Subcellular Localization in Bacteria: From EnvZ/OmpR to Transertion

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Subcellular Localization in Bacteria: From EnvZ/OmpR to Transertion

Abstract
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An unexplained subcellular localization was reported for a functional fluorescent protein fusion to the response regulator OmpR in Escherichia coli. The pronounced regions of increased fluorescence, or foci, are dependent on OmpR phosphorylation, and do not occupy fixed, easily identifiable positions, such as the poles or midcell. Here we show that the foci are due to OmpR-YFP binding specific sites in the chromosome. By measuring OmpR-YFP localization at the ompF and ompC promoters under increasing levels of OmpR phosphorylation, we demonstrate support for a model of hierarchical binding to these promoters. Our results explain the inhomogeneous distribution of OmpR-YFP fluorescence in cells and further demonstrate that for a transcription factor expressed at wild-type levels, binding to native sites in the chromosome can be imaged and quantified by fluorescence microscopy.

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Mark Goulian

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SUBCELLULAR LOCALIZATION IN BACTERIA: FROM ENVZ/OMPR TO TRANSERTION

Elizabeth A. Libby

A DISSERTATION

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SUBCELLULAR LOCALIZATION IN BACTERIA: FROM ENVZ/OMPR TO TRANSERSION

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Elizabeth Abigail Libby
To Alex and Daniel who saw the humor in everything…
Acknowledgments

This work would not have been possible without my advisor Mark Goulian, who in addition to patiently guiding another physicist into biology, gave me the freedom to pick my projects and follow them wherever they led. He also tirelessly worked with me on several projects, including troubleshooting data analysis algorithms and writing image analysis scripts.

A huge thank you to all the Goulian lab members past and present – Eric Batchelor, whose strains and amazingly complete lab notes proved invaluable time and time again; Paige Derr, who always had the appropriate inappropriate remark and taught me an astonishingly large amount about troubleshooting experiments; Albert Siryaporn, who got me started on experiments in the lab and provided a valued critical eye for results great and small; Tim Miyashiro for all the helpful discussions; Andrew Lippa for his deep appreciation of both long shot experiments and practical jokes; Sriram for great conversations about science and sports; Melissa Lasaro for always sharing thoughts and advice; Manuela Roggiani for her great scientific eye and amazing microscopy music playlists; Manan Shah for working with me on fun spin-off projects; David Chow for fun conversations over coffee; and Seda Ekici, a Goulian lab member briefly, who worked with me on developing the chromosomal labeling tool, and was an amazing roommate for two years.
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My friends, in Philadelphia and elsewhere, have been the greatest. In particular, I don’t think grad school would have been the same without Lauren Willis.
ABSTRACT

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Elizabeth A. Libby

Mark D. Goulian

The internal structures of the bacterial cell and the associated dynamic changes as a function of physiological state have only recently begun to be characterized. Here we explore two aspects of subcellular localization in E. coli cells: the cytoplasmic distribution of the response regulator OmpR and its regulated chromosomal genes, and the subcellular repositioning of chromosomal loci encoding membrane proteins upon induction. To address these questions by quantitative fluorescence microscopy, we developed a simple system to tag virtually any chromosomal location with arrays of lacO or tetO by extending and modifying existing tools.

An unexplained subcellular localization was reported for a functional fluorescent protein fusion to the response regulator OmpR in Escherichia coli. The pronounced regions of increased fluorescence, or foci, are dependent on OmpR phosphorylation, and do not occupy fixed, easily identifiable positions, such as the poles or midcell. Here we show that the foci are due to OmpR-YFP binding specific sites in the chromosome. By measuring OmpR-YFP localization at the ompF and ompC promoters under increasing...
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It has long been hypothesized that subcellular positioning of chromosomal loci in bacteria may be influenced by gene function and expression state. Here we provide direct evidence that membrane protein expression affects the position of chromosomal loci for two different membrane proteins. In derived systems in which a cytoplasmic protein is produced, a shift was not observed. Antibiotics that block transcription and translation similarly prevented locus repositioning towards the membrane, suggesting that both transcription and translation of a membrane protein are required. We also found that repositioning occurs remarkably rapidly, and is observable within a few minutes following induction. As membrane protein encoding genes are distributed throughout the chromosome, this may reveal an important mechanism for maintaining the bacterial chromosome in an expanded and dynamic state.
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**Chapter 1: Introduction and Overview**

With the emerging use of fluorescent markers in bacterial cells, it became rapidly clear that the bacterial cell exhibits striking examples of subcellular organization, many examples of which are also connected to cellular physiological function. For example, one of the most extensively studied examples in *E. coli* is the assembly of the cell division ring at midcell during cell division. It has been demonstrated that a ring of FtsZ proteins forms at midcell and is carefully positioned by spatial gradients and oscillations of the Min system proteins (1-3), and that the subsequent sequential localization of associated proteins forming the division ring complex is likely established by diffusion and capture to the site of the Z-ring (FtsZ) (4, 5). This, and other examples relating to what may be loosely termed bacterial cell biology, have provided evidence that bacterial cells can create intracellular organization, even in the absence of bounded compartments (For a recent review see (6)).

Study of the internal structure of living bacterial cells is necessarily complicated by their small size. *E. coli*, arguably one of the most well characterized bacteria, measures only approximately 1 µm wide by 2 µm long (a roughly cylindrical geometry with end caps), a size on the order of visible wavelengths of light. Due to this, and the lack of readily identifiable intracellular landmarks such as organelles found in eukaryotic cells, high precision spatial studies of the interior of bacterial cells tend to require the use of a reference position. In bacteria such as *Caulobacter crescentus*, which can differentiate into two morphologically distinct cell types by undergoing asymmetric division, or in *Bacillus subtilis*, which can initiate spore formation at one pole, the asymmetry itself can provide a spatial landmark (for a review of examples of polarity in these organisms see (7)). In the case of *E. coli*, however, which undergoes symmetric division, available cellular landmarks are more limited, and generally include the poles, the septum, and
the membrane. It has also been recently demonstrated that *E. coli* chromosomes have a linear superstructure with specific genetic loci in non-replicating chromosomes appearing at reproducible loci along the long axis of the cell (8, 9).

As the questions we were interested in addressing required performing high resolution studies of the subcellular chromosomal positions of specific genetic loci under various physiological conditions and in different genetic backgrounds, we created an efficient tool for targeted placing of chromosomal labels by extending existing labeling methods (10). This tool enables the recombination of an array of *tet* or *lac* operators into the *E. coli* chromosome at any non-essential site. This tool is also particularly useful as the recombination event recognizes a particular sequence (FRT) for which there is a collection of single gene knockouts containing this sequence for all non-essential genes (11). In addition, chromosomal FRT sites can be easily engineered in many organisms, e.g. by lambda-Red mediated recombination (12). As this greatly increased the efficiency of labeling specific regions of chromosomal DNA, we used this system to examine two open questions concerning the internal organization of the *E. coli* cell.

### 1.1 Imaging OmpR Binding to Native Chromosomal Loci

It was previously observed that fluorescently tagged versions of the transcription factor OmpR of the two component signaling system EnvZ/OmpR exhibited phosphorylation dependent localization patterns within in the *E. coli* cytoplasm (13). This was particularly interesting as EnvZ/OmpR is a well studied model system, which regulates the expression of the major outer-membrane porins, *ompF* and *ompC*, in *E. coli*. When activated, EnvZ, the inner membrane bound histidine kinase, autophosphorylates at a histidine residue (hence histine kinase) and subsequently phosphorylates OmpR, the cytoplasmic response regulator, at an aspartate residue. Although
EnvZ is frequently annotated as an osmosensor, the precise signal and its mechanism of interaction with EnvZ remains unknown. In addition to osmolarity, a small membrane protein MzrA and some lipophilic compounds such as procaine have been shown to activate the EnvZ/OmpR system (14-19). Furthermore, as phosphorylation activates OmpR and causes it to bind DNA and act as a transcription factor, the production of the OmpR regulated genes *ompF* and *ompC* have been used to infer OmpR-P levels. It has been shown *in vitro*, in what has been termed the hierarchical binding model, that the *ompF* promoter region may have both high and low affinity sites for OmpR, activating and repressing OmpF expression respectively. In contrast, the *ompC* promoter region was shown to have comparatively lower affinity activating sites (20-22). However this model is controversial as a similar study by a different group observed no significant affinity difference between the sites at these promoter regions (23).

In addition to the unexplained subcellular patterns observed for OmpR-GFP, several other studies of fluorescently labeled transcription factors in bacteria have demonstrated evidence of pronounced subcellular localization. However, the interpretation and origin of these localization patterns is not always clear. For example, a fluorescently tagged PhoP (the response regulator of the PhoQ/PhoP magnesium responsive two component signaling system) was observed to form distinct foci of unknown origin at the cell poles in response to signal (24). In contrast, some major chromosomal structural proteins HU, H-NS, Dps, StpA have been observed to colocalize with the nucleoid regions of cells, and the SeqA protein which regulates the initiation of chromosomal DNA replication, was observed in distinct well localized subcellular locations (25). The emergence of high throughput studies of fluorescently tagged proteins has also advanced the study of protein localization in bacteria. In *E. coli*, a library was created with GFP fusions to each predicted open reading frame, and the resulting clones and sample of images of the fluorescence distribution made freely available (26). Additionally, a high throughput study
was recently completed on *Caulobacter crescentus* in which approximately 300 proteins were identified that showed distinctive subcellular localizations, and the origin of these patterns for many of the proteins remains to be characterized (27).

As OmpR, the response regulator, is phosphorylated by EnvZ in response to signal, the presence of phosphorylation dependent OmpR-GFP foci suggested that the active form of OmpR interacted preferentially with specific regions of the cell. OmpR is known to interact with EnvZ at the membrane and to act as a transcription factor regulating gene expression at various sites within the chromosome, but the full extent of the OmpR regulon likely remains uncharacterized, as microarrays and other experiments have identified inconsistencies depending on the growth condition (28). As we observed diffuse fluorescence signal even at high levels of OmpR-P, and we knew that the postulated number of OmpR molecules per cell far exceeded the number of known binding sites within the chromosome, the localization pattern was not obviously due to OmpR binding at individual promoters. However, by artificially causing the cells to filament and therefore increasing the spacing between the nucleoid region and the inner membrane, we were able to observe that local increases in OmpR-YFP fluorescence preferentially colocalized with regions stained with the DNA specific dye, DAPI. This suggested that the observed OmpR-YFP foci were due to DNA binding.

As the OmpR-YFP foci were observed in growing culture, a condition of continuously replicating chromosomes, it was not possible to use their relative positions along the long axis of the cells to identify chromosome regions of interest. Instead, as we hypothesized that multiple OmpR binding sites would be required to observe such readily apparent foci, we marked the locations of two well characterized OmpR regulated genes, *ompF* and *ompC*, which are each known to have multiple OmpR binding sites (29-33). Once we determined that the predominant signal of the OmpR-YFP was caused by binding to the *ompF* and *ompC* promoter regions, we
sought to extend this observation to further characterize the EnvZ/OmpR signaling system as well as to study of the regulation of \textit{ompF} and \textit{ompC} in individual live cells.

As we were able to observe OmpR-YFP localization at the \textit{ompF} and \textit{ompC} promoter regions \textit{in vivo}, we were able to provide support for the hierarchical binding model in live cells. Furthermore, we were able to shed some more light on why experiments attempting to prove that EnvZ is an osmosensor have produced problematic results, by demonstrating that OmpR binding to the \textit{ompC} promoter under increasing osmolarity can be dependent on the carbon source of the growth media. However, when cells were treated with the anesthetic procaine (Novocaine), which is also a known stimulant of EnvZ, we observed a carbon source independent increase in OmpR binding. This suggested that the observed behavior is not simply a carbon-source-dependent block of OmpR binding at the \textit{ompC} promoter, and is instead consistent with a model in which EnvZ senses a carbon source-dependent-factor responding to osmolarity.

\subsection{Membrane Protein Expression Triggers Chromosomal Locus Repositioning in Bacteria}

The second project described here addresses a separate open question regarding a possible connection between the localization of a protein and the subcellular positioning of the encoding genetic locus. As studies of several bacteria, including \textit{E. coli}, have demonstrated that chromosomal genetic loci appear at reproducible locations along the long axis of the cell, it has been speculated that the cell physiology may exploit chromosomal organization and conformation. Although experiments have indicated an ordered superstructure of bacterial chromosomes, they have also revealed a dynamic structure, in principle capable of allowing
individual loci to interact with each other, or with specific cellular regions (34, 35). On a comparatively long scale in actively growing cells, the chromosome is actively replicating and segregating, a process that results in the directed motion of the entire chromosome approximately 2µm over approximately 15 minutes (9, 34, 36, 37). On a fine scale, individual loci have been shown to execute subdiffusive motion of approximately equal magnitude, throughout the chromosome. (For a given locus $\text{MSD} = 4D\tau^\alpha$ with $\alpha \sim 0.39$ was experimentally measured (38).)

There is a long standing hypothesis that genetic loci encoding membrane proteins may reposition to the membrane due to co-transcriptional, co-translational insertion of the protein into the membrane (39). This in turn has been speculated to influence the targeted insertion of membrane proteins (6, 40), as well as a multitude of cellular processes involving chromosome structure and dynamics, including chromosome segregation, gross transcriptional control, autocatalytic gene activation, regulation of nucleoid structure, assembly of macromolecular complexes, and the formation of membrane and supercoiling domains (39-50). However, there is little direct evidence that the production of a specific membrane protein has any influence on the subcellular position of the encoding locus.

If co-transcriptional, co-translational, insertion of membrane proteins does occur, it would require several known processes – i.e. transcription, translation, and insertion - to become coupled specifically in the case of membrane proteins. In bacteria, there are two pathways for the insertion of membrane proteins, one of which has been shown to occur co-translationally. In this pathway, the signal recognition particle (SRP) targets a nascent polypeptide emerging from a translating ribosome to the membrane bound Sec insertion machinery (51). Interestingly, this co-translational system is highly conserved between E. coli, yeast, and humans, but with some modifications (52). The bacterial SRP is comprised of a complex of the protein Ffh bound to a 4.5 S RNA, which in turn binds to the signal sequence emerging from a translating ribosome.
Although the precise signal sequence for co-translational insertion is not highly conserved, SRP has been shown to primarily bind highly hydrophobic sequences which are naturally occurring in at least some membrane proteins with transmembrane domains (53) (54). Proteins that are not hydrophobic enough to engage the co-translational pathway, instead use a post-translational pathway where the ribosome completes translation and the released preprotein is targeted for secretion. (For reviews see (52, 55)).

The evidence, however, that transcription and translation are coupled for membrane proteins, is less clear cut. Early experiments using electron microscopy demonstrated that transcription and translation can, in general, be linked based on the isolation of DNA-RNAP and RNAP-RNA-ribosome complexes (56). Transcription rates are generally measured in the range of 20-80 nt/sec, which vary based on growth rate and the gene being transcribed (57). It was recently shown that the rate of RNA elongation and the speed of the translating ribosome may be tightly coupled, particularly in the case of highly expressed genes (58). However, several other experiments suggested that in the case of most transcripts, transcription and translation may not be coupled. For example, a study examining the spatial distribution of fluorescently labeled RNAP and fluorescently labeled ribosomes, concluded that in B. subtilis, the distribution of RNAP and ribosomes did not show significant overlap (59). This was based on the observation that ribosomes are predominantly found at the cell periphery, whereas RNAP was predominantly associated with the interior of the nucleoid. However, the same work also demonstrated the presence of some RNAP at the membrane, which would be consistent coupled transcription and translation for some chromosomal locations at the periphery of the nucleoid.

In order for transcription to be coupled to co-translational insertion, at least some of the mRNA of the membrane protein would be expected to remain in the vicinity of the encoding locus. Although, in principle, a single mRNA transcript still attached to chromosomal DNA
would be sufficient to create effective tethering between the chromosome and the membrane, the basic question of whether mRNA in the bacterial cell significantly disperses from its encoding chromosomal locus remains debated. Several studies have measured differing values for the apparent diffusion constants for mRNA in bacterial cells. A study using plasmids observed that on a case by case basis the mRNA may diffuse throughout the cell, or may preferentially remain near the site of the encoding locus (60). However, a subsequent study using specific chromosomal loci, instead observed that the mRNA does not significantly diffuse from the site of the encoding gene (61). In disagreement with this, however, a high throughput study of E. coli chromosome reported mRNA dispersion from chromosomal loci (62).

The other evidence supporting a model of co-transcriptional, co-translational insertion of membrane protein is the general collapse of the bacterial nucleoid away from the cell periphery when treated with either translation or transcription inhibitors (e.g. (48, 63-66)). This indicates that transcription and translation affect chromosome conformation, but the mechanisms may be indirect or be the result of specific anchoring proteins or non-specific effects from macromolecular interactions between cellular components. Further complicating this evidence is a recent report that the mRNA of some membrane proteins repositions to the cell periphery in a translation independent fashion (67). Taken together, these results suggested that although “transertion” is widely presumed to occur, it was by no means obvious that for a specific chromosomal locus there would be an observable repositioning towards the membrane that is membrane protein, transcription, and translation dependent.

We were therefore interested in testing whether we could observe loci encoding specific membrane proteins repositioning to the membrane upon induction, and determining whether the repositioning was dependent on both transcription and translation. We chose two E. coli inner membrane protein encoding genes, lacY encoding lactose permease, and tetA, encoding a
tetracycline efflux pump. Both proteins are believed to contain multiple transmembrane domains, and LacY has been shown to be co-translational inserted (68). We studied the effects of induction of LacY at its native location within the chromosome, and found that not only does the lac locus reposition towards the membrane when LacY is induced, the repositioning is strikingly apparent during visual inspection of images, as it has a comparable magnitude to the width of the cell. In order to make quantitative measurements of the repositioning of the lac locus, we used a chromosomal marker (TetR-YFP binding a short array of tetOs), whose location can easily be measured to subpixel accuracy, in combination with the peak fluorescence of the membrane stain FM4-64. As a result, we were able to obtain a precise measurement (to within ~60 nm) of the relative spacing between the chromosomal location and the membrane (projected into the plane of focus). This particular measurement also appears relatively insensitive to fluctuations in cell widths found across a population of E. coli cells. Furthermore, we determined that the repositioning was not simply due to active transcription at this locus, as replacement of LacY with a cytoplasmic protein did not result in a repositioning of the locus.

In addition to observing the repositioning of the lac locus, we also sought to explore whether this phenomenon could be observed in other portions of the chromosome, and with TetA induction. Furthermore, as tetA induction can be tuned by varying the amount of tetracycline in the growth medium, we were able to characterize the repositioning of the tet locus at a chromosomal location far from the lac locus, and as a function of induction. These results, coupled with the observation that the onset of repositioning was remarkably rapid following addition of inducer, suggested that chromosomal loci may reposition towards the membrane dynamically as a function of protein produced, but do not remain there.

To attempt to address the mechanism of repositioning for membrane proteins, we treated cells with the transcription inhibitor rifampicin, and the translation inhibitor kasugamycin.
Previous studies have cited the collapse of the bacterial nucleoid upon treatment with transcription and translation inhibitors (48, 63-66) as evidence that the nucleoid is maintained in an open conformation by co-transcriptional, co-translational insertion of membrane proteins (“transertion”). Here we demonstrate specifically that rifampicin, which interferes with RNA polymerase, and kasugamycin, which prevents the initiation of translation, separately prevents repositioning of the membrane protein encoding locus, suggesting that both transcription and translation are required for locus repositioning. As membrane protein encoding loci are predicted to occur throughout the *E. coli* chromosome, this would suggest that repositioning by this mechanism may be an important factor in maintaining the bacterial nucleoid in an open and dynamic conformation.
1.3 A brief note on notation

As the work discussed here commonly requires a careful distinction between genetic loci and the proteins produced, every effort has been made to use standard and consistent notation throughout and suppress extraneous details of genotypes for clarity.

In standard notation a gene is denoted italics beginning with a lowercase letter. The protein produced by that gene is denoted using a capital first letter, no italics.

E.g. for ompR encoding OmpR we can have

Table 1.1

<table>
<thead>
<tr>
<th>Notation</th>
<th>Meaning</th>
</tr>
</thead>
<tbody>
<tr>
<td>ompR</td>
<td>Refers to the gene (With an allele number this indicates a mutation at that gene locus – e.g. ompR101.)</td>
</tr>
<tr>
<td>OmpR</td>
<td>Refers to the protein OmpR</td>
</tr>
<tr>
<td>ompR::cat</td>
<td>The gene ompR has been interrupted by the sequence for the gene cat.</td>
</tr>
<tr>
<td>ompR</td>
<td>Phenotype is an OmpR deletion.</td>
</tr>
<tr>
<td>Δ(ompR)::cat</td>
<td>ompR has been deleted and replaced by cat.</td>
</tr>
<tr>
<td>ompR⁺⁻yfp⁺</td>
<td>A transcriptional or translational fusion of OmpR to YFP. Here we always include a symbol distinguishing the two. (see below)</td>
</tr>
<tr>
<td>Ψ(ompR⁺⁻yfp⁺)</td>
<td>Our notation. Translation fusion. Produces a fusion of the OmpR protein to YFP.</td>
</tr>
<tr>
<td>Φ(ompR⁺⁻yfp⁺)</td>
<td>Our notation. Transcriptional fusion. Produces OmpR and YFP as two separate proteins from the same transcript.</td>
</tr>
</tbody>
</table>
For strain and plasmid notation the following conventions are used:

Strains: person’s initials followed by strain number: e.g. EAL179. Plasmids: “p” followed by the plasmid number: e.g. pEL12. Therefore, strain EAL179 harboring the plasmid pEL12 is denoted EAL179/pEL12. Occasionally (and in the work described here, frequently) the presence of a plasmid may modify the strain itself. The two cases described in this paper, the harboring of pCP20 and pEL8, result in the recombination of particular sequences (FRT sites) within the chromosome. I.e the Keio strain JW0334 has FRT-kan-FRT in place of lacY in the chromosome (ΔlacY::[FRT-kan-FRT]), and is therefore kanamycin resistant. JW0334/pEL8 (my notation) is actually ΔlacY::FRT and is kanamycin sensitive. As those constructs are always intermediates, this notation was chosen to avoid unnecessary strain confusion.
1.4 References


Chapter 2: Imaging OmpR Binding to Native Chromosomal Promoters in E. coli


2.1 Introduction

Several fluorescence microscopy studies of transcription factors in bacteria have revealed tantalizing evidence of subcellular localization of these proteins (1-6). However, in most cases the biological significance and underlying mechanism of localization are not well understood. At least part of the difficulty in interpreting the distribution of intracellular fluorescence is due to the lack of readily available landmarks within the bacterial cytoplasm. Here we extend standard tools for tagging the E. coli chromosome to demonstrate that fluorescent foci formed by a YFP fusion to the transcription factor OmpR co-localize with specific genes. We also show that these foci are likely due to occupation of OmpR-binding sites on the DNA and therefore provide a means for studying OmpR binding to native sites in vivo.

The response regulator OmpR and its partner histidine kinase EnvZ are a particularly well-characterized two-component system. The signals that directly stimulate EnvZ have not been established, however changes in extracellular osmolarity by inner
membrane impermeable osmolytes, treatment with some lipophilic compounds such as procaine, and expression of a small membrane protein MzrA can activate the EnvZ/OmpR system (7-12). The best-studied OmpR-regulated genes in *E. coli* are *ompF* and *ompC*, which encode the classical porins (8, 9). At high levels of OmpR phosphorylation (OmpR-P), *ompF* transcription is repressed and *ompC* transcription is activated. The expression of these genes has been used to infer changes in OmpR phosphorylation and to study EnvZ/OmpR signaling under various physiological conditions.

Previously, a subcellular localization was reported for a functional fluorescent protein fusion of YFP or GFP to OmpR in *E. coli* (2). In cells expressing OmpR-YFP at roughly wild-type levels (2), most of the fluorescence appears uniformly distributed throughout the cytoplasm. However, on top of this diffuse background, distinct foci of fluorescence are clearly visible (2) and (Fig. 2.1). Under conditions associated with increased OmpR phosphorylation, the intensity and number of foci increase. Furthermore, they disappear completely in conditions of low OmpR-P (e.g. in *envZ* strains - Fig. 2.1) (2). These foci do not appear at fixed, easily identifiable cellular positions, such as the poles or mid-cell, and they can be eliminated by over-expression of unlabeled OmpR (2). Taken together, prior work suggested that the foci are due to a phosphorylation-dependent increased local concentration or clustering of OmpR-YFP. However, the significance and origin of these foci were not understood.

In this work, we tested the hypothesis that the foci are due to OmpR-YFP binding to the chromosome. The *ompF* and *ompC* regulatory regions contain four and three
OmpR binding sites, respectively; each site is bound by an OmpR dimer (13-17). We hypothesized that binding of multiple OmpR-YFP molecules at these sites could produce observable foci. To test this, we marked specific sites in the *E. coli* chromosome with *lac* and *tet* operators, using a simple system for integrating these markers. We show that fluorescent foci are indeed observable at *ompF* and *ompC* and depend on the presence of OmpR binding sites.

2.2 Results

**A simple system for inserting lacO and tetO arrays into the chromosome**

To facilitate the targeted insertion of *lac* and *tet* operators into the chromosome, we constructed conditionally-replicative plasmids – one containing arrays of *lacO* repeats (18) and a kanamycin resistance gene, and the other containing *tetO* repeats (19) and a chloramphenicol resistance gene (Fig. 2.2). These plasmids also contain recognition sequences (FRT sites) for the FLP recombinase. The plasmids can therefore be stably integrated into FRT sites in the *E. coli* chromosome by expressing FLP (20).

Chromosomal FRT sites can be easily engineered in many organisms, e.g. by lambda-Red mediated recombination (21). For *E. coli* K-12, strains are available with a FRT site inserted in virtually any non-essential gene (22). Therefore, with this system one can easily target arrays of operators to almost any location in the *E. coli* chromosome and move the subsequent construct to other strain backgrounds by P1 transduction. Additionally, both *lacO* and *tetO* repeats can be moved into the same strain to simultaneously label two chromosomal locations.
Co-localization of OmpR-YFP foci with chromosomal loci

To determine if the brightest OmpR-YFP foci co-localize with either \(ompF\) or \(ompC\), chromosomal \(lacO\) repeats were inserted in place of \(ompF\), \(ompC\), or \(lacI-lacA\) (as a negative control) in a strain containing an \(ompR-yfp\) translational fusion (Fig. 2.3A). CFP-LacI was expressed from a plasmid. A comparison of sample CFP and YFP fluorescence images is shown in Fig. 2.3B. From visual inspection, a significant number of the OmpR-YFP foci show striking co-localization with \(ompF\). The foci appear to be occasionally co-localized with \(ompC\), as shown in the upper-most cell in Fig. 2.3B, 3\(^{rd}\) row. Co-localization with the \(lacI-lacA\) region, which has not been reported to have adjacent OmpR binding sites, appears to be less frequent. To quantitatively analyze the extent of co-localization, we developed software to identify the centroid of the brightest OmpR-YFP spot in each cell, and determine its distance from the centroid of the nearest CFP-LacI spot. The resulting histogram (Fig. 2.3C) is consistent with impressions from visual inspection of the images: the brightest OmpR-YFP foci show striking co-localization with \(ompF\) and relatively little co-localization with \(lacI-lacA\) and \(ompC\).

For the microscope used in these experiments, there was a time lag of approximately two seconds between the acquisition of CFP and YFP images. This complicates the analysis of co-localization as individual chromosomal loci move within the cell over this time interval. Over the same time interval, the boundaries of cells do not show detectable motion, indicating that the observed motion is not due to drift of the sample. To characterize the drift of a chromosomal location, two successive CFP images of a field of cells were acquired two seconds apart. When repeated over many fields, the
resulting distributions of displacements were found to be similar for the \textit{ompF}, \textit{ompC}, and \textit{lacI-lacA} regions. The distribution for \textit{ompF} is shown in Fig. 2.3C (dashed line). 73\% of the \textit{ompF} displacements are separated by less than 2.25 pixels (~180 nm). We therefore chose a cutoff of 2.25 pixels as a conservative criterion for co-localization of OmpR-YFP and CFP-LacI foci. Thus, CFP and YFP fluorescence maxima were scored as co-localized if they were separated by a distance less than or equal to 2.25 pixels.

With this co-localization criterion, approximately 70\% of the brightest OmpR-YFP foci co-localize with \textit{ompF}, compared with approximately 15\% co-localization with \textit{lacI-lacA} (Fig. 2.3C – inset). The \textit{ompC} locus shows an intermediate amount of co-localization with the brightest OmpR-YFP foci, at about 23\%. Furthermore, we note that the distribution of \textit{ompF} drift distances shows remarkable agreement with the distribution of distances between \textit{ompF} locations and the brightest OmpR-YFP foci (Fig. 2.3C – compare red solid and black dashed lines). This indicates that the most prominent OmpR-YFP spots are in the vicinity of \textit{ompF}.

**Proximity of \textit{ompF} to \textit{ompC} and \textit{lacI-lacA}**

The porin genes \textit{ompF} and \textit{ompC}, which share several common regulators, are separated by approximately 1300 kb, and are roughly symmetrically located around \textit{terC} (Fig. 2.4B, inset). The \textit{lac} locus is roughly half the distance to \textit{ompF} (630 kb). To analyze the extent of \textit{ompF} co-localization with \textit{ompC} and \textit{lac}, \textit{ompF} was labeled with \textit{tetO} repeats and \textit{ompC} or \textit{lac} was labeled with \textit{lacO} repeats (Fig. 2.4A). Analysis of the
distances between the chromosomal labels indicates that, with the same co-localization criteria described above, roughly 13% of the ompF labels co-localize with an ompC label and roughly 10% co-localize with a lac label (Fig. 2.4B). This suggests a significant fraction of the lac and ompC co-localization with OmpR-YFP in Fig. 2.3C can be accounted for by the co-localization of these loci with ompF.

**OmpR-YFP co-localization is OmpR binding site dependent**

To test if the co-localization of the brightest OmpR-YFP foci with ompF is due to OmpR binding, we deleted the OmpR binding sites F1-F4 upstream of ompF. This resulted in a marked shift in the distribution of distances between ompF and the brightest OmpR-YFP foci when compared with the corresponding distribution in a strain with the binding sites intact (Fig. 2.4C). In particular, deletion of the binding sites caused the brightest OmpR-YFP foci to fall farther from ompF on average; approximately 4% of the foci co-localize with ompF in the binding site deletion strain whereas approximately 60% co-localize with ompF when the binding sites are intact (Fig. 2.4C). From visual inspection, we found that deletion of F1-F4 resulted in many cells that did not show clear OmpR-YFP foci. This observation formed the basis for the analysis in the following sections. We also note that in Fig. 2.4C we restricted the analysis to only those cells with distinct foci (see Materials and Methods and the legend to Fig. 2.4C).

**OmpR-YFP fluorescence at ompF and ompC is OmpR binding site dependent**

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The OmpR-YFP foci correspond to a local increase in fluorescence (e.g. Fig. 2.1B). By studying their positions, we found the brightest foci are usually in the vicinity of \( ompF \). In addition, this co-localization depends on adjacent OmpR binding sites. To further explore the association of OmpR-YFP with these sites, we measured the YFP fluorescence in the neighborhoods of \( ompF \) and \( ompC \).

When \( ompF \) and \( ompC \) are spatially well-separated, deletion of the OmpR binding sites at \( ompF \) should decrease the OmpR-YFP fluorescence at \( ompF \) while leaving the fluorescence at \( ompC \) unchanged. To test this, and also to determine whether there is a similar effect at \( ompC \), we compared four strains, corresponding to intact OmpR binding sites at \( ompF \) and \( ompC \), deletion of the sites at \( ompF \) - \( \Delta(F1-F4) \), deletion of the sites at \( ompC \) - \( \Delta(C1-C3) \), and the double deletion \( \Delta(F1-F4) \Delta(C1-C3) \). Fluorescence images were analyzed to determine the local YFP fluorescence in the neighborhood of \( ompF \) and \( ompC \) (see Materials and Methods for details).

When the binding sites at \( ompF \) were deleted, the fluorescence at \( ompF \) decreased but had relatively little effect on the fluorescence at \( ompC \) (Fig. 2.5). The effect of deleting binding sites at \( ompC \), on the other hand, was relatively weak. When the binding sites at both \( ompF \) and \( ompC \) were deleted, the local OmpR-YFP fluorescence was comparable for the two locations. This suggests the average OmpR-YFP occupancy at the \( ompC \) promoter is lower than the occupancy at \( ompF \) for the growth conditions used in these experiments (minimal glucose medium). We also note that \( ompC \) transcription under these growth conditions is relatively low but can be significantly increased by stimulating the EnvZ/OmpR system with procaine (Fig. 2.6A—right panel). If the
increased ompC transcription from procaine stimulation were due to significantly increased OmpR binding to the ompC promoter, then we should also observe increased OmpR-YFP fluorescence at ompC. This is indeed the case, as is evident in Fig. 2.6B (right panel). Treatment with 10 mM procaine resulted in local fluorescence at ompC that was comparable to the level at ompF in untreated cells (Fig. 2.6B, compare left and right panels). Furthermore, the increase in local fluorescence at ompC was eliminated when the OmpR binding sites C1-C3 were deleted. Procaine stimulation also produced an increase in fluorescence at ompF. The fold change is lower than for ompC, but the overall levels of fluorescence are higher Fig. 2.6B (left panel). Procaine stimulation has the effect of repressing ompF transcription (Fig. 2.6A—left panel), as expected from higher levels of OmpR-YFP phosphorylation.

**Carbon Source Dependence of OmpR Binding to ompC**

To explore the effects of osmolarity on OmpR binding to DNA, we focused on ompC because OmpR-YFP shows relatively little localization at this site under conditions of low osmolarity (Fig. 2.6B and 2.8B). This is consistent with the correspondingly low level of ompC transcription (Fig. 2.8A). We measured the OmpR-YFP fluorescence in the vicinity of an ompC label as described above. We used a strain in which the OmpR binding sites at ompF were deleted in order to provide greater sensitivity and eliminate potential difficulties from OmpR-YFP foci localized at ompF. The distributions of peak OmpR-YFP fluorescence intensity in the vicinity of ompC across populations of cells
grown under different conditions is shown in Fig. 2.8B. In minimal media supplemented with glucose, increasing concentrations of sucrose resulted in a marked increase in local OmpR-YFP at $ompC$ (Fig. 2.8B, left). Previous work imaging OmpR-YFP binding to multicopy plasmids suggested that osmolarity had relatively little effect (23). However, those experiments were performed using glycerol as a carbon source. In agreement with these observations, when we repeated our measurements with cells growing in minimal glycerol media, we found that the addition of sucrose did not significantly increase OmpR-YFP fluorescence at $ompC$ (Fig. 2.8B, right). Also consistent with the results of (2) we observed a strong increase in OmpR-YFP fluorescence at $ompC$ for cells treated with procaine. Importantly, for cells expressing OmpR-YFP and growing in either glucose or glycerol, osmolarity and procaine both have a significant effect on porin transcription (Fig. 2.8A and (2)). In addition, the changes in transcriptional reporter expression in response to stimuli are comparable in the two carbon sources.

### 2.3 Discussion

Our results indicate that the observed subcellular localization of OmpR-YFP results from OmpR-YFP binding specific sites in the chromosome. The wild-type expression level of OmpR-YFP gives considerable diffuse fluorescence in the cytoplasm, which would make single-molecule imaging difficult (Fig. 2.1). Single molecules of LacI-YFP have been imaged, but this required very low fluorescent protein expression levels (~3 repressors/cell) (24). From our results, it appears that the multiple binding sites
upstream of the porin genes \textit{ompF} and \textit{ompC} (which are maximally occupied by 8 and 6 OmpR molecules, respectively), can give sufficiently high local concentrations of OmpR-YFP to be easily detected by fluorescence microscopy (Fig. 2.1). We also note that when the binding sites at \textit{ompF} and \textit{ompC} were deleted, additional OmpR-YFP foci were evident (data not shown). It seems likely that these reflect OmpR-YFP binding regulatory sites at other OmpR-regulated genes in the chromosome. Exploring this question further would require testing other members of the OmpR-regulon for co-localization. Procaine induction data (Fig. 2.6B) suggest that even when OmpR binding sites are not fully occupied, a measurable local increase in OmpR-YFP can be observed at \textit{ompF} and \textit{ompC}. We can therefore use this local fluorescence to infer the relative extent of OmpR-YFP binding at these sites.

A model of hierarchical OmpR binding at the porin promoters has been proposed to account for the differential regulation of \textit{ompF} and \textit{ompC} (25, 26). In this model, the \textit{ompF} regulatory region contains high and low affinity binding sites for OmpR-P and the \textit{ompC} region contains only low affinity sites (Fig. 2.7). OmpR-P primarily binds the high affinity sites (at \textit{ompF}) when its concentration is low. With increasing OmpR-P, additional binding occurs at the low affinity sites (at \textit{ompF} and \textit{ompC}). Occupation of the high affinity sites results in activation of \textit{ompF} transcription. Occupation of the low affinity sites results in repression of \textit{ompF} and activation of \textit{ompC} transcription. This model is consistent with \textit{in vitro} studies reporting hierarchical and cooperative binding at the porin promoters (27-29).
Based on the following observations, our data provide in vivo support for the above model. First, for the growth conditions used here—minimal glucose medium with or without procaine—we observed more OmpR-YFP at \textit{ompF} than at \textit{ompC}. This is consistent with greater overall OmpR-YFP binding at \textit{ompF}. Second, stimulation of the EnvZ/OmpR system with procaine produced a larger fold-change in fluorescence at \textit{ompC} than at \textit{ompF} (Fig. 2.6B), indicating the relative increase in OmpR-YFP binding at \textit{ompC} is greater than at \textit{ompF} when OmpR phosphorylation is greatly increased. Such behavior is consistent with a larger proportion of low affinity OmpR binding sites at \textit{ompC} than at \textit{ompF}. A sketch of these observations, interpreted in the context of the hierarchical binding model, is shown in Fig. 2.7.

Most studies of the EnvZ/OmpR system in \textit{E. coli} have used porin expression to infer OmpR activity. However, \textit{ompF} and \textit{ompC} expression are also modulated by numerous other factors (23, 30), making it difficult to determine whether specific stimuli act through EnvZ or other regulators. A previous study of OmpR-YFP, using \textit{E. coli} growing on glycerol as the carbon source, suggested that changes in osmolarity had little effect on the association of OmpR with \textit{ompF} and \textit{ompC} promoters (2). This observation was surprising in light of the generally accepted role of the EnvZ/OmpR system in osmoregulation of porin expression. In the present study, we noticed that at low osmolarity OmpR-YFP foci were significantly more intense in cells growing on glucose than in cells growing on glycerol. This motivated us to re-examine the role of increasing osmolarity on OmpR-YFP localization.
Remarkably, we observed markedly different effects for cells growing on glucose and glycerol, even though an ompC transcriptional reporter shows similar behavior for cells growing in either carbon source. For cells growing in glucose, OmpR-YFP localization at ompC increased significantly when cells were treated with procaine or increasing concentrations of sucrose. This is consistent with a model of EnvZ/OmpR signaling in which procaine and high osmolarity both result in increased OmpR phosphorylation, which in turn binds to the ompC promoter and activates ompC transcription. The results are also consistent with an earlier in vitro study of the effects of osmolarity on OmpR binding to DNA, which made use of extracts from cells growing in glucose minimal media (31). Our data for the same experiments repeated in minimal medium supplemented with glycerol, on the other hand, are in agreement with previous studies imaging OmpR binding to ompC and ompF promoters on plasmids (2). At the native ompC promoter in the chromosome we did not observe strong increases in OmpR-YFP localization. The small increase observed for increasing concentrations of sucrose was strikingly different from the large increase observed for treatment with procaine, even though the transcriptional response for treatment with procaine was smaller than the corresponding response for treatment with sucrose (Fig. 2.8A). These results suggest that the role of OmpR in porin osmo-regulation is media dependent. In particular, for growth on glycerol, factors other than increased OmpR binding to DNA are likely to be important for activation of ompC expression at high osmolarity.

Our work demonstrates that the fluorescently labeled transcription factor OmpR-YFP, expressed at roughly wild-type levels, can be imaged binding native chromosomal
loci. By quantifying this fluorescence localization at labeled sites of interest, we were able to study OmpR activity in live cells. This method may be applicable to the study of other transcription factors for which functional fluorescent protein fusions are available. We note that the quantification of fluorescence does not depend on the existence of distinct foci. However it is necessary to know the approximate location where the transcription factor binds. The method is likely to be particularly useful at loci containing multiple binding sites, where the fluorescence of bound transcription factor is most likely to give a significant signal. We also developed a simple system for inserting arrays of lac and tet operators into chromosomal FRT sites. By using these constructs in conjunction with the Keio collection of E. coli deletion strains, one can readily insert lacO and tetO arrays at virtually any location of the chromosome. The large collection of marked strains that can be rapidly constructed by this method will be useful for studies of other DNA binding proteins and chromosome organization.

2.4 Materials and Methods

E. coli strains were grown at 37 °C except when propagating plasmids with temperature sensitive origins of replication (pEL8 and pCP20), which was carried out at 30 °C. Plasmids containing the oriR6Kγ origin of replication were propagated in the pir+ E. coli strain PIR2 (Invitrogen). Plasmids and strains used in this work are listed in Table 2.1.
Construction of the CFP-LacI and TetR-mCherry expression plasmid pEL7

The Tn10 tetR gene was amplified from the plasmid pAS02 (2) using the primers
5’ CGAGCCGTCGACAGGAAACAGACCATGTCTAGATTAGATAAAAG -3’ and
5’- CAGTTAGGTACCAGACCCACTTTTCACATTTAAG-3’ and digested with SalI and
KpnI. The mCherry gene was amplified from pRSETb-mcherry using primers 5’-
GAATTAGGTACCGTGAGCAAGGGCGAGGAGG -3’ and 5’-
GGCCTCAAGCTTTAATTTGATACGCTCCATG -3’ and digested with KpnI and
HindIII. The above two fragments were ligated to SalI and HindIII digested pEB96 (2).
The resulting plasmid, pEL7, expresses cfp-lacI and tetR-mCherry translational fusions
under control of an arabinose-inducible promoter.

Construction of the tetO array insertion plasmid pEL5

An array of tetO2 operators was isolated from p306tetO224 by digesting the
plasmid with SacI and KpnI. This was ligated to a fragment of pCAH63 (32) (containing
cat and oriR6Kγ) that was amplified using the primers 5’-
GCATTAGGCTCGAAGTTCTATTTACTAGAAAGAATAGGAACCTTCGAGCA
GGGAGGCAAACAATG-3’ (the FRT site is underlined (20) ) and 5’-
TGTTCGAGCAGAAAGCAGACC-3’ and digested with SacI and KpnI. A clone with
approximately 100 copies (~4 kb) of tetO2 repeats was designated pEL5.
**Construction of the lacO array insertion plasmid pSE1**

A fragment containing approximately 10 kb of lacO repeats was cut from pEB127 (E. Batchelor and M. Goulian unpublished) by digestion with SalI and BamHI and ligated to a fragment of pCE40 (20) (containing FRT, kan, and oriR6Kγ) that was amplified using the primers 5’- caggatccCGTCGTCAGGTGAATG -3’ and 5’- gagtcgacGGCGATTAAGTTGGTAACG -3’. A clone with a size consistent with approximately 10 kb of lacO repeats was designated pSE1. This plasmid was constructed with Seda Ekici.

**Construction of pEL8, a cat’ derivative of pCP20**

The cat gene in pCP20 (33) was deleted by digestion with SmaI and NcoI, treatment with T4 DNA polymerase to blunt the ends, and ligating the DNA. The resulting plasmid, pEL8, no longer confers chloramphenicol resistance but is otherwise isogenic with pCP20.

**Chromosomal integration of pEL5 and pSE1.**

Plasmids were integrated into chromosomal FRT using a protocol similar to that described in (20). To integrate pEL5, a strain containing a chromosomal FRT site and the plasmid pEL8 was transformed with pEL5 by electroporation and grown on LB plates.
containing 15 µg/ml chloramphenicol at 37 °C. Selected colonies were streaked on LB/chloramphenicol and grown at 37 °C. Colonies were then re-streaked on LB without antibiotic and grown at 37 °C and in parallel streaked on LB with 50 µg/ml ampicillin and grown at 30 °C to test for loss of pEL8. The plasmid pSE1 was integrated by essentially the same procedure, except in some cases pCP20 was used for expression of FLP recombinase instead of pEL8, and selective plates consisted of LB agar with 25 µg/ml kanamycin. Insertions in ompF and ompC were constructed in the Keio collection strains JW0912 and JW2203 (22) or, for the case of OmpR binding site deletions, in the strains EAL81 and EAL96. The insertions in lac were constructed in the strain EPB238 (2). Integrated plasmids were moved by P1 transduction as needed.

**Deletion of OmpR binding sites at ompF and ompC**

A deletion of ompC and the three upstream OmpR binding sites was constructed by lambda-Red-mediated recombination as in (21). The primers 5’ -

GTGCTGTCAAATACTTAAGAATAAGTTATTGATTCCGGGGATCCGACC - 3’ and 5’-CGCAGGCCCTTTGTGCATATCAATCGAGA

GTGTAGGCTGGAGCTGCTTC-3’ were used to amplify the FRT-kan-FRT cassette from pKD13. The underlined sequences in the primers denote sequence upstream of the OmpR binding sites and downstream of the ompC gene, respectively. The PCR product was transformed by electroporation into BW25113/pKD46 as in (21) and the deletion was verified by PCR, resulting in strain EAL81.
To delete *ompF* and the four upstream OmpR binding sites, the same procedure was used as outlined above, but with the primers 5’-

\[\text{TCAAGCAATCTATTTGCAACCCCAGCATAAAATTTCCGAGGATCGGCTCGACC-3’}\]

and 5’-

\[\text{GAACTGGTAAACGATACCCAGAAGGTGGTGAGGCTGGAGCTGGTC-3’}\]

which removes the OmpR binding sites F1-F4, through to the last 7 amino acids of *ompF*. The underlined sequences in the primers denote sequences upstream of the OmpR binding sites and the end of *ompF*, respectively. This strain was named EAL96.

*Construction of EAL97*

EPB240 contains the chloramphenicol resistant plasmid pEB55 integrated at the lambda phage attachment site. The *cat* antibiotic resistance gene in this integrated plasmid was deleted by lambda-Red-mediated recombination with a *FRT-kan-FRT* cassette, which was amplified from pKD4 (21) using the primers 5’-

\[\text{ATATCCCAATGGCATCTAGTAAACATTTTGAGGCAGCAGTCGCTCGCTGCTGCTGCTTCGAA-3’}\] and 5’-

\[\text{ATGAAACCTGAATCGCCAGCGCATCAGCAGCTTGGTCGCTTTCGTGATAATATG}\]

\[\text{AATATCCTCCTCCTTAG-3’}\]. The underlined sequences in the primers denote sequences in the *cat* gene. The resulting integrated plasmid (which now confers kanamycin resistance) was moved into a clean EPB240 background by P1 transduction. The *kan* cassette was then removed using pEL8, resulting in EAL97.
Microscopy

Cultures were grown overnight to saturation in minimal A medium \(^{34}\) containing 0.2% glucose and 50 µg/mL ampicillin for plasmid maintenance, when necessary. Cultures were then diluted at least 1:1000 into the same media. When the optical density at 600 nm reached approximately 0.1, procaine was added as indicated. To induce production of LacI-CFP and TetR-mCherry, arabinose was added to 10 mM.

Microscopy was performed on live cells at 37 °C essentially as described in \(^{2}\) except that the objective lens was Olympus UPLFLN 100XO2PH (100X, NA 1.3, with a phase ring). In addition, a cube containing a Chroma HQ575/50X excitation filter, HQ640/50M dichroic, and Q610 LP emission filter was used for mCherry fluorescence imaging. A phase contrast image was first acquired, followed by a 750 msec YFP image, followed by a 400 msec CFP image, and finally a 400 msec mCherry image as indicated.

Image Analysis

Image analysis was based on methods previously outlined in \(^{2}\), with the exception that phase contrast images were used to identify cell boundaries. To find the brightest OmpR-YFP focus in a cell, the software identified the brightest pixel in the YFP image, subject to the restriction that the pixel value must be at least two standard deviations above the average cellular fluorescence and also above a preset value of 55. These restrictions were chosen to minimize false positives. The location of the OmpR-YFP focus was determined by computing the centroid of a 5x5 array of pixels centered...
on this maximum. To identify the positions of (at most) four CFP-LacI foci, pixel values were required to be above 100 and restricted to at least 1.8 standard deviations above the mean. The CFP maxima were required to be separated by at least 3.5 pixels to be scored as separate foci. For mCherry fluorescence analysis, similar conditions were used, but with the restrictions that the pixel values were greater than 200 and 1.9 standard deviations above cellular background. Fewer than four foci were identified if the top four maxima did not all pass the restrictions on pixel value.

To determine the YFP fluorescence in the neighborhood of CFP-LacI and TetR-mCherry foci, up to four maxima were identified in the CFP and mCherry images, and their positions were determined by the procedure described above. For each of these maxima, the software searched for the brightest YFP pixel within a 5X5 array of pixels centered on the CFP or mCherry maximum. A 5x5 array of pixels centered on this YFP maximum was then fit to a Gaussian \( C_0 \exp(-C_1 r^2) + C_2 \) as in (2); \( C_0, C_1 \), and \( C_2 \) are fitting parameters and \( r \) is the radius from the center of the array. The amplitude of this fit, \( C_0 \), was used as the measure of the local fluorescence.

For the data in Fig 2.4C, we discarded cells that lacked distinct foci. This was determined for each cell by performing a Gaussian fit, as above, centered on the brightest YFP pixel. From visual inspection of images, distinct foci were determined to be those for which the quantity \( C_1 \) was within the range (0.35, 2.3).

For Figures 2.5 and 2.6, cells for which \( \text{ompF} \) and \( \text{ompC} \) loci fell within a drift radius of each other were discarded as follows. For each CFP or mCherry maximum in a
given cell, the software reported the distance to the nearest maximum in the other fluorescence channel. CFP and mCherry maxima whose separations were within the drift radius of a chromosomal location over the longest time scale of image sequence collection were discarded as not being sufficiently well separated. Fluorescence intensities were normalized across different days using the parameter C0 from Gaussian fits to YFP fluorescence at random locations that were at least a distance of 5 pixels from the CFP-LacI and TetR-mCherry foci. We required random locations to fall within the region that was typically occupied by *ompF* and *ompC*, which was roughly the middle 70% of the major and minor axes. More precisely, we required the major and minor axis coordinates to fall within the region that is occupied by *ompF* and *ompC* 98% of the time (determined empirically for each growth condition). These Gaussian fits to random locations were used to background subtract and rescale the data. Images for figures were prepared using ImageJ (National Institutes of Health) and Matlab. Images in Fig. 2.1 were normalized to the same average cellular fluorescence. Brightness and contrast levels were identical for all of the images in Fig. 2.1, and were adjusted in Fig. 2.3 to maximize visibility of localized fluorescence. Image analysis software was developed in-house using Labview.

*Porin transcription fluorescence assay*

CFP fluorescence expressed from the *ompC* promoter, and YFP fluorescence expressed from the *ompF* promoter, for cultures growing with or without procaine were determined by following the procedures described in (2).
2.5 Figures

Figure 2.1. Fluorescence micrographs of live cells displaying OmpR-P dependent foci of OmpR-YFP fluorescence. A) Left: EAL97 (ompR-yfp⁺). Right: EPB238 (ompR-yfp⁺ envZ⁻). Scale bars represent 2 µm. Cells were grown in minimal glucose medium. B) Three-dimensional representations of the intensity distributions for the single cells shown in A, displayed as a function of image coordinates. For comparison purposes, the images were normalized to have the same average cellular fluorescence.
Figure 2.2. A system for inserting arrays of lac operators (lacO) and tet operators (tetO) into chromosomal FRT sites.

A) Plasmids pSE1 and pEL5 contain an R6Kγ origin of replication, a selectable marker conferring chloramphenicol or kanamycin resistance, and a FRT site, which is recognized by the FLP recombinase. Plasmid construction details are in Materials and Methods.
Figure 2.2 (continued). A system for inserting arrays of *lac* operators (*lacO*) and *tet* operators (*tetO*) into chromosomal FRT sites.

B) Fluorescence micrographs of cells containing both *tetO* and *lacO* arrays (TetR-mCherry fluorescence—left and CFP-LacI fluorescence—right). The strain EAL105 has pSE1 inserted at *ompC* and pEL5 inserted at *ompF*. (The plasmids were integrated into separate strains and then moved by P1 transduction.) TetR-mCherry and CFP-LacI were expressed from the plasmid pEL7.
Figure 2.3. OmpR-YFP localization at \textit{ompF}, \textit{ompC}, and \textit{lac}.

A) Constructs integrated in the chromosome at \textit{ompF} (EAL70), \textit{ompC} (EAL62), or \textit{lacI-lacA} (EAL73) to assay co-localization with OmpR-YFP foci. The regulatory regions upstream of \textit{ompF} and \textit{ompC} have 4 and 3 OmpR binding sites, respectively. Each site is bound by an OmpR dimer.
**Figure 2.3 (continued).** OmpR-YFP localization at \textit{ompF}, \textit{ompC}, and \textit{lac}.

B) Images of OmpR-YFP (left), CFP-LacI (middle), and OmpR-YFP with the CFP-LacI local maxima from the middle image identified by red dots (right) for the three different strains described in (A). LacI-CFP was expressed from pEB96. Scale bar represents 2 \( \mu \text{m} \).
Figure 2.3 (continued). OmpR-YFP localization at *ompF*, *ompC*, and *lac*.

C) Co-localization of chromosomal loci with the brightest OmpR-YFP spot in each cell. Histograms of distances between the brightest OmpR-YFP spot and the closest labeled chromosomal location (CFP-LacI) in strains with labels at *ompF*, *ompC* or *lacI-lacA*. The drift of the *ompF* locus (CFP-LacI) over two seconds is also shown for comparison (see text for discussion). Based on the drift distribution, co-localization was defined to be a YFP-CFP distance of less than 2.25 pixels. Inset: Percentage of cells where OmpR-YFP is counted as co-localizing with a given gene locus. Each distribution represents the means of two independent experiments, and the bars denote the range. For each experiment, the distribution was determined from at least 130 cells. Strains and growth conditions are as in (B). 1 pixel = 80 nm.
Figure 2.4.

A) Constructs integrated in the chromosome at *ompF* and *ompC* (EAL105) or *ompF* and *lac* (EAL85) to simultaneously image two loci. CFP-LacI and TetR-mCherry were expressed from pEL7.
Figure 2.4(continued)

B) Distribution of distances between chromosomal labels. Inset: chromosomal map positions of the three loci. The distributions give an \(\text{ompF-ompC}\) mean separation of 6.3 pixels \(\approx 0.5\ \mu\text{m}\) and an \(\text{ompF-lac}\) mean separation of 7.3 pixels \(\approx 0.6\ \mu\text{m}\). In addition, approximately 13% of the distances between \(\text{ompF}\) and \(\text{ompC}\) and 10% of the distances between \(\text{ompF}\) and \(\text{lac}\) fall within 2.25 pixels (the cutoff distance used to score for co-localization—see text for details).
Figure 2.4(continued)

C) Effect of deleting the OmpR binding sites at \(ompF\) (F1-F4) on the localization of the brightest OmpR-YFP foci. Histograms of distances between the brightest OmpR-YFP spot and the closest \(ompF\) chromosomal location (labeled with TetR-mCherry) in strains +/- OmpR binding sites F1-F4. The percentages of OmpR-YFP spots co-localizing (out to 2.25 pixels) with the nearest chromosomal locus are approximately 60% and 4% for the strains with and without sites F1-F4, respectively. Strains are EAL105 (F1-F4 intact) and EAL111 (F1-F4 deletion). CFP-LacI and TetR-mCherry were expressed from pEL7. To confine the analysis to the \(ompF\) locus, we excluded cells in which \(ompF\) and \(ompC\) (labeled with CFP-LacI) fell within 3.5 pixels of each other. Cells were further restricted to those with distinct foci, as described in materials and methods. This resulted in 136 distances from 182 cells of EAL105 and 73 distances from 196 cells of EAL111.
Figure 2.5. Quantification of OmpR-YFP Fluorescence at Chromosomal Locations

OmpR-YFP fluorescence in the neighborhoods of \textit{ompF} and \textit{ompC}, in the presence or absence of OmpR binding sites. The local YFP fluorescence was determined from a Gaussian fit in the neighborhood of either \textit{ompF} (gray) or \textit{ompC} (white), as described in Materials and Methods. The data represents the mean and range of two independent experiments. Each mean value was computed from at least 130 measurements. Strains are, from left to right, EAL105, EAL111, EAL112, EAL113. CFP-LacI and TetR-mCherry were expressed from pEL7.
Figure 2.6. Effects of EnvZ/OmpR stimulation on OmpR-YFP fluorescence at \textit{ompF} and \textit{ompC}.

A) Effect of procaine on \textit{ompF} and \textit{ompC} transcription measured in the \textit{ompF-yfp} and \textit{ompC-cfp} reporter strain EPB273a.

B) Local OmpR-YFP fluorescence in the neighborhood of \textit{ompF} (left) or \textit{ompC} (right) +/- 10mM procaine. OmpR-YFP fluorescence was determined as in Fig. 5. The data represents the means and ranges of two experiments. Strains are EAL105, EAL111 (F1-F4 deletion), EAL113 (C1-C3 deletion).
**Figure 2.7:** Model of Hierarchical OmpR-P Binding at *ompF* and *ompC*.

The regulatory region for *ompF* has a mixture of high affinity and low affinity OmpR binding sites; *ompC* only has low affinity sites. A sketch of the resulting occupancy of the *ompF* and *ompC* promoters as a function of OmpR-P is shown on the right. The dashed lines represent OmpR-P occupancy consistent with our observations for growth +/- 10 mM procaine.
Figure 2.8

Effects of carbon source, osmolarity, and procaine on OmpR-YFP localization at *ompC*.  

A) Effects of osmolarity (10% and 15% sucrose) and procaine (10 mM) on *ompC-cfp* expression in EPB273a. Cultures were grown in minimal A medium supplemented with 0.2% glucose (left) or 0.2% glycerol (right).

B) Distributions of peak OmpR-YFP fluorescence (as shown averaged in Fig. 4C) measured in the neighborhood of *ompC* loci for cells (EAL111) growing in minimal A medium supplemented with 0.2% glucose (left) or 0.2% glycerol (right). The data (black) for cells growing in medium without procaine or sucrose is shown in the upper and lower panels for comparison.
Table 2.1. Strains and plasmids used in this study.

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2.6 References

Chapter 3: Membrane Protein Expression Triggers Chromosomal Locus Repositioning

3.1 Introduction

Studies of several different bacteria have revealed that their chromosomes are organized structures, with genetic loci occupying relatively defined positions along the long axis of the cell (1-6). However, the potential impact of gene function and expression state on chromosome organization and on subcellular positioning of specific loci remains relatively unexplored. Such modulation of the spatial organization of the chromosome may affect numerous cellular processes, including the regulation of gene expression, gross transcriptional control, assembly of macromolecular complexes and domains, and the generation of cellular asymmetry (7-13).

One long standing hypothesis posits that genes actively expressing membrane proteins are localized to regions proximal to the membrane (7, 10, 14). To date, however, there is no direct evidence for specific locus repositioning resulting from membrane protein expression. We therefore sought to test directly whether expression of a membrane protein affects the cellular localization of its encoding gene in Escherichia coli. For the two loci that we tested, we show that induction of membrane protein expression rapidly results in a dramatic repositioning towards the membrane. This shift is a significant perturbation on the scale of the cell, and may therefore be a major determinant of chromosome conformation.
3.2 Results

We first tested the effect of inducing the lac operon on the intracellular position of the chromosomal lac locus. The operon lacZYA encodes the membrane protein lactose permease (LacY), in addition to two cytoplasmic proteins, beta-galactosidase (LacZ) and galactoside acetyltransferase (LacA). To follow the intracellular position of lacZYA, we inserted an array of Tet repressor binding sites (tetO) in the gene cynX, which is adjacent to lacA and approximately 2 kb from lacY. TetR-YFP binding to this array produces foci that are easily identified by fluorescence microscopy (15, 16). Cell membranes were labeled with the fluorescent dye FM4-64, and the distances of chromosomal loci to the membrane (peak FM4-64 fluorescence) were determined (Fig. 3.1A). Since each two-dimensional fluorescence image is a projection of a three dimensional cell, we refer to the measured distances as projected distances. For the approximately cylindrical geometry of the E. coli cell, chromosomal loci that fall on average closer to the membrane will have distributions of projected distances, measured from fluorescence images of a cell population that are relatively enhanced at the membrane. Therefore, a decrease in the distance between a chromosomal locus and the membrane across a population of cells will appear as a shift of the distribution of projected distances towards the membrane.

We found that induction of LacY expression produced such a shift. In a population of cells growing in the absence of inducer, the position of the native lac locus showed a distribution that was biased towards midcell, away from the cell membrane (Figs. 3.1 B,C). Induction of the lac operon shifted the distribution to smaller projected distances, indicating a shift of the lac locus towards the membrane. In contrast, for a
strain in which \textit{lacY} was replaced with a gene encoding a cytoplasmic protein (\textit{aadA}, encoding spectinomycin adenylyltransferase), there was no significant difference in the distribution of \textit{lac}-membrane projected distances for cells growing in the presence or absence of inducer (Fig. 3.S1). IPTG induction of this mutated \textit{lac} operon resulted in levels of beta-galactosidase that were in the same range as those expressed from the wild-type strain (Fig. 3.S2). To compensate for cell-to-cell variation in cell widths in each sample population, we also normalized the measured distances by cell radius (one half of the peak to peak FM4-64 distance – see Methods). Distributions with this normalized distance similarly demonstrate a LacY-expression-dependent shift of the locus towards the membrane (Fig. 3.1B), whereas the three cases in which a membrane protein is not produced, or produced only at very low levels, showed remarkably similar spatial distributions (Fig. 3.1D).

To test a second membrane protein, we used the tetracycline efflux pump, TetA, derived from the transposon Tn10 (17). A DNA segment encoding the tetracycline inducible repressor TetR and a fusion of TetA to the fluorescent protein mCherry (\textit{tetR tetA-mcherry}) was inserted in the chromosome at the phage lambda attachment site (\textit{attB}). An array of LacI binding sites (\textit{lac} operators) was integrated just downstream of \textit{tetA-mcherry} and labeled with LacI-YFP (16, 18). Full induction of TetA-mCherry expression with anhydrotetracycline (aTc) produced a significant shift in the distribution of \textit{tet}-membrane projected distances, indicating repositioning of the \textit{tet} locus towards the membrane (Figs. 3.2A, 3.S3A). In contrast, in a strain where the \textit{tetA-mcherry} coding sequence was replaced with \textit{mcherry} (encoding a cytoplasmic protein), no such shift was
observed (Fig. 3.2B, 3.S3B). We note that TetA-mCherry and mCherry expression levels for the two systems were in the same range (Fig. 3.S4). We also found that induction of TetA-mCherry did not have a significant effect on the localization of the lac locus, which is roughly 440 kb from attB (Fig. 3.2C), indicating that TetA-mCherry induction does not cause repositioning throughout the entire chromosome.

TetA expression level can be continuously tuned by varying the amount of tetracycline in the growth medium (19). To compare the extent of chromosomal localization near the membrane for different levels of TetA-mCherry expression, we measured the fraction of tet loci that were within a distance of 0.3R from the FM4-64-labeled membrane in fluorescence images, where R is the cell radius. We observed that membrane localization increased in a graded fashion with increasing TetA-mCherry expression, reaching a maximum of approximately 0.3 at full protein induction (100 ng/mL aTc) (Fig. 3.3A). Note that if a locus were localized to the membrane of a cylindrical cell, the locus would appear within 0.3R of the membrane in approximately 50% of the two-dimensional fluorescence images. Hence, the maximal value for this measure of membrane localization (for a cylindrical shape) is 0.5.

We also sought to characterize the time scale over which the tet locus repositions towards the membrane following maximal induction. The process was remarkably rapid; a significant change was detectable at the first measurement interval, spanning 1 to 3 minutes following the addition of aTc (Fig. 3.3B). This behavior is consistent with the rapid onset of TetA-mCherry protein expression, which by two minutes post induction
shows detectable protein expression in roughly 30 percent of the population, and has reached a maximal rate of expression by four minutes (Fig. 3.S5).

To explore the roles of transcription and translation in locus repositioning, we examined the effects of specific antibiotics. Treatment with the transcription inhibitor rifampicin abrogated repositioning of the \textit{tetA} locus in response to induction with aTc (Fig. 3.4A). Treatment with the translation inhibitor kasugamycin similarly prevented localization to the membrane (Figure 3.4B). Although there was a small level of repositioning in response to aTc, the same behavior was observed when the cytoplasmic protein mCherry was expressed, indicating this behavior is not specific to membrane proteins and presumably reflects some other effect of aTc on kasugamycin-treated cells. These results indicate that the chromosomal repositioning described here requires both transcription and translation.

### 3.3 Discussion

It has been hypothesized that for membrane proteins, transcription, translation, and insertion into the membrane are concurrent (termed transertion), and would therefore lead to membrane localization of the encoding genes (7, 10, 14) (Fig. 3.5A). To our knowledge, transertion has never been demonstrated, however our results are consistent with such a mechanism. Recently it was shown that mRNA encoding the membrane protein BglF localizes to the membrane in \textit{E. coli} independently of translation, indicating that the membrane targeting information is encoded directly in the \textit{bglF} mRNA (20).
Our observation that repositioning of the *tet* locus requires translation suggests that such a mechanism alone cannot account for the chromosomal repositioning that we observe here, although it may play a role. We also note that our results do not necessarily imply a direct physical association between the chromosomal loci and the membrane. For example, it is possible that loci reposition to the surface of the bacterial nucleoid, which would have the effect of moving these loci closer to the membrane. However, based on our results, the mechanism for this repositioning would have to be specific to loci encoding membrane proteins.

It has been argued that most mRNA species in the bacterial cell are likely to be mature transcripts that are detached from RNA polymerase (21). However, a single nascent transcript that is physically associated with its encoding gene could in principle be sufficient to reposition a local region of the chromosome. Our observation that the extent of localization near the membrane increases continuously with increasing protein expression level does not contradict this view, especially if one takes into account that transcription can occur in bursts (22, 23). If the frequency of such bursts increases with protein expression level, and if the chromosomal locus is most likely to occupy positions proximal to the membrane only during transcription, then this would be expected to give the behavior observed in Fig. 3.3A. The rapid increase in localization near the membrane following addition of saturating concentrations of inducer (Fig. 3.3B) is consistent with this model as well. We also note that the observed chromosome repositioning does not require an active process for movement towards the membrane, and could instead be mediated by a process of diffusion. For example, the requirement that a transcribing
DNA locus move a distance of order the radius of the cell (~400 nm) in two minutes is within the range of apparent diffusion constants measured for mRNA and DNA in *E. coli* (21, 24-26).

*E. coli* K-12 has roughly 1000 genes spread throughout the chromosome that are predicted to encode inner membrane proteins (Fig. 3.5B), and it has been argued that a significant fraction are expressed under standard laboratory culture conditions (27). Based on the above results, and as previously hypothesized (6, 7, 10, 28), membrane protein expression across the entire genome is likely to play a key role in shaping chromosome conformation. Our results further suggest that repositioning at any given locus is likely to be transient, occurring concomitantly with bursts of transcription. The resulting movement towards and away from the membrane at points distributed around the chromosome may be an important mechanism for maintaining the nucleoid in a sufficiently dynamic state to ensure accessibility to regulatory proteins, ribosomes, and RNA polymerase. Many other effects of chromosome-membrane associations have been proposed (7-11, 14, 29-31), but will require further experiments to determine whether membrane protein expression plays a direct role.
3.4 References


3.5 Figures

Figure 3.1.

LacY expression triggers chromosomal repositioning at the native locus.

(A) Measurement of distances between chromosomal loci and the membrane. Left: Sample false color composite image of an E. coli cell labeled with FM4-64 (Red) and YFP (green). Right: The intensity profile along the dashed line for the two fluorescence channels. Distances were measured between the peak YFP and nearest peak FM4-64 fluorescence as described in the Methods. All such distances reflect projections onto the plane of focus and are therefore referred to as projected distances. 1 pixel = 80 nm. Scale bar = 1 µm.
Figure 3.1 (continued): LacY expression triggers chromosomal repositioning at the native locus.

B) Distribution of projected distances of the lac locus to the membrane across a population of cells growing in the absence or presence of inducer (2 mM IPTG) for wild-type lac. The upper distribution shows distances measured in nanometers; the lower distribution represents the same data but with each distance normalized by the cell’s radius (cell width/2—see Methods). Each distribution is comprised of measurements of at least 300 loci.
Figure 3.1 (continued): LacY expression triggers chromosomal repositioning at the native locus.

(C) Sample images of induced cells from (B). Fluorescence profiles from line scans and the corresponding projected distance to the membrane are also indicated. Note that for the growth conditions used here, cells had on average two copies of the chromosomal region containing the lac locus.
Figure 3.1 (continued): LacY expression triggers chromosomal repositioning at the native locus.

(D) Distribution of projected distances of the lac locus to the membrane across a population of cells growing in the absence or presence of inducer (2 mM IPTG) for a mutated lac operon with the coding sequence of lacY replaced with the corresponding sequence of aadA, which encodes a cytoplasmic protein. For comparison, (D) also includes the data from (B) for lacY+ cells without inducer. Each distribution is comprised of measurements of at least 250 loci.
Figure 3.2: Chromosomal repositioning from TetA expression.

Distributions of projected distances of the *tet* locus to the membrane across a population of cells growing in the presence or absence of inducer (100 ng/ml anhydrotetracycline - aTc), for (A) *tetA-mcherry* and (B) *mcherry*. The plot in (B) also includes data from (A), *tetA-mcherry* without inducer, for comparison.
Figure 3.2 (continued): Chromosomal repositioning from TetA expression.

(C) Effect of tetA-mcherry induction on the distribution of projected distances of the lac locus (~440 kb away tet) to the membrane. Note that for all measurements, fluorescence from TetA-mCherry and mCherry was negligible compared with FM4-64 fluorescence. Each distribution is comprised of measurements of at least 350 loci.
Figure 3.3: Dose-dependence and kinetics of chromosomal repositioning.

The fraction of loci proximal to the membrane is defined to be the fraction of loci that are within 0.3R of the membrane (R = cell radius). (A) Steady-state membrane localization as a function of TetA-mCherry expression, at various levels of induction (from left to right: 0, 0.5, 1, 10 µg/ml tetracycline and 100 ng/ml aTc).
Figure 3.3 (continued): Dose-dependence and kinetics of chromosomal repositioning.

(B) Kinetics of locus repositioning following addition of aTc to 100 ng/ml. Points and vertical bars denote the mean and range of two measurements from at least 150 loci each. The horizontal bars in (A) also denote the range of two measurements. The horizontal bars in (B) denote the time interval of each measurement.
Figure 3.4: Transcription and translation inhibitors block chromosomal repositioning.

(A) Effect of the transcription inhibitor rifampicin on the localization of the tetA locus. The fraction of tetA loci proximal to the membrane (within 0.3R of the membrane) are shown for:
- (untreated)-no inducer or antibiotics;
- (rif)-no inducer, treated with rif;
- (aTc)- growth in aTc;
- (rif + aTc)- aTc added immediately after addition of rif.
Figure 3.4 (continued): Transcription and translation inhibitors block chromosomal repositioning.

(B) Effect of the translation inhibitor kasugamycin on the localization of the tet locus. The fraction of tet loci (expressing TetA-mCherry or mCherry) proximal to the membrane are shown for cells treated with kasugamycin and/or aTc as indicated.

Data shown in (A) and (B) are the means and ranges of two measurements, comprised of at least 95 loci.
Figure 3.5: The “Transertion” Model and Distribution of Predicted Membrane Proteins throughout the Chromosome.

A) Schematic of the co-transcriptional, co-translational insertion (“transertion”) of a membrane protein resulting in an effective association between the encoding chromosomal locus and the membrane.

B) Number of predicted membrane proteins found throughout the E. coli chromosome as a function of map position. An unknown number of these are candidates for co-translational insertion.
Figure 3.S1. Distribution of projected distances of the mutated lac locus to the membrane across a population of cells growing in the absence or presence of inducer (2 mM IPTG). The distributions show distances measured in nanometers, and reflects the same data sets shown normalized by cell width in Fig. 3.1D.
**Figure 3.S2.** Beta-galactosidase assay comparing LacZ activity for *lacY* and *aadA* strains with or without inducer (2 mM IPTG). Means and standard deviations were computed from three replicates. Strains were grown as in Figure 3.2, but without the addition of FM4-64.
Figure 3.S3: Distribution of projected distances (in nanometers) of the *tet* locus to the membrane across a population of cells growing in the absence or presence of inducer (100 ng/ml aTc). The distributions reflect the same data sets shown normalized by cell width in Fig. 3.2A,B.
Figure 3.S4. Average mCherry cellular fluorescence for tetA-mcherry⁺ and mcherry⁺ strains growing with or without 100 ng/ml aTc. Strains were grown as in Figure 3.3, but without the addition of FM4-64. Each average was computed from at least 300 cells. Error bars represent the range of means across two independent experiments.
Figure 3.S5. Average TetA-mCherry cellular fluorescence as a function of time post induction with 100 ng/ml aTc. Cells were grown as in Figure 3.4B, but without the addition of FM4-64. Points and bars represent the means and ranges of two experiments, respectively.
3.6 Materials and Methods

See Table 3.S1 for a list of strains and plasmids used in this study.

Media and Growth Conditions

*E. coli* strains were grown at 37°C in minimal A medium (1) supplemented with either 0.2% glucose (for *tet* induction experiments) or 0.2% glycerol + 0.1% Casamino acids (for *lac* induction experiments). For maintenance of plasmids or selection for chromosomal markers, antibiotics were used at the following concentrations: 50 µg/ml ampicillin, 20 µg/ml chloramphenicol, 20 µg/ml kanamycin, 100 µg/ml spectinomycin. For cells growing in Minimal A glucose medium, 10 mM arabinose was used to induce LacI-YFP expression (from pEL12), as needed. Strains containing *lacO* arrays and harboring pEL12 were always maintained in the presence of 1 mM IPTG to avoid replication fork stalling (2, 3). For MSS81 derivatives growing in Minimal A glycerol medium, TetR-YFP expression was induced with 5 mM arabinose. We found that it was not necessary to add anhydrotetacycline to strains containing *tet* operator arrays in MSS81-derived strains. This was likely due to the lower expression of TetR-YFP and the relatively small number of *tetO* sites that we observed in these strains.

Tetracycline (Sigma-Aldrich) and anhydrotetracycline (Acros Organics) were used at the concentrations indicated. For all experiments, cultures were grown to saturation overnight and then diluted at least 1:500 into fresh medium. For steady state experiments, inducers were added when the cultures were diluted (tetracyclines) or at least 4 hours prior to measurement (IPTG). Arabinose was added 2 to 3 hours prior to measurement. To stain cell membranes, FM4-64
(Invitrogen) was added to 2 µM one hour prior to measurement. Relative TetA-mCherry expression level (Figures 3.4A, 3.S2) was determined from mCherry fluorescence of cells grown under the same conditions but without the FM4-64 membrane stain, which would otherwise dominate the fluorescence signal.

For the kinetics of chromosomal repositioning (Figure 3.4B) samples were grown as for the steady state experiments except aTc was added prior to measurement at the times indicated. (Inductions were staggered across samples so all samples were measured at comparable ODs.) Samples were measured over the time interval post-induction indicated by the horizontal bars in Figure 3.4B.

For the kinetics of protein induction (Figure 3.S3), samples were grown as in Fig. 3.4B, except that the FM4-64 was not added. Upon reaching the appropriate OD, each culture was induced with 100 ng/ml aTc. At the times post induction indicated, 20 µl of the culture were removed and rapidly cooled in an ice slurry. To allow time for mCherry folding, the samples were kept on ice overnight before measurement of cellular fluorescence.

For the effects of rifampicin and kasugamycin treatment (Figures 3.S4 and 3.S5), samples were grown as in Figure 3.3, except that rifampicin was added to 250 µg /ml or kasugamycin to 10 mg/ml prior to measurement. Locations were measured over a ~10 minute period beginning approximately 10 minutes post antibiotic treatment. For the experiment labeled (rif + aTc), aTc was added within 30 seconds after the addition of rif. For the experiment labeled (ksg + aTc), cells were pretreated with kasugamycin for 9 minutes, followed by the addition of aTc.
Microscopy

Microscopy was performed on live cells at 37 °C, essentially as described in (4) for all experiments except for measurements of mCherry fluorescence for Figure 3.S3 (see above), which was performed at room temperature. Fluorescence images in YFP and FM4-64 channels were aligned in software using images of 140 nm SPHERO Multi-Fluorophore Particles (Spherotech, Inc.)

Image Analysis

Locations of LacI-YFP and TetR-YFP foci were determined by the same general method as in (4), however image sizes were increased by a factor of four using a cubic spline interpolation. In these 4x interpolated images, chromosomal locations were calculated as the center of mass of 19x19 pixel arrays centered on well-isolated maxima (separated by at least 19 pixels). Once a location was identified, the closest maximum in the FM4-64 image was determined. The distance from the chromosomal location to this point and to the adjoining two pixels on either side in the FM4-64 rim stain contour were then averaged. This quantity was taken to be the distance between a chromosomal location and the membrane. Cell width was calculated from two lines that were perpendicular to the major axis of the cell and positioned at the 1/4 and 3/4 points along the major axis. The distance between the two maxima in the FM4-64 intensity profile along each of these lines was computed and the resulting two distances were averaged together and divided by two to approximate the cell radius.
Strains used for Data in Figures

(See Table 3.S1 for all strains and plasmids.)

**Figure 3.1:** EAL173 (lacY'), and EAL183 (aadA').

**Figure 3.2:** EAL179/pEL12 (tetA-mcherry'), EAL210/pEL12 (mcherry'), and EAL214/pEL12 (aadA', tetA-mcherry').

**Figure 3.4:** EAL179/pEL12 (tetA-mcherry').

**Figure 3.S1:** EAL173 (lacY'), and EAL183 (aadA').

**Figure 3.S2:** EAL179/pEL12 (tetA-mcherry') and EAL210/pEL12 (mcherry')

**Figure 3.S3:** EAL179/pEL12 (tetA-mcherry')

**Figure 3.S4:** EAL179/pEL12 (tetA-mcherry')

**Figure 3.S5:** EAL179/pEL12 (tetA-mcherry') and EAL210/pEL12 (mcherry')

*Construction of EAL173*

A DNA segment consisting of ΔcynX::(FRT-kan-FRT) and surrounding region in the Keio knockout strain JW0332(5) was amplified with the primers 5'-ATATCTGCCGACCAAACC-3' and 5'-GATCTACATTAGCCGCATCC-3' and electroporated into MG1655/pKD46 as in (6). (Note that since JW0332 has transcriptional terminators inserted at the end of lacZ, which is tightly linked to cynX, we used lambda-Red mediated recombination rather than P1 transduction.) The resulting kanamycin resistant strain was verified to have an insertion in cynX by PCR using primers outside of the original amplified region, and also verified to be ampicillin sensitive. The kanamycin resistance gene was then removed by transforming the strain with pEL8 and growing on LB with 50 µg/ml ampicillin at 30°C degrees. The plasmid
pEL5 was then integrated into the chromosome, resulting in EAL170. The chromosomally integrated pEL5 was moved from EAL170 into MSS81 by P1 transduction and selecting for chloramphenicol resistance. This strain was named EAL173.

Construction of EAL183

The gene lacY, from the start codon to the stop codon, was replaced with the corresponding region of aadA by lambda-Red mediated recombination. The aadA was amplified from pRSM2832 with the primers 5’-

AATAACCGGGCAGGCCATGTCTGCCCGTATTTCGCGTAAGGAAATCCATTGTGAGGA
GGATATATTTGA
-3’ and 5’-

GTTGGTCGGATAAGCGTGCGCCGCATCCGACATTGATTGCTTATAATTTT TTTAATCT
TGTTATTTAAAATAG
-3’ (the underlined sequences denote homology to aadA). The PCR product was transformed into EAL174/pKD46 as described in (6) with selection on LB containing 100 µg/ml spectinomycin and 1 mM IPTG, resulting in EAL181. The region containing \( \Delta(\text{lacY})::\text{aadA} \ \Delta(\text{cynX})::\text{pEL5} \) was moved into MSS81 by P1 transduction, creating EAL183.

Construction of EAL179

DGC2 contains the tetR tetA genes from the transposon Tn10 integrated at the phage lambda attachment site in MG1655. The strain also contains a translational fusion of mcherry to the 3’ end of tetA, a cat chloramphenicol resistance gene adjacent to tetR, and a FRT-kan-FRT cassette downstream of mcherry. The kan gene was removed by transforming DGC2 with pCP20 and selecting on ampicillin at 30°C. An array of lacO \( ([\text{lacO}]_n) \) was then integrated at the
residual FRT. The resulting kanamycin resistant strain was named EAL66. The region containing cat tetR tetA-mcherry FRT::pSE1 was then moved to EPB255 by P1 transduction, creating EAL179.

**Construction of EAL210**

To construct a strain expressing mcherry in place of tetA-mcherry in EAL66, the tetA gene was deleted by lambda-Red mediated recombination. The primers: 5’- 
AATTCCTAATTTTGTGACACTCTATCATGATAGGGATTATTTTACCACCCTAGAATT
AAAGAGGAGAAATTAAGC-
3’ and 5’-
GAACTCCTTGATGATGGCCATGTTATCCTCCTCGGCCCTTGCTACCATGGCTTAATT
CCTCTTTAATTCTAGG- 3’ were annealed to each other and amplified by PCR. The resulting 125 bp product was electroporated into EAL66/pKD46 as in (6) and cells were selected for resistance to fusaric acid as in (7). The resulting strain was verified by sequencing the P_{tetA-} mcherry region in the chromosome and verifying that mCherry fluorescence was localized to the cytoplasm. The region containing cat tetR P_{tetA-mcherry} FRT::SE1 was then moved into EPB255 by P1 transduction. When the resulting strain was transformed with pEL12, we were unable to detect fluorescent foci from LacI-YFP binding to the lacO array, which likely reflects the loss of most of the operator array in the course of the above manipulations. The integrated copy of pSE1 was therefore replaced by transforming the strain with pEL8, isolating a kanamycin sensitive colony, and retransforming with pSE1, creating EAL210.

**Construction of EAL214**

EAL181 was transformed with pEL8 to remove pEL5 and pSE1 was then integrated at the residual FRT site in cynX, creating EAL211. DGC2 was transformed with pCP20 to remove
the *kan* resistance gene and the sequence containing *cat tetR tetA-mcherry* FRT was then moved to EAL211 by P1 transduction, resulting in EAL214.

**Construction of pEL12**

The plasmid pLAU53 contains $P_{\text{ara}}$( *lacI-cfp tetR-yfp*) with two XhoI sites: one at the *lacI-cfp* fusion, and one at the *tetR-yfp* fusion (8). To create a *lacI-yfp* fusion, pLAU53 was cut with XhoI, to remove the *cfp* and *tetR*. The remaining ~6.3 kb fragment was self-ligated, and transformed into Top10 (Invitrogen) and selected on LB supplemented with 50 µg/ml ampicillin and 1 mM arabinose. The transformation plate was screened for colonies that were CFP- and YFP+, producing pEL12.
<table>
<thead>
<tr>
<th>Strain or Plasmid</th>
<th>Relevant Genotype</th>
<th>Source or Reference (Construction Summary)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG1655</td>
<td>E. coli</td>
<td>E. coli Genetic Stock Center, CGSC no. 7740</td>
</tr>
<tr>
<td>EAL66</td>
<td>MG1655 attL::[cat tetR tetA-mcherry FRT::pSE1]</td>
<td>This study.</td>
</tr>
<tr>
<td>EAL170</td>
<td>MG1655 Δ(cynX)::pEL5</td>
<td>This study.</td>
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<tr>
<td>EAL173</td>
<td>MSS81 Δ(cynX)::pEL5</td>
<td>This study. (P1: EAL170 x MSS81)</td>
</tr>
<tr>
<td>EAL174</td>
<td>MG1655 Δ(cynX)::pEL5</td>
<td>This study. (P1: EAL170 x MG1655)</td>
</tr>
<tr>
<td>EAL179</td>
<td>MG1655 Δ(lacI-lacA) attL::[cat tetR tetA-mcherry FRT::pSE1]</td>
<td>This study. (P1: EAL66 x EPB255)</td>
</tr>
<tr>
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<td>MG1655 Δ(lacY)::aadA Δ(cynX)::pEL5</td>
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</tr>
<tr>
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<td>MSS81 Δ(lacY)::aadA Δ(cynX)::pEL5</td>
<td>This study. (P1: EAL181 x MSS81)</td>
</tr>
<tr>
<td>EAL210</td>
<td>MG1655 Δ(lacI-lacA) attL::[tetR P_{tetA-mcherry} FRT::pSE1]</td>
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<tr>
<td>EAL211</td>
<td>MG1655 ΔcynX::pSE1 Δ(lacY)::aadA</td>
<td>This study.</td>
</tr>
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<td>EAL214</td>
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</tr>
<tr>
<td>Strain</td>
<td>Description</td>
<td>Reference</td>
</tr>
<tr>
<td>----------</td>
<td>-------------------------------------------------------------------------------------------------------</td>
<td>----------------------------------------------</td>
</tr>
<tr>
<td>EPB255</td>
<td>MG1655 Δ(lacl-lacA)</td>
<td>E. Batchelor and M. Goulian, unpublished.</td>
</tr>
<tr>
<td>JW0332</td>
<td>BW25113 Δ(cynX)::kan</td>
<td>(5)</td>
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<tr>
<td>MSS81</td>
<td>MG1655 Δ(lacl-lacA) attλ([P ara-(lacI-cfp tetR-yfp) Δcat::FRT])</td>
<td>M. Shah, E. Libby, and M. Goulian, unpublished.</td>
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<tr>
<td></td>
<td>The P ara-(lacI-cfp tetR-yfp) is derived from pLAU53</td>
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### Plasmids

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<tr>
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<th>Description</th>
<th>Source</th>
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<tr>
<td>pCP20</td>
<td>λplc-FLP λcl857 Rep + bla cat</td>
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</tr>
<tr>
<td>pEL5</td>
<td>oriR6Kγ[tetO2]n FRT cat</td>
<td>(4)</td>
</tr>
<tr>
<td>pEL8</td>
<td>λplc-FLP λcl857 Rep + bla</td>
<td>(4)</td>
</tr>
<tr>
<td>pEL12</td>
<td>pBAD24 P ara-[lacI-eyfp]</td>
<td>This study</td>
</tr>
<tr>
<td>pKD46</td>
<td></td>
<td>(6)</td>
</tr>
<tr>
<td>pLAU53</td>
<td>pBAD24 P ara-(lacI-cfp tetR-yfp)</td>
<td>(8)</td>
</tr>
<tr>
<td>pRSM2832</td>
<td>FRT-aadA-FRT</td>
<td>(10)</td>
</tr>
<tr>
<td>pRSETb- mcherry</td>
<td>mcherry</td>
<td>R. Tsien</td>
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<tr>
<td>pSE1</td>
<td>oriR6Kγ kan FRT [lacO]n</td>
<td>(4)</td>
</tr>
</tbody>
</table>
Methods References:


Chapter 4: Special Experimental Considerations

This section contains notes on experimental details and procedures that have been crucial to making the successful measurements described here. It is important to note that these are simply procedures and algorithms that worked (i.e. worked in the context of the controls!) and not unique methods. After all, when we developed many of these procedures, there were no rules, just a lot of trial and error.

4.1 Dual chromosomal labeling with tetO and lacO arrays

In Chapter 2 we describe the use and creation of tetO and lacO arrays that can be simply integrated at any non-essential position in the E. coli chromosome. These tetO and lacO arrays are carried on conditionally replicative plasmids pEL5 (tetO cat FRT) and pSE1 (lacO kan FRT) which recombine into the chromosome at the location of a chromosomal FRT scar in the presence of FLP recombinase (1). This section expands on the experimental methods outlined therein.

The FRT scar is two 13 bp recognition sites for the FLP recombinase separated by a 6 bp spacer (2). As also described in this reference, the sequence of this spacer determines the orientation of the FRT recombination event – parallel or antiparallel. While this is relevant for designing a PCR for verification of pEL5 and pSE1 recombination events into the chromosome, it should not have an impact on the functionality of the array insertion itself for chromosomal labeling purposes.
Integration of the plasmids into the chromosome at the location of a FRT scar can be robustly accomplished (i.e. done with high probability of success) if the following experimental guidelines are followed:

Reasonably high yield minipreps of pSE1, pEL5, and pEL8 are required. pEL8, which is a chloramphenicol sensitive version of pCP20, tends to give particularly low yield minipreps. For all of the plasmids, I therefore recommend growing 4 ml of LB culture for at least 18 hours in the presence of the appropriate antibiotic and at the appropriate temperature (pEL8 amp-50 at 30°C; pSE1 kan-25 at 37°C; pEL5 cam-20 at 37°C). Minipreps of plasmid DNA are done by applying all 4 ml to a single column, and eluting in 50 ul 1:10 EB. For particularly good yield, I also recommend pre-warming the EB for optimal elution of larger plasmids, eluting 2x with 25 ul 1:10 EB, and using a microcentrifuge instead of a vacuum manifold. If the 3-5 ul of the plasmid preparations are run out on a gel, they should produce easily visible bands.

There are two common starting situations for insertion of the arrays. In the first case, the target location in the chromosome has a single FRT scar, in the second case there are two FRT scars separated by an antibiotic resistance marker. Both situations are the same fundamentally, however, it is always a good idea to test for loss of the FRT flanked antibiotic resistance marker after transformation with pEL8, just in case. All transformation steps outlined below should be performed with electro-competent bugs for optimal integration. Chemical transformations do work – most of the time - however, in our experience they tend to yield strains with smaller number of operator repeats. As life is short, I recommend the “Wash and Zap” which is simply 2-4 ml of SOB culture (with antibiotics as needed) grown to an OD$_{600}$~0.3-.5, placed on ice for at least 1 hour, and then spun down gently (~9 krpm or so) at 4°C and washed 2-3x with ~1 ml 10% ice cold glycerol. This generally yields 1-2 aliquots. If properly prepared, transformation with 1ul of a pBR322 based supercoiled plasmid should yield a lawn. At each step where multiple
candidates are suggested, it is not necessary to explicitly test each one unless there is a problem with the end product.

**Integration procedure**

1) Transform the target strain with pEL8 (or pCP20 if only using pSE1). Recover from the transformation at 30°C. Plate on LB amp-50 at 30°C. The resultant strain is TARGET/pEL8.

2) Restreak TARGET/pEL8 on LB amp-50 at 30°C. (2 candidates are sufficient.)

3) Grow TARGET/pEL8 for competent bugs at 30°C and transform with pEL5 or pSE1. Recover from the transformation at 37°C. Plate on LB cam-15 (pEL5) or LB kan-20 (pSE1) at 37°C. Note that you can use higher antibiotic concentrations in a pinch (cam-20 or kan-25) but I would not go any higher than that, as the efficiency suffers.

4) Restreak TARGET/pEL8 (?) + pEL5 or TARGET/pEL8(?) + pSE1 on the same selective media. (I recommend at least 4 candidates. Here the question marks are my notation indicating the probable, but as yet unverified, loss of the plasmid.)

5) Verification: restreak again using a single colony on the following: LB amp-50 at 30°C (testing for loss of pEL8) and LB at 37°C.

6) At this point there are two choices of approximately equal effort to look for successful operator arrays (the project generally dictates which is more efficient): you can either transform with a plasmid such as pEL7 (CFP-LacI, TetR-mCherry), or you can make a P1 lysate and P1 transduce into a strain with similar fluorescence constructs (MSS81, EPB255/pEL12 etc.) I recommend screening at least 4 candidate operator array insertions in the presence of the appropriate fluorescent protein fusion for bright fluorescent foci.
4.2 Testing and Using Integrants

Using product strains to visualize chromosomal loci is a highly reproducible process. Once established for a given growth condition, we have found that the induction timings of the LacI and TetR fluorescent fusions, as well as the need to pre-treat with aTc or IPTG to prevent replication fork stalling the procedure produces little day to day variability. It is therefore important to establish a procedure using the exact experimental procedure that will eventually be used – i.e. starting an overnight culture from a plate, and the precise dilution factor from the overnight culture into the experimental culture. That being said, strains can be preliminarily tested (i.e. for the presence of a sufficiently large operator array) by simply grabbing colonies off of plates and estimating induction parameters.

Generally, in growth media where the arabinose operon is repressed (e.g. in Minimal glucose or LB) induction of the fluorescent fusion should be done approximately 1-2 hours prior to visualizing samples. This involves the addition of saturating amounts of arabinose – a final concentration of 10-20mM. In LB, a supplementary addition of arabinose (to 20-40 mM) may be necessary within 30 minutes of measurement time. There also appears to be an optimal culture density for induction -slightly less than OD<sub>600</sub> ~ 0.1. For a 1:500 dilution from a saturated overnight culture, this will occur approximately 3 hours post dilution. (Growth in MinA/glucose + amp-50 µg/ml.) Similarly, if colonies are picked off a plate for testing, or grown in a different medium, induction around this OD will produce reasonable results.

It is also important to always maintain cells with long arrays in the presence of IPTG or aTc to prevent fork stalling, even when the LacI or TetR proteins are not induced. Failure to do so appears to preferentially select against the ability to eventually induce the binding proteins (no
fluorescence will be observed in a subset of the cells in culture) and select against the presence of the operator array (resulting in the observation of diffuse fluorescence). Initial indications of a problem may appear as ~10% of the cells producing long non-septating filaments. This is less of a problem with pEL7 (and N-terminal LacI fusions) in which the fluorescent protein interferes with the DNA binding domain of LacI. Rare (<1/100) filamentous cells appearing in a culture is normal and such cells are excluded from analysis.

It is very difficult to control the induction of the arabinose inducible LacI and TetR fusions under conditions where the arabinose operon is not under repression (i.e minimal glycerol medium). The resulting protein expression from even small additions of arabinose can easily cause toxicity and the foci due to the array to be overwhelmed. This is a particular issue when LacI or TetR fusions are expressed from plasmids. We have used two approaches with varied success under these conditions. The first approach is to induce with a small amount of arabinose (500 µM) immediately prior to measurement (i.e. induction and measurement must occur within 10 minutes.) The second approach is to add a small amount (~1 mM) of arabinose to the saturated overnight culture and allow an additional 20 minutes of growth before diluting the culture into media without arabinose. This method produces a large amount of protein which is then divided out as the cells divide.

4.3 Image collection and data quality considerations

One obstacle in precision multi-channel image collection is alignment between the fluorescence channels. While the slight misalignment between channels due to the difference in wavelengths used (e.g. the chromatic aberration caused by the difference between red (mCherry) ~ 610 nm emission and blue (CFP) ~ 475 nm emission), are generally attempted to be corrected
for by special design of the microscope objective, the following are much larger concerns for our setup: the relative alignment of the image on the CCD due to light passing through separate filter sets (essentially a separately aligned optical path), and any vibrations or movement between successive image acquisitions. While the first problem can produce systematic errors, it can be fixed in software, as discussed below, the second problem is more likely to produce random noise, and cannot be simply corrected for.

To check data sets for vibrations and movement, it is easy to construct image stacks in image analysis software (i.e. ImageJ) using the calculated shift between channels, and check for gross misalignment. Generally, if a given slide has an alignment problem due to sample movement, it occurs in the first few acquisitions, as the agarose pad may have been slightly stretched by the process of removing the coverslip and putting down the sample. Misalignments due to sample drift tend to be on the order of a hundred nanometers, and are readily apparent by eye. Most sample vibrations due to the building cannot be so easily avoided, but are generally damped by the air table, and produce smaller, non-systematic, problems.

The relative alignment of the channels is generally determined by examining a multispectral sample in multiple channels. If possible, this sample should be checked across the image to rule out significant differences in the shift across the field. We have found that the 140 nm SPHERO Multi-Fluorophore Particles (Spherotech, Inc.) were the best choice for alignments that required YFP fluorescence. As the particles have a distribution of sizes, and are not equally fluorescent in each fluorescent channel, exposure times were adjusted to produce a high contrast image in each channel. Images were then acquired sequentially in each fluorescence channel, repeating the first channel at the end of the acquisition to verify that the misalignment of channels is not due to sample drift or movement. If subpixel alignment is required, the images should be interpolated before attempting to determine the correct shift. The correct shift can then be
determined by taking line scans of several particles in each channel and attempting to match peak positions.
4.4 References
