Light Microscopy Techniques For Bacterial Cell Biology

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INTRODUCTION

Bacteria have typically been viewed as poor candidates for the techniques employed by eukaryotic cell biologists to localize subcellular factors. At a practical level, their small size (1 to 2μm on average) makes bacteria less than ideal subjects for light microscopy. Furthermore, in the absence of membrane bound organelles, prokaryotes have often been portrayed as "sacs of enzymes" exhibiting little if any organization within the confines of their plasma membrane.

Electron microscopy did much to dispel the myth that bacterial cells are intrinsically uninteresting at the subcellular level. Gifted electron microscopists, such as Eduard Kellenberger and Antoinette Ryter, used transmission electron microscopy to create exquisite images of bacterial cells during growth and differentiation. Their work provided fundamental insights into the subcellular organization of the bacterial cell, including the nature of the bacterial nucleoid, the structure of the bacterial cell wall, and the morphological changes *Bacillus subtilis* cells undergo during spore development (Kellenberger and Ryter, 1958; Robinow and Kellenberger, 1994; Ryter, 1964). Immunoelectron microscopy, which uses antibodies conjugated to colloidal gold particles to localize factors of interest in thin sections of cells prepared for EM, also advanced our understanding of bacterial cells. For instance, immunoelectron microscopy first revealed cell type-specific gene expression in developing *B. subtilis* cells, the polar localization of the chemoreceptor MCP in *Escherichia coli*, and the ring structure formed by the bacterial cell division protein FtsZ (Bi and Lutkenhaus, 1991; Maddock and Shapiro, 1993; Margolis et al., 1991).

Ultimately, it was the adaptation of immunofluorescence microscopy (IFM)—a technique routinely used for localizing proteins within eukaryotic cells and tissues—for prokaryotic systems that overcame the notion that bacterial cells are too small to be practical candidates for

light microscopy. In contrast to immunoelectron microscopy, which requires highly specialized expertise and equipment, IFM is accessible to any microbiologist seeking to determine the subcellular localization pattern of a protein of interest. Consequently, IFM has been used in a wide range of applications, for instance localizing cell type determinants in *Caulobacter crescentus*, elucidating genetic hierarchies in the localization of cell division proteins to the nascent septal site in *E. coli*, and pinpointing the timing of gene expression in individual *B. subtilis* cells in response to developmental signals (Shapiro and Losick, 2000).

Once the presumption that bacterial cells were too small was disproven, other light microscopy techniques developed for use in eukaryotic cells were rapidly adapted for bacterial systems. These techniques included the use of BrdU (a fluorescent nucleotide analog) to label replicating DNA, and fluorescent *in situ* hybridization (FISH) (Lewis and Errington, 1997; Niki and Hiraga, 1997). The most successful of these adaptations has been the use of the green fluorescent protein (GFP) from the jelly fish *Aequoria victoria* in bacterial systems. GFP can be used both as a transcriptional reporter and as a tag for subcellular localization of both proteins and chromosomal and plasmid DNA (Belmont and Straight, 1998; Tsien, 1998). Since it was first used in bacterial cells, GFP has been widely employed to confirm and expand upon data originally obtained by immunoelectron microscopy or IFM (Margolin, 2000; Webb et al., 1995). Moreover, in contrast to immunoelectron microscopy and IFM, GFP permits the real time localization of proteins of interest and even regions of the bacterial chromosome in live cells. As a result of this property, GFP fusions have been the basis for experiments demonstrating the rapid movement of newly replicated chromosomal origins and plasmids to opposite poles of the cell during the course of the cell cycle as well as the pole-to-pole oscillations of the bacterial cell division protein MinD (Raskin and de Boer, 1999; Webb et al., 1998).

Together IFM and GFP have fundamentally changed our view of bacterial cells. Far from amorphous bags of DNA and protein, bacteria—with their own highly organized genetic material and cytoskeletal structures—are now considered to have a level of subcellular organization approaching that of eukaryotic cells (Shapiro and Losick, 2000). The question of whether any subcellular ultrastructure exists has been answered. Accordingly, bacterial cell biologists have shifted the focus of their research to investigate how bacterial cells maintain such an elaborate level of organization without membrane bound organelles and in the apparent absence of motor proteins and cytoskeletal-based trafficking systems.

This chapter provides an overview of the uses of immunofluorescence microscopy and GFP in bacterial systems. The techniques presented require a microscope equipped for epifluorescence and a high power (60X or 100X) DIC objective with a high numerical aperture. Depending on the brightness of the sample, images can be captured either with a standard 35mm camera mounted on the microscope or with a high resolution, high sensitivity CCD (charged coupled device) camera in conjunction with a digital imaging system. A list of sources of materials and reagents as well as a table of filter sets for visualizing the different fluorophores are provided at the end of the chapter. Although these protocols were originally developed for use in *E. coli* and *B. subtilis*, with some small modifications they should be easily adapted for use with other bacterial species.

IMMUNOFLUORESCENCE MICROSCOPY

Although it provides neither the resolution of electron microscopy, nor information about protein localization during real time like GFP, IFM offers several advantages over both techniques. First, due to the relatively gentle fixation required to prepare samples and the use of whole cells instead of thin sections, IFM is significantly more sensitive than immunoelectron

microscopy. It is possible to visualize proteins that are present at as few as 100 molecules per cell and perhaps those of even lower abundance using IFM (Lemon and Grossman, 1998; Weiss et al., 1997). Moreover, the wide range of available fluorophores facilitates the simultaneous localization of as many as four or five factors of interest in a single cell.

The preparation of cells for immunofluorescence microscopy requires three basic steps: fixation, permeabilization, and staining. Fixation is the means by which growing cells are preserved for IFM. Once fixed, cells are stable for up to a week, allowing analysis at a time that is convenient to the investigator. Permeabilization makes cells porous enough to allow antibodies access to cytoplasmic components. After permeabilization, antibodies conjugated to fluorophores are used to stain cells for factors of interest. If desired, cell wall material and the bacterial nucleoid can also be labeled during this third and final step using wheat germ agglutinin (lectin) conjugated to fluorophore and a nucleic acid stain such as 4',6-Diamidino-2-phenylindole (DAPI).

Fixation

Although a multitude of variations exist, there are essentially two methods for fixing cells prior to IFM—methanol (Angert, personal communication; Hiraga et al., 1998) (Protocol 1) and gluteraldehyde/paraformaldehyde (Addinall et al., 1996; Harry et al., 1995; Levin and Losick, 1996; Pogliano et al., 1995) (Protocol 2). Each method has its own advantages and disadvantages. For example, although methanol fixation is more gentle, it does not preserve cell wall material as well as gluteraldehyde/paraformaldehyde. The choice of fixative is, therefore, best made empirically.

When using gluteraldehyde/paraformaldehyde, cells grown in rich medium [such as Luria-Bertani (LB) broth] typically require a higher concentration of gluteraldehyde than those

grown in defined minimal medium. However, it should be noted that the concentration of gluteraldehyde used in Protocol 2 can effect the sensitivity of the technique. At higher concentrations of gluteraldehyde, background fluorescence increases and antibody-antigen recognition decreases, seriously compromising the sensitivity of the technique. For these reasons it is very important to do a thorough titration of gluteraldehyde when optimizing the fixation protocol. Finally, the repeated centrifugations required to remove fixative can lead to the mislocalization of certain subcellular factors (Lemon and Grossman, 1998). If this is a concern, Protocol 3 provides an alternative means to wash cells following fixation.

Protocol 1: Methanol Fixation

1) Drop 1ml of culture into 10ml of ice cold 80% MeOH

2) Let stand for 60' at room temperature.

3) Add 200µl of 16% paraformaldehyde (this step fixes the nucleoids so that they remain intact during permeabilization and staining.)

4) Let stand for 5' at room temperature.

5) Spin down cells at relatively low speed (3500RPM in a Sorvall T6000D centrifuge) and resuspend gently (avoid vortexing...finger flicking is best) in 1ml of ice cold 80% MeOH. The cells will tend to clump together at this point. This is not a problem as the clumps will disperse when the cells are adhered to the slide. Cells will keep for up to one week in methanol at 4°C.

Solutions:

80% MeOH -20°C

16% Paraformaldehyde

Protocol 2: Gluteraldehyde/Paraformaldehyde Fixation

1) While cells are growing prepare fixative. The gluteraldehyde concentration needs to be optimized for each protein of interest, however, 1.5 to 3µl of 25% gluteraldehyde per ml of 16% paraformaldehyde is typical. Store on ice.

2) Aliquot 20µl of 1M $NaPO_4$ pH 7.4 into 1.5 ml microfuge tubes and label.

3) Immediately prior to sampling add 100µl of fix into each prepared tube.

4) Take 500µl aliquots of each culture and add to appropriately labeled tube. Invert tubes 1 or 2 times.

5) Incubate 15 minutes at room temperature followed by 30 minutes on ice.

6) Wash cells three times by pelleting in microfuge at approximately 20,000 x g and resuspending in 1 ml PBS. After the final PBS wash, resuspend pellets in GTE to a final OD of approximately A600=0.200.

Solutions

16% paraformaldehyde

25% gluteraldehyde

Phosphate Buffered Saline (PBS)

50mM Glucose 25mM Tris 8.0 10mM EDTA 8.0 (GTE)

1M $NaPO_4$ pH 7.4

Protocol 3: Filter Wash

1) Proceed with fixation according to either protocol 1 or 2 up to point of spin.

2) Pre-wet filter in holder with PBS. Handle filter with forceps. Do not let filter or cells dry out.

3) Instead of spinning to remove fix from cells and concentrate them, filter the cells through a low protein binding filter using a 5ml syringe and filter holder. Be gentle when pushing the fixed cells, and later the wash, through the filter.

4) Wash cells on filter with 3ml of PBS.

5) Resuspend cells in GTE by placing filter in microfuge tube with 200-400µl of GTE and flicking the tube. Pull out the filter after resuspending cells.

Note: Fixed and filtered cells are best for immunofluorescence when used the same day. However, if necessary, they can be stored for up to 3 days at 4°C.

Equipment

Filter Holder 13mm SST Swinney Syringe Holder

0.1µm VVPP Durapore Membrane Filters

Solutions

PBS

GTE

Permeabilization and staining

The permeabilization step is critical to the success of IFM. It is important to achieve adequate breakdown of the cell wall to allow entry of antibodies, while at the same time maintaining cell ultrastructure. *B. subtilis* and *E. coli* cells are adhered to the microscope slide and permeabilized using an isotonic lysozyme solution. In general, *E. coli* require significantly less lysozyme for permeabilization than *B. subtilis* (Addinall et al., 1996); growth conditions

and strain background can also affect the length of time required for effective permeabilization. Thus, titrating both the concentration of lysozyme (or other lytic agent) and/or the incubation time to optimize permeabilization is strongly advised.

While lysozyme is sufficient to permeabilize *E. coli* and *B. subtilis* cells, other species may require additional treatment. Esther Angert found that *Metabacterium polyspora* cells are resistant to standard lysozyme treatment. She, therefore, developed a two step permeabilization protocol in which the *M. polyspora* cells were subjected to two, two hour incubations—first in mutanolysin and then in lysozyme (Angert and Losick, 1998).

In the protocol for IFM presented below (Protocol 4), staining is a two step process requiring both primary antisera against the protein of interest [or an epitope tag such as c-Myc, FLAG, or hemagglutinin (HA)] and a secondary antibody conjugated to a fluorophore. It is possible to eliminate the second staining step if primary antibodies conjugated to fluorophores are available. The steps at which cell wall and nucleic acid stains can be added are noted in parentheses. If polyclonal antibodies are used, affinity purification of antisera (Protocol 5) can significantly reduce background staining. Protocol 6 is a simple method for fixing and staining bacterial nucleoids that does not require permeabilization.

Protocol 4: Immunofluorescence microscopy

1) Prepare 15 or 8 well slides. Put 10µl of 0.1% poly-L-lysine solution into each well. Let stand five minutes and aspirate off. Wash once with distilled sterile water and let air dry. (All washes are done with a Pasteur pipette that has been hooked up to an aspirator with a trap. It is important not to touch the wells with the pipette tip during all washes.) Store slides in empty petri dishes to protect them from dust, etc. Use slides within one hour of poly-L-lysine treatment for best results.

2) While slides are drying prepare 2mg/ml* lysozyme solution in GTE and store on ice.

3) Drop ~10µl of fixed cells onto well. Let stand 2 minutes. Aspirate and wash once with 10µl of PBS. Let air dry. It is important NOT to aspirate too forcefully or touch the wells with the pipette tips.

4) Re-wet cells for 5 minutes with 10µl of PBS.

5) Aspirate off PBS and put 10µl of lysozyme solution onto each well. Incubate 1 to 7 minutes at room temperature (incubation time may vary depending on strain background and growth conditions).

6) Wash wells once with PBS.

7) Add 10µl of 2% BSA solution to each well as a blocking agent. Incubate 10 minutes at room temperature.

8) Meanwhile dilute primary antibody appropriately in PBS-2% BSA. For untested sera, a titration of between 1:50 to 1:10,000 is generally a good place to start.

9) Aspirate off BSA solution (do not let wells dry!) and add 10µl of diluted antibody to each well. The incubation period varies but it should be at least one hour. If incubating overnight or longer it is best to put the slide at 4°C. To prevent drying, place the slide and a wet piece of tissue (not touching the slide) in a petri dish and seal with parafilm.

10) After primary incubation, wash wells 10 to 25 times with PBS (increasing the number of washes can decrease background) and apply secondary, fluorophore conjugated antibody. Secondary antibodies should be diluted in 2% BSA solution.** Incubate 30 minutes to 1 hour at room temperature. [Wheat germ agglutinin (lectin) conjugated to the fluorophore of choice can be added at this point to stain cell wall material.]

11) Wash wells 10 to 25 times with PBS. Apply 1 drop of Slow Fade equilibration buffer from Slow Fade kit (Molecular Probes) to each well and let stand 5 minutes. (Alternatively, 1µg/ml DAPI in Slow fade equilibration buffer can be added at this point to stain nucleoids.)

12) Aspirate off and apply 1 drop of Slow Fade in glycerol to each well in the uppermost row. Carefully put on cover slip, angling it so it covers first the top wells and then the middle and bottom wells. Make sure not to get any Slow Fade on top of the cover slip and avoid creating bubbles in the wells below. Aspirate off any extra Slow Fade, as it will make the slide hazy when viewed through an oil objective. Cover slip can be replaced with a fresh one if necessary.

13) The slide is now ready to be examined. Store slides in foil wrapped petri dishes at -20°C.

Note: This procedure can be done on a coverslip instead of an eight or fifteen well slide.

* For *E. coli* much less lysozyme is needed. Typically 1-10µg/ml is sufficient, however, a lysozyme titration is recommended to account for differences between strains.

** Titrating fluorophore conjugated secondary antibodies will ensure optimal results. However, a 1:100 to 1:500 dilution of a 1mg/ml solution of fluorophore conjugated secondary antisera is typical.

Solutions:

PBS

GTE

2% BSA in PBS

0.1% poly-L-lysine

2mg/ml lysozyme in GTE

1mg/ml DAPI stock solution

Supplies:

Slow Fade kit

8 or 15 well slides

large cover slips

FITC, Cy3, Cy5, etc. conjugated secondary antibodies

Protocol 5: Affinity Purification of Polyclonal Antisera

1) Run denaturing polyacrylamide gel with 10µg to 100µg of the purified protein used to generate antisera. Typical load is 1-10µg per well or 10 to100µg total in one large well.

2) Transfer the protein to PVDF membrane using standard procedure for immunoblotting.

3) Stain the membrane with Ponceau S. Rinse with ddH_2O to destain. Using a razor blade or X-acto knife cut out the region of the membrane containing the protein trying to get as thin a strip as possible.

4) Block the membrane strip with 5% dry milk in PBS for 20 min at RT.

5) Wash membrane strip 2 by 2 minutes in PBS. Cut strip in half. Note: If affinity purifying ≤ 300µl of sera it is possible to use only half of the membrane and save half, tightly wrapped in plastic, at -20°C. Re-wet frozen membrane in MeOH and rinse in PBS before using.

6) Incubate the strip of membrane containing protein with 300µl of antisera for 1 hour at room temperature. It is helpful to cut the strip of membrane into smaller pieces and put them into a microfuge tube for incubation purposes.

7) After incubation, pull off the depleted antisera and set aside. Antisera may retain a relatively high titer of antibodies and can be subjected to further affinity purification should serum be in short supply.

8) Wash the membrane 2 by 15 minutes in 5ml of PBS.

9) Meanwhile, aliquot 100µl of $NaPO_4$ pH 8.0 into 3 microfuge tubes.

10) To strip membrane add 300µl of 5mM glycine 150mM NaCl pH 2.4.

11) Incubate 30 seconds at room temp.

12) Pull off supernatant and put into microfuge tube with $NaP0_4$ solution to neutralize.

13) Repeat steps 10-12 two more times.

14) Combine all three tubes to equilibrate the antibody levels and then divide into fresh aliquots. Generally, dialysis of affinity purified antisera does not appear to be necessary. Store the affinity purified antisera at 4°C as freezing sometimes damages the antibody. Sodium azide can be added to 1mM.

Note: Yields vary so it is important to titer the antibody after each affinity purification.

<u>Solutions</u>

PBS

Ponceau S

(10X solution: 2% Ponceau S in 30% trichloroacetic acid, 30% sulfosalicylic acid)

5% dry milk in PBS

Stripping solution

(5mM glycine, 150mM NaCl pH2.4)

1M NaPO4

Supplies

polyacrylamide gel

PVDF membrane

Protocol 6: Quick method for visualizing bacterial nucleoids

1) Immediately prior to fixing cells prepare a poly-L-lysine coated slide:

- Put 10-20µl drops of poly-L-lysine into each well and let stand 2 minutes.
- Wash once with ddH_2O.
- Aspirate to dry completely.

2) Add culture to each well for 30 seconds to 2 minutes. Time varies depending on the density of the cell culture. Aspirate and dry with vacuum.

3) Overlay cells with EtOH for 1 to 2 minutes. Aspirate.

4) Wash 3 times in 10 to 20 µl ddH_2O. Aspirate and dry.

5) Add DAPI (1µg/ml) and coverslip.

Supplies &Solutions

8 or 15 well glass slides

0.1% poly-L-lysine solution

1µg/ml DAPI solution in ddH_20

100% EtOH

ddH_2O

THE GREEN FLUORESCENT PROTEIN

In contrast to IFM, in which sample preparation involves both a fixation step and a lengthy staining procedure, the preparation of cells expressing GFP for microscopy is trivial. At its most basic, sample preparation requires only a glass slide, a coverslip, and a microscope equipped with the appropriate filter set (see Table 1). Furthermore, because GFP permits the visualization of proteins of interest in unfixed cells, it can be used for real time studies of live cells both in culture and, in the case of pathogens and plant symbionts, in host tissues (Cheng and Walker, 1998; Kohler et al., 2000). Finally, in contrast to other fluorescent proteins, GFP fluoresces without the addition of a substrate or co-factor (Tsien, 1998). For these reasons, GFP has become one of the favorite tools of bacterial cell biologists.

GFP was originally isolated as a protein that co-purified with the chemiluminescent protein aequorin from the jelly fish *Aequoria victoria* (Tsien, 1998). However, because of its inability to fold well at temperatures over 25°C, native GFP proved to be less than ideal for use in most eukaryotic and prokaryotic systems. Moreover, the peak excitation frequency of 395nm for native GFP (in the UV range) was damaging both to the specimen and to the investigator in the absence of proper eye wear or a UV absorbing screen. To circumvent these problems, several labs developed allelic variants of GFP that increased the folding efficiency of the chromophore at higher temperatures and altered the excitation spectra of the molecule, shifting it towards a peak of 470 nm (Cormack et al., 1996; Tsien, 1998). These so-called "red-shifted" variants made it possible to use GFP as a transcriptional reporter in lieu of *lacZ* and have facilitated the use of GFP fusions for the subcellular localization of proteins of interest (Kohler et al., 2000; Lemon and Grossman, 1998). Additional site directed mutagenesis of the chromophore led to the development of blue, cyan, and yellow variants of GFP (BFP, CFP, and YFP respectively); these

provide a means of localizing two factors of interest simultaneously in the same cell (see below) (Tsien, 1998).

General considerations

Whether using GFP as a transcriptional reporter or as a tag for the subcellular localization of a target protein, maximizing expression is central to the success of the experiment. For this reason, when making an N-terminal GFP fusion, and in situations where GFP will be used as a transcriptional reporter, it is important to ensure that the Shine-Dalgarno sequence is optimized for the system in which the GFP moiety will be expressed. Codon usage is also an important consideration. Many of the commercially available GFP molecules have been optimized for expression in mammalian cells (so-called "humanized" GFP). However, trial and error indicates that the "native" versions of GFP are expressed significantly better in these bacterial systems than the commercially available GFP variants (Lemon, personal communication).

GFP fusion proteins

At 22kD, the addition of a GFP moiety can significantly alter protein folding and activity *in vivo*. To circumvent problems associated with constructing a functional fusion protein, the GFP moeity can be fused to either the N-terminus or C-terminus of the protein of interest. Additionally, the length and composition of the polypeptide linker that joins the native protein to GFP can also have a surprisingly large effect on protein function (P.A.L. unpublished data; Lindow, personal communication).

Because of the difficulties associated with making a functional GFP fusion, it is advisable to determine if a GFP fusion protein is able to complement a null mutation to ensure that protein function is not disturbed. Similarly, whenever possible, the localization pattern of a GFP fusion

protein should be confirmed by immunofluorescence microscopy. Polyclonal sera against GFP is available commercially from several sources should it be required (see Table 3).

If a GFP fusion protein is incapable of complementing a null mutation, it is possible to place the fusion under the control of an inducible/repressible promoter in the presence of the wild type allele (Levin et al., 1999). In this configuration, the fusion protein is expressed at low levels and is used to "trace" the location of the target molecule without disturbing its activity.

Colored variants of GFP and vital stains for cell membrane

Although the number of colors available for GFP fusions is limited compared to IFM, mutagenesis of the chromophore has led to the development of cyan, blue, and yellow variants (CFP, BFP, and YFP) (Tsien, 1998). To avoid overlapping emission and excitation spectra, these variants can only be used in one of two combinations: BFP and GFP; or CFP and YFP. As with GFP, codon usage is an important consideration when selecting one of the colored variants for expression in bacterial cells. If the non-humanized version is preferred, it is possible to use site directed mutagenesis to make the changes in key codons of GFP necessary to generate BFP, CFP or YFP (See Table 2) (Levin et al., 1999; Lemon and Grossman, in press; Gueiros-Filho, personal communication). Although a red fluorescent protein (DsRed) has recently become available commercially, experimental and anecdotal evidence indicates that its folding time is too long to be useful for most experiments (Baird et al., 2000; Hahn, personal communication).

Vital membrane stains provide an convenient way to visualize the periphery of live cells in the presence of a GFP fusion. The red membrane stain, N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl) hexatrienyl) pyridinium dibromide (FM 4-64, Molecular Probes), which has an absorbance of 543nm, has been used in a wide variety of studies to determine cell boundaries and to track membrane dynamics during sporulation in *B. subtilis* (King et al., 1999;

Lemon and Grossman, 1998; Pogliano et al., 1999). FM 4-64 is visible in the filter set most commonly used for GFP fusions and, therefore, may not be the best choice of stain for fainter GFP fusions that localize to the periphery of the cell. The blue membrane stain, 1-(4-trimethylammoniumphenyl) -6-phenyl-1,3,5-hexatriene p-toluenesulfonate (TMA-DPH Molecular Probes), which absorbs at 355 nm, can be used as an alternative (although not in conjunction with the blue nucleic acid stain DAPI) (Gueiros-Filho, personal communication).

Other Applications

In addition to serving as a marker for protein localization and as a transcriptional reporter, technology developed by Andrew Belmont and Aaron Straight permits GFP to be used to localize plasmids and regions of chromosomal DNA. Belmont and Straight tagged a region of yeast chromosomal DNA with a tandem array of lactose operator sites and visualized this region by fluorescence microscopy in live cells using GFP fused to the lactose repressor protein (Straight et al., 1996; Belmont and Straight, 1998). This technology has subsequently been adapted for bacterial cells, where it has been used to visualize the position of chromosomal origin and termini, to measure the timing of origin separation during the cell cycle in *B. subtilis*, and to trace the localization of plasmids in *E. coli* cells (Gordon et al., 1997; Webb et al., 1997). A similar technology has also been developed that takes advantage of the tetracycline repressor and its binding site (Michaelis et al., 1997).

The colored variants of GFP provide a means of exploring protein-protein interactions *in vivo* using fluorescence resonance energy transfer (FRET). Due to overlapping emission and excitation spectra, CFP can, following excitation, transfer its energy to a YFP molecule provided that the donor and recipient proteins are in close proximity (10-50Å). The degree of FRET between two fusion proteins may be measured either *in vitro* with a fluorimeter or *in vivo* with a

fluorescence microscope (Heim and Tsien, 1996; Pollok and Heim, 1999; Tsien, 1998). [Although it is possible to use BFP and GFP as a FRET pair, CFP and YFP are strongly preferred due to the relative sensitivity of BFP to photobleaching (Tsien, 1998).] While this technique has not yet been applied to bacterial cells, FRET has been used to determine the oligomeric state of the G-protein-coupled receptor for yeast alpha-factor and to explore interactions between nuclear pore proteins in *Saccharomyces cerevisiae* (Overton and Blumer, 2000; Damelin and Silver, 2000). These results in a similarly sized organism support the idea that FRET will be a powerful method with which to investigate dynamic protein interactions in bacterial cells.

Optimizing growth conditions for GFP

While bright GFP fusions are visible under most growth conditions, fainter fusions may require special attention. In general, it is best to grow cells in minimal defined medium whenever possible to keep background low. Cells grown in Luria-Bertani broth or other rich medium tend to have a faint green background even in the absence of a GFP fusion. This background can interfere with the signal from weaker GFP fusion proteins, making them extremely difficult to see. Temperature is also an important consideration, as even those variants of GFP that have been optimized for higher temperature applications are brighter when cells are grown at 30°C or below. Finally, oxygen is required for maturation of the GFP chromophore (Tsien, 1998). Thus, adequate aeration during growth is critical for maximizing the intensity of any given GFP fusion. (For this reason, it is unlikely that the GFP chromophore would be able to develop under anaerobic conditions, making GFP impractical for use in obligate anaerobes.)

Methods for visualizing GFP fusions in live cells

Protocol 6 presents two methods for preparing live cells for fluorescence microscopy. The first method is the simplest—requiring only pressure to immobilize the cells on the slide. In

the second method, an agarose pad is used to provide a solid surface for bacterial adhesion. The agarose pad stabilizes the cells to some degree, making it the method of choice for cells that will be examined over any length of time. In addition, different reagents—isopropylthio-beta-D-galactoside (IPTG), antibiotics, vital dyes, etc.—can be added to the agarose pad during the course of the experiment; this allows the investigator to examine the effect of different chemicals on gene expression patterns or protein localization in real time.

Protocol 7 is to be used when staining live cells with vital membrane dyes and nucleic acid stains (typically DAPI, which effectively stains the nucleoids of live bacterial cells.) As discussed above the red membrane stain, FM 4-64, which emits both strong red and faint green fluorescence, is visible in the filter set most commonly used for GFP. To circumvent this problem, the filter set recommended for fluorescein-isothiocyanate (FITC), which eliminates the red fluorescence, can be used instead of the GFP filter set. It should be noted, however, that the FITC filter set is not as sensitive as the long pass GFP filter set, which may be a problem with dimmer fusions. Alternatively, the FM 4-64 solution can be added to the agarose pad after the GFP image has been collected (Sharp and Pogliano, 1999).

In general it is best to look at unfixed, untreated cells to ensure that the GFP fusion is as bright as possible. Although some fusions are visible after fixation, both methanol and gluteraldehyde/paraformaldehyde significantly lower the brightness of most GFP fusion proteins. Poly-L-lysine, the reagent used to adhere fixed cells to the slide for immunofluorescence, can also have a deleterious effect on the brightness and localization pattern of GFP.

Microscopy

A drawback of GFP and its variants is their relatively high sensitivity to photobleaching by the high intensity light required for excitation. Though bright fusions and fusions to highly

expressed proteins are less susceptible than their dimmer counterparts, exposure time should be minimized. An electronic shutter system, in which the shutter is controlled by a computer instead of manually, can significantly shorten the period in which cells are exposed to high intensity light. In the same vein, when taking multiple images of the same field of cells using different filter sets, it is best to start looking at fluorophores that excite at the longer (red) wavelengths and work towards the lower end of the spectrum (blue). This approach avoids photobleaching those fluorophores that excite at the shorter wavelengths (CFP and BFP), which tend to be the most sensitive.

To maximize the brightness of a GFP fusion it is best to use a microscope equipped with a high power DIC objective. The polarizing filter can be left in for focusing on a plane of cells and then pulled out to collect GFP images. Phase contrast objectives, although sufficient for the brightest GFP fusions, absorb too much light to be useful for visualizing fusions of average and below average brightness.

Protocol 6: Preparing live cells for fluorescence microscopy

A. Untreated slides

1) Drop five microliters of culture onto middle of glass slide.

2) Carefully put a coverslip on top of drop. Avoid creating bubbles if at all possible.

3) Using a tissue or a paper towel carefully press down on cover slip to remove extra liquid.

4) Examine cells as soon as possible after preparing slide.

B. Slide with agarose pad

1) Make an agarose pad by dropping ~20 to 50µl of 1% agarose onto a glass slide. To make surface flat, use two slides, one untreated and one wrapped in tape at either end and coated with

rain-X or other siliconizing substance. Drop agarose on treated slide between taped region. Cover with second, untreated slide. Let harden. Lift off taped slide.

2)Drop 10 to 20 microliters of cell culture onto agarose pad. Let stand for five minutes.

3) Aspirate excess liquid.

4) Place coverslip gently over agarose pad.

Protocol 7: Staining live cells with DAPI and vital membrane dyes

1) Dilute FM 4-64 or TMA-DPH 1:100 in PBS or dH_2O.

2) Make an agarose pad as described in protocol 6.

3) Incubate 200µl of fresh cells with 2-10µl of membrane dye for two minutes.

4) Spot cells onto agarose pad.

5) Leave for 5 to 10 minutes at room temperature.

6) Aspirate excess liquid.

7) If using FM 4-64, a small quantity of DAPI solution in GTE, dH_2O, or minimal medium can be added at this point to stain the nucleoids. Cover with cover slip. Remove any excess liquid.

Note: Cells must be growing prior to adding the stain to ensure that it is incorporated efficiently. The stain of interest can also be added to the media in which the cells are growing 30 minutes prior to microscopy at a 1:10,000 dilution. Finally, if the membrane stain masks the GFP signal it is possible to add the dye after collecting GFP images. See Sharp *et al.* (Sharp and Pogliano, 1999).

Solutions:

1% agarose in minimal media or GTE

1μg/ml DAPI in PBS

FM 4-64 1mg/ml in ddH20

TMA-DPH 1mg/ml in DMF

CONCLUSION

The protocols presented here represent the collected efforts of investigators at all levels working on a diverse array of biological problems. Largely through these efforts bacteria are no longer viewed as the proverbial swimming pool of enzymes; instead they are seen as possessing a level of subcellular organization as complex as that of any eukaryotic cell. These labors, therefore, have been the basis for what can be viewed as a revolution in bacterial cell biology.

While each method has been presented in as complete a manner as possible, the protocols should not be taken as gospel. Instead, these protocols are better seen as simply a place to start. It is likely that modifications will be required in order to optimize each protocol for both the model system and for the application. Do not be afraid to experiment!

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TABLES

Table 1: Filter Set Specifications. Filter sets are available from several sources, however, Chroma is one of the largest supplier and has excellent service and support.

Primary Use	Excitation	Beam	Emission	Chroma #
DAPI	D360/40x	400DCLP	D460/50m	31000
BFP	D380/30x	420DCLP	D460/50m	31041
CFP	D436/20x	455DCLP	D480/40m	31044 v. 2
GFP	HQ480/40x	Q505LP	HQ510LP	41012
FITC	HQ480/40x	Q505LP	HQ535/50m	41001
YFP	HQ500/20x	Q515LP	HQ520LP	41029
Rhodamine	HQ545/30x	Q570LP	HQ620/60m	41002c

Table 2: Key mutations affecting GFP folding, excitation and emission spectra. This is a table of those GFP variants that have been shown to fold and be expressed well in *E. coli* and *B. subtilis*. For a more detailed discussion of the classes of GFP molecules see Tsien (1998). Amino acids that are identical to wild type GFP are left blank.

Position	**64**	**65**	**66**	**67**	**68**	**69**	**70**	**71**	**62**	**203**	**Reference**
Wild type	Phe	Ser	Tyr	Gly	Val	Gln	Cys	Phe	Ser	Thr	(Tsien, 1998)
mut1	Leu	Thr									(Cormack et al., 1996)
mut2		Ala			Leu				Ala		(Cormack et al., 1996)
mut3		Gly							Ala		(Cormack et al., 1996)
BFPmut2		Ala	His		Leu				Ala		(Levin et al., 1999; Tsien, 1998)
CFPmut2		Ala	Trp		Leu				Ala		(Gueiros-Filho, personal communication; Tsien, 1998)
YFPmut2		Ala			Leu				Ala	Tyr	(Lemon and Grossman, in press; Tsien, 1998)

This table is based on Table 1 from Cormack *et. al* (1998).

Table 3: Suppliers

Product	**Supplier**	**Telephone in US**
Filter Sets for Fluorescence Microscopy	Chroma	800-824-7662
15 or 8 Well Slides	ICN	800-854-0530
16% Paraformaldehyde	Electron Microscopy Sciences	800-523-5874
25% Gluteraldehyde	Electron Microscopy Sciences	800-523-5874
0.1% Poly-l-Lysine	Sigma	800-325-3010
Slow Fade Kit	Molecular Probes	541-465-8300
FM 4-64	Molecular Probes	541-465-8300
TMA-DPH	Molecular Probes	541-465-8300
DAPI	Molecular Probes	541-465-8300
Conjugated Antibodies	Jackson Immunoresearch	800-367-5296
Low Protein Binding Filters	Millipore	800-645-5476
Filter Holder	Millipore	800-645-5476
Polyclonal anti-GFP serum	Clontech	800-662-2566