{700} > 0.5$) when crowding between high and low light cells becomes increasingly distinctive.

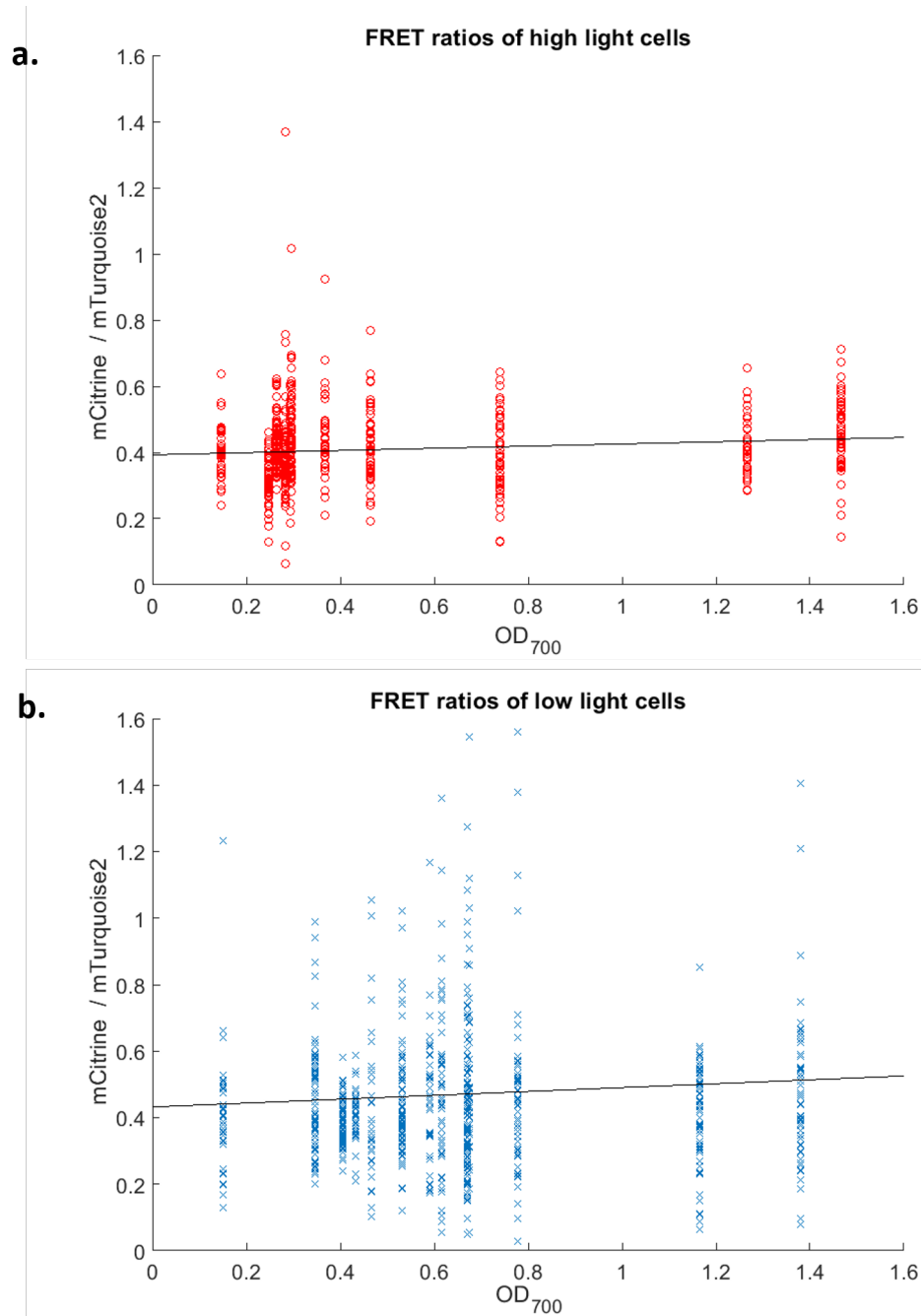


Figure 29: The ratiometric FRET ratio of high light and low light *R. sphaeroides* over OD_{700} as measured by confocal fluorescence microscopy. The fluorescence intensity of mCitrine channel (508 - 600 nm) was divided by the intensity of mTurquoise2 channel (449 - 506 nm) to determine the FRET ratio of single cells. The FRET ratios over OD_{700} for both growth conditions were fitted with linear least squares regression using MATLAB. **a.** FRET ratios for high light *R. sphaeroides* remained constant over increasing OD_{700} ($n = 633$ cells). **b.** FRET ratios for low light *R. sphaeroides* increased with crowding and OD_{700} ($n = 768$ cells).

3.4 Discussion

Quantification of macromolecular crowding in living cells is challenging due to the complex and dynamic nature of the cytoplasm. Previous studies tried to mimic the effects of crowding *in vitro* by using artificial and natural crowders such as Ficoll and cell extracts. However, *in vitro* approaches could not reproduce the dynamic and heterogeneous environment of the bacterial cytoplasm. It is also difficult to determine the right concentration of crowders to use in the system and the crowding agents may have unspecific interactions with the system or with other crowders. In addition, self aggregation may occur when high concentration of crowders were used.

The photosynthetic purple non-sulfur bacterium *R. sphaeroides* develops varying numbers of ICM vesicles when it is grown photoheterotrophically under different light intensities, making it an excellent candidate to study crowding *in vivo*. In this chapter, the excluded volume effects by these 70 nm diameter vesicles in *R. sphaeroides* grown under high light and low light intensities were quantified *in vivo* using a genetically encoded FRET probe with references to crowding measurements by BChl extraction.

BChl extraction showed that there is a huge contrast between the number of ICM vesicles in low light and high light cells. In low light cells, the average number of vesicles per cell was around 1200 and the number increased up to 1700 as the cells reached higher optical density. This increase in the number of vesicles was due to the lower amount of light absorbed by the cells as an effect of self shading. High light cells, however, had a constant number of vesicles of around 400 vesicles. In general, low light cells contained around three to four times more vesicles as compared to high light cells.

In order to estimate the proportion of the cytoplasm occupied by the vesicles under the two conditions, the cell volume and vesicle volume under low light and high light conditions were estimated. The average cell volume (V) for both high light and low light cells were calculated by assuming the cells have the shape of a cylinder capped by two half-spheres using the formula, $V = \pi w^2(l - w/3)/4$, where l refers to cell length and w refers to cell width [148]. The length and width for low light cells were 1.631 ± 0.038

μm and $0.901 \pm 0.016 \mu\text{m}$ ($n = 12$ cells), and for high light cells $1.738 \pm 0.083 \mu\text{m}$ and $0.890 \pm 0.017 \mu\text{m}$ ($n = 13$ cells). Based on the measured cell length and width, the calculated cell volume was $0.848 \mu\text{m}^3$ for low light cells and $0.897 \mu\text{m}^3$ for high light cells. These values were slightly larger than the previously estimated volume of $0.7 \mu\text{m}^3$ for *R. sphaeroides* cells [141]. Another study by Adams and Hunter estimated the size and volume of single chromatophores using TEM and AFM. Low light and high light *R. sphaeroides* vesicles were found to have a volume of $136 \pm 23 \times 10^3 \text{ nm}^3$ and $126 \pm 28 \times 10^3 \text{ nm}^3$, respectively[2]. Using the estimated vesicle volume from [2], a typical high light cell containing 400 vesicles would have around $0.0504 \mu\text{m}^3$ of the cytoplasmic volume being taken up by the vesicles alone. This corresponded to around 5.6% of the total cytoplasmic volume, assuming that this high light cell contained 400 vesicles and had a cell volume of $0.897 \mu\text{m}^3$. For a low light cell with a volume of $0.848 \mu\text{m}^3$ and contained 1200 vesicles, around 19.2% ($0.1632 \mu\text{m}^3$) of the total cell volume would be occupied by the vesicles. As low light cells reached higher optical density, up to 25.1% ($0.2312 \mu\text{m}^3$) of the cytoplasm could be occupied by the vesicles, assuming that these low light cells contained 1700 vesicles on average.

Even though BChl concentration provided a good estimate of the crowding level in photoheterotrophic *R. sphaeroides*, the reported values were part of a bulk measurement that disregarded cell-to-cell variations and heterogeneities. To determine crowding on the single cell level, a FRET probe was expressed from an inducible plasmid in *R. sphaeroides* for direct quantification and the cells were grown under high and low light. Low light cells were found to have a higher FRET ratio than high light cells, and the FRET ratio increased with increasing OD_{700} in low light cells. These results were consistent with the known *in vitro* crowding level measured by BChl extraction. As discussed above, low light cells contained three to four times more vesicles compared to high light cells that could take up almost 25% of the cytoplasmic volume, assuming there were 1700 vesicles in a low light cell. It is possible that in low light cells, a more crowded environment was created due to the presence of large amount of vesicles that decreased the cytoplasmic

volume significantly. This may increase the overall crowding by the rest of the cytoplasmic contents and result in an increase in FRET signal in low light cells.

A previous study [14] showed an increase in FRET ratio in *E. coli* during an osmotic upshift; by recovering the osmotically stressed cells in low-osmolarity medium, the FRET ratio lowered to the initial value. This study has shown that the FRET probe is functional in *R. sphaeroides* cells. Since *R. sphaeroides* is physiologically and metabolically distinctive to *E. coli*, there are a few aspects that need to be carefully considered when expressing the probe in *R. sphaeroides*. Firstly, FRET efficiency is strongly affected by the maturation rates of the fluorophores [116]. This could be an issue as the pigmentation and ICM vesicles in *R. sphaeroides* are induced by low oxygen tension and presence of light [23]. The anaerobic growth condition of *R. sphaeroides* may disrupt maturation and folding of fluorophores on the FRET probe. The previous design of the FRET probe (crGE) with the slow maturing donor mCerulean led to artificially high FRET ratios in *E. coli* under inducible expression [73]. To prevent any artefacts, an improved FRET sensor crTC2 with the combination mTurquoise2-mCitrine that gave a more reliable FRET ratio [73] was used in this study. Before imaging, *R. sphaeroides* cells were also added with chloramphenicol to arrest protein synthesis and incubated for 30 minutes with vigorous shaking to maximize fluorescent protein maturation.

A detailed analysis of the FRET probe linker design was also made by the authors [71]. By introducing structural variations on the linker, they found that the α -helix region was essential for compression by macromolecular crowding when expressed in live *E. coli* cells [71]. Compression of the FRET probes was also found to be dependent on the size of probe and crowder in *in vitro* systems with added crowders such as Ficoll, Dextran, BSA, and PEG [71][14]. *In vitro* characterization using crowders with radius up to 10 nm suggested that the degree of compression became greater with increasing crowder hydrodynamic radius until around 2 - 4 nm where the compression stopped increasing [71]. Therefore, large crowders such as ICM vesicles (diameter = around 70 nm) from *R. sphaeroides* will probably give a similar level of compression on the FRET probe.

This could be measured by isolating ICM vesicles from *R. sphaeroides* and incubating with purified sensor. By calibrating the sensor in known Ficoll concentrations, *E. coli* expressing the crGE probe grown in a standard MOPS medium was found to have a similar crowding level as a 19 % Ficoll PM70 solution [14]. It would also be useful to convert the *in vivo* FRET ratios in *R. sphaeroides* into known % Ficoll PM70 solution, so a comparison of macromolecule fraction between *R. sphaeroides* and *E. coli* can be made.

Since the main role of the FRET probe was to measure excluded volume, it was designed to have minimal chemical interactions with the cell components and other chemicals [14]. The probe was not sensitive to high concentrations of sodium chloride, small metabolites and ions, pH higher than 7, DNA, and glycerol [14][72]. In buffers with increased viscosity through the addition of glycerol, FRET ratio was found to decrease possibly due to stabilization of the probe in the extended conformation; whereas in crowded environment added with synthetic crowder Ficoll-70, the FRET efficiencies were found to increase [66]. These findings show that the FRET probe is sensitive to excluded volume effects instead of viscosity.

In this study, high light cells were found to have a higher concentration of FRET probe in the cytoplasm but a lower FRET ratio compared to low light cells. This suggests that the probe was not a significant proportion of the total protein concentration and probably did not affect the FRET signal. This agrees with previous experiment that found the expression of maltose binding protein from an inducible promoter did not increase FRET ratio in *E. coli* [73]. Overexpression of the FRET probe in *E. coli* with 1 mM IPTG led to the formation of fluorescent inclusion bodies at the cell poles [73]. However, *R. sphaeroides* cells expressing the FRET probe showed homogeneous fluorescence with no local differences in crowding within the cytoplasm, thus eliminating the possibility of inclusion bodies formation due to overexpression. In low light *R. sphaeroides*, ICM vesicles filled the cytoplasm evenly as shown by the uniform BChl autofluorescence [64] and cell sections of transmission electron microscopy [2]. Therefore, even distribution of fluorescence by the FRET fluorophores was expected in *R. sphaeroides*. This is in

contrast to several studies looking at starved or stressed cells, where non-homogeneous distribution of fluorescence was observed within the cytoplasm due to the presence of non-diffusive barriers or confinement [98][82].

In summary, a compressible FRET probe has been shown to generate accurate measurements of macromolecular crowding in *R. sphaeroides* for the first time. In low light cells where crowding is greater, a higher FRET ratio was measured as predicted by BChl extraction. Future experiments to convert *in vivo* FRET ratios in *R. sphaeroides* to *in vitro* fluorescence FRET measurements may be conducted by purifying the FRET probe and adding crowding agents. In addition to the use of FRET sensor, diffusion of biomolecules within the cells was also commonly used by the scientific community to infer crowding. Reduced molecular diffusion rates in crowded cellular environments were observed previously. A detailed discussion on the effects of crowding on the diffusion of different protein molecules in *R. sphaeroides* is presented in Chapter 4.

4 Studying crowding effects on protein diffusion *via* Photoactivated Localization Microscopy (PALM)

The effects of macromolecular crowding in the bacterial cytoplasm can be inferred by:

- Expressing a FRET probe *in vivo* and analyzing the FRET ratios
- Measuring the diffusion of different-sized protein molecules *in vivo*

In this chapter, the effects of crowding induced by ICM vesicles in photoheterotrophic *R. sphaeroides* on protein diffusion were investigated using Photoactivated Localization Microscopy (PALM). The response regulator CheY₆ and ribosomal protein L1 were tagged with photoactivatable mCherry (PAmCherry) and expressed from the IPTG inducible pIND4 plasmid to give individual tracks. The apparent diffusion coefficients of PAmCherry, PAmCherry-CheY₆, and PAmCherry-L1 were measured in aerobic, anaerobic high light, and anaerobic low light *R. sphaeroides*. A comparison of the diffusion rates showed that diffusion of protein molecules >27 kDa decreased following the increase in ICM vesicles. In low light *R. sphaeroides* where the crowding level is high, protein molecules were found to have slower diffusion compared to the diffusion in aerobic and high light cells.

4.1 Functionality of CheY₆ fusions

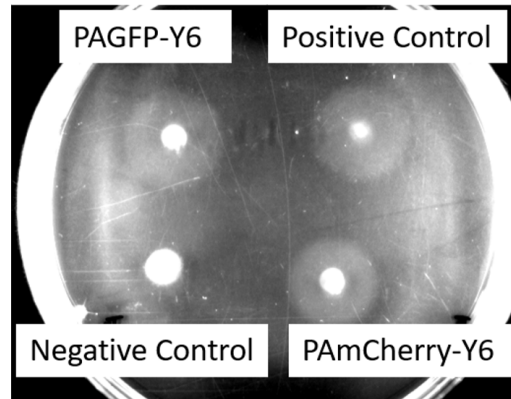
CheY₆ is a small, well-studied response regulator protein that diffuses freely in the cytoplasm and interacts with various chemosensory components localized to different regions of the cell. Therefore, it is interesting to understand whether crowding induced by various number of ICM vesicles has an impact on the diffusion and spatial organization of CheY₆ molecules in *R. sphaeroides*.

Previously, N- and C-terminal CheY₆ genomic fusions to YFP were made but the strains were nonchemotactic [102]. Even though the N-terminal CheY₆ fusion had similar *in vitro* phosphorylation rates to wild type CheY₆, western blot showed that expression

of CheY₆ fusions was very low compared to wild type [102]. This suggests that the integration of a fluorophore tag into the genome may have affected the expression of the native operon. Therefore, N-terminal CheY₆ fusions were placed onto an inducible pIND4 plasmid to achieve better control of expression level for PALM experiments in this work.

To assess the functionality of CheY₆ fusions, PAGFP and PAmCherry fusions were constructed and expressed from pIND4 [54] in a nonmotile CheY₆(D56N) mutant background. The motility and chemotactic ability of these fusions were analyzed using swim plate assays. Low percentage agar plates containing propionate as a single attractant were made and stationary *R. sphaeroides* cultures were spotted onto the plates. When the carbon source was depleted, a concentration gradient was established and the cells could swim from low to high concentration of attractant and form an expanding ring (if they were chemotactic).

The CheY₆(D56N) strain is a phosphorylation site mutant with a stopped phenotype, meaning that the mutant is nonmotile and unable to form an expanding colony or ring on swim plates [102]. It was suggested that CheY₆(D56N) mimics the phosphorylated state and binds to stop the motor until it is competed off the motor by wild type CheY₆. Through expression of the CheY₆ fusions from pIND4, the CheY₆(D56N) mutant was able to recover its motility (Figure 30). As a positive control, an empty pIND4 vector was placed in wild type WS8N background and the strain was used as a reference for motility. The CheY₆(D56N) strain containing an empty pIND4 vector was used as a nonmotile negative control. The strain expressing PAmCherry-Y₆ regained a comparable colony diameter to wild type when it was induced with at least 10 μ M IPTG. The pIND-PAmGFP-Y₆ strain formed a smaller colony at 10 μ M IPTG but fully recovered its motility at higher IPTG concentrations (100 μ M, 1 mM, and 10 mM). The swim plate assays showed that both the PAGFP and PAmCherry fusions were functional.



0.25% agar in M22

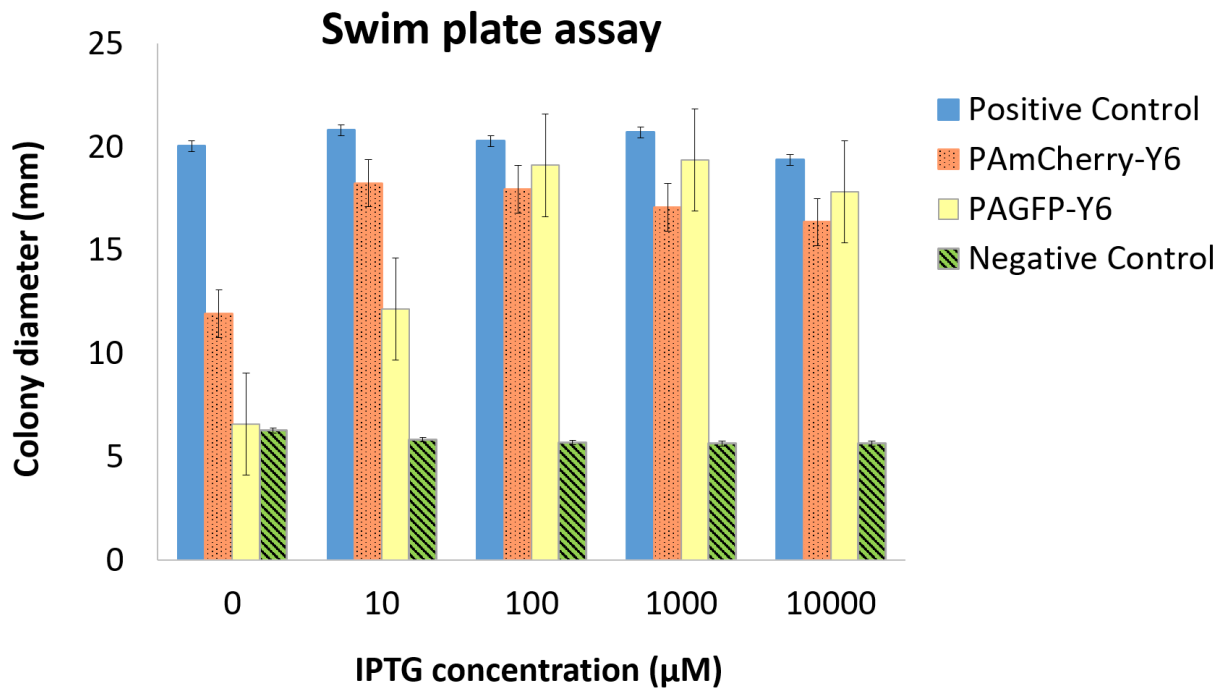


Figure 30: Swim plates showed that CheY₆ fusions are functional and can recover motility in *R. sphaeroides*. The top diagram shows an example of a swim plate spotted with wild type WS8N (positive control), CheY₆(D56N) (nonmotile negative control), pIND-PAGFP-Y₆, and PAmCherry-Y₆ in CheY₆(D56N) background. The colony diameters were measured after 48 hours incubation under 100 μM IPTG induction. The bottom diagram shows the average colony diameters of strains expressing CheY₆ fusions compared to positive and negative control. The strain containing pIND-PAmCherry-Y₆ recovered chemotaxis with the addition of 10 μM IPTG. The pIND-PAmGFP-Y₆ strain had comparable swim diameters to the wild type during 100 μM IPTG induction. Two repeats were performed in triplicates. The error bars refer to the standard error.

4.2 Choosing the right photoactivatable fluorescent protein for PALM

The main goal of this chapter was to measure protein diffusion using single molecule tracking in high light and low light *R. sphaeroides* where crowding levels are different. However, one of the challenges of performing single molecule tracking was the low signal-to-noise ratio caused by high autofluorescence of photoheterotrophic *R. sphaeroides*. The background fluorescence caused by the high levels of cellular BChl molecules made imaging of single fluorophores in low light cells extremely difficult. Previously, BChl autofluorescence has been used to visualize the spatial organization of ICM in several purple nonsulfur bacteria without the need of using fluorescent protein tags [64]. To choose the most suitable fluorescent label for *R. sphaeroides*, the emission spectrum of its autofluorescence was characterized by using a laser scanning confocal Zeiss 880 microscope (Figure 31). The cells were found to emit the strongest autofluorescence at around 690 nm upon excitation with the 405, 488, 561, and 633 nm lasers, respectively. Therefore, fluorophores that emit near the far red spectrum should be avoided to prevent overlaps with the autofluorescence.

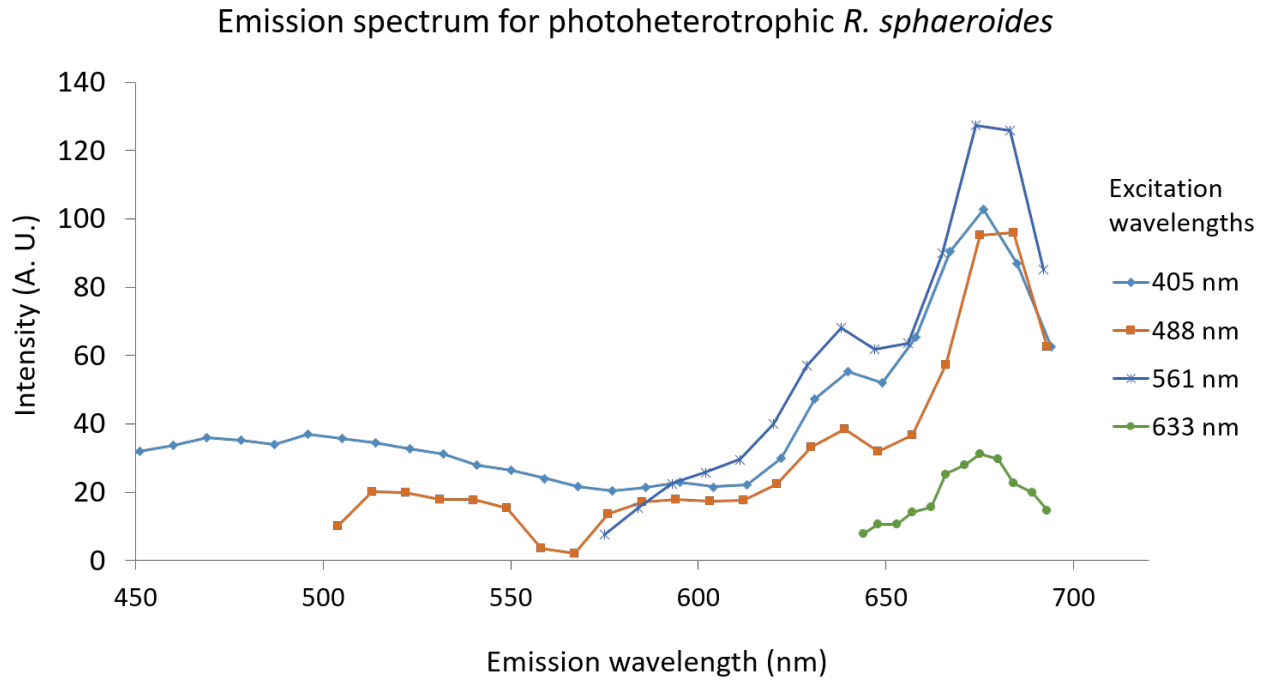


Figure 31: Emission spectrum for photoheterotrophic *R. sphaeroides* (WS8N) between the range of 450 to 700 nm. The cells were excited using the 405, 488, 561, and 633 nm lasers separately on a laser scanning confocal Zeiss 880 microscope. The emission spectrum generated by each laser was recorded and the intensities were analyzed in ImageJ. The intensities of the emission are non-comparable but all the excitation wavelengths produce an autofluorescence peak at around 690 nm.

PALM was found to be a good technique for minimizing autofluorescence in *R. sphaeroides* cells because it allowed a prebleach step using the activation laser before imaging. Photoactivatable FPs had been used to study molecular dynamics and interactions at the single molecule level in bacteria [69][45]. Single molecule PALM in this work was performed using a PALM/TIRF microscope in the Micron Advanced Imaging Consortium, University of Oxford. Therefore, the fluorescent protein must also be compatible with the filter sets and lasers of the microscope. Both PAmCherry-Y₆ and PAmGFP-Y₆ fusions were tested and PAmCherry was selected as the fluorescent tag for PALM in this work. PAmCherry is able to convert from its initial dark state to a fluorescent state following irradiation with the 405 nm laser. Continuous 561 nm laser is then used to excite and image the activated PAmCherry for single molecule tracking. PAmCherry has an excitation/emission maxima at 564/595 nm [133] that does not overlap with the *R. sphaeroides* autofluorescence peak.

4.3 Autofluorescence

During imaging of aerobic *R. sphaeroides* cells, I observed a gradual increase in cellular background fluorescence (including negative control cells) in the green and red channel on agarose pads. In contrast to PAmCherry molecules, the autofluorescence was visible when illuminating with a 561 nm laser, even in the absence of 405 nm photoactivation. These molecules started to appear as randomly diffusing species in the cytoplasm usually after around an hour of imaging, until the entire cell was filled with diffuse fluorescence. This made single molecule tracking challenging as these autofluorescent spots were almost indistinguishable from the intensity of PAmCherry.

I hypothesized that the autofluorescence was caused by low oxygen level within the agarose pads. In the past, melted agarose were placed in a gene frame on a glass slide and immediately covered with a clean coverslip to flatten the surface. In this case, the agarose pads were not exposed to air and probably had a low oxygen content. To test this hypothesis, once the agarose pads started to solidify, coverslips were removed and agarose pads were left in a humidity chamber for 30 minutes to be fully oxygenated. Freshly grown aerobic *R. sphaeroides* WS8N were placed on both oxygenated and unoxygenated agarose pads and imaged under 561 nm excitation. At this stage, the cells had very low background autofluorescence and the cells could still obtain limited oxygen from the suspension medium and agarose pads. After incubating the cells on agarose pads for 1.5 hour, an increased background fluorescence was observed in cells on non-oxygenated agarose pads but not in the oxygenated agarose pads (Figure 32). This observation shows that in contrast to PAmCherry and other FPs where oxygen is required for full maturation, the production of autofluorescence molecules in aerobic *R. sphaeroides* is related to lack of oxygen.

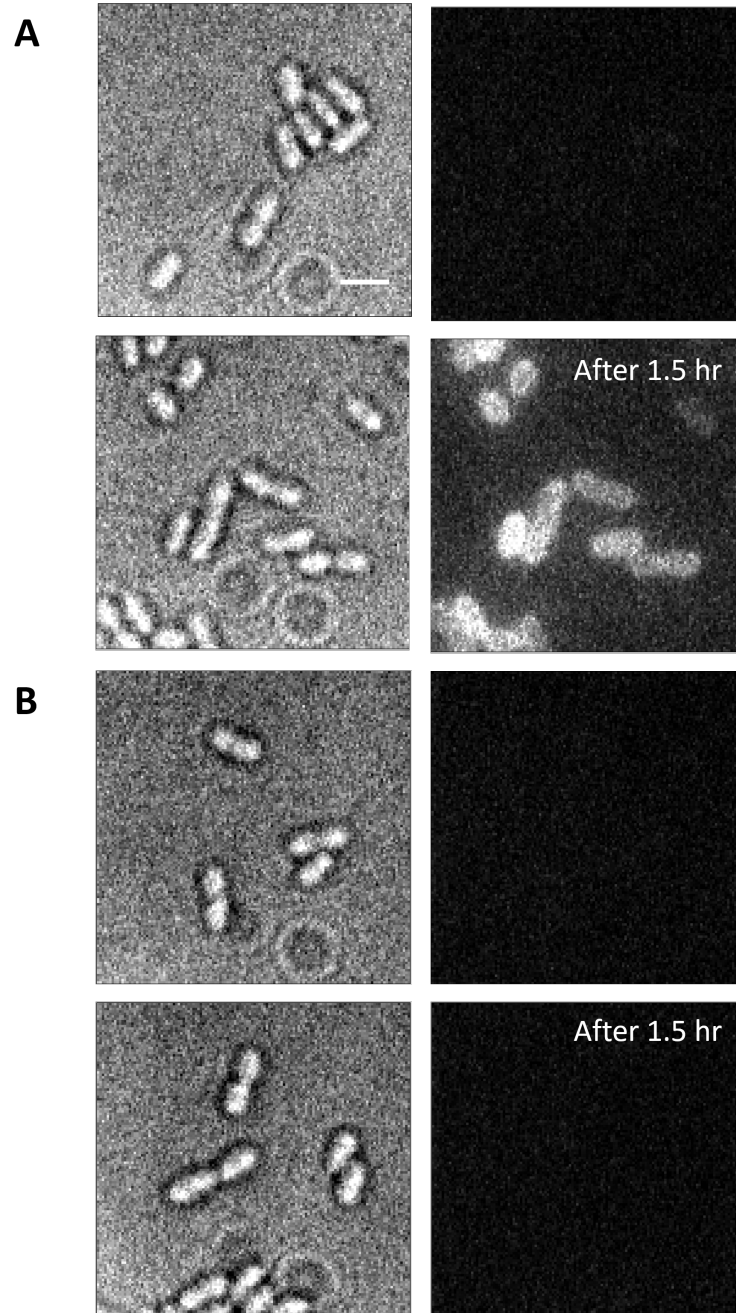


Figure 32: Autofluorescence in aerobic *R. sphaeroides* was triggered by low oxygen level. Freshly grown aerobic *R. sphaeroides* WS8N were placed on (A) non-oxygenated and (B) oxygenated 1% agarose pad in SUX. The panels on the left are the brightfield images and the panels on the right are the fluorescent images. High autofluorescence was observed in (A) but not (b). Brightness and contrast of the fluorescent images are displayed at the same levels using ImageJ for comparison. 70% 561 nm laser illumination. PAmCherry filter sets ET600/50. Cycle time 7.75 ms. Scale bar 2 μm

To investigate whether the source of autofluorescence is endogenous or exogenous, the same experiment was repeated with the addition of chloramphenicol in the growth medium and agarose pads to inhibit translation. Aerobic *R. sphaeroides* cells were resuspended in SUX medium added with chloramphenicol and imaged using 561 nm excitation. After 1.5 hours, no significant increase in autofluorescence was observed in the majority of the cells. This result suggests that the appearance of autofluorescence was dependent on protein synthesis.

A recent study reported the observation of unknown autoblinking molecules in the cell walls of *Deinococcus* strains during single molecule PALM imaging [43]. These fluorescent molecules were acquired from growth medium and they could be excited by 488nm, 561 nm, and 643 nm laser to form two populations of immobile and slow diffusing molecules [43]. However, the increased autofluorescence observed in *R. sphaeroides* was not due to exogenous factors such as dust on coverslips or fluorescent molecules in the medium as it required protein synthesis. In addition, *R. sphaeroides* were grown in SUX medium that is non-fluorescent compared to other rich media such as LB and SOC. Previous study showed that exposure to cell wall synthesis inhibitor ampicillin led to increased green endogenous autofluorescence in *E. coli*, possibly due to the enhanced production of flavins as one of the stress responses [135]. Other sources of natural cellular autofluorescence include aromatic amino acids (Phe, Tyr, Trp), NAD, bacteriochlorophyll, and carotenoids. The identity of these *R. sphaeroides* autofluorescence molecules is unclear but the results suggest that the production was triggered by low oxygen level in the agarose pads.

Characterizations of these autofluorescence molecules is important for discriminating the autofluorescence molecules from actual fluorescent proteins. Since autofluorescence could overlap with the emission spectrum of targeted fluorescent protein tags, minimal background fluorescence during data acquisition is essential to reduce false localizations and trajectories. This is especially important in single molecule studies where the photon count of each fluorophore is low.

4.4 Cell viability and phototoxicity

The PALM technique includes a prebleach step to reduce cellular autofluorescence for single molecule tracking. However, prolonged exposure to near UV laser irradiation could lead to light-induced damage [151]. Intense laser irradiation especially in the violet (405 nm) and blue (445 - 457 nm) light channel is damaging to bacterial cells and may lead to cell death and growth arrest, whereas green light (YFP channel) was found to be less toxic to live cells [36]. Therefore, the effects of phototoxicity were investigated by monitoring cell growth after prolonged exposure to the 561 nm laser.

Wild type *R. sphaeroides* WS8N was placed on SUX medium agarose pads with gridded coverlips and a bright field image was taken. The cells were then exposed to 70% (9.5 mW) 561 nm laser illumination for 30, 20, and 10 seconds and then allowed to recover for 12 hours in the light cabinet. After recovery, the cells from the same FOV were imaged using bright field to check for cell division. Figure 33 shows bright field images of cells dividing after exposed to 20 s laser illumination and recovered for 12 hours.

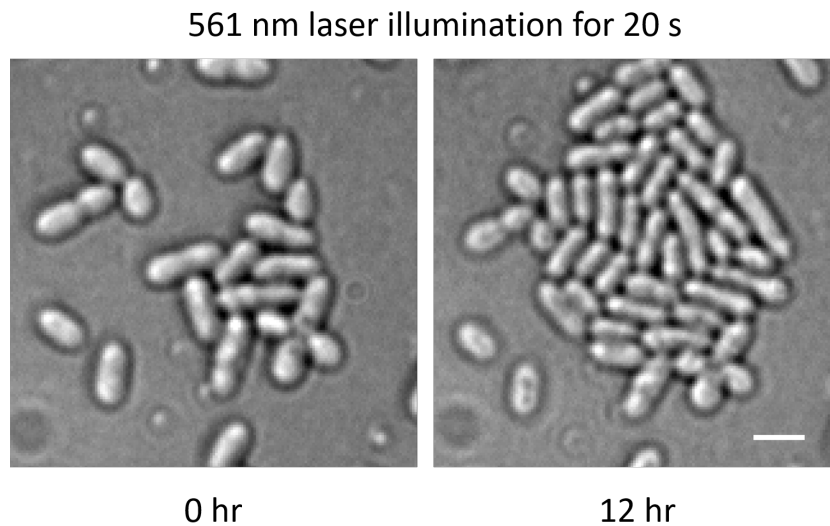


Figure 33: Bright field images of *R. sphaeroides* cells dividing after being exposed to the 561 nm laser. Wild type *R. sphaeroides* WS8N cells were subjected to 70% (9.5 mW) 561 nm laser illumination for 20 seconds. After recovering in the light cabinet for 12 hours, the cells continue to divide. Scale bar 2 μ m.

It was found that *R. sphaeroides* cells continue to divide after exposure to <30 s of

561 nm irradiation, even though the cells grew at a slower rate compared to controls that were not exposed to the 561 nm laser. Once the irradiation time reached 40 s, the cells stopped growing and remained in an ‘intact’ state without obvious changes in cell morphology or cell lysis.

These results suggest that the imaging conditions in this study were not detrimental, although it is possible that some cells suffered negative effects on growth and light-induced cellular damage. The effects of the 405 nm laser was not investigated since a low intensity (around 336 μ W for a few seconds) was used for photoactivation and probably have less impacts on cell viability and growth. For PALM experiments on live cells, care must be taken to avoid prolonged laser exposure that causes cell death.

4.5 Diffusion of PAmCherry in *E. coli* and aerobic *R. sphaeroides*

To verify the accuracy and limits of the PALM technique, I decided to measure the diffusion of single PAmCherry in *E. coli* and aerobic *R. sphaeroides* where the background autofluorescence is low. Typically, the diffusion of free fluorescent protein was measured using bulk fluorescence technique such as FRAP in *E. coli* cells, elongated by treatment with cephalixin to prevent division [39][87]. Single molecule tracking of a fluorescent protein is challenging because of the shorter fluorescent lifetime compared to other commercial dyes and the difficulty to achieve accurate localizations due to the fast diffusion.

4.5.1 Experimental setup for PALM

In this work, the IPTG inducible pIND4 plasmid expressing free PAmCherry was transformed into *E. coli* S-17 and conjugated into wild type *R. sphaeroides* WS8N. The cells were immobilized on 1% agarose pads and imaged on a custom-built PALM/TIRF microscope. PAmCherry filter sets (ET600/50, emission 575 - 625 nm) and near-TIRF microscopy were used to improve the signal-to-noise ratio. To acquire a PALM movie, a 64×64 pixels field of view (FOV) containing one or two well-isolated cells was selected in brightfield. The small FOV allowed an exposure time of 7 ms and a cycle time of 7.75 ms. The high frame rate of around 129 frames per second was essential for tracking single molecules with high diffusion coefficients. The 561 nm laser was used to perform a quick pre-bleach to remove any cellular autofluorescence. During data acquisition, the cells were imaged using continuous 561 nm laser excitation at 70% for 800 frames (around 6.2 s). A low 405 nm laser intensity was selected to ensure activation of one or two PAmCherry molecules each time. The intensity of the 405 nm laser was increased gradually as the PAmCherry pool started to photobleach over time. Around 6 movies were recorded for each FOV. The same acquisition setting was used for both *E. coli* and *R. sphaeroides*.

4.5.2 Data analysis using Stormtracker

Stormtracker [142], an automated single molecule analysis software, modified by Dr. Matthew Stracy and Dr. Helen Miller was used to analyze the PALM movies. Single PAmCherry spots were localized through a self-defined threshold that was affected by the signal-to-noise ratio. False positive localizations may appear in the background when the threshold is too low. If the threshold is too high, it may lead to false negatives or biasing of slow molecules. A relatively low threshold was selected for tracking PAmCherry molecules due to the lower photon counts of the fluorophore. Localized spots were then linked into trajectories using a tracking window of 8 pixels. The tracking window sets the upper limit on how far a molecule can diffuse in one frame. Since PAmCherry has a fast diffusion coefficient, a relatively large tracking window ($0.768\ \mu\text{m}$) was selected to avoid splitting of a single molecular trajectory, resulting in an artificially high number of trajectories. A small tracking window is more useful when the diffusion of molecule is slower or when the density of spots in the FOV is high.

Figure 34 shows the procedures of the tracking analysis for an *E. coli* cell. A brightfield image of the cell was first taken and all localizations within the FOV were generated using localization intensity threshold of 20 (shown in Figure 34B). The localizations with 4 steps or more were linked to form a track that represents the motion of a single PAmCherry molecule in the cell. More tracks were recorded in the same cell by continuously activating and exciting the fluorophores.

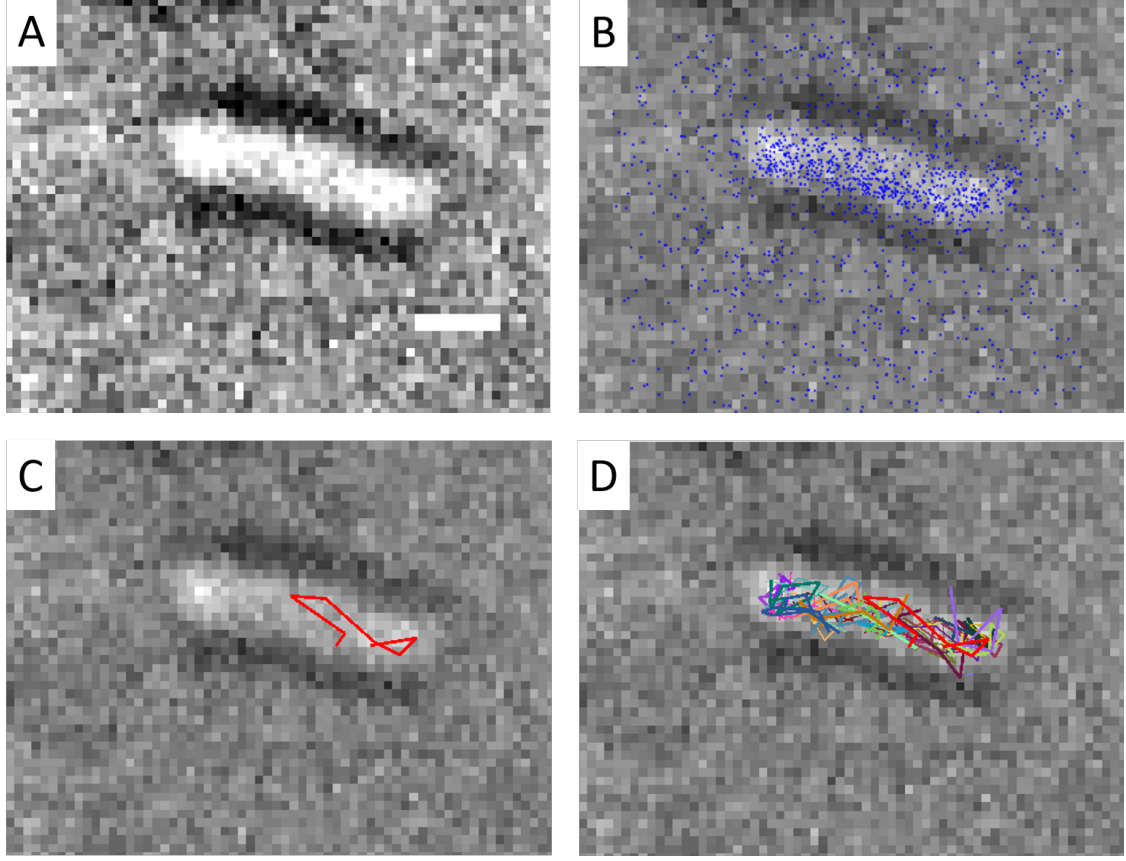


Figure 34: Single molecule tracking of free PAmCherry molecules in an *E. coli* cell. (A) A brightfield image was taken before data acquisition for determining the cell outline. (B) By using a localization intensity threshold of 20, localized spots (blue) were generated in the FOV. The localizations were linked through a tracking window of 8 pixels to form single tracks. (C) An example of a single PAmCherry molecule track (red) in the cell. (D) All tracks with 4 steps or more were shown, colour coded by trajectory number. PAmCherry molecules were able to diffuse freely in the cytoplasm. Scale bar 1 μm .

The apparent diffusion coefficient, $D = \text{MSD}/4\Delta t$ for PAmCherry was calculated from the mean squared displacement (MSD) for each trajectory with a minimum steps of four (five localizations). The MSD for each trajectory was calculated using the formula:

$$\text{MSD} = \frac{1}{N-1} \sum_{i=1}^{N-1} [(x_{i+1} - x_i)^2 + (y_{i+1} - y_i)^2]$$

where N is the number of localizations [142]. In this work, $N = 5$ and $\Delta t = 7.75$ ms. The D values here provide useful insights regarding the diffusive behaviors of molecules,

but they do not represent the accurate diffusion coefficients because the calculation did not correct for localization precision. Localization precision can be obtained by imaging stationary PAmCherry molecules (bound to an immobile structure or component) in live cells through the same data acquisition settings. The apparent D values generated from all the trajectories were plotted on a histogram. Figure 35 shows an example of multiple PAmCherry tracks in an aerobic *R. sphaeroides* cells and the apparent D histogram generated with those same tracks.

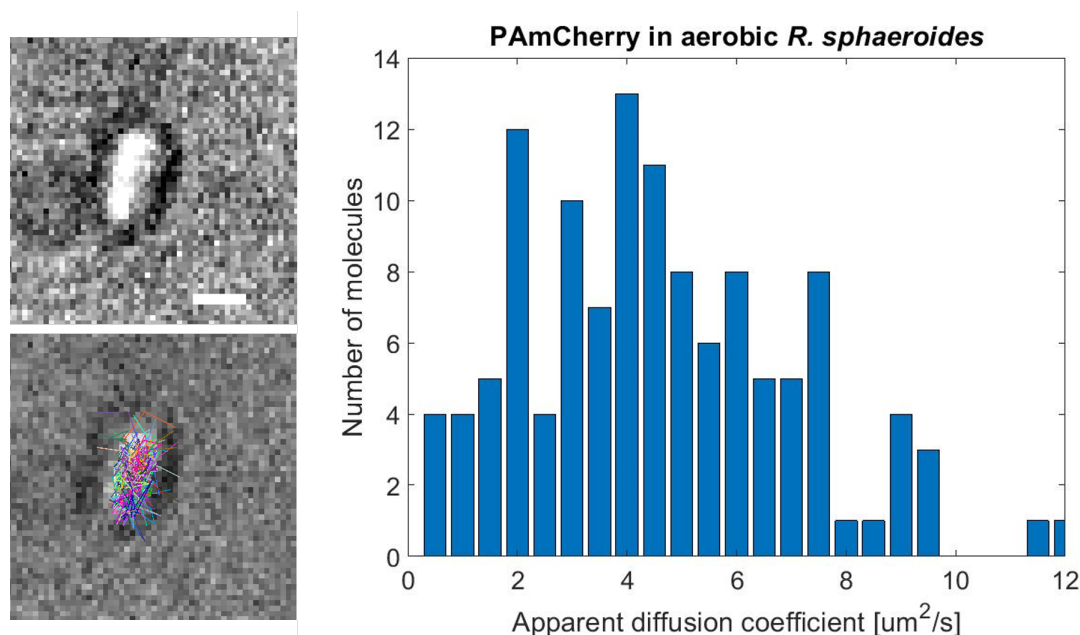


Figure 35: The distribution of apparent diffusion coefficients for free PAmCherry molecules in an aerobic *R. sphaeroides*. All the tracks were arbitrarily coloured and PAmCherry molecules were able to diffuse freely in the cytoplasm. The histogram shows the apparent D values for all tracks with 4 steps or more. Scale bar 1 μm .

All the apparent diffusion coefficients of PAmCherry were combined and fitted to give an estimated D value. The area of the fit was constrained to have the same area as the distribution. For this analysis, longer tracks were truncated to four steps. The calculated apparent D of PAmCherry in *E. coli* and aerobic *R. sphaeroides* were 3.53 ± 0.13 and $3.62 \pm 0.23 \mu\text{m}^2\text{s}^{-1}$, respectively (Figure 36). The standard errors on the fitted D values were determined by bootstrapping. These apparent D values are on the same order of magnitude as the diffusion coefficients of GFP in bacterial cytoplasm measured

by other techniques ($D = 3 - 14 \mu m^2 s^{-1}$) [39][87][60][82][123]. The PALM tracking of free PAmCherry in *E. coli* and aerobic *R. sphaeroides* shows the reliability of our experimental setup and data analysis method. Since any protein labelled with a PAmCherry will have a slower diffusion rate compared to a free fluorescent protein, single molecule tracking of the small CheY₆ fused to PAmCherry should be achievable.

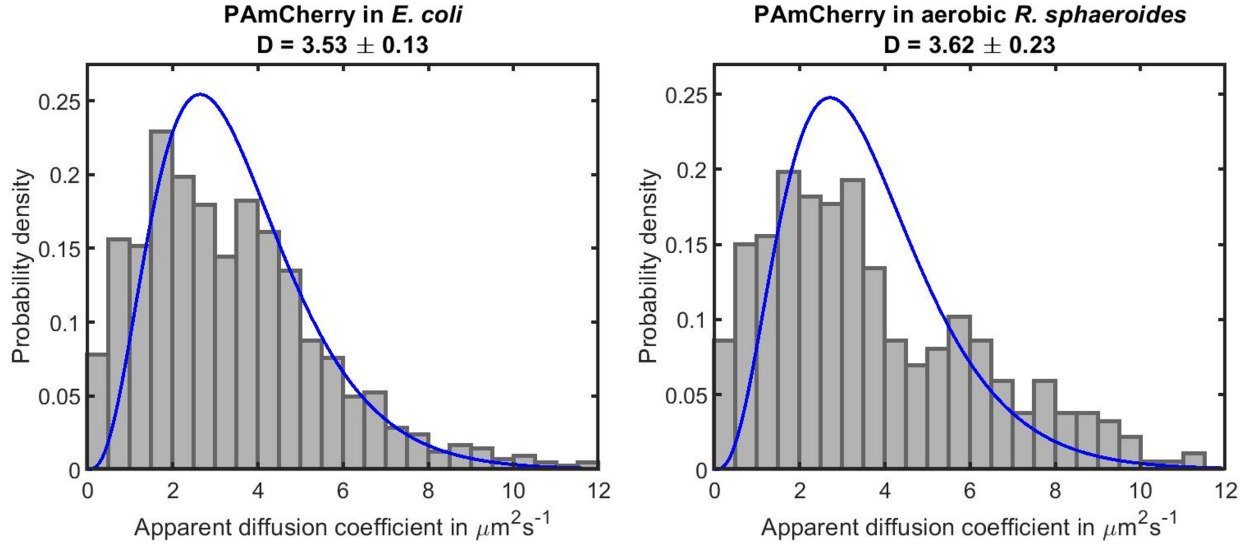


Figure 36: The apparent diffusion coefficients D of free PAmCherry molecules in *E. coli* ($n = 847$ molecules) and aerobic *R. sphaeroides* ($n = 374$ molecules). The D values were calculated using all tracks with 4 steps (longer trajectories were truncated) and fitted with a least squares gamma distribution.

4.6 Diffusion of PAmCherry in high light and low light *R. sphaeroides*

The diffusion coefficients of free PAmCherry molecules were also measured in high light and low light *R. sphaeroides* cells. PAmCherry is a good tracer molecule for studying crowding as it is foreign to the cell and is unlikely to have specific interactions with the cytoplasmic components. For low light cells, a longer prebleach step with the 561 nm excitation laser was required because of the higher background fluorescence. A higher localization intensity threshold between the range of 30 - 40 was also used for photoheterotrophic cells due to the lower signal-to-noise ratio.

Interestingly, there seemed to be more than a single diffusing species in high light cells. By analyzing the diffusion profiles of PAmCherry in individual high light cells, a small percentage of the population was found to have mean D of around $3.3 \mu m^2 s^{-1}$ (similar to the diffusion in aerobic cells) while the majority of the cells had slower mean D of around $2.49 \mu m^2 s^{-1}$ or $1.1 \mu m^2 s^{-1}$. Figure 37 showed three representative cells each with a slow diffusion profile (Example 1), fast diffusion profile (Example 2), or with a mixture of both (Example 3). In both cases, PAmCherry can freely explore the cytoplasm without being confined to any specific region. These observations show the ability of single molecule tracking to capture cell-to-cell variations and heterogeneities within a cell population. The higher diffusion rate in some cells may have contributed to the overall D of PAmCherry in high light cells (shown in Figure 37E). As expected, the PAmCherry D values in high light cells fitted poorly to a single gamma distribution for one diffusing species (Figure 37F).

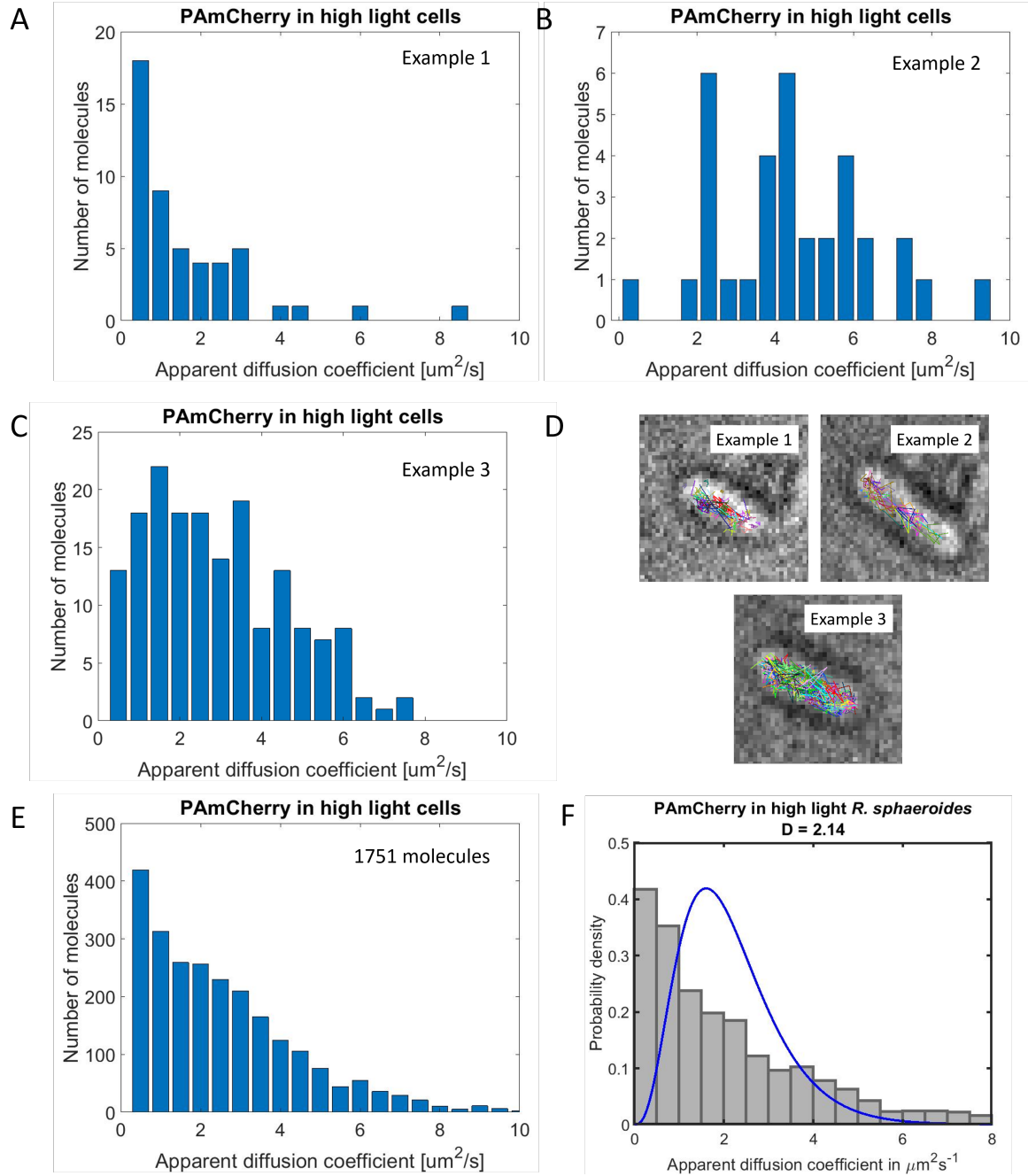


Figure 37: The diffusion profiles of free PAmCherry molecules in high light *R. sphaeroides* cells. (A), (B), and (C) show the different diffusive behaviors of PAmCherry observed in three representative cells. In (A), the diffusion rate was slower with a mean D of $1.127 \mu\text{m}^2\text{s}^{-1}$. The cell in (B) had a mean D of $3.3 \mu\text{m}^2\text{s}^{-1}$ that was similar to the measured diffusion in aerobic cells. (C) show a cell with a mean D of $2.49 \mu\text{m}^2\text{s}^{-1}$, possibly containing a mixture of both the fast and slow diffusing species. (D) show the brightfield images of the three cells from (A), (B), and (C). (E) is a histogram with the combined apparent D values from all high light cells. Mean $D = 2.06 \mu\text{m}^2\text{s}^{-1}$. The D values were calculated using all tracks with steps longer than 4. (F) The D values were found to fit poorly to a single gamma distribution, suggesting that there was more than a single diffusing species in the cells.

To identify the most suitable fit for the data, a reduced chi-squared test was also performed on the PAmCherry D distribution in high light cells to determine the goodness of fit for the one-species and two-species distribution. A better fit (reduced chi-squared = 6.53) was obtained for the two-species model compared to the fit for a single diffusing species (reduced chi-squared = 334.44). Therefore, the PAmCherry apparent D data for high light cells were fitted with two gamma distributions to give a larger fraction (65.6 %) of fast diffusing molecules, $D = 2.46 \pm 0.09 \mu m^2 s^{-1}$ and smaller fraction (34.4 %) of slow diffusing molecules, $D = 0.50 \pm 0.03 \mu m^2 s^{-1}$ (Figure 38). Two diffusing species were also observed in the PAmCherry D distribution for low light cells, as suggested by the reduced chi-squared value of 24.14 for two-species fit and a much higher reduced chi-squared value for one-species fit. In contrast to the result of high cells, around half of the PAmCherry fraction (52%) was diffusing with much lower D of $0.28 \pm 0.03 \mu m^2 s^{-1}$ in low light cells, while 48% had $D = 0.94 \pm 0.10 \mu m^2 s^{-1}$.

These data show that *R. sphaeroides* cells grown under photoheterotrophic conditions contained a distinct slow diffusing species of PAmCherry with $D < 0.5 \mu m^2 s^{-1}$; whereas in aerobic cells, there was a predominant fast diffusing species with a D of $3.62 \pm 0.63 \mu m^2 s^{-1}$. In general, free PAmCherry was found to have lower apparent D under high light and low light growth conditions, even for the fast species. As the number of ICM vesicles increases, the fraction of slow PAmCherry also increases from 34.4% to 52 %, possibly due to the larger excluded volume effects in low light cells. These data also show that the effects of crowding in low light cells could be so strong that it decreased the diffusion rate of a 27 kDa protein by an order of magnitude.

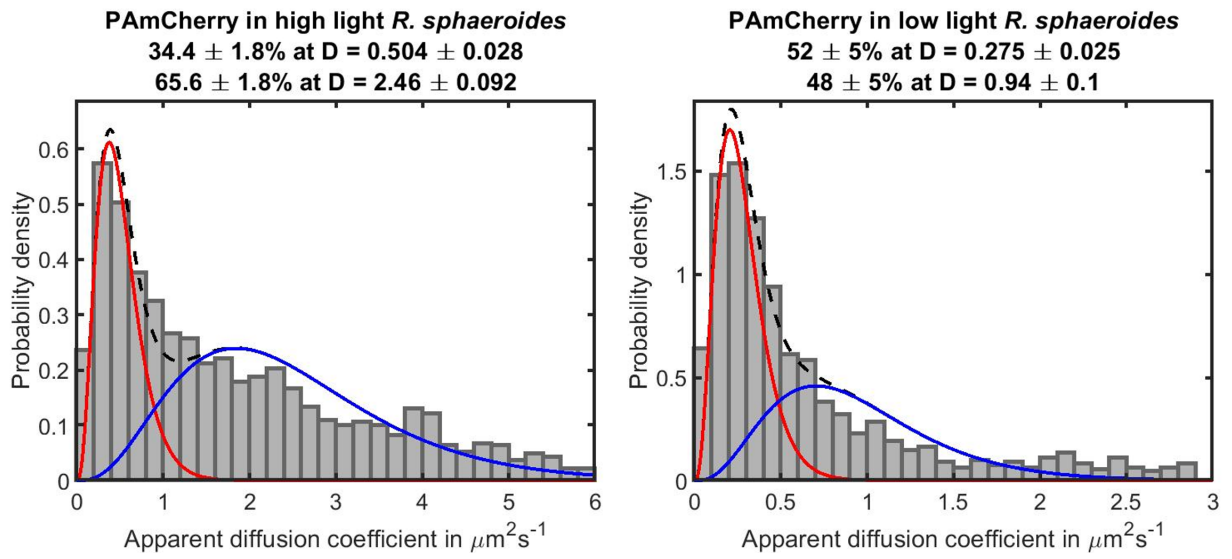


Figure 38: The apparent diffusion coefficients D of unbound PAmCherry molecules in high light ($n = 1751$ molecules) and low light *R. sphaeroides* ($n = 1188$ molecules). The D values were calculated using all tracks with 4 steps (longer trajectories were truncated) and fitted with a least squares curve.

4.7 Diffusion of PAmCherry-Y₆ in *E. coli* and *R. sphaeroides*

The same experimental configuration was used to measure the apparent D of PAmCherry-Y₆ in *E. coli* and *R. sphaeroides* with different crowding levels. In *E. coli* and aerobic *R. sphaeroides*, the apparent D of 3.19 ± 0.083 and $3.23 \pm 0.18 \mu\text{m}^2\text{s}^{-1}$ were obtained for PAmCherry-Y₆, respectively. These values are very similar to the diffusion coefficient of PAmCherry, suggesting that the addition of a 14 kDa protein to the 30 kDa PAmCherry tag would not have a huge impact on diffusion in an less crowded cellular environment. The D values here also agree with the diffusion coefficient of CheY-GFP, $D = 4.6 \pm 0.8 \mu\text{m}^2\text{s}^{-1}$ measured by the fluorescence correlation spectroscopy (FCS) technique in *E. coli* [21].

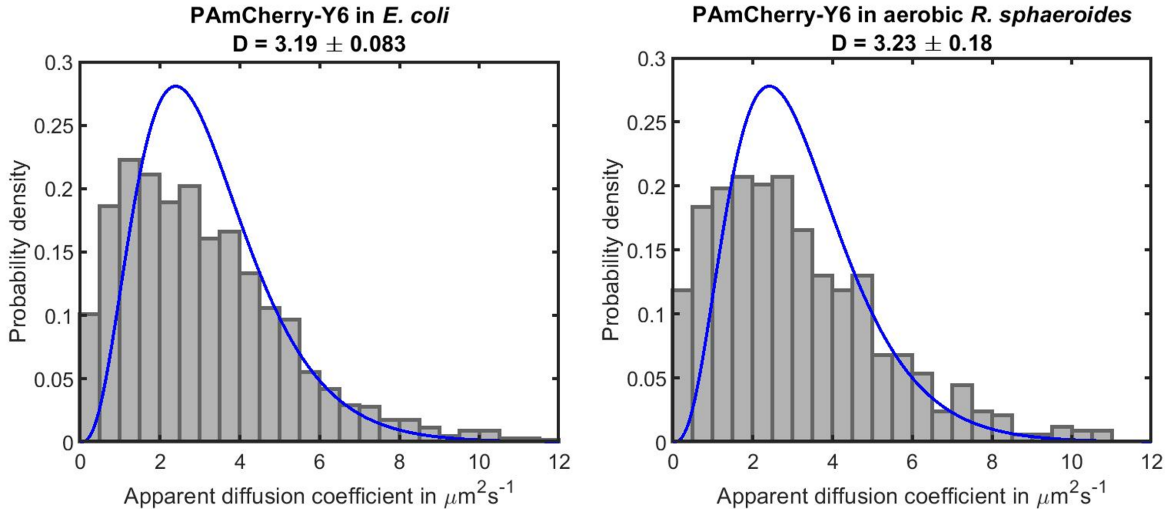


Figure 39: The apparent diffusion coefficients D of unbound PAmCherry-Y₆ molecules in *E. coli* ($n = 1390$ molecules) and aerobic *R. sphaeroides* ($n = 678$ molecules). The D values were calculated using all tracks with 4 steps (longer trajectories were truncated) and fitted with a least squares curve.

Next, the diffusion of PAmCherry-Y₆ in photoheterotrophic *R. sphaeroides* was measured. Similar to PAmCherry, the D distributions of PAmCherry-Y₆ in high light and low light conditions were fitted with two gamma distributions (Figure 40). In high light cells, 44.5 % PAmCherry-Y₆ had $D = 2.01 \pm 0.16 \mu\text{m}^2\text{s}^{-1}$ and 55.5 % was diffusing with smaller D of $0.46 \pm 0.03 \mu\text{m}^2\text{s}^{-1}$. The low light cells also had a similar percentage of fast and slow diffusing molecules: 45.9 % was diffusing at $D = 1.07 \pm 0.20 \mu\text{m}^2\text{s}^{-1}$ and 54.1 % of the molecules had $D = 0.29 \pm 0.03 \mu\text{m}^2\text{s}^{-1}$.

Overall, PAmCherry-Y₆ was found to exhibit lower diffusion coefficients in *R. sphaeroides* grown under high light and low light growth conditions compared to aerobic condition. The molecules were able to freely diffuse within the cytoplasm without obvious signs of binding to any cellular components. Again, a sequential decrease in diffusion coefficients for the fast diffusing fraction of PAmCherry-Y₆ was observed. The apparent D decreased from 3.23 to $2.01 \mu\text{m}^2\text{s}^{-1}$, and finally to $1.07 \mu\text{m}^2\text{s}^{-1}$ as the number of vesicles in *R. sphaeroides* increased from aerobic to photoheterotrophic growth conditions. This suggests that the increase in macromolecular crowding induced by ICM vesicles could lower the diffusion rate of a 44 kDa protein within the cytoplasm.

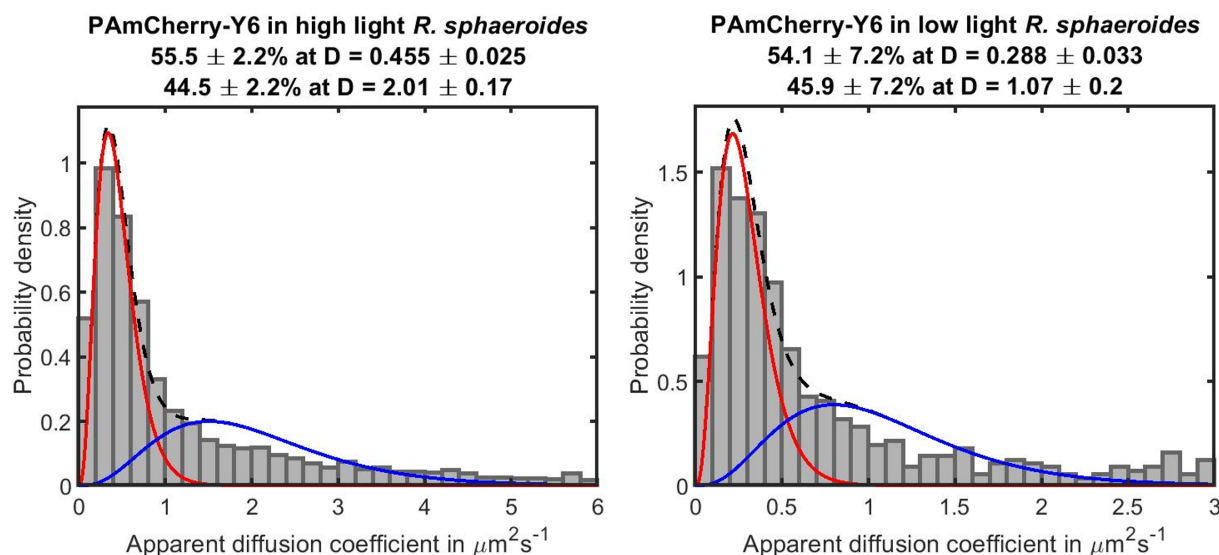


Figure 40: The apparent diffusion coefficients D of PAmCherry-Y₆ molecules in high light (n = 2211 molecules) and low light *R. sphaeroides* (n = 624 molecules). The D values were calculated using all tracks with 4 steps (longer trajectories were truncated) and fitted with a least squares curve.

Since CheY₆ is able to interact with the flagellar motor and chemosensory clusters, epifluorescence microscopy was also performed using CheY₆ fused to YFP [96] and expressed from pIND4 plasmid to study its overall spatial distribution in the cytoplasm. The functionality of this YFP-Y₆ fusion was also confirmed by swim plates using the non-motile CheY₆(D56N) strain. *R. sphaeroides* WS8N containing pIND-YFP-Y₆ was grown under aerobic and photoheterotrophic conditions with the induction of IPTG. Fluorescence microscopy showed that the majority of cells have a localized spot possibly at the cytoplasmic cluster and diffuse fluorescence within the cytoplasm (Figure 41). Around 81.4% aerobic cells (n = 113 cells), 88.9% high light cells (n = 180 cells), and 79.2% low light cells (n = 149 cells) had a cytoplasmic spot. Localization to the flagellar motor was not observed since there are thousands of CheY₆ molecules within a cell, and visualizing the relatively small amount of CheY₆ binding to the flagellar motor is difficult.

These data show that YFP-Y₆ molecules have similar spatial organization regardless of its growth conditions (mostly freely diffusing). The slower PAmCherry-Y₆ diffusion measured by single molecule tracking in low light cells is unlikely to be caused by binding or interactions with the motor or chemoreceptors. Analysis of the single molecule tracks did not find PAmCherry-Y₆ to localize to any specific region of the cell. CheY₆ is a soluble, response regulator signalling protein that is not known to interact with other cytoplasmic components. In addition, PAmCherry-Y₆ molecules were overexpressed from pIND4 for the PALM studies and the majority of the population should be diffusing randomly. Therefore, the decreased diffusion rate is most probably due to the increased excluded volume effect (crowdedness) of photoheterotrophic *R. sphaeroides*.

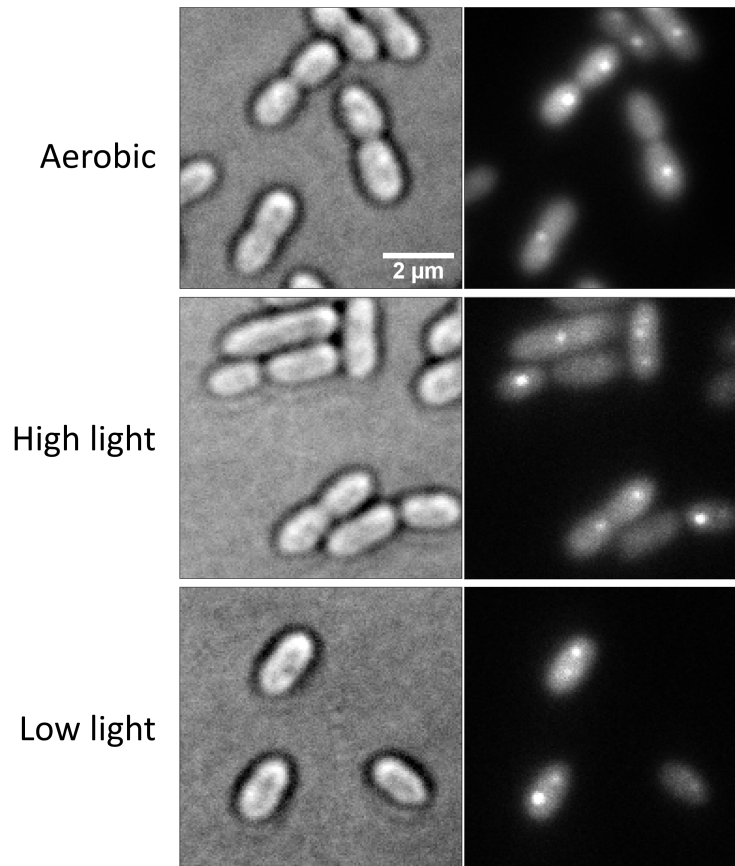


Figure 41: The spatial distribution of YFP-Y₆ in aerobic and photoheterotrophic *R. sphaeroides* WS8N expressed from the inducible pIND4 plasmid. 81.4% of aerobic cells has a CheY₆ spot at the cytoplasmic cluster (n = 113 cells). For photoheterotrophic cells, 88.9% high light cells (n = 180 cells) and 79.2% low light cells have a fluorescent spot (n = 149 cells) in addition to the diffuse fluorescence within the cytoplasm.

4.8 L1-PAmCherry fusions

To obtain information about the diffusion of large complexes in *R. sphaeroides* with different crowding levels, single molecule tracking was performed on *R. sphaeroides* ribosomal protein L1 that was labelled with PAmCherry. Unfortunately, all attempts to create L1-YFP and L1-PAmCherry fusions through homologous recombination were unsuccessful. I therefore decided to use the protein overexpressed in a wild type background as, even if not completely incorporated into the ribosome, the L1 protein is 24 kDa and its diffusion can be used to measure crowding. However, expression of L1 from pIND did lead to a reduction in growth rate and cell elongation, suggesting that it may be incorporated and interfere with protein synthesis. In this section, L1-PAmCherry was expressed from pIND4 plasmid in wild type *R. sphaeroides* WS8N and imaged using the same imaging settings.

The diffusion of L1-PAmCherry was first measured in *E. coli* as a control (Figure 42). The D distribution of L1-PAmCherry suggests that there was more than a single diffusing species within the same *E. coli* cells, which had also been observed in previous studies [9][11]. I therefore decided to fit all the D data for L1 with two gamma distributions. Two fractions of molecules diffusing at $0.44 \pm 0.05 \mu\text{m}^2\text{s}^{-1}$ (41.2%) and $1.22 \pm 0.12 \mu\text{m}^2\text{s}^{-1}$ (58.8%) were observed.

According to the work of Bakshi *et al.*, an actively translating ribosome in *E. coli* (bound to mRNA) had an estimated diffusion of $0.04 \mu\text{m}^2\text{s}^{-1}$ and its diffusion increased to $0.6 \mu\text{m}^2\text{s}^{-1}$ following the treatment of rifampacin (rif) and the depletion of mRNA [9]. Based on the diffusion of the 40 kDa PAmCherry-Y₆, the estimated diffusion coefficient of the free L1-PAmCherry (50 kDa) is around $2 \mu\text{m}^2\text{s}^{-1}$, which is 4 times faster than the observed diffusion for the slow diffusing species and almost twice as fast as the fast diffusing species. Therefore, this implies that 41.2% of the non-native L1 protein (the slow diffusing species) was incorporated into the ribosome, or was interacting with nucleic acids or other ribosomal subunits but was not involved in translation. On the other hand, the faster species was likely to be the free L1-PAmCherry molecules. Regardless of whether

the L1 molecules are being incorporated, the tracking of L1-PAmCherry provides insights on the diffusion rate of a molecule larger than both PAmCherry and PAmCherry-Y₆.

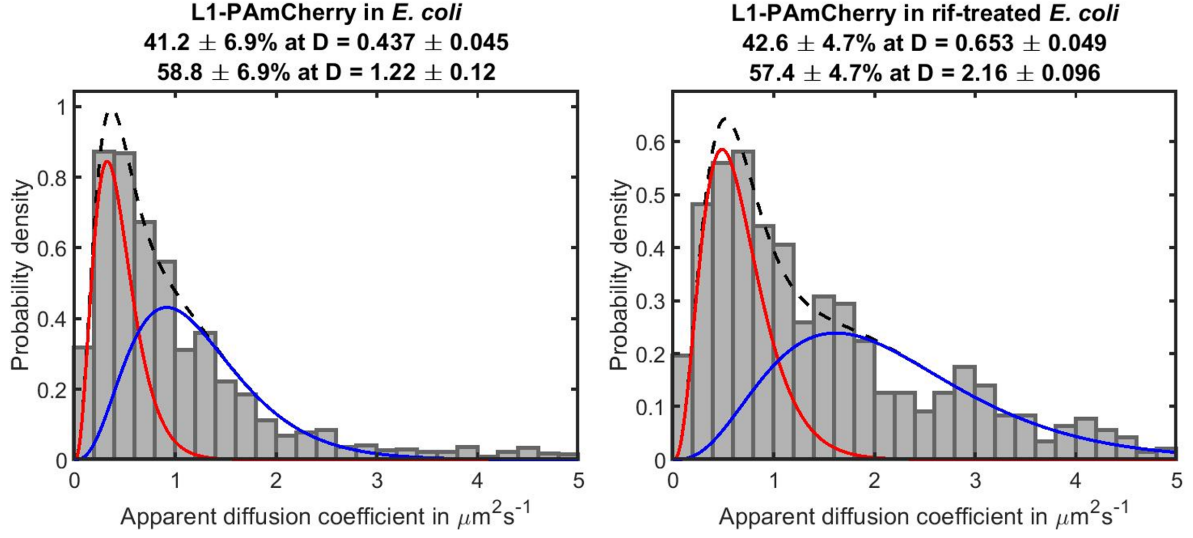


Figure 42: The apparent diffusion coefficients D of L1-PAmCherry molecules in *E. coli* ($n = 1421$ molecules) and rifampicin-treated *E. coli* ($n = 744$ molecules). The D values were calculated using all tracks with 4 steps (longer trajectories were truncated) and fitted with a least squares curve.

Previous studies showed that rifampicin (rif) could inhibit transcription and affect the structure of the nucleoid, causing a change in the diffusion of ribosomes in *E. coli* [9] and *Caulobacter crescentus* [11]. Therefore, *E. coli* expressing L1-PAmCherry was also treated with rif to investigate its effect on L1 diffusion. Surprisingly, rifampicin treated cells suggested that there may be an increase in diffusion for both the fast and slow diffusing species. For the fast diffusing species, the apparent D increased from 1.22 ± 0.12 (wild type) to $2.16 \pm 0.10 \mu\text{m}^2\text{s}^{-1}$ after the rif treatment. The shift in diffusion was probably due to the presence of faster molecules with $D > 2 \mu\text{m}^2\text{s}^{-1}$ in the rif-treated cells. The slow species also showed a slight increase in D value (from 0.44 ± 0.05 to $0.65 \pm 0.05 \mu\text{m}^2\text{s}^{-1}$). However, in this case the difference between the two slow species was probably not significant given the high error values.

4.9 Diffusion of L1-PAmCherry in *R. sphaeroides*

Preliminary results show that in aerobic cells, 57.1% of L1-PAmCherry molecules has an apparent D of $0.64 \pm 0.06 \mu m^2 s^{-1}$ and 42.9% has an apparent D of $2.49 \pm 0.29 \mu m^2 s^{-1}$ (Figure 43). Upon treatment with rif, there was a slight increase in the fast fraction from 42.9% to 51.9%, while the slow fraction decreased from 57.1% to 48.1%. In contrast to the observed nucleoid-ribosome segregation in *E. coli*, DNA in *R. sphaeroides* is dispersed throughout the cytoplasm and the spatial distribution of ribosomes relative to the DNA is unknown. Therefore, the diffusion of ribosomes in another α -proteobacterium, *Caulobacter crescentus* was also used as a reference.

Previously, 3D single molecule tracking of L1-YFP in *C. crescentus* found that the majority of the population (89%) had a D of $0.039 \mu m^2 s^{-1}$, while the rest of the population was shown to have a faster D of $3.23 \mu m^2 s^{-1}$ [11]. Again, the slower fraction was hypothesized to be fully assembled ribosomes involved in active translation, while the slower fraction was believed to be free ribosomal subunit L1 molecules [11]. After treating the cells with rif, Bayas *et al.* found that the D of the slow L1 population increased from 0.039 to $0.215 \mu m^2 s^{-1}$. The increase in D was similar to the values measured in both *E. coli* and aerobic *R. sphaeroides*, and it illustrated the diffusion of incorporated L1 which was unbound to mRNA.

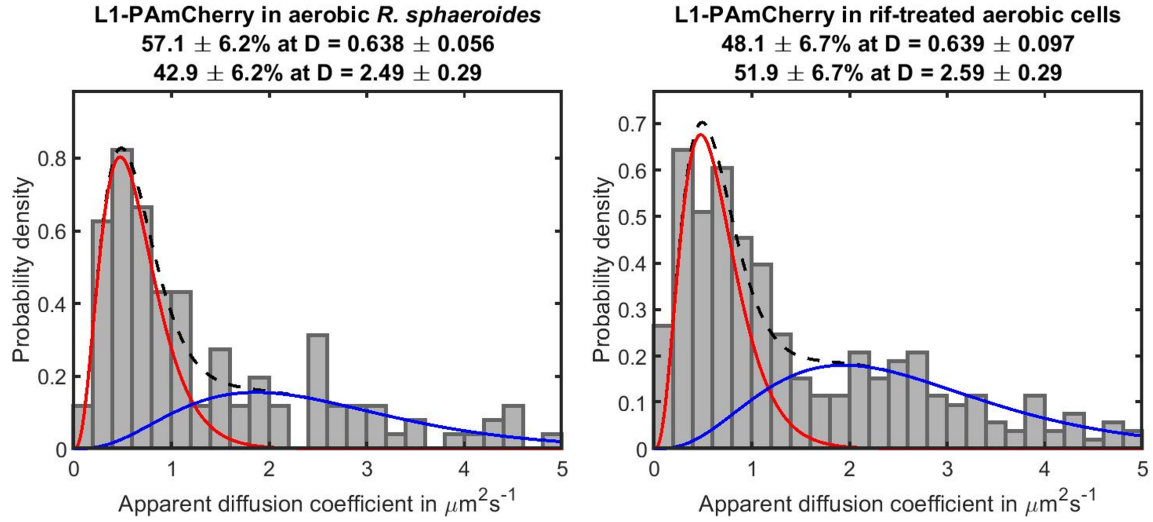


Figure 43: The apparent diffusion coefficients D of L1-PAmCherry molecules in aerobic cells ($n = 135$ molecules) and rif-treated aerobic *R. sphaeroides* ($n = 286$ molecules). The D values were calculated using all tracks with 4 steps (longer steps were truncated) and fitted with two least squares curve fits.

The results from aerobic *R. sphaeroides* and *E. coli* suggest that in a less crowded cellular environment, a 50 kDa molecule has a D of around $2.49 \pm 0.29 \mu\text{m}^2\text{s}^{-1}$, whereas a fully assembled ribosome has a D of around $0.64 \pm 0.06 \mu\text{m}^2\text{s}^{-1}$. Next, I measured the diffusion of L1-PAmCherry in high and low light *R. sphaeroides* where crowding levels are expected to be higher. It was found that high and low light cells have overall lower apparent D values compared to aerobic cells (Figure 44). The histograms of L1-PAmCherry for both photoheterotrophic growth conditions show a shift from fast to slow diffusion compared to aerobic cells, and low light cells were found to have two populations of slow diffusing molecules with $D > 0.5 \mu\text{m}^2\text{s}^{-1}$. These results indicate that crowding effects by ICM vesicles can also lower the diffusion of larger molecules. In the case of low light where the cytoplasm is most crowded, any free L1 subunits and fully assembled ribosomes seemed to be moving at the same apparent diffusion coefficient.

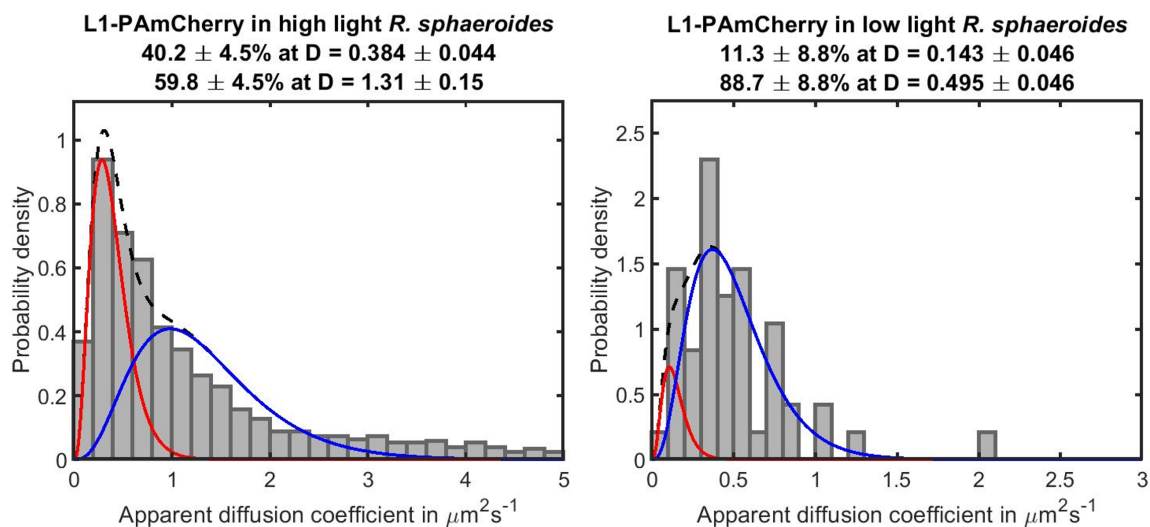


Figure 44: The apparent diffusion coefficients D of L1-PAmCherry molecules in high light ($n = 1102$ molecules) and low light *R. sphaeroides* ($n = 50$ molecules). The D values were calculated using all tracks with 4 steps (longer steps were truncated) and fitted with a least squares curve.

4.10 Discussion

In this chapter, the effects of *R. sphaeroides* ICM vesicles on the diffusion of different sized proteins were investigated using single molecule tracking. Chapter 3 showed that the increase in number of chromatophores in photoheterotrophic *R. sphaeroides* generated higher macromolecular crowding levels measurable by a FRET sensor. Therefore, the goal of this chapter was to determine whether the increase in crowding, as the cells transitioned from aerobic to photoheterotrophic growth, was also reflected by the decrease in protein mobility. PAmCherry was used to visualize the motion of single tracer molecules in aerobic, high light, and low light *R. sphaeroides* and a comparison of the apparent diffusion coefficients was made. Table 11 provides the apparent diffusion coefficients of PAmCherry, PAmCherry-CheY₆, and L1-PAmCherry calculated under different crowding conditions.

Histograms of diffusion coefficients for PAmCherry and PAmCherry-Y₆ in aerobic *R. sphaeroides* suggested that there was only one predominant population diffusing with an apparent D of 3.62 ± 0.23 and $3.23 \pm 0.18 \mu\text{m}^2\text{s}^{-1}$, respectively. These data agree well with the diffusion coefficients measured in *E. coli*, since both the cytoplasm of *E. coli* and aerobic *R. sphaeroides* do not contain any chromatophores and should be relatively less crowded.

Interestingly, single molecule tracking of PAmCherry and PAmCherry-Y₆ in photoheterotrophic *R. sphaeroides* showed the presence of a second slow-diffusing species within the same cells with apparent D $< 0.5 \mu\text{m}^2\text{s}^{-1}$ that was not found in aerobic *R. sphaeroides* and *E. coli*. Previous studies described the effects of metabolic state and impermeable barriers on molecular motion [98][144]. For instance, tracer molecules could be trapped in microcompartments or undergo binding to structures such DNA, resulting in reduction of mobilities. However, PAmCherry was not expected to bind or be associated with any cytoplasmic components since it was foreign to *R. sphaeroides*. In addition, all single molecule tracking work was conducted in cells undergoing exponential growth. Therefore, the difference in diffusion coefficients was not caused by decreased

cytoplasmic fluidity as a result of metabolic arrest. The slow-diffusing species observed in high light and low light cells probably arise from excluded volume effects exerted by the ICM vesicles of photosynthetic *R. sphaeroides*. Since ICM vesicles contribute to high level of autofluorescence, in the future it may be possible to measure the autofluorescence level in each cell to estimate the amount of chromatophores per cell.

The decrease in protein diffusion in photoheterotrophic cells suggested that the presence of chromatophores may occupy significant proportion of the cytoplasmic volume and increase crowding by compressing the rest of the cytoplasmic contents. In this study, ICM vesicles were also considered as static, impermeable obstacles that may lead to a drop in apparent diffusion coefficients. Even though there was evidence suggesting that *R. sphaeroides* chromatophores exist as discrete, spherical structures in the cytoplasm [141] [93], these 70 nm vesicles probably have very low mobilities *in vivo* due to the large size and crowdedness of the cytoplasm. In addition, a short frame rate of 7.75 ms was used to image PAmCherry and the diffusion coefficients were calculated based on 5 localizations (4 steps). The motion of chromatophores over the period of around 39 ms would be close to stationary.

Under high light condition, *R. sphaeroides* cells start to generate around 400 ICM vesicles per cell (as shown in Chapter 3) that could take up around 5.6% of the total cytoplasmic space, thus creating a higher crowding level in the cytoplasm compared to aerobic cells. 66% of PAmCherry and 45% of PAmCherry-Y₆ molecules were diffusing at around $D = 2 \mu m^2 s^{-1}$ in high light cells, which was slightly slower than the diffusion coefficient in aerobic cells. This suggested that a reduction of around 5.6% of the total cytoplasmic space in high light cells was enough to cause a measurable difference in apparent diffusion coefficient. In addition, it is possible that some PAmCherry and PAmCherry-Y₆ molecules had decreased ability to diffuse around freely due to these impermeable obstacles or became confined in microcompartments surrounded by chromatophores, forming a second population of slow-diffusing species with $D < 0.5 \mu m^2 s^{-1}$. The high light cells also had the most heterogeneous diffusion profiles and the largest

spread in D distribution. The heterogeneity in D values may also be contributed by cells being in different developmental stages during imaging.

As the cells filled the cytoplasm with more vesicles under low light condition, the available space accessible by the protein molecules decreased and PAmCherry and PAmCherry-Y₆ molecules showed further reductions in diffusion coefficients. As mentioned in Chapter 3, 1200 ICM vesicles could take up around 19% of the cytoplasmic volume in low light cells and increase crowding by concentrating the rest of the biomolecules in the cell. Multiple diffusing species were observed but both populations had estimated apparent D values of $< 1 \mu m^2 s^{-1}$, and around 50% of the molecules was diffusing at around $D = 0.3 \mu m^2 s^{-1}$. Both molecules experienced up to 10 times reductions in apparent D (from around 3 to $0.3 \mu m^2 s^{-1}$) as the cells transitioned from aerobic to low light condition (Table 11).

Table 11: Apparent diffusion coefficients of different-sized protein molecules in aerobic and photoheterotrophic *R. sphaeroides* measured by single molecule tracking. As crowding increases (low light > high light > aerobic), the apparent diffusion coefficient D decreases.

Molecule	Size (kDa)	Apparent diffusion coefficient ($\mu m^2 s^{-1}$)		
		Aerobic	High light	Low light
PAmCherry	27	3.62 ± 0.23	66%: 2.46 ± 0.09 34%: 0.50 ± 0.03	48%: 0.94 ± 0.10 52%: 0.28 ± 0.03
PAmCherry-Y ₆	40	3.23 ± 0.18	45%: 2.01 ± 0.17 55%: 0.46 ± 0.03	46%: 1.07 ± 0.20 54%: 0.29 ± 0.03
L1-PAmCherry	>50	43%: 2.49 ± 0.29 57%: 0.64 ± 0.06	60%: 1.31 ± 0.15 40%: 0.38 ± 0.04	89%: 0.50 ± 0.05 11%: 0.14 ± 0.05

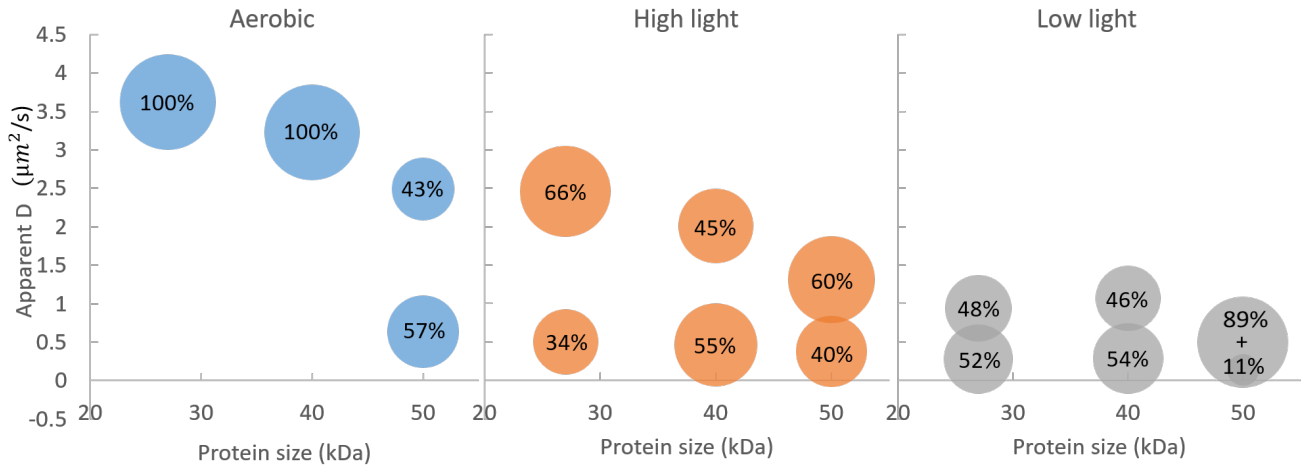


Figure 45: The apparent diffusion coefficients ($\mu m^2 s^{-1}$) of protein molecules were affected by the molecular size and crowding level. In general, the apparent D decreases with increasing size and crowding level.

A similar reduction in diffusion coefficient was also observed for L1-PAmCherry. Previously, fully assembled ribosomes were found to be confined to the nucleoid-free regions in *E. coli* cells [111][8]. However, the individual trajectories for L1 did not show specific confinement within the cell as *R. sphaeroides* has a diffuse nucleoid that fills the cytoplasm. In addition, the chromatophores were also not known to localize to any specific cell region, although the ICM network may be denser towards to the cell periphery during the earlier development stages when the vesicles were still attached to the cytoplasmic membrane.

The results of this chapter provide useful insights regarding the effects of crowding on the diffusion of proteins larger than 27 kDA. By comparing each protein's highest and lowest diffusion coefficients under aerobic and low light condition, I found that protein diffusion *in vivo* could be decreased by up to 10 times through crowding effects of ICM vesicles (Table 11). Figure 45 also shows the relationship between diffusion coefficient, protein size, and crowding levels. It is interesting to see that the addition of a small CheY₆ could cause a significant difference in D between free PAmCherry and PAmCherry-Y₆. It is possible that the attachment of CheY₆ changes the hydrodynamic radius of the molecule as both molecules are more similar in size; whereas the addition of a relatively small FP tag to a ribosome does not change the overall shape of the large molecule and has less effect on its diffusion.

Another factor that could affect protein diffusion is the possible interactions between the tracer molecules and the *R. sphaeroides* cellular components. Previous studies suggested that protein molecules, especially proteins that are native to the cytoplasm, could interact specifically or nonspecifically with other cellular components, resulting in large reduction in molecular diffusion *in vivo* [32][90][159]. For example, a study showed that positively charged GFP could bind to the surface of ribosomes and experienced a dramatic decrease in mean D down to $0.14 \mu\text{m}^2\text{s}^{-1}$ in the cytoplasm of *E. coli* and *Lactococcus lactis* [113]. In this work, PAmCherry is not known to have strong association to large cell structures, as binding to ribosomes or DNA would lead to an obvious decrease in

PAmCherry mobility in aerobic *R. sphaeroides* and *E. coli*.

In this study, the decrease in protein mobility is in parallel with the increase in chromatophores and membrane surface area within the cytoplasm. Therefore, possible binding to the membrane should also be considered as it can contribute to a reduction in D . The single molecule trajectories of PAmCherry, PAmCherry-Y₆, L1-PamCherry did not indicate binding to the plasma membrane. However, the chromatophore membrane of *R. sphaeroides* has a distinctive protein composition that is different from the plasma membrane. As the main photosynthetic membrane system in *R. sphaeroides*, the surface of ICM vesicles is packed with light harvesting (LH) complexes, reaction center, cytochrome bc_1 complexes, and ATPases to facilitate efficient photosynthesis [22][86]. This specialized membrane architecture may affect the subcellular distribution of cellular components in bacterial cell. For instance, the thylakoid membrane of some cyanobacteria species was known to be associated with photosynthetic mRNA [79]. Currently, it is unclear whether there is a difference in the distribution of ribosomes between aerobically and photoheterotrophically grown *R. sphaeroides*. However, *R. sphaeroides* chromatophores were not known to be associated with ribosomes. Fluorescence microscopy using YFP-Y₆ showed localization to the cytoplasmic chemosensory cluster but not to the chromatophores. However, it is not possible to rule out the presence of weak, unspecific interactions between the tracer molecules and the chromatophores. Future work on the properties and surface charges of these proteins are required to better understand molecular diffusion in the presence of ICM vesicles.

Understanding how crowding levels affect the diffusion and mobility of proteins is important for diffusion-dependent signalling pathways such as chemotaxis. Under normal conditions, studies suggested that CheY diffusion from the chemoreceptor cluster to the flagellar motor in *E. coli* is not limiting due to the small size of the bacterial cell and the rapid diffusion of CheY. However, most studies did not consider the effects of crowding on protein diffusion. In this study, a significant reduction in PAmCherry-Y₆ diffusion coefficient was measured in photoheterotrophic cells where crowding is high, suggesting

that the response and adaptation time of chemotaxis in photoheterotrophic *R. sphaeroides* may be different.

However, the addition of genetically encoded fluorescent protein tags may affect the functionality and diffusion of target proteins. Previous study by Nenninger *et al.* suggested that GFP tagging of target proteins between 27 to 84 kDa has low effect on protein mobility [90]. However, the addition of a 27 kDa PAmCherry tag to the small 14 kDa CheY₆ will definitely reduce its diffusion within the cytoplasm. Larger molecules are known to diffuse significantly slower under crowding effects because they experience larger excluded volume. It is possible that the actual diffusion of a small 15 kDa protein will not be affected by macromolecular crowding effects in photoheterotrophic *R. sphaeroides*. The procedures for labeling the 14 kDa CheY₆ with a small <1 kDa organic dye and internalizing the labelled protein into live cells were discussed in Chapter 4.

In the future, using an organic dye with a longer fluorescent lifetime will enable longer tracking before the fluorophore photobleaches. Having a longer tracking time may enable the visualization of proteins transitioning between different diffusion modes. For example, a molecule could shift from free diffusion to confined or binding motion as it became caged within a local crowded region.

5 Electroporation of maleimide dye labelled CheY₆

The effects of macromolecular crowding in the bacterial cytoplasm can be inferred by:

- Expressing a FRET probe *in vivo* and analyzing the FRET ratios
- Measuring the diffusion of different-sized protein molecules *in vivo*

In this chapter, the diffusion of single CheY₆ proteins, labelled with a fluorescent organic dye, in *R. sphaeroides* and *E. coli* was investigated using single molecule tracking. Compared to genetically encoded fluorescent proteins, fluorescent organic dyes are smaller in size, brighter, and more photostable [30][119]. However, protein-dye conjugation requires laborious *in vitro* protein purification and labelling process. Native CheY₆ protein has very low labelling efficiency to maleimide fluorescent dyes because it only has one cysteine residue that is buried in the protein structure. In this work, cysteine-modified CheY₆ mutants with enhanced labelling yield have been generated and labelled with a best suited organic dye. A novel technique of protein internalization into live bacteria cells *via* electroporation [26] was used to study the behaviours of CheY₆ *in vivo* using a custom built microscope.

5.1 Generation of cysteine-modified CheY₆ mutants for enhanced labelling

The thiol or sulfhydryl group (SH) on a cysteine residue is commonly used in protein labelling to attach maleimide organic dyes to protein molecules. However, the thiol groups on cysteine residues can form disulfide bonds (S-S) with each other and become unavailable for maleimide reaction. In addition, oxidation of the thiol (SH) side chain leads to the formation of sulfenic acid (SOH) that is unreactive to maleimide dyes. Therefore, thiol reducing agents such as dithiothreitol (DTT) and β -mercaptoethanol (BME) are used to reduce the thiol groups / native disulfide bonds in a protein molecule, allowing the cysteine residues to react with organic fluorophores to form stable covalent bonds.

R. sphaeroides CheY₆ has one native cysteine (residue 80) that is inaccessible to the maleimide reaction. Therefore, one cysteine residue was introduced to the CheY₆ C terminus position 134 by quick-change PCR to generate a cysteine-modified mutant CheY₆(A134C) (M. W. Smith, 2015) (Figure 46). CheY₆(A134C) contained a 6× His-tag for purification and was successfully labelled with Atto647 dye. However, CheY₆(A134C) was known to have low labelling yield because the carboxyl group at the C terminus, being negatively-charged, may be unfavourable for cysteine-maleimide crosslinking (Prof. C. Redfield, personal communication). In addition, unfolding of the CheY₆(A134C) was found to enhance the labelling yield, suggesting that the inserted cysteine was inaccessible.

I therefore made a new CheY₆ mutant with better labelling performance. Discussion with Prof. C. Redfield suggested that the addition of cysteine on the more exposed N terminus, in particular position 3 or 4 (glycine or serine), before the 6× His-tag sequence, may improve labelling (Figure 46). Cysteine insertion at the N terminus should not impact the protein structure or functionality since it is away from the interaction site (asp56) of CheY₆ and CheA₃P1. The presence of a positively charged methionine on the N terminus may even encourage the labelling reaction (Prof. C. Redfield, personal communication).

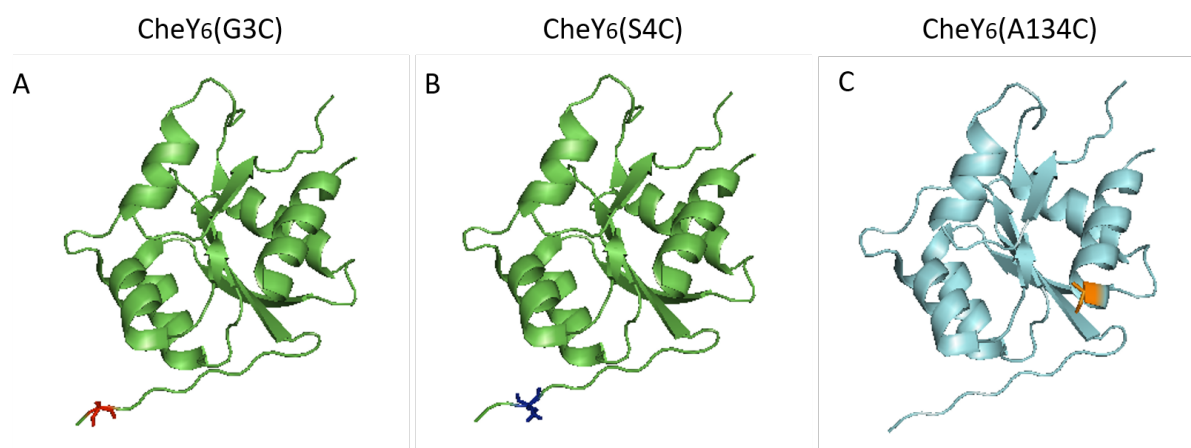


Figure 46: The structure of CheY₆ with a 6× His-tag sequence. (A) CheY₆(G3C) shows the 3rd residue on the His-tag, glycine, coloured in red. (B) CheY₆(S4C) shows the 4th residue on the His-tag, serine, coloured in blue. (C) The alanine on position 134 is shown in orange. To generate CheY₆ mutants CheY₆(G3C) and CheY₆(S4C), quick change was used to change the glycine and serine residue to cysteine. (PDB: 3KYI).

Quick change PCR was used to generate the cysteine-modified mutants, CheY₆(G3C) and CheY₆(S4C) using specific primers (see Methods). The degree of labelling (DoL) of the two mutants was compared to the previous CheY₆(A134C) mutant in Figure 47. Unfolded and native CheY₆ mutants were each labelled with 5, 2, 1, 0.75, and 0.5× molar excess Atto647 dye and loaded on SDS-PAGE gels to allow separation of labelled CheY₆ and free dye. The gels were then scanned using UV-Vis scanner at 647 nm excitation and stained with Coomassie Blue. The relative DoLs (arbitrary units) were determined by comparing the intensity of the fluorescent bands of CheY₆-Atto647 (normalized to the intensity of the coomassie bands to prevent unequal loading error).

CheY₆(A134C) had very low labelling yield in its native form across all concentrations of Atto647, as shown by the very low intensities of CheY₆-Atto647 fluorescent bands in Figure 47, 3A. The DoL for CheY₆(A134C) was improved significantly when the protein was unfolded, probably due to the increased accessibility of the buried cysteine. CheY₆(G3C) and CheY₆(S4C), on the other hand, had better DoL even in their native form. Their labelling efficiency increased with the increasing concentrations of dye added, suggesting that a higher molar excess of dye was required for improving the DoL for CheY₆ mutants. This was not too surprising as most protocols recommended a 5× molar excess dye for maximum labelling efficiency.

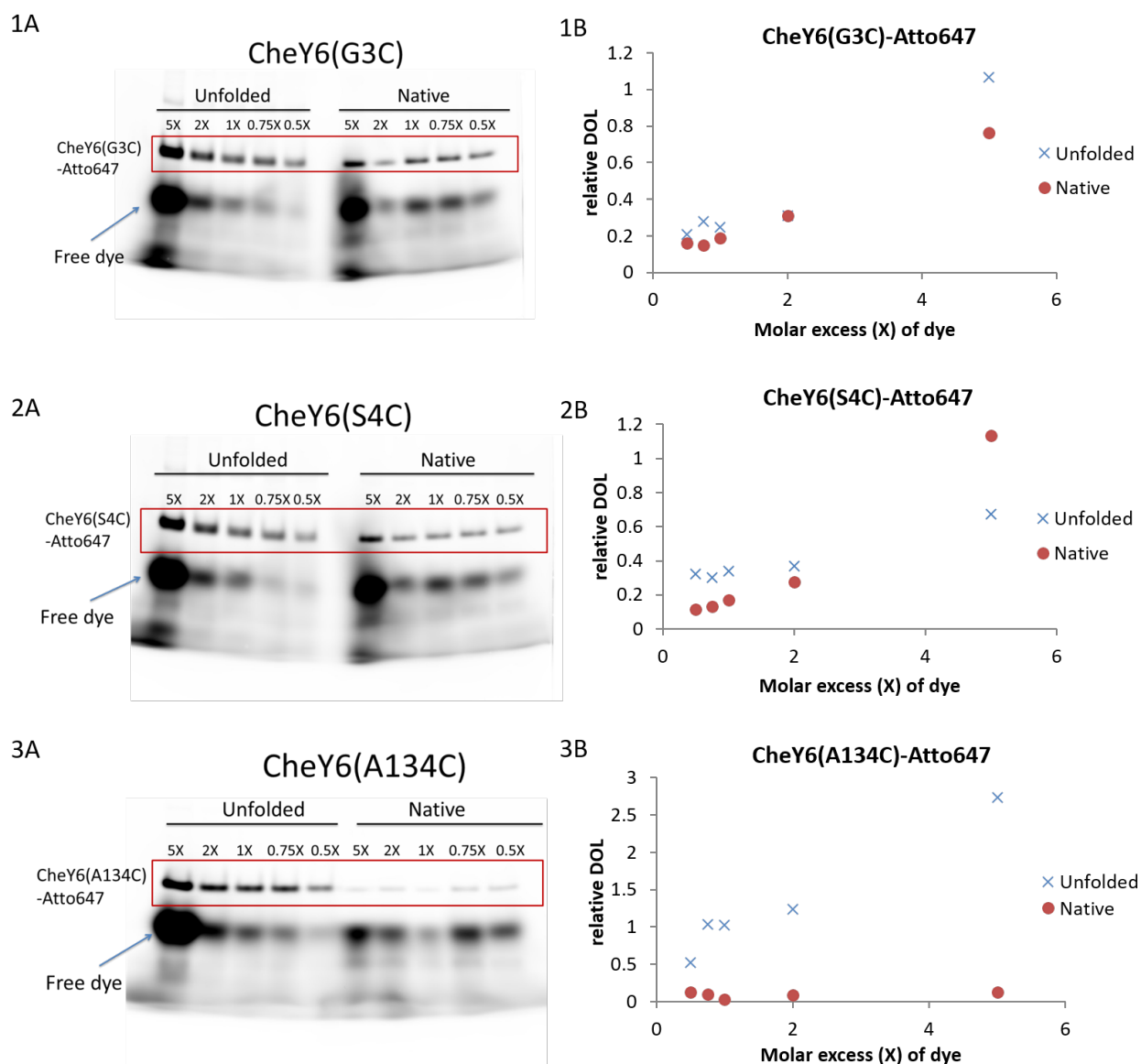


Figure 47: SDS-PAGE of CheY₆-Atto647 samples scanned using UV-Vis scanner at 647 nm excitation. Native and unfolded CheY₆ mutants were labelled with varying concentrations of Atto647 and loaded on SDS-PAGE. The relative DoLs (arbitrary units) were determined by comparing the intensities of the fluorescent bands with the total amount of protein loaded (obtained from the intensities of coomassie bands, not shown). The relative DoLs for native CheY₆(A134C) when its labelled with 0.5 – 5× molar excess dye were low (<0.15 A. U.). However, when CheY₆(A134C) was unfolded and labelled with 5× molar excess dye, the DoL increased from 0.12 A. U. to 2.73 A. U., suggesting that the low DoL is caused by the inaccessibility of the cysteine residue for labelling. Atto647 has an excitation maximum at 643 nm.

Circular dichroism (CD) for CheY₆(G3C) and CheY₆(S4C) were conducted according to the parameters in Methods to confirm that the two mutants were folded (Figure 48). Mutant CheY₆(G3C) was chosen for the rest of the electroporation experiments because its overall relative DoL was slightly higher than the DoL of CheY₆(S4C).

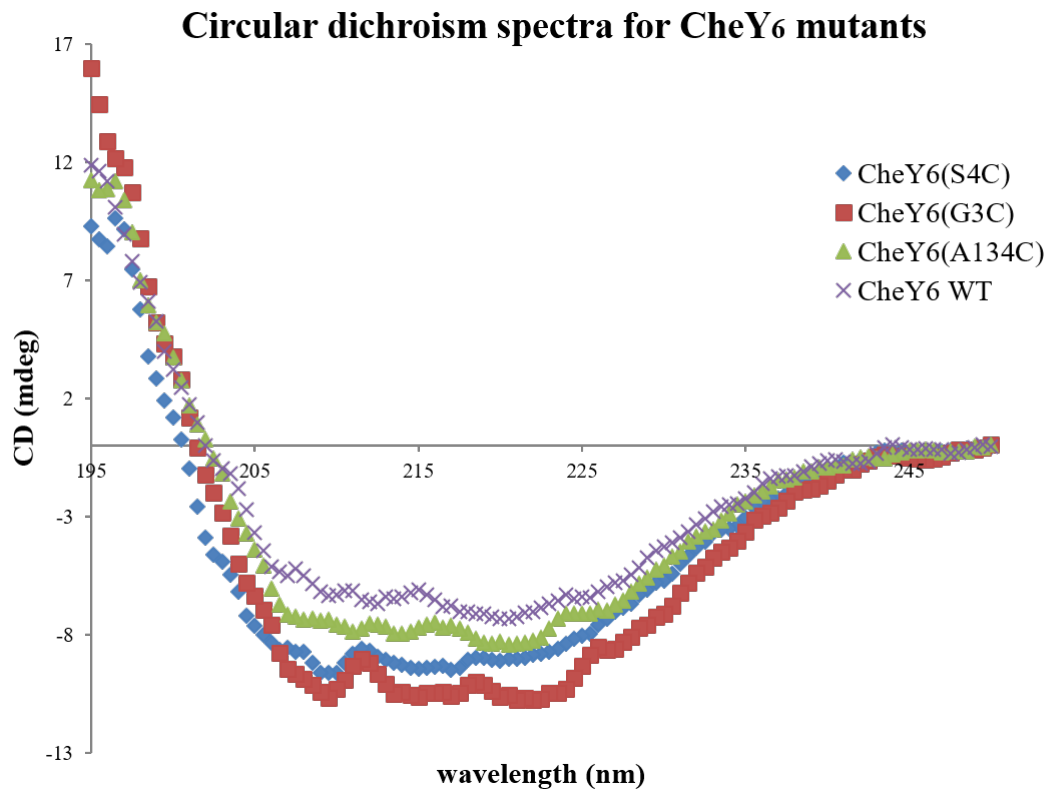


Figure 48: Circular dichroism (CD) traces of three CheY₆ mutants were compared to CheY₆ wild type spectrum. The presence of α -helices in these proteins shows that the proteins were folded.

5.2 Dye candidates

Previously, CheY₆ and CheY were labelled with Atto647 and electroporated into aerobic *R. sphaeroides* (M. W. Smith, 2015) and *E. coli* [31]. Atto647 was bright and photostable, making it a good candidate for single molecule tracking of rapidly diffusing molecules. However, preliminary labelling experiments with Dr. Helen Miller found that it was difficult to separate free Atto647 dye from labelled CheY₆. Unbound dyes, although in low concentration relative to labelled protein, were able to diffuse into the periplasmic and cytoplasmic fractions of the cell and introduced artefacts for the single molecule tracking results. Figure 49 shows free Atto647 dye taken up by non-electroporated *E. coli* RP437 and *R. sphaeroides* WS8N. Empty *R. sphaeroides* cells (- dye) had autofluorescence but cells exposed to free Atto647 dyes were brighter. Interestingly, *R. sphaeroides* cells seemed to be more permeable to free dye compared to *E. coli*. Around half of the *E. coli* cells did not take up free dye; whereas all *R. sphaeroides* cells were heavily loaded with Atto647. Size exclusion chromatography also showed that CheY₆ formed aggregates and multimer with Atto647 during labelling (Figure 50), lowering the final yield of labelled CheY₆ available for electroporation.

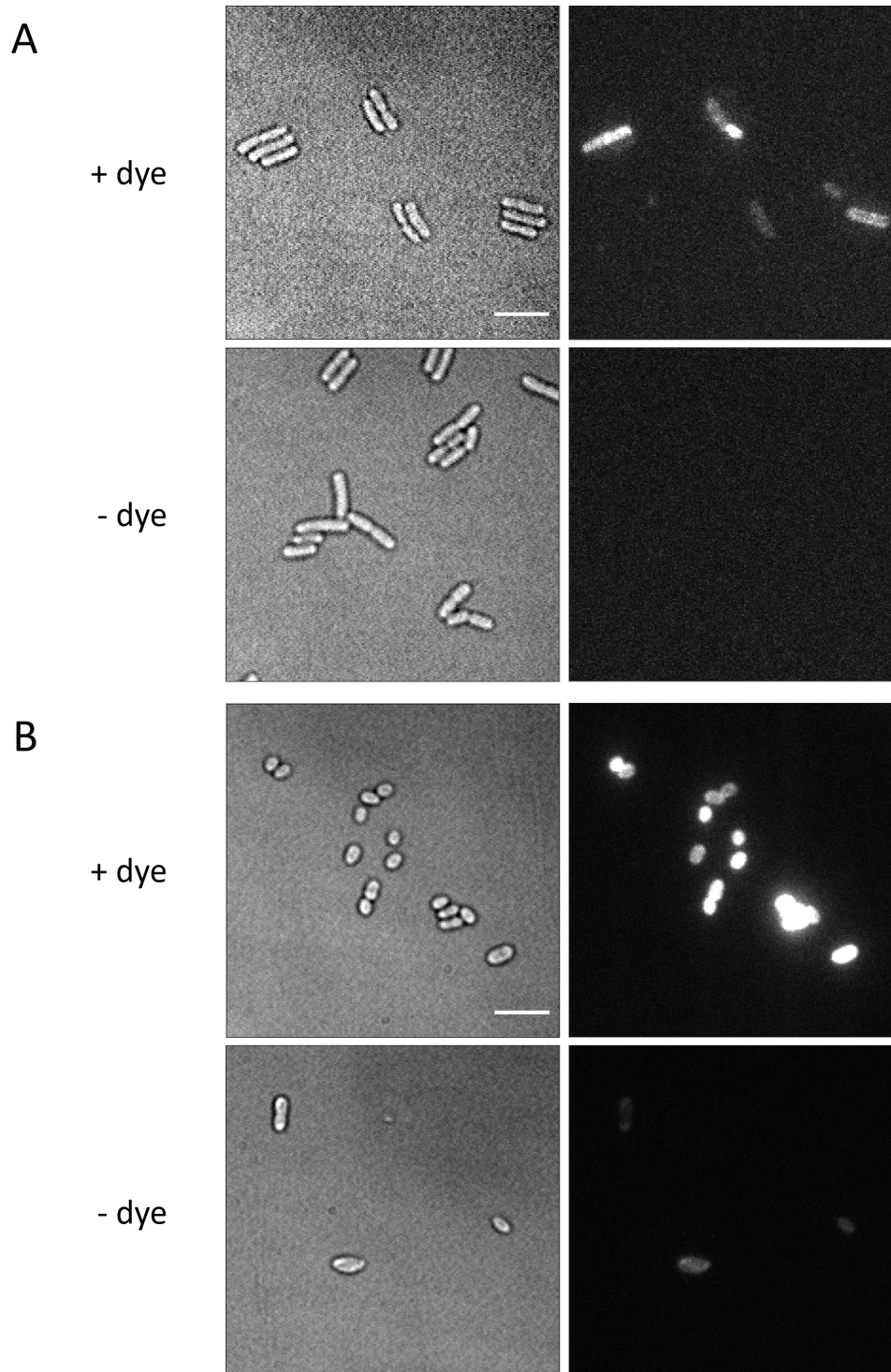


Figure 49: Free Atto647 dye was able to internalize or bind to (A) *E. coli* RP437 and (B) *R. sphaeroides* WS8N cells. Around 50 % of the *E. coli* cells and all of the *R. sphaeroides* cells took up free dye. Autofluorescence could be observed in empty *R. sphaeroides* cells that were not added with free dye. Brightness and contrast of the two fluorescent images for *E. coli* and *R. sphaeroides* were displayed at the same levels respectively using ImageJ for comparison. Excitation: 620 - 640 nm. Exposure: 42 ms. Power: 0.417mW. Scale bar 3 μm .

To overcome the issue of free dye contamination and protein aggregation, different labelling conditions were tried:

- Protein-dye conjugation reaction was allowed to take place in the dark for 1 hour at room temperature or at 4°C overnight
- Different labelling buffer pHs were tested
- DMSO concentration was limited to less than 4%
- Different dye to protein ratio was tested by using 1 to 5 $\times$ molar excess dye
- Lower protein concentration and larger reaction volume were tested
- Different size exclusion column was used: Superdex 75 10/300 GL and HiLoad 16/600 Superdex 75 pg were used for free dye removal
- Overnight dialysis was used to remove excess free dye after labelling

Unfortunately, no significant improvement in labelling efficiency was observed. There seemed to be a rather strong nonspecific interaction between the Atto647 dye and CheY₆ sample. Previously, the hydrophobicity of an organic dye was found to correlate with the dye's nonspecific adhesion to other substrates [162]. In addition, several organic red dyes such as Atto647N maleimide, Atto647 NHS-ester, and Alexa633 maleimide were found to have strong or moderate interaction with lipid bilayers, although the factor that determined this level of membrane-dye interaction was unclear [53]. If Atto647 has the tendency to bind nonspecifically to CheY₆, it may also interact with other cellular components, which is not ideal for studying protein diffusion.

Therefore, a hydrophilic maleimide dye, iFluor633 was selected based on its brightness and ease of conjugating to CheY₆. iFluor633 has a size of around 1 kDa, which is much smaller than the typical 27 kDa fluorescent protein and ideal for tracking of the 14 kDa CheY₆. Alexa Fluor633 was found to have a similar brightness compared to Atto647 and iFluor633 but was not selected because it had a higher blinking frequency and was often used in STORM studies.

5.3 Optimizations to standard labelling and purification protocols

The labelling and purification procedures for CheY₆-iFluor633 were optimized. In brief, 200 μ M protein sample aliquot was diluted with labelling buffer to a reaction volume of 2 mL and a final concentration of 20 μ M. 10 μ L of 20 μ M dye resuspended in DMSO was added to the reaction sample drop by drop with gentle mixing. The conjugation reaction was allowed to proceed in the dark at room temperature for 1 hour before adding DTT to quench the reaction.

Previously, the conjugation mixture containing excess dye was loaded onto a size exclusion chromatograph column directly to remove free dye. However, residual dye molecules were found to trap within the resin and contaminate other protein samples, probably due to the hydrophobicity of the Atto647 dye. Therefore, the protein-dye mixture was first added to a nickel column and washed with at least 30 column volume of washing buffer. Since CheY₆ contained a His-tag, it would bind to the resin while the excess free dye got washed away. Then, the CheY₆ sample was eluted from the nickel column and loaded on size exclusion chromatograph to further remove any free dye from the labelled protein. By labelling CheY₆ with iFluor633 using the optimized protocols, CheY₆ aggregation and free contamination were reduced significantly (Figure 50).

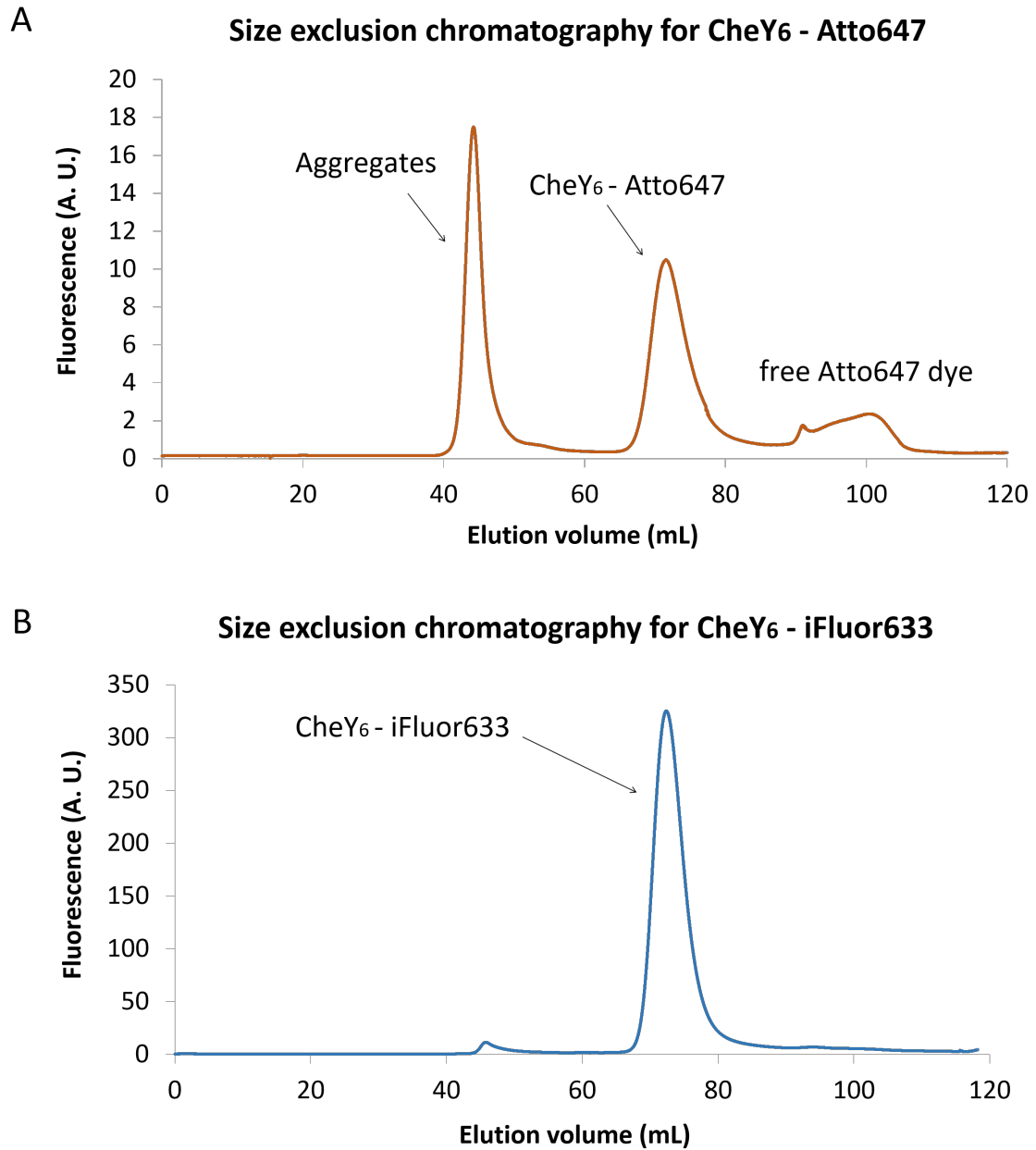


Figure 50: Size exclusion chromatograph (SEC) was used to remove free dye molecules. CheY₆-dye conjugate was purified using a gel filtration column (HiLoad 16/600 Superdex 75 pg) attached to a UV detector with the suitable excitation and emission wavelengths. (A) CheY₆ formed aggregates and multimer when it was labelled with Atto647. SEC settings: Excitation: 645 nm. Emission: 669 nm. Sensitivity: 1 (high). Gain: 1. Flow rate: 1.0 mL/min. (B) Purification of CheY₆-iFluor633 showed minimal aggregation and free dye contamination. SEC settings: Excitation: 638 nm. Emission: 655 nm. Sensitivity: 2 (medium). Gain: 2. Flow rate: 0.8 mL/min. The elution protein-dye samples were collected using a 96-well sample collector and stored at -80°C.

After removal of free dye by size exclusion chromatograph, an SDS-PAGE was ran and imaged using a UV-Vis scanner to assess any free dye contamination. Purified protein sample was then concentrated to a final volume of 500 μ L and stored at -80°C. Labelling yield could also be quantified by comparing the concentration of unlabelled protein and the protein-dye conjugate, using the degree of labelling (DoL) formula:

$$\frac{Dye}{Protein} = \frac{A_{max}\epsilon_{Protein}}{[A_{280} - (correctionfactor)A_{max}]\epsilon_{Dye}}$$

where A_{max} is the maximum absorption of the dye at its excitation peak, A_{280} is the maximum absorption of the protein at 280 nm, $\epsilon_{Protein}$ and ϵ_{Dye} are the protein's and the dye's extinction coefficients ($M^{-1}cm^{-1}$) respectively, and the correction factor accounts for absorption of the dye at 280 nm.

5.4 Optimized electroporation of protein into *E. coli* and *R. sphaeroides*

Electrocompetent *E. coli* and *R. sphaeroides* were made according to the protocols in Chapter 2. 2D imaging of CheY₆ in *E. coli* and *R. sphaeroides* was conducted in collaboration with Dr. Helen Miller. 3 samples were prepared before imaging the cells:

- Electroporated (EP) sample: Electrocompetent cells were incubated with CheY₆-iFluor633 in a pre-chilled cuvette and an electric field was applied to internalize the labelled CheY₆. The cells were allowed to recover in SOC medium for *E. coli* or succinate (SUX) medium for *R. sphaeroides* for at least 10 minutes. The number of internalized protein *via* electroporation can be improved by increasing the amount of labelled protein added and using higher voltage electrical pulse. However, by using a high voltage, the viability of cells after electroporation will decrease. Non-internalized protein was removed by washing the cells before imaging.
- Non-electroporated (NEP) sample: Same treatment as the EP sample minus the electric pulse. This sample served as a control to measure the efficiency of the electroporation technique and was used to control against false positives due to contamination of free dye.
- Negative control (EMPTY): Electrocompetent cells that were not incubated with the fluorophores or labelled protein.

After recovery, cells were washed with MB buffer for *E. coli* or SUX for *R. sphaeroides*. Washed pellets were then resuspended in 500 μ L buffer. A 1:5 dilution was made by taking 100 μ L of resuspended cells and adding with 400 μ L buffer to reach an OD₆₀₀ of 0.5. The resuspended cells were then washed 3 times using 0.22 μ m Ultrafree-MC Centrifugal Filter (Merck) with hydrophilic PVDF and 0.5 mL volume. For each wash, the filters were added with 500 μ L buffer and centrifuged at 7000 rpm. After the washes, a final spin for 1 minute was used to remove any residual buffer. The washed cells were

resuspended in 50 μ L buffer and placed on 1% agarose pads with a burnt coverslip for imaging.

Single molecule tracking was performed on a custom built fluorescence microscope from the Berry Group. Several optimizations were made to the microscope setup to maximize signal-to-noise ratio for iFluor633. These include experimenting with different filter sets, allowing a larger excitation band, increasing the laser power, and decreasing the field of view to allow high speed imaging. The washing protocol and microscope optimizations were developed by Dr. Helen Miller.

Previously, visualising electroporated labelled CheY₆ was extremely difficult due to low concentration of labelled protein being added to the electroporation procedure. Achieving a good concentration of dye labelled CheY₆ was challenging as a result of the low labelling yield of the previous CheY₆ mutant. Figure 51 showed an example of electroporated *E. coli* cells that did not show fluorescent signals due to the addition of insufficient CheY₆. Single molecule tracking was not possible since the fluorescent images of EP sample looked identical to the images of EMPTY sample. However, this observation also suggested that the electroporation technique would not lead to autofluorescence in cells. Previous studies using the electroporation technique also did not report the technique as a contribution of autofluorescence in live cells [136][149][31].

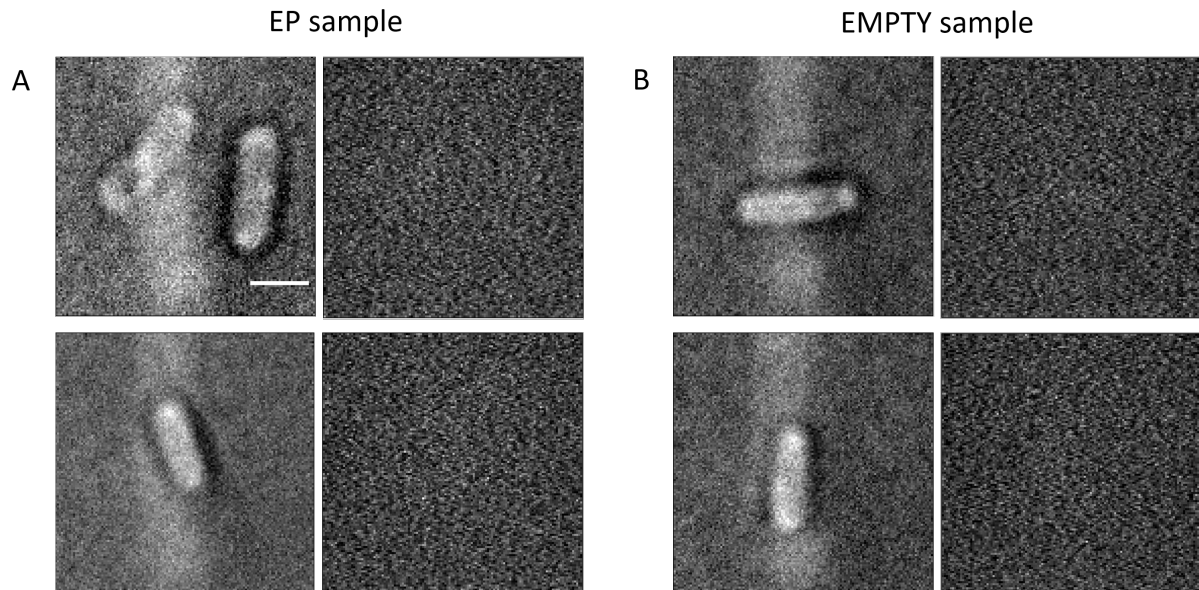


Figure 51: (A) No fluorescent signals could be observed in the electroporated (EP) sample when an insufficient amount of labelled CheY₆ was added. The panels on the left show the brightfield image and the panels on the right show the fluorescent images. The EP sample (A) looked similar to the EMPTY sample (B), with a clean background with no autofluorescence. This also showed that electroporation did not trigger autofluorescence in *E. coli* cells. Brightness and contrast of the fluorescent images were displayed at the same levels respectively using ImageJ for comparison. Exposure time 15 ms. Scale bar 1.5 μm .

After increasing the labelling yield for CheY₆, CheY₆-iFluor633 was successfully electroporated into *E. coli* and aerobic *R. sphaeroides* cells. Figure 52 showed an example of single CheY₆ molecules diffusing in the *E. coli* EP sample. The EMPTY sample from the same session had a clean background with low or no autofluorescence. This suggested that the observed fluorescent signal came from CheY₆ and not false positives due to background. Previously, fluorescence signals were observed in NEP cells incubated with CheY₆-Atto647, suggesting that free dye in the labelled protein sample could diffuse across the cellular membrane. However, using the optimized labelling and washing protocols with iFluor633, both the NEP and EMPTY samples gave no or very weak fluorescence signals. To prevent any false positives, the EMPTY and NEP samples were imaged using the same settings as the EP sample at the same time. If autofluorescence was observed in the EMPTY or NEP sample, the EP data from the same session would be

discarded. Figure 53 shows 3D fluorescence images of CheY₆ in aerobic *R. sphaeroides*.

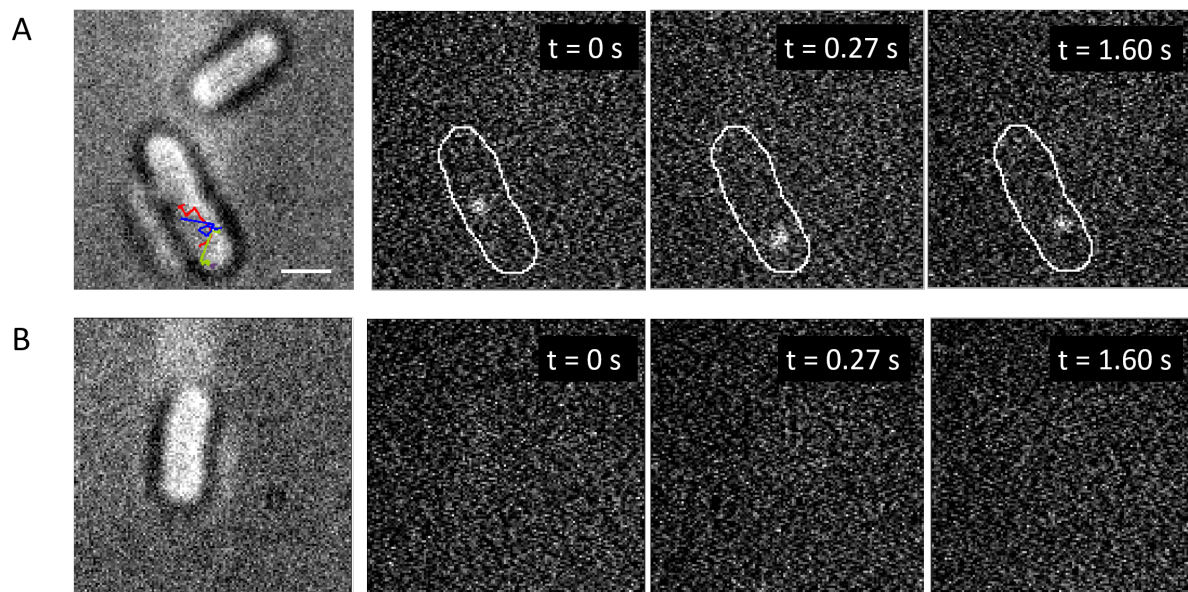


Figure 52: (A) Single CheY₆-iFluor633 diffusing in *E. coli* EP sample. The tracks on the brightfield image were generated by Stormtracker [142]. By using an organic dye, long tracking (over 1 s) with constant laser illumination was possible. (B) Negative control (EMPTY) cell showed a clean background with no autofluorescence. Brightness and contrast of the fluorescent images were displayed at the same levels respectively using ImageJ for comparison. Cycle time 19.53 ms. Scale bar 3 μm .

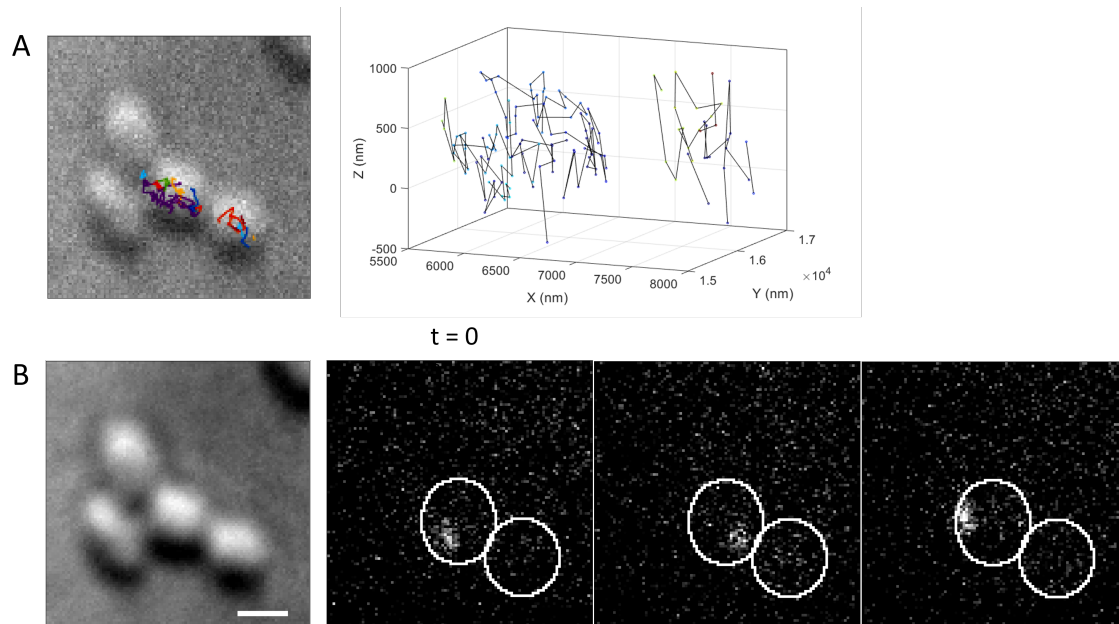


Figure 53: Single molecule tracking of single CheY₆-iFluor633 molecules diffusing in aerobically grown *R. sphaeroides*. (A, left) shows the brightfield image of *R. sphaeroides* cells and individual trajectories of CheY₆-iFluor633 molecules superimposed on the brightfield image. (A, right) 3D perspective view of the same trajectories in the left image. (B) Fluorescence images of single CheY₆-iFluor633 molecules diffusing in the cells in (A). Scale bar 1 μm . Data and figure provided by Dr. Helen Miller.

Even though CheY₆-iFluor633 was electroporated in aerobic *R. sphaeroides*, there were two major caveats that raised concerns in the tracking and data interpretation of CheY₆-iFluor633 in *R. sphaeroides*. First, most of the CheY₆-iFluor633 molecules detected in the EP sample were slow moving particles and achieving single molecule resolution for rapidly diffusing CheY₆ was technically challenging. In addition, I observed diffusing fluorescence spots in EMPTY *R. sphaeroides* cells that emitted strong signals in the far red channel. These autofluorescence molecules were of unknown origin and they were indistinguishable from the signals of iFluor633 (Figure 54). Preliminary tracking of these autofluorescence molecules found that they were diffusing at apparent diffusion coefficients between 0.5 - 2.5 $\mu\text{m}^2\text{s}^{-1}$ (Figure 55). It was not until I moved on to the PALM project (Chapter 5) when I realized that these autofluorescence molecules were triggered by low level of oxygen. Please refer to Chapter 5 for more details on the autofluorescence properties of *R. sphaeroides*. To avoid artefacts from these long-lived autofluorescence molecules, the agarose pads were aerated before imaging and the cell samples were imaged in the order of EP, NEP, and EMPTY. If autofluorescence molecules were observed in EMPTY, all imaging data from the same session was discarded. For the reasons mentioned above, I decided to shift focus onto my PALM project of tracking single CheY₆ labelled with photoactivatable mCherry, which was beginning to show promising results. Despite the caveats that appeared during the earlier stage of the project, the electroporation technique had began to show reliable result with good signals in the EP sample and clean background (minimum autofluorescence) in the EMPTY and NEP samples by using oxygenated agarose pads.

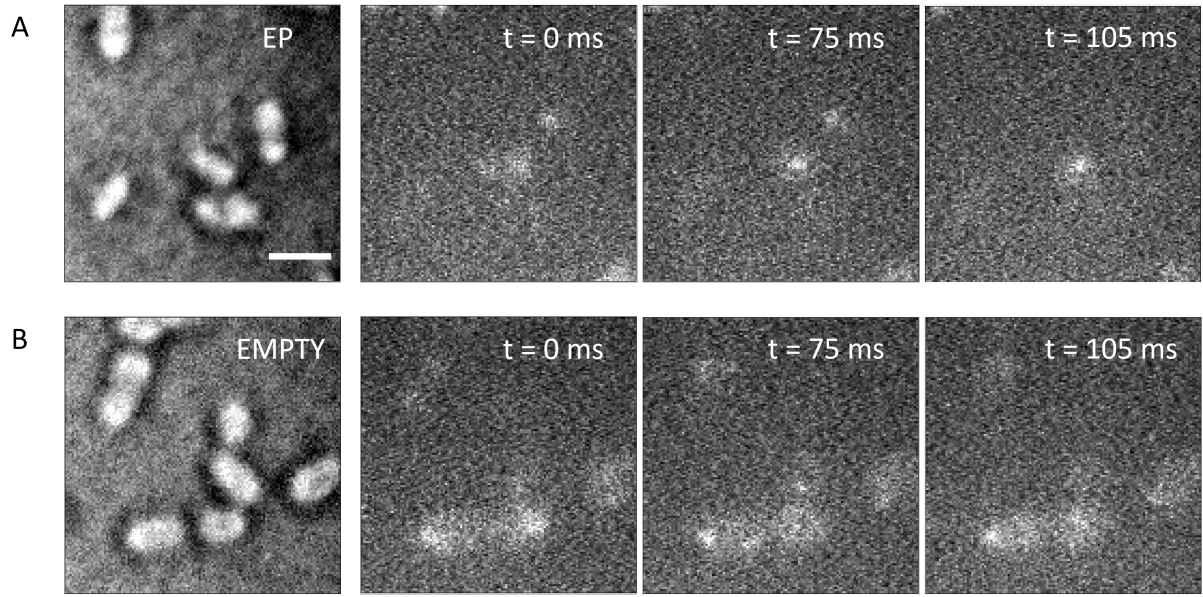


Figure 54: (A) EP and (B) EMPTY *R. sphaeroides* cells showed similar degree of fluorescence signals on agarose pads. (A) Electrocompetent cells were electroporated with CheY₆-iFluor633 before imaging. (B) EMPTY cells were never in contact with the labelled CheY₆ sample but showed high level of autofluorescence. Brightness and contrast of the two fluorescent images for both EP and EMPTY cells were displayed at the same levels respectively using ImageJ for comparison. Excitation: 620 - 640nm. Exposure: 15 ms. Power: 0.417mW. Scale bar 1.5 μ m.

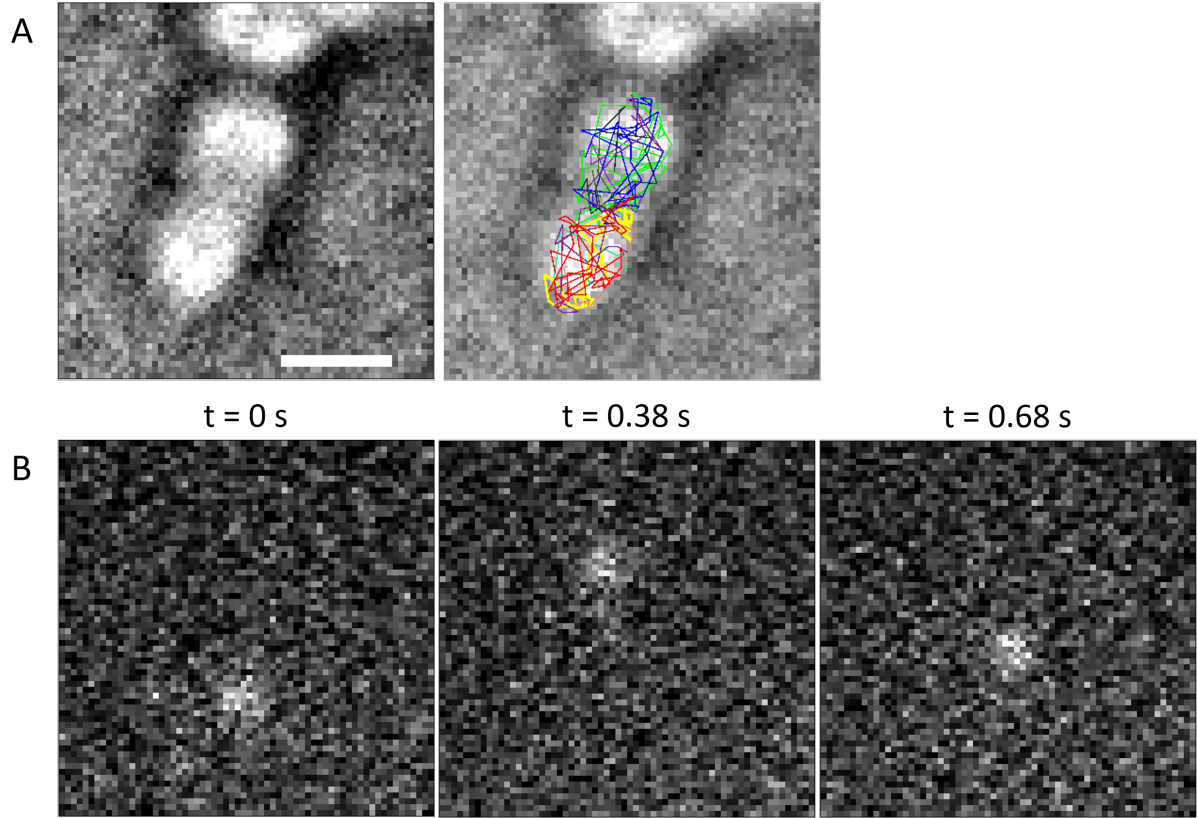


Figure 55: Single molecule tracking of autofluorescence molecules diffusing in aerobically grown *R. sphaeroides*. (A) shows the brightfield image of a *R. sphaeroides* cell and individual trajectories of autofluorescence molecules superimposed on the brightfield image. The apparent diffusion coefficients of these molecules generated by Stormtracker were between $0.5 - 2.5 \mu m^2 s^{-1}$. Localization threshold: 20. Tracking window: 15 pixels. (B) Fluorescence images of live, unlabelled aerobic cells over time. These autofluorescence molecules had a long fluorescence lifetime of over 1 second. Excitation: 620 - 640 nm. Exposure: 15 ms. Power: 0.417mW. Scale bar $1 \mu m$.

5.5 Discussion

Genetically encoded fluorescent proteins have been the choice for studying protein behaviour and motion in live cells. However, fluorescent proteins emit weak signals above background noise and they rapidly photobleach, which are common problems in single molecule tracking experiments. In addition, the 27 kDa fluorescent proteins are bulky and they may affect the functionality of the targeted protein. On the other hand, organic dyes are smaller, brighter, and more photostable than the common fluorescent proteins. Some studies have relied on the use of HaloTag and fluorescent dyes [10][75][48], but HaloTag is slightly larger than FP and may affect diffusion of small proteins. Unfortunately, conjugating fluorescent dyes to proteins often require *in vitro* labelling and purification procedures. In the past, the electroporation technique has been successfully used to introduce DNA into bacterial cells. More recently, the Kapanidis group developed an electroporation method [26] to internalize fluorescently-labelled protein molecules into live cells. The electroporation technique was then extended by the Berry group and Armitage Lab to examine the behaviour of *E. coli* CheY [31] and *R. sphaeroides* CheY₆ *in vivo* (M. W. Smith, 2015).

In this chapter, CheY₆-iFluor633 was successfully electroporated into *E. coli* and aerobic *R. sphaeroides*. I was able to minimize autofluorescence of the cells by using oxygenated agarose pads and optimised washing protocols. In general, the majority of the CheY₆ molecules were found to be quite stationary or have very slow diffusion in cells, probably due to limitation of the microscope to capture the fast diffusing species. The slow diffusing CheY₆ molecule observed could be interacting or binding with the chemosensory clusters or flagellar motor. This observation agreed with the work of M. W. Smith (2015) that showed colocalization of electroporated CheY₆ molecules with FliM foci and cytoplasmic cluster *in vivo*. However, it was unclear what percentage of the CheY₆ molecules were free diffusing in Smith's work. A similar result was also obtained in *E. coli*: the estimated diffusion coefficient for CheY-Atto647 measured by single molecule tracking was $0.14 \pm 0.03 \mu\text{m}^2\text{s}^{-1}$, most likely due to interactions with the

flagellar motor or chemosensory cluster [31]. However, previous study in *E. coli* reported a much higher estimated diffusion coefficient of $4.6 \pm 0.8 \mu m^2 s^{-1}$ for CheY-GFP measured by the fluorescence correlation spectroscopy (FCS) [21]. It is possible that single molecule tracking of the fast population for CheY₆-iFluor633 was difficult to achieve due to motion blur. Therefore, only slow moving particles were observed.

The aim of this chapter was to investigate the effects of crowding by ICM vesicles on the diffusion of CheY₆ in different growth states. However, I was unable to measure the diffusion coefficient for CheY₆ in *R. sphaeroides* reliably due to a few factors. First, photoheterotrophic *R. sphaeroides* cells have high autofluorescence that makes single molecule tracking difficult. Photoactivatable organic dyes [49] may be used since they allow a prebleach step to minimize cellular background noise before excitation. However, choosing a new dye will require additional optimizations to the current labelling protocols and imaging setup, and there was insufficient time to repeat all the procedures mentioned above to complete this study on time.

In addition, CheY₆ is a small molecule and the total size of CheY₆-iFluor633 is smaller than a 27 kDa fluorescent protein. Therefore, faster speeds are required to track electroporated CheY₆-iFluor633 than free fluorescent proteins. A short exposure time is required to calculate its rapid diffusion coefficient, and this further reduces its signal-to-noise ratio. Even though the tracking of CheY₆-iFluor633 should better reflect the diffusion of native CheY₆ molecules, it was beyond the technical limitations of the microscopes I had access to (discussion with Dr. Helen Miller). Finally, there were also some concerns on the electrostatic properties and “stickiness” of the dyes used to investigate on the mobility of labelled CheY₆. It is important to investigate whether certain dyes would interact with lipid bilayers or other cellular components nonspecifically and how this characteristic would affect the diffusion in photoheterotrophic *R. sphaeroides* where there was extended invaginations of cytoplasmic membranes.

In summary, this chapter describes the development of the labelling, purification, and electroporation protocols for *R. sphaeroides* CheY₆. New CheY₆ mutants with

enhanced labelling yield were generated by quick-change and a modified protocol for protein labelling and purification to minimize free dye contamination was developed. iFluor633 was found to be a good dye candidate for labelling CheY₆ and electroporation. CheY₆-iFluor633 molecules were successfully electroporated and tracked in *E. coli* and *R. sphaeroides* cells. Single molecule tracking using organic dye conjugation is a powerful technique for investigating protein behaviour in a bacterium, but special care needs to be taken to avoid potential artefacts in single molecule experiments. In the future, it may be useful to conduct a sphaeroplast experiment to check that the dye labelled protein molecules were successfully internalised into the cytoplasm. Finally, the electroporation project also showed promising preliminary data for 3D single molecule tracking in *R. sphaeroides* for the first time (work by Dr. Helen Miller). The result would provide insight into the spatial organization of CheY₆ at high resolution and may capture interaction of the response regulator with the *R. sphaeroides* chemotaxis components (chemoreceptor clusters and flagellar motor).

6 Summary and implications

This project measured the effects of macromolecular crowding by ICM vesicles on protein diffusion in photoheterotrophic *R. sphaeroides* using single molecule tracking and a FRET probe to sense changes in excluded volume.

The cytoplasm of *R. sphaeroides* develops invaginations of intracytoplasmic membrane (ICM) vesicles when the bacterium is grown photosynthetically and also anaerobically in the dark. The number of ICM vesicles per cell is inversely correlated to light intensity; with an estimate of around 1500 vesicles for low light cells and 270 vesicles for high light cells, calculated using acetone / methanol extraction and transmission electron microscopy [2]. For high light cells, 270 vesicles would correspond to around 4.9% ($0.0340 \mu\text{m}^3$) of occupied cytoplasmic volume based on previously estimated vesicle volume [2] and cell volume [141]. Low light cells would have a higher percentage of 29% ($0.204 \mu\text{m}^3$), assuming that the cells contained 1500 vesicles per cell.

Previously, studies have used stress conditions to induce macromolecular crowding effects in non-photosynthetic species, but the role of ICM vesicles as potential natural crowding agents in a typical *R. sphaeroides* cell is not known. By combining the use of FRET-based crowding sensor and single molecule tracking, this thesis suggests that low light *R. sphaeroides* cells have higher crowding than high light cells, as shown by the higher FRET ratio and decreased protein mobility observed in the cytoplasm of low light cells (Figure 56). High concentrations of macromolecules is known to induce conformational changes or compaction of protein molecules [164][83] and decrease molecular diffusion [60][143]. Therefore, by comparing the FRET ratios and protein diffusion coefficients obtained from high light and low light cells, this work proposed that *R. sphaeroides* grown under low light intensity with large number of ICM vesicles would have significantly higher macromolecular crowding level than high light grown cells.

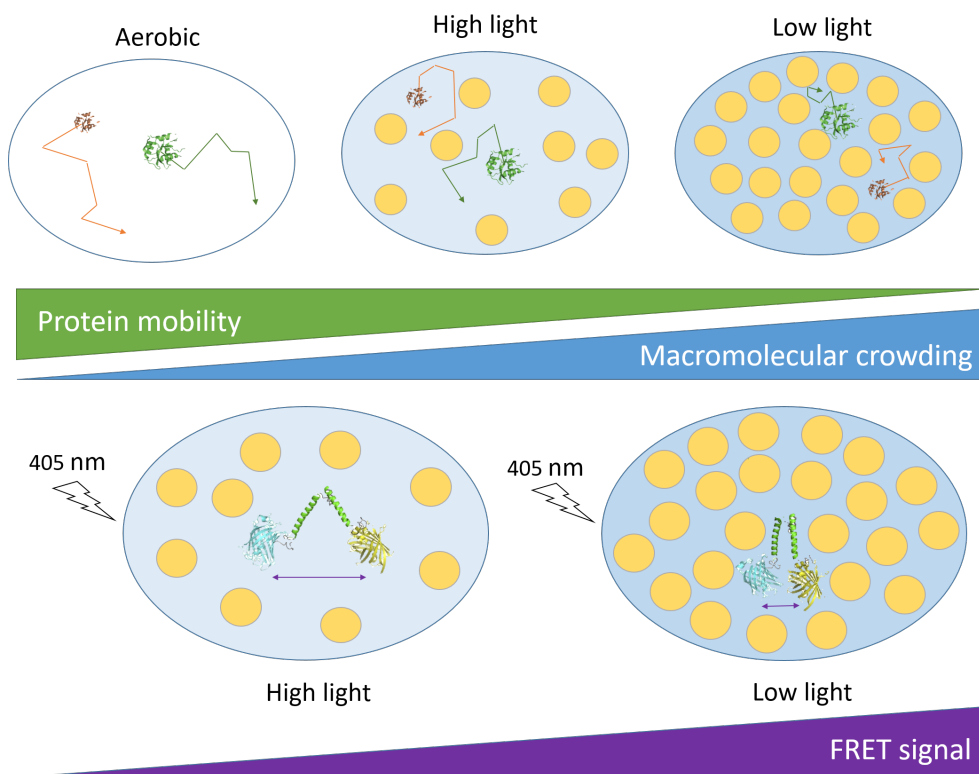


Figure 56: Proposed model of macromolecular crowding in live *R. sphaeroides* cells. The result of this work suggested that ICM vesicles act as natural macromolecular crowders that can decrease diffusion of protein > 26 kDa and increase the FRET signal of a crowding sensor in the cytoplasm of *R. sphaeroides*. Single molecule tracking of protein molecules diffusing in *R. sphaeroides* showed that the apparent diffusion coefficient of proteins decreased as the bacterium transitioned from aerobic to photoheterotrophic growth. Under photoheterotrophic condition, the cells develop ICM vesicles that reduce the cytoplasmic volume, resulting in higher crowding level and lower protein diffusion in the cytoplasm. It is also possible that some vesicles act as physical barriers to the movement of protein. A further reduction in protein mobility was observed in low light cells where the greatest number of ICM vesicles were found. The increase in crowding in low light cells was also measured *via* a genetically encoded FRET probe. In low light cells where the cytoplasm is highly crowded with ICM vesicles, the CFP-YFP probe connected by a flexible linker adopts a more condensed conformation, resulting in higher FRET ratio readouts compared to high light cells. Figure not to scale.

6.1 FRET sensor to study macromolecular crowding

A recently developed FRET probe sensitive to changes in excluded volume effects [14] was used in this work to study crowding in photoheterotrophic *R. sphaeroides*. Previously, FRET sensors were used in osmotically stressed *E. coli* to quantify crowding level as water left the cytoplasm by osmosis, thus lowering the volume of the cell and increasing crowding [72][14].

In this work, it was found that FRET sensor could detect a change in crowding level in *R. sphaeroides* under normal growth conditions without the use of osmotic upshift. By using confocal fluorescence microscopy, photoheterotrophic cells grown under high light ($800 \mu\text{mol photons } m^{-2}s^{-1}$) and low light intensity ($15 \mu\text{mol photons } m^{-2}s^{-1}$) that contained the FRET sensor were imaged. High light cells were found to have a mean FRET ratio of 0.3907 ($n = 633$ cells), which was lower than the mean FRET ratio of 0.4028 for low light cells ($n = 768$ cells), $p\text{-value} = 0.000227$, suggesting that crowding was higher in low light cells. This observation was also supported by the BChl extraction result that estimated a 3 to $4\times$ more vesicles in low light cells (around 1200 vesicles per cell) compared to high light cells (around 400 vesicles per cell). I estimate that ICM vesicles would occupy 5.6% of the cell volume in high light cells and 19.2% of the cell volume in low light cells as discussed in Chapter 3. A trend of increasing FRET ratios and vesicle number over increasing optical density was also observed in low light cells. These result showed that even under normal growth condition, the cytoplasm of *R. sphaeroides* could undergo dramatic morphological change that resulted in a change in crowding level measurable by the FRET sensor.

In the past, typical crowding studies have relied on cell lysis to estimate the fraction or mass of macromolecules (such as protein, DNA, RNA) in a cell *in vitro*, but these methods could not capture the dynamics and heterogeneity of the intracellular environment. By combining the BChl extraction result with direct imaging of the FRET sensor *in vivo*, it was possible to obtain bulk measurements of crowding *in vitro* and monitor changes in crowding in real time on a single-cell level.

Previous studies have reported the presence of non-diffusive barriers or confinement in stressed cells [98][82]. However, the imaging setup used in this work did not have single molecule sensitivity and an even distribution of fluorescence signal was observed in *R. sphaeroides*. Therefore, only the average ratiometric FRET ratio for single cells was used to compare crowding between high light and low light cells. In the future, it would be useful to understand whether the FRET sensor could provide insights on local crowdedness or heterogeneous crowding of the cytoplasm. For example, it may be possible to visualize microcompartments that are dilute or supercrowded by monitoring differences in FRET ratios at different regions of the cell. Other future experiments include a more quantitative comparison between the crowdedness of the *R. sphaeroides* cytoplasm with the cytoplasm of *E. coli* and other synthetic system.

6.2 Single molecule tracking for studying crowding and diffusion *in vivo*

Molecular diffusion and motion of tracer molecules are often used as a tool to study macromolecular crowding and the structure of cytoplasmic components. Usually, a decrease in diffusion coefficient indicates an increase in excluded volume effect due to steric repulsion by macromolecules such as proteins, ribosomes, and DNA. The effects of crowding also depend on the size of the tracer molecule: smaller molecules are less affected by crowding as they have more accessible volume; while larger molecules have a higher possibility of bumping into neighbouring crowders and become confined in microcompartments by diffusive barriers.

The work in this thesis compared the diffusion coefficients of different-sized protein molecules in *R. sphaeroides* under different growth conditions and crowding levels. Instead of ensemble microscopy techniques such as FRAP, single molecule tracking was used to directly visualize the motion of proteins labelled with a fluorescent organic dye or a photoactivatable fluorescent protein. In aerobic *R. sphaeroides*, the target proteins

were found to have similar mobilities as the ones in *E. coli*. Interestingly, single molecule PALM revealed that a second, slow-diffusing species was observed in photoheterotrophic cells following the development of ICM vesicles in the cytoplasm. These observations suggested that the presence of ICM vesicles in *R. sphaeroides* may reduce the cytoplasmic volume and lead to increased crowding of the cytoplasm that lowered protein diffusion. It is also possible that some vesicles acted as obstacles to hinder diffusion of protein molecules > 27 kDa.

6.2.1 Electroporation of small, fluorescently labelled protein

R. sphaeroides CheY₆ was labelled with an organic dye and electroporated into live cells to allow continuous, high speed imaging of the small 15 kDa protein over a long period of time. Previously, the motion and localization of CheY proteins *in vivo* had been studied by adding a genetically encoded fluorescent protein tag [102][21]. Recently, some studies had also utilized HaloTag [75][67] or SNAPTag [56] to label the target protein with fluorescent dyes. However, the use of membrane permeable, self-labeling dyes may be problematic in *R. sphaeroides* and bacteria with heavily invaginated membrane system. In addition, the use of bulky fluorescent protein fusions or labelling tags could affect the diffusion coefficient and functionality of the target protein. Therefore, one of the aims of this work was to infer the native diffusion coefficient of CheY₆ in live cells by following the trajectories of single CheY₆ conjugated with a small fluorescent dye.

However, at the beginning of this work, I was unable to observe any fluorescent signals in the electroporated (EP) sample for both *R. sphaeroides* and *E. coli*, due to the low yield of fluorescent dye labelled CheY₆. Therefore, optimizations to the *in vitro* labelling and purification protocols were made to improve the yield of labelled CheY₆. A cysteine residue was introduced onto the His-tag located at the N-terminal of CheY₆ to enable labelling with a maleimide dye. SDS-PAGE and fluorescence microscopy were used to check the efficiency of free dye removal by size exclusion chromatography. By improving the yield of labelled CheY₆, CheY₆-iFluor633 was successfully electroporated into aer-

obic *R. sphaeroides* and *E. coli*. A strict data analysis process was carried out for the electroporation project to eliminate false positives that may occurred due to the autofluorescence of *R. sphaeroides*. Negative control (EMPTY) sample and non-electroporated (NEP) sample were imaged under the same setting as the EP sample at the same time as described in Chapter 5. If autofluorescence was observed in either EMPTY or NEP sample, the EP data for the session would be discarded. The electroporation technique was also not known to trigger autofluorescence because no fluorescent signal was observed in electroporated cells that were incubated with low concentration of labelled CheY₆. In those cases, the EP sample looked identical to the EMPTY sample, with no fluorescent spots and a clean background. Previous work by M. W. Smith in 2015 also reported that electroporation did not lead to autofluorescence in *R. sphaeroides*. In the future, it may be useful to conduct a sphaeroplast experiment to confirm that the dye labelled protein molecules were electroporated into the cell cytoplasm.

By electroporating a higher concentration of CheY₆ labelled with iFluor633 into *R. sphaeroides* and *E. coli*, I was finally able to observe fluorescent signals in the EP sample. Fluorescence microscopy revealed that most CheY₆-iFluor633 molecules were stationary or had low mobility in the cytoplasm, suggesting that the molecules were interacting with cytoplasmic components. This observation agreed with previous studies that showed colocalization of dye-labelled CheY₆ / CheY and the flagellar motor in *R. sphaeroides* (M. W. Smith, 2015) and *E. coli* [31], respectively.

The excellent photon counts and photostability of fluorescent dyes also allowed 3D tracking of single CheY₆ molecules in aerobic *R. sphaeroides* for the first time (work by Dr. Helen Miller). By investigating the motion of CheY₆ in live cells, the roles of CheY₆ as a response regulator and its interactions with the flagellar motor FliM protein and the chemoreceptor clusters could be understood. However, the result of this section also demonstrated the challenges of the electroporation technique and the technical difficulties of tracking rapidly-diffusing 15 kDa molecules in live cells. The predicted diffusion coefficient for CheY₆ was around 10 to 15 $\mu\text{m}^2\text{s}^{-1}$ based on its molecular weight and

hydrodynamic radius, but this high diffusion coefficient population was not observed, possibly due to motion blur and low signal-to-noise ratio. In addition, the high autofluorescence found in photosynthetic cells also prevented the use of single molecule tracking for measuring diffusion coefficient, in contrast to ensemble techniques such as FRAP where D was calculated from signals contributed by thousands of fluorescent proteins.

In the future, a new dye candidate that does not overlap with the *R. sphaeroides* BChl emission spectrum should be selected. Photoactivatable dyes such as the Janelia Fluor dyes [50][49] may also be used to minimize cellular autofluorescence by having a prebleach step. This work does however show promise for tracking studies using molecules smaller than 27 kDa *in vivo* in the future.

6.2.2 Tracking of different-sized protein using PALM

The apparent diffusion coefficients of three different-sized protein molecules expressed from an inducible plasmid in *R. sphaeroides* and *E. coli* were measured using single molecule PALM. This technique allowed a prebleach step before imaging to overcome the autofluorescence issue found in the Electroporation project. To test the reliability of the experimental setup and tracking analysis, the apparent diffusion coefficient of PAmCherry was calculated in both *E. coli* and *R. sphaeroides* and compared to known D values for fluorescent proteins in the literature. The apparent D for PAmCherry in *E. coli* and aerobic *R. sphaeroides* was $3.53 \pm 0.13 \mu\text{m}^2\text{s}^{-1}$ and $3.62 \pm 0.23 \mu\text{m}^2\text{s}^{-1}$, respectively. These values were on the same order of magnitude with diffusion coefficients of unbound fluorescent proteins from other studies [39][60][82][87].

In photosynthetic cells grown under high light or low light, different-sized protein molecules were found to exhibit decreased mobilities as the cytoplasm was filled with varying amount of chromatophores that could act as diffusive barriers. The majority of PAmCherry had an apparent D of $2.46 \pm 0.09 \mu\text{m}^2\text{s}^{-1}$ in high light cells, but a second, slow-diffusing population with $D = 0.50 \pm 0.03 \mu\text{m}^2\text{s}^{-1}$ was also observed. This slow-diffusing species ($D = 0.28 \pm 0.03 \mu\text{m}^2\text{s}^{-1}$) was also observed in low light cells but was

absent in aerobic cells.

A reduction in apparent diffusion coefficient was also observed using PAmCherry-Y₆ as the cells transitioned from aerobic growth to high light and low light photosynthetic growth. The overall diffusion of PAmCherry-Y₆ under different growth conditions was slightly slower than PAmCherry due to the addition of a small, 14 kDa CheY₆. To determine the effects of crowding on the motion of larger proteins, the diffusion of L1-PAmCherry was also measured in *R. sphaeroides*. In aerobic cells, around 57% of L1-PAmCherry molecules had an apparent D of $0.64 \pm 0.06 \mu\text{m}^2\text{s}^{-1}$ and 43% had an apparent D of $2.49 \pm 0.29 \mu\text{m}^2\text{s}^{-1}$. The presence of heterogeneous diffusion coefficients suggested that not all L1 subunits were incorporated into the ribosome. Overexpression of L1-PAmCherry resulted in a fast species formed by free L1 subunits diffusing randomly in the cell, while the rest of the L1 subunits were most probably part of a fully assembled ribosome.

In photoheterotrophic cells, the diffusion of L1 protein was again reduced. In high light cells, the fast species was diffusing at $D = 1.31 \pm 0.15 \mu\text{m}^2\text{s}^{-1}$ while the slow species had $D = 0.38 \pm 0.04 \mu\text{m}^2\text{s}^{-1}$. Interestingly, all the L1 molecules were diffusing at $D < 0.5 \mu\text{m}^2\text{s}^{-1}$ in low light cells and the fast species observed in aerobic and high light cells was absent. This suggested that under extremely crowded condition, molecules > 50 kDa would exhibit slow diffusion despite having large differences in molecular weight. All three proteins did not show confinement to any specific regions of the cell.

By using a relatively straightforward single molecule tracking analysis, this work showed that crowding in *R. sphaeroides* resulted in subpopulations of protein molecules with decreased apparent diffusion coefficients. The ability to observe multiple diffusive species demonstrated the benefits of single molecule microscopy over conventional ensemble techniques. This thesis proposed that the diffusion of protein > 27 kDa could be reduced by around 10 times due to excluded volume effects of ICM vesicles. In future studies, it would be interesting to compare the diffusion coefficients of molecules smaller than 27 kDa under the same crowding conditions.

6.3 Future work

There are currently few studies which directly demonstrate the biological impacts of macromolecular crowding *in vivo*. In fact, many studies ignored the effects of macromolecular crowding when they study molecular diffusion or interactions with other molecules. However, this work has shown that crowding can have a huge impact on protein diffusion in *R. sphaeroides* under different, normal growth conditions. This finding is especially important as there are diffusion-limited processes in bacteria [114]. For example, the diffusion of response regulator CheY from the chemoreceptor cluster to the flagellar motor in *E. coli* could lead to a difference in chemotaxis response time [117][126][44].

A study found that compared to aerobic *R. sphaeroides* cells, a lower percentage of photoheterotrophic cells was responsive to drop in attractant concentration [59]. This decreased sensitivity to chemosensory stimulus could be caused by a lower diffusion coefficient of the CheYs in photosynthetic *R. sphaeroides* due to increased crowding. Modelling work suggested that a reduction in CheY diffusion by 2-fold due to macromolecular crowding in *E. coli* could lead to a significant increase in chemotactic response time [70]. However, it is not known whether the native, 14 kDa CheY₆ (without the fluorescent protein fusion) would experience a similar reduction in diffusion due to crowding by ICM vesicles. Future studies can include a tethered cell assay on high light and low light cells to study whether there is a difference in chemotactic response time and adaptation time to step changes in stimuli. These data should be complemented by a predictive model on *R. sphaeroides* chemotaxis, taking into account any differences in protein levels, rate constants, and sensitivity of chemosensory proteins.

6.4 Implications

The intracellular environment affects the diffusion coefficients and spatial organization of biomolecules within the bacterial cytoplasm. Previous modeling work suggested that macromolecular crowding plays an important role in genetic networks as it reduces molecular diffusion and can alter the search time of DNA-binding proteins such as transcription factor for their target sites [68]. Reduction in diffusion coefficients due to crowding may also affect the efficiency of DNA repair mechanism in bacteria. Using single molecule tracking, a study showed that DNA repair proteins RecN, RecJ, and RecO exhibited two diffusive states, DNA-bound or freely-diffusing, in live *B. subtilis* cells [108]. This suggested that the majority of the Rec proteins were mobile and able to diffuse through the nucleoid to scan for DNA damage sites, until a DNA double strand break site was identified and the Rec proteins would assemble on the chromosome. It is possible that under crowded condition, the Rec proteins would have slower diffusion and longer search time for DNA damage sites, thus affecting the efficiency of the DNA repair kinetics. These Rec proteins may also spend a longer time interacting non specifically with chromosomal DNA. To compensate for the reduction in diffusion coefficient, the total number of Rec proteins may increase in the cell.

In addition to the effect on molecular diffusion, macromolecular crowding may also be important for the organization and assembly of large protein complexes. Previous study found that in the absence of molecular crowding agents, the *R. sphaeroides* cytoplasmic chemoreceptor array collapsed upon cell lysis while the transmembrane array remained intact [17]. To visualize the structure of the cytoplasmic cluster, *R. sphaeroides* cells expressing a YFP-labelled chemoreceptor protein TlpT were lysed and resuspended in buffers containing artificial crowding agents, polyethylene glycol (PEG) or polyvinylpyrrolidone (PVP) to prepare for imaging. Correlative cryo-fluorescence light microscopy (FLM) found that TlpT-YFP foci were observed only if crowding agents were added [17]. Using electron cryo tomography (ECT), the study also showed high resolution images of cytoplasmic arrays displaying hexagonally packed trimers-of-dimers complexes in the

presence of crowding agents. This suggests that cytoplasm density or macromolecular crowding may be important for stabilizing the *R. sphaeroides* cytoplasmic chemoreceptor cluster and other cellular structures.

Another example of cellular structure that could be affected by crowding is the FtsZ ring, one of the most critical components of the bacterial division machinery. FtsZ is a tubulin homologue that forms a ring-like structure in the cytoplasm to initiate membrane constriction and cytokinesis. Depending on the concentrations of crowding agents added, FtsZ subunits were able to assemble into single filaments or large bundles of supramolecular structures *in vitro* [100]. During cell division, FtsZ ring localizes at mid-cell and coordinates with chromosome segregation to ensure proper distribution of genetic materials between the daughter cells. *R. sphaeroides* has two chromosomes and it relies on both division proteins FtsZ and MipZ to control DNA segregation and cell division. Current model for *R. sphaeroides* chromosome segregation described MipZ oscillating between the cell poles before forming a ring at mid-cell close to the FtsZ ring until cell division finishes [34]. It would be interesting to study whether higher crowding in the *R. sphaeroides* cytoplasm can affect the dynamics of the division complex or lead to compaction of the nucleoid. The movement and localization of key proteins such as MipZ, FtsZ, and ParB under the effects of crowding may provide insights on the mechanisms of chromosomes segregation.

There were few studies that investigated the role of ICM vesicles as crowding agents and how they could affect the formation and segregation of large complexes such as the chemosensory cluster or the FtsZ ring. Future studies could compare the spatiotemporal dynamics of these large structures under different subcellular architecture in aerobically and photosynthetically grown *R. sphaeroides* cells.

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Appendix

Growth media

Luria-Bertani Broth (LB)

Ingredient	Quantity per litre
Yeast extract	5 g
Tryptone	10 g
NaCl	5 g

Made up to 1 litre with MilliQ water. Adjusted to pH 7.0 and autoclaved.

LB agar

Ingredient	Quantity per litre
Agar	16 g
Yeast extract	5 g
Tryptone	10 g
NaCl	5 g

Made up to 1 litre with MilliQ water. Adjusted to pH 7.0 and autoclaved.

Tryptone broth

Ingredient	Quantity per litre
Tryptone	10 g
NaCl	10 g

Made up to 1 litre with MilliQ water. Adjusted to pH 7.0 and autoclaved.

2 × TY

Ingredient	Quantity per litre
Yeast extract	10 g
Tryptone	16 g
NaCl	5 g

Made up to 1 litre with MilliQ water. Adjusted to pH 7.0 and autoclaved.

Succinate medium

Ingredient	Quantity per litre
1 M Phosphate buffer pH 7.0	20 mL
Concentrated base (see below)	20 mL
Growth factors (see below)	2 mL
(NH ₄) ₂ SO ₄	0.5 g
NaCl	0.5 g
Sodium succinate	2 g
Casamino acids	1 g

Made up to 1 litre with MilliQ water. Adjusted to pH 7.2 with KOH and autoclaved.

M22

Ingredient	Quantity per litre
1 M Phosphate buffer	20 mL
Concentrated base (see below)	20 mL
Growth factors (see below)	2 mL
(NH ₄) ₂ SO ₄	0.5 g
NaCl	0.5 g
Sodium succinate	2 g

Made up to 1 litre with MilliQ water. Adjusted to pH 7.2 with KOH and autoclaved.

0.25 % M22 agar (swim plate)

Ingredient	Quantity
M22	1 L
Agar	2.5 g
Propionate in MiliQ (0.22 μ m filtered)	concentration varies

Autoclaved.

SOC

Ingredient	Quantity
Glucose	20 mM
MgCl ₂	10 mM
MgSO ₄	10 mM
KCl	2.5 mM
NaCl	10 mM
Tryptone	20 g / L
Yeast extract	5 g / L

Autoclaved.

GYT medium

Ingredient	Quantity
Glycerol	10 %
Yeast extract	0.125 % (w/v)
Tryptone	0.25 % (w/v)

0.22 μ m filtered and stored in 1 mL aliquots at 4 °C..

Buffers and solutions

Lysis buffer

Ingredient	Concentration
Glycerol	10 %
Tris-HCl	50 mM
NaCl	150 mM
Imidazole	25 mM

Made up to 1 litre with MilliQ water. Adjusted to pH 8.0 and 0.2 μm filtered.

Elution buffer

Ingredient	Concentration
Glycerol	10 %
Tris-HCl	50 mM
NaCl	150 mM
Imidazole	500 mM

Made up to 1 litre with MilliQ water. Adjusted to pH 8.0 and 0.2 μm filtered.

Labelling buffer

Ingredient	Concentration
Glycerol	10 %
Tris-HCl	50 mM
NaCl	150 mM
TCEP	0.1 mM

Made up to 1 litre with MilliQ water. Adjusted to pH 7.5 and 0.2 μm filtered. Degassed.

Electroporation buffer

Ingredient	Concentration
Glycerol	10 %
Tris-HCl	50 mM
NaCl	25 mM
TCEP	0.1 mM

Made up to 1 litre with MilliQ water. Adjusted to pH 7.5 and 0.2 μm filtered. Degassed.

2 $\times$ non-stained SDS protein loading dye

Ingredient	Concentration
Glycerol	20 %
Tris-HCl	250 mM
SDS	2 %
DTT	1 mM

Made up to 1.5 mL with MilliQ water. Adjusted to pH 7.5 and 0.45 μm filtered.

Motility buffer

Ingredient	Concentration
K ₂ HPO ₄	6.2 mM
KH ₂ PO ₄	3.8 mM
EDTA	0.1 mM

Made up to 1 litre with MilliQ water. Adjusted to pH 7.0 and 0.45 μm filtered. Autoclaved.

10 × TBE

Ingredient	Quantity
Tris base	108 g
Boric acid	55 g
EDTA pH 8.0	7.4 g

Made up to 1 litre with MilliQ water. Adjusted to pH 8.3 and autoclaved.

TFB I

Ingredient	Concentration
Potassium acetate	30 mM
RbCl ₂	100 mM
CaCl ₂ 2H ₂ O	10 mM
MnCl ₂ 4H ₂ O	50 mM
Glycerol	15% (v/v)

Adjusted to pH 5.8 with 0.2 M acetic acid and filtered.

TFB II

Ingredient	Concentration
CaCl ₂ 2H ₂ O	75 mM
RbCl ₂ 2H ₂ O	10 mM
PIPES	10 mM
Glycerol	15% (v/v)

Adjusted to pH 5.8 with 0.2 M acetic acid and filtered.

1M Phosphate buffer pH 7.0

Ingredient	Quantity per litre
1M K ₂ HPO ₄	174.18 g
1M KH ₂ PO ₄	136.09 g

Adjusted to pH 7.0 (KOH), autoclaved and stored at 4 °C.

Concentrated base

Ingredient	Quantity per litre
Metals 44 solution	25 mL
MgSO ₄ 7H ₂ O	14.5 g
Nitrilotriacetic acid (diNa salt)	5.94 g
CaCl ₂ 2H ₂ O	1.67 g
FeSO ₄ 7H ₂ O	50 mg
Ammonium molybdate 4H ₂ O	4.6 mg

Dissolved diNa first and adjusted to pH 5.0 using KOH. This allowed FeSO₄ to dissolve. All other ingredients were added and adjusted to pH 6.8 (KOH). Autoclaved and stored at 4 °C.

Growth factor

Ingredient	Quantity per litre
Niacin	1 g
NaHCO ₃	500 mg
Thiamine HCl	500 mg
Biotin	20 mg

Autoclaved and stored at 4 °C.

Metals 44 solution

Ingredient	Quantity per litre
ZnSO ₄ 7H ₂ O	11 g
FeSO ₄ 7H ₂ O	5 g
MnSO ₄ 4H ₂ O	3 g
Tetrasodium versenate (EDTA)	2.5 g
3M sulphuric acid	1.5 mL
CuSO ₄ 5H ₂ O	0.39 g
CoCl ₂ 6H ₂ O	0.2 g
H ₃ BO ₃	0.12 mg

Filter sterilized and stored at 4 °C.

20 × Rapid reducing buffer for SDS-PAGE

Ingredient	Concentration
MOPS	62.8 g
Tris base	72.6 g
SDS	10 g
Sodium bisulfite	6.5 g

Add up to 500 mL with MiliQ water.

Circular dichroism (CD) buffer

Ingredient	Concentration
Potassium phosphate	10 mM
NaCl	20 mM

Made up to 2 L with MilliQ water.