MiniReview

Green fluorescent protein – a bright idea for the study of bacterial protein localization

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Abstract

Use of the green fluorescent protein (GFP) of Aequorea victoria as a reporter for protein and DNA localization has provided sensitive, new approaches for studying the organization of the bacterial cell, leading to new insights into diverse cellular processes. GFP has many characteristics that make it useful for localization studies in bacteria, primarily its ability to fluoresce when fused to target polypeptides without the addition of exogenously added substrates. As an alternative to immunofluorescence microscopy, the expression of gfp gene fusions has been used to probe the function of cellular components fundamental for DNA replication, translation, protein export, and signal transduction, that heretofore have been difficult to study in living cells. Moreover, protein and DNA localization can now be monitored in real time, revealing that several proteins important for cell division, development and sporulation are dynamically localized throughout the cell cycle. The use of additional GFP variants that permit the labeling of multiple components within the same cell, and the use of GFP for genetic screens, should continue to make this a valuable tool for addressing complex questions about the bacterial cell. ß 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Green fluorescent protein; Protein localization; Gene fusion; Hybrid protein; Fluorescence microscopy

1. Introduction

The use of lacZ gene fusions, encoding hybrid proteins composed of a target polypeptide fused to Escherichia coli β-galactosidase, has revolutionized our ability to study bacterial gene expression and protein export [1]. A similar impact is being made by application of green fluorescent protein (GFP) from Aequorea victoria to study macromolecular localization in living cells, including bacteria [2]. Many important questions about the subcellular organization of bacterial cells have been difficult to address due, in part, to their small size. GFP has, however, provided new experimental approaches to investigate the localization of both protein and DNA molecules in prokaryotes.

The traditional approach to study macromolecular localization in bacteria includes techniques of immunogold labeling and immunofluorescence microscopy (IFM). Although these methods have been effectively used to visualize the targeting of specific cellular components, their use can be limited by the requirement for antibodies directed against the target protein. Antibodies against proteins found in low abundance can be difficult to obtain, and the level of detection of this class of protein is relatively insensitive. They also require cell fixation that precludes investigation of living cells. Moreover, these techniques can be technically demanding and costly.

Gene fusions using gfp, on the other hand, can be constructed with relative ease (as discussed further below), and their use, in general, heightens the sensitivity of molecular detection. Since the GFP chromophore is formed by autocatalytic cyclization of three amino acids [3], and fluorescence is triggered by excitation with specific wavelength light, neither invasive sample preparation nor substrate addition is required, making it possible to study protein localization in living cells. In addition, since GFP is relatively small (238 amino acids) and can remain functional even when fused to a target protein, it allows protein localization to be monitored in real time.

As a result, studies using GFP have bolstered our understanding of a variety of cellular processes such as cell division, chromosome replication and partitioning,
sporulation, development and signal transduction, and have changed our view of the organization of the bacterial cell in general. The bacterial cell, once considered by analogy to be a ‘bag of enzymes’, is now known to accommodate the segregation and sequestration of a variety of molecules into specific subcellular regions. Surprisingly, many of the cellular components are dynamic in nature, assembling and disassembling into higher order structures, and migrating in a directed manner between distinct locations in the bacterial cell, revealing more similarities between prokaryotes and eukaryotes that had previously been imagined [4–6].

2. Creation and use of GFP fusions

Early studies in which wild-type GFP was expressed in *E. coli* revealed a number of problems that hindered its use as a reporter for protein localization. For example, formation of the GFP chromophore is slow, requiring up to 2 h post-synthesis to form [7]. Wild-type GFP also tends to form inactive inclusion bodies, especially at elevated temperatures. In addition, the fluorescent intensity of GFP is relatively low, and the protein has a major absorbance peak at 395 nm, a value that requires specialized filter sets for optimal excitation.

Fortunately, a number of variants of GFP have been isolated that overcome many of these limitations [7–11]. Cormack et al. [10] isolated a particularly valuable set of GFP variants by screening for mutants that showed increased brightness upon excitation at 488 nm. In addition to improved solubility in *E. coli*, an amino acid change within the chromophore resulted in a ‘red-shift’ in excitation from 395 to 488 nm [11,12]. These variants can be detected with good sensitivity by fluorescein isothiocyanate filter sets commonly found on fluorescence microscopes. Derivatives of these mutants are used almost exclusively in protein localization studies.

A number of vectors have been constructed to facilitate creation of gene fusions with different gfp variants. Many of these vectors have either been published [8,13,14], or are commercially available (e.g. Clontech, Palo Alto, CA, USA). A general strategy for constructing GFP fusions is to use PCR, or other cloning strategies [15] to engineer restriction enzyme sites into both gfp and the target gene to create in-frame fusions on recombinant plasmids. The gfp reporter may be fused to either the 5’ or 3’ end of the target gene, to generate either amino- or carboxy-terminal fusions, respectively. This choice is often made by predicting which region of the protein will most likely tolerate the addition of extra amino acids and still remain functional. Also, when fusing GFP to membrane proteins, GFP must be joined to a domain that is exposed to the cytoplasmic side of the inner membrane. Where necessary, linker sequences may also be added between the target protein and GFP to improve fluorescence output [16].

GFP is not without its limitations, however. For example, formation of the chromophore requires molecular oxygen, and all forms of GFP display reduced fluorescence at elevated growth temperatures [7]. Consequently, GFP cannot be used under all growth conditions under which microorganisms normally grow. Certain types of proteins also are not suitable for detection by GFP. For example, because of the relatively slow formation of the chromophore, even in the GFP variants, proteins with short half-lives may not be detectable. Also, proteins must still be present in sufficient levels since detection of only a few molecules of GFP is technically difficult, with results easily misinterpreted.

As with any fusion protein, caution must also be used when interpreting the results of protein localization studies since the addition of the GFP moiety can alter the function, or location of the target polypeptide. A genetic test for altered function is to determine if expression of a GFP fusion can complement a knockout mutation in the gene encoding the target protein. Fortunately, there are several examples, as discussed below, of proteins that remain fully functional, as determined by complementation studies, when fused to GFP. There are also instances where GFP does disrupt normal protein function without interfering with its localization. It is difficult to assess from the literature, however, the number of instances where GFP fusion proteins are not active, or have altered localization properties, and each case must be assessed empirically.

Foci of GFP fluorescence must also be carefully scrutinized to ensure that they are the result of proteins targeted to a specific subcellular location, and not merely caused by protein aggregation. Also, fluorescent intensity may be artificially skewed by elevated concentrations of GFP within small volumes of subcellular compartments [17].

Another complication that can arise is that synthesis of elevated levels of hybrid proteins can yield incorrect or ambiguous interpretations about protein localization. For example, overexpression of cell division proteins can themselves disrupt normal cell division. In addition, high-level protein expression can result in protein aggregation or mislocalization. One solution has been provided by Boyd et al. [18] who developed a convenient method to introduce plasmid-borne gfp constructs to the *E. coli* chromosome, thus allowing expression of the fusions at more physiological relevant levels.

Inconsistencies in results between laboratories using GFP fusions have also been reported (compare [19,20] and [21,22]). It is likely that differences in bacterial strains, growth conditions, growth rates, or fusion constructs may explain these discrepancies. In these cases the use of alternative techniques, such as IFM, may be helpful to test conclusions reached by the use of GFP.

These limitations notwithstanding, GFP has been successfully used to study protein and DNA localization in a variety of different microbial systems. A summary of these applications is shown in Table 1 with many of the specific
GFP fusions discussed in further detail in the following sections. Further discussion of experimental methods for using GFP in macromolecule localization studies in bacteria has been previously published [23].

3. Sporulation

Among the first applications of GFP in bacteria was to characterize sporulation in *Bacillus subtilis* [24–26]. Sporulation in *Bacillus* involves formation of a polar septum that partitions the sporangium (the developing cell) into the forespore and the mother cell. Assembly of these distinct subcellular compartments requires both differential gene expression and protein localization for which the use of GFP is well suited. For example, GFP fusions between CotA, a protein involved in formation of the spore coat, and DacF, a protein known to be localized to the prespore compartment, were targeted as faithfully as the wild-type proteins [25,26].

SpoIVA is a protein important for development of the mature spore and whose localization was studied using time-lapse photomicroscopy, in combination with deconvolution microscopy. This approach provided a spatio/

### Table 1

GFP fusions used to characterize bacterial cellular processes

<table>
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<th>Target protein and function (source)</th>
<th>Reference</th>
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<td>DacF – spore-specific protein (<em>B. subtilis</em>)</td>
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<td></td>
<td>SpoIE – phosphatase (<em>B. subtilis</em>)</td>
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<td>SpoIIG – protease (<em>B. subtilis</em>)</td>
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<td></td>
<td>SpoIF – spore development (<em>B. subtilis</em>)</td>
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<td>RNA polymerase subunit β' (<em>B. subtilis</em>)</td>
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temporal perspective of SpoIVA localization during sporulation and revealed that SpoIVA localized in a shell-like structure at the mother cell membrane that surrounds the forespore [27]. Localization was found to begin at the time of polar division and continued until completion of the forespore engulfment by the mother cell membrane. SpoIVA was also shown to be present in the mature spore. These studies provided an explanation for the role of SpoIVA in recruitment of coat proteins to the surface of the developing spore [27].

The sporulation-specific transcription factors σE and σF are responsible for induction of gene expression in the mother cell and forespore, respectively. Although both of these sigma factors are synthesized in the sporangium, their activation in their respective cellular compartments requires proper localization of additional components of the sporulation process. One of these components includes SpoIE, a membrane-bound phosphatase that functions in activation of σF within the forespore. SpoIE–GFP fusions were used to develop models to explain how σF is selectively activated in the developing spore [17,24,28,29]. Time-lapse observation of these fusions confirmed that SpoIIIE was localized near both cell poles and is further subject to additional stages of regulation to ensure that the phosphatase activity is triggered only in the appropriate compartment. Continued use of GFP fusions, in combination with genetic and biochemical techniques, should further help to understand how SpoIIIE activity is sequestered in the developing spore.

σE is activated in the mother cell by proteolytic cleavage of an amino-terminal extension that tethers the transcription factor to the cytoplasmic membrane, and eventually to the polar septum. Activation of σE is performed by a specific membrane-bound protease, SpoIIGA. Consistent with its role in σE activation, SpoIIGA–GFP was localized to the asymmetric septum separating the mother cell from the forespore [30]. It has further been reported that fusions between sigE, encoding σE, and gfp direct synthesis of a product that is sequestered to the mother cell side of the asymmetric septum [31], thus providing an explanation of how induction of gene expression can occur specifically in the mother cell. Additional insights into sporulation provided by GFP are discussed further below in Section 6, and are summarized in Table 1.

4. Cell division

Perhaps some of the most surprising new insights into cellular processes, especially with respect to proteins that are dynamically localized throughout the cell cycle, have resulted from the ability to directly visualize components of the cell division machinery. Among the first applications of GFP to study cell division was its application to characterize localization of FtsZ, a highly conserved component of the cell division machinery. Previous studies using IFM had shown that FtsZ forms a ring at the mid-cell division site [32]. FtsZ–GFP fusions, likewise, formed ring-like structures at the site of septal ring formation in living cells [33]. Moreover, subsequent studies were used to monitor the rapid assembly and disassembly of FtsZ polymers in individual, growing cells throughout the complete cell cycle [34].

In E. coli, at least nine different proteins are known to be important for construction of the cell division septum. Although the function of most of these proteins in cell wall synthesis remains unclear, the use of GFP fusions has provided means to determine their order of assembly, and to identify features of the proteins important for proper targeting. Ma et al. [33], for example, reported that FtsA colocalized with FtsZ to the site of cell division at mid-cell. Expression of deletion derivatives of an ftsA–gfp gene fusion further revealed structural features of FtsA important for its proper localization.

GFP, along with IFM, has contributed to determining the order of assembly of the cell division apparatus by expressing chimeric proteins in different mutant backgrounds, or in the presence of cell division inhibitors. For instance, proper targeting of fusions between GFP and the cytoplasmic domains of FtsI [35], FtsL [36], FtsK [37], FtsQ [38], and ZipA [39] all required prior localization of FtsZ, consistent with this protein’s involvement early in the cell division process. In most of these cases the GFP fusions were fully functional in haploid, further supporting the experimental conclusions. The result that ZipA can assemble in the absence of the other cell division proteins, but does require FtsZ [39], indicated that ZipA is an early recruit to the cell division site, but apparently does not function as the initiator of FtsZ ring formation as had been previously proposed [40]. The signal to determine the potential site of cell division remains unknown. FtsL and FtsI are considered to be late additions to the septal ring, since both required that the remaining cell division components are expressed for their proper localization.

Fig. 1 shows an example of how GFP fusions can be used to investigate cell division in E. coli. Although disruption of the ftsE gene results in elongated cell morphology, the role of this gene in proper cell division is not known [41]. To better characterize this mutant, localization of ZipA–GFP was monitored in an ftsE mutant. As shown, ZipA is localized to discrete foci in both wild-type and mutant cells, indicating that the block in cell division in the mutant does not occur at an early stage in the cell division process. The use of additional GFP fusions will be helpful to identify the defect in cell division imposed by the ftsE mutation. A similar approach has been taken to identify the block in cell division imposed by mutants that lacked phosphatidylyethanolamine [42].

Characterizing E. coli strains expressing GFP fusions to the MinC, D, and E proteins led to the striking discovery that these cell division components are dynamically local-
5. Replication, transcription and translation

Due to the lack of adequate experimental tools, a fundamental question that has remained unanswered for decades is whether the replication machinery moves along a stationary chromosome (perhaps the most common way of visualizing the process used in textbooks), or does the replication complex remain at a fixed position with the chromosomal DNA threaded through the polymerase ‘factory’ style [48]? Lemon and Grossman [22] gained a unique perspective of DNA synthesis by the visualizing GFP-tagged replication proteins in living cells. A gene fusion between polC, encoding a subunit of DNA polymerase, and gfp was introduced to the B. subtilis chromosome. The PolC–GFP protein functioned normally in DNA replication even when the hybrid represented the sole source of PolC in the cell. Remarkably, discrete fluorescent foci were visualized near the mid-point of exponentially growing cells. More fluorescent foci were observed as the cellular growth rate increased, consistent with the requirement for multiple replication forks in rapidly growing cells. Similar results were also seen when two additional DNA polymerase subunits were fused to GFP. From these observations, the authors concluded that the DNA polymerase complex of B. subtilis is found at fixed positions near mid-cell rather than being distributed throughout the nucleoid. Although other studies did not yield identical results [21], additional studies by Lemon et al. [49] incorporating a reversible block to DNA replication further supported the stationary polymerase complex model.

Upon release of the replication block, regions of the newly replicated chromosomes, also visualized by GFP, were oriented away from the centrally located replication complex and toward opposite poles of the cell. Also, in a synchronous population of cells, the chromosomal region located between the origin and terminus migrated toward the replication machinery prior to duplication, again consistent with a model of DNA synthesis where the chromosomal DNA is the dynamic component of the process. These findings are significant in that they facilitate development of specific theoretical models of replication and chromosome segregation that can be subject to further testing [50].

Using GFP to monitor localization of the transcriptional and translational machinery has provided new insights into these fundamental cellular processes as well. Inspection of RNA polymerase–GFP fusions expressed in B. subtilis revealed that transcription occurred primarily within the nucleoid, rather than on the periphery [51]. At high growth rates, RNA polymerase became further concentrated at distinct foci, likely representing areas of rRNA synthesis and reminiscent of nucleoli in eukaryotic systems. By contrast, translation, as monitored by ribosomal protein–GFP fusions, was found localized to subcellular regions distinct from transcription, including the cell poles and future division sites [51]. These observations suggest that the bacterial cell is organized in a manner more like that of eukaryotic cells than previously thought. Further work will be required to determine the biological significance of the spatial separation between transcription and translation in bacteria.
6. Chromosome and plasmid segregation

The segregation of chromosomes and plasmids is a highly accurate process in bacteria, ensuring that sister molecules are accurately distributed to newly divided cells. Understanding how this process unfolds in living cells has been difficult to determine because of the small size of the bacterial cell, and the dynamic nature of the chromosome during replication and cell division. Two strategies to investigate chromosome behavior in bacteria include the use of GFP-fused partitioning proteins that bind specifically to the origin of replication (ori), and by using a GFP-LacI reporter/repressor hybrid protein. This latter protein retains the ability to bind to lacO operator sites that are introduced in tandem to specific locations on the bacterial chromosome, or on plasmids [52]. It has been used to visualize directly DNA molecules as they undergo segregation, revealing that chromosomal partitioning remains a highly ordered process throughout the cell cycle [53–56].

Fluorescent foci representing lacO sequences introduced near the ori of both B. subtilis [54] and E. coli [53] revealed that the ori is preferentially associated at or near the cell poles early in the cell cycle, while a region near the chromosome terminus remained near mid-cell [53]. Observing the kinetics of chromosome motion through a complete cell cycle revealed that, following duplication, one sister ori stayed near the cell pole while the other migrated toward the site of formation of the new pole in the daughter cell [53,57]. In contrast, the low copy number E. coli plasmids F and P1 were found at mid-cell in newly divided cells and then abruptly migrated to the quarter cell positions following replication, suggesting different mechanisms for partitioning of the chromosome and plasmids [53].

Models of segregation of high copy number plasmids suggest that they are free to diffuse throughout the cytoplasm. However, recent observations by Pogliano et al. indicated that GFP-tagged high copy number plasmids also appeared as distinct foci in the cells at mid-cell or near one of the quarter cell positions, rather than being distributed throughout the cytoplasm [58]. Moreover, foci migrated away from each other during cell growth, reminiscent of the behavior of lower copy number plasmids with active partitioning systems. The correlation between the location and dynamics of high copy plasmids with that observed for the replication machinery is striking, suggesting plasmids may associate with replication proteins to partition themselves efficiently. These results have prompted reevaluation of models of passive segregation of high copy number plasmids.

In B. subtilis, sporulation is intimately linked with chromosome segregation to ensure that the replicated chromosomes are appropriately localized to opposite cell poles prior to asymmetric cell division. A system that coordinates chromosome segregation and expression of sporulation-specific genes utilizes SpoOJ and Soj, a pair of proteins with homologies to plasmid partitioning proteins. SpoOJ–GFP foci were localized to specific sites near the ori and did so in both vegetatively growing cells as well as cells undergoing asymmetric cell division during sporulation [56,59,60]. The localization and movement of SpoOJ mimic that of the ori [55] and were mobilized to opposite poles during the cell cycle [56]. SpoOJ has been proposed to compose a mitotic-like apparatus for proper orientation and segregation of the Bacillus chromosome [60].

The role of Soj has also been investigated by determining the cellular location of GFP–Soj hybrid proteins. It was found that the protein localized near the cell poles, as well as being distributed freely throughout the cytoplasm of vegetative cells [61,62]. However, the hybrid protein, in a SpoOJ-dependent manner, was found to oscillate between the poles in a manner reminiscent of MinCD, described above. Soj is apparently a dual functioning protein, required both for aggregation of SpoOJ–chromosome complexes, as well as serving as a repressor of sporulation genes. Although it remains unclear how the oscillatory behavior of Soj contributes to its function in sporulation, it is yet another example of how events that comprise a complex cellular process can be coordinated by the dynamic motion of specific proteins.

The SeqA protein of E. coli binds to hemi-methylated DNA at GATC adenine methylation sites of newly replicated DNA to sequester the ori from continually initiating new rounds of replication that are out of synchrony with the cell cycle. Similar to that observed with DNA polymerase, SeqA foci were localized near the mid-point of growing cells and subsequently migrated to fixed positions one quarter of the distance to the cell poles [20]. Subsequent observations suggested that the location of SeqA coincided with that of the replication forks as predicted by the factory model of DNA replication [19]. Although additional experiments will be required to reconcile differences in the dynamic localization of SeqA in independent studies [19,20,63], the results are consistent with the function of membrane-bound SeqA in tethering the replication forks to the cell center to ensure accurate segregation of the nucleoid by directing newly replicated chromosomal DNA away from the replication machinery [19].

7. Signal transduction

Bacteria respond rapidly to changes in environmental conditions both by immediately changing patterns of gene expression, as well as their direction of motility. Many bacteria have the ability to migrate either toward an attractant, or away from a toxic repellent by continuously monitoring their surrounding environment and respond accordingly. A number of membrane-bound proteins function as receptors for specific attractants and repellent molecules. These receptors, in association with additional cellular components, initiate a phospho-relay...
system that results in altered cell motility. To better understand the function of this response system in E. coli, GFP fusions were constructed to determine the localization of components of the chemotaxis machinery. Sourjik and Berg [64] used a version of GFP emitting yellow light (YFP) to tag three chemotaxis proteins, CheA, CheY and CheZ, as well as the motor component FliM. The chemotaxis proteins were localized predominantly to the cell poles in association with the chemotaxis receptor proteins, consistent with previous IMF studies. By expressing the Che-YFP fusions in different mutant backgrounds, the YFP fluorescence was shown to be dependent upon other components of the chemotaxis complex, including the chemoreceptors.

Differential protein localization is also known to be important for controlling prokaryotic development in Caulobacter crescentus. CtrA is a master regulatory protein that controls a number of events in the cell cycle of C. crescentus, and whose activity is controlled by a membrane-bound histidine kinase, CckA. The spatial distribution of CcKA–GFP fusions was found to alter in different cell cycle stages, becoming localized to a cell pole in early predivisional cells, and then dispersing upon cell division [65]. CckA apparently regulates progression through the developmental cycle of Caulobacter by differential localization of histidine kinase activity. Additional two-component regulatory proteins that undergo differential localization include the histidine kinases DivJ and PleC [66], and the response regulatory DivK [67]. All of these proteins show polar localization at specific stages in the cell cycle, with DivJ and PleC being responsible for phosphorylation of DivK. Importantly, these results indicate that spatial control of the two-component regulatory proteins is superimposed on temporal regulation as a means to coordinate key events in the Caulobacter cell cycle.

8. Protein export

In addition to visualizing location of specific classes of proteins within the confines of the bacterial cytoplasm, GFP has also been used to study the export of proteins outside of the cytoplasm. Gene fusion technology has played important roles in dissecting bacterial protein export pathways. Fusions between an exported protein and β-galactosidase, for example, have provided new genetic selections and screens to isolate mutants defective in the export process. Using a similar approach, Feilmeier et al. [68] constructed fusions between GFP and proteins destined for localization to the periplasmic space. In contrast to the studies reported above, GFP was inactive in the periplasmic space due to its inability to fold properly in this subcellular compartment. The authors exploited this phenotype to isolate mutants that blocked export of the fusion [68]. Future work will likely see further exploitation of the green fluorescence phenotype in genetic approaches to identify mutants expressing altered levels of GFP [69].

In contrast to the results just described, when GFP was fused to proteins exported via the twin-arginine (TAT) pathway, fluorescence was observed in the periplasmic space [70]. This result is consistent with the prediction that the TAT pathway exports fully folded proteins, and that GFP can function in the periplasmic space if delivered in a properly folded conformation.

9. Conclusions

Improvements in GFP technology are continually being made that enhance the sensitivity and utility of this reporter system, including new GFP variants that display an expanded spectrum of excitation and emission wavelengths. These variants have successfully been used to study localization of different cellular components within the same cell [49,71]. In addition, fluorescent proteins from other species have been identified to further expand the repertoire of reporter systems available for localization studies [72,73]. These variants, coupled with improvements in image analysis [74,75], should continue to enhance the sensitivity of GFP detection, further eliminating artifacts resulting from overexpressed or aggregated proteins. Clearly, the continued use of GFP as a localization reporter, in combination with other proven techniques, such as IFM, cell fractionation, and genetic approaches using other reporter genes, will result in new insights about the architecture and machinery of the bacterial cell, in addition to raising new questions about the function of some of its most fundamental components.

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