

[4] Green Fluorescent Protein as a Marker for Conditional Gene Expression in Bacterial Cells

By ROY J. M. BONGAERTS, ISABELLE HAUTEFORT, JULIE M. SIDEBOTHAM,
and JAY C. D. HINTON

Introduction

Bacterial pathogenesis results from a complex adaptation of the pathogen to its host. The necessity to resist the mammalian immune response has led to the selection of bacteria that have developed sophisticated virulence determinants. Expression of these determinants occurs in response to various environmental signals and is tightly regulated by a complex regulatory cascade.¹ Until recently, most virulence genes were identified from studies based on *in vitro* systems, which had little relevance to the true *in vivo* situation of bacterial infection. This has been remedied by the development of several powerful techniques to identify *in vivo* induced (*ivi*) genes such as *in vivo* expression technology (IVET)² and signature tagged mutagenesis (STM).³ However, these two techniques do not yield detailed information about the expression levels of *ivi* genes during infection. To enable the study of spatial and temporal expression of *ivi* genes in the host, analysis at the mRNA or protein level is required. Traditional reporter systems have been used for many years to study bacterial gene expression, but we now need to develop new accurate reporter systems that allow the monitoring of gene expression at the individual bacterial cell level. Since most virulence genes respond to environmental signals, *ivi* gene expression is likely to vary with the stage of infection, the spatial localization within the host, and the particular cell type within that host tissue. Overall, the monitoring of virulence gene expression requires sensitive reporter systems to show up- and down-regulation and transient and low levels of virulence gene expression.

Green Fluorescent Protein as Reporter System to Monitor *in Vitro* and *in Vivo* Gene Expression

The green fluorescent protein (GFP) of the marine invertebrate *Aequorea victoria* is a single autofluorescent, acidic, compact, globular polypeptide with a molecular mass of 26 kDa. Assembly of the GFP fluorophore requires a series

¹ B. B. Finlay and S. Falkow, *Microbiol. Mol. Biol. Rev.* **61**, 136 (1997).

² M. J. Mahan, J. M. Slauch, and J. J. Mekalanos, *Science* **259**, 686 (1993).

³ M. Hensel, J. E. Shea, C. Gleeson, M. D. Jones, E. Dalton, and D. W. Holden, *Science* **269**, 400 (1995).

of posttranslational intramolecular reactions, involving cyclization and autoxidation of amino acids Ser⁶⁵-Tyr⁶⁶-Gly⁶⁷. Mature GFP emits green light (508 nm) when excited with ultraviolet light (395 nm). Because no exogenous substrates or cofactors are required for its activity, it is a unique tool for monitoring gene expression, protein localization, and protein dynamics in both prokaryotic and eukaryotic living cells.^{4,5}

Advantages and Drawbacks of Green Fluorescent Protein

GFP has been intensely studied in recent years and different types of GFP variants with altered characteristics have been developed. Wild-type GFP has a neutral excitation peak of 395 nm with a minor peak at 475 nm, and an emission peak at 508 nm. Classes of spectral variants include GFPs with shifted emission and excitation wavelengths, and higher and lower intensities of fluorescence compared to wild-type GFP.⁶ A significant proportion of wild-type GFP molecules fail to fold and cyclize properly when synthesized at 37° and another class of GFP derivatives correct this problem. These thermostable variants fold correctly and are therefore significantly brighter. Combinatorial incorporation of multiple mutations of this class of variants has led to a substantial increase in fluorescence,⁷⁻⁹ exemplified by a mutagenesis study in *Escherichia coli* which resulted in the isolation of three distinct classes of GFP variants, all having red-shifted excitation maxima and folding more efficiently than the wild-type protein. The GFPs from these three classes contained the amino acid substitutions F64L and S65T for GFPmut1; S65A, V68L, and S72A for GFPmut2; and S65G and S72A for GFPmut3.⁷ These variants have proved to be particularly useful for *in vivo* studies¹⁰ because they are ideal for microscopic and flow cytometric analyses. Another class of GFP variants possesses enhanced levels of intracellular fluorescence without changes in the amino acid sequence through optimization of codon bias. These variants produce mRNA species that are more efficiently translated than mRNA produced from the wild-type coding sequence resulting in increased GFP production. Combining the mutations from these different classes has produced distinct GFP reporters with greatly enhanced levels of fluorescence. Because only one single fluorophore is formed from each GFP molecule synthesized, such increases in GFP fluorescence

⁴ M. Chalfie, Y. Tu, G. Euskirchen, W. W. Ward, and D. C. Prasher, *Science* **263**, 802 (1994).

⁵ M. B. Elowitz, M. G. Surette, P. E. Wolf, J. Stock, and S. Leibler, *Curr. Biol.* **7**, 809 (1997).

⁶ R. Heim, D. C. Prasher, and R. Y. Tsien, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 12501 (1994).

⁷ B. P. Cormack, R. H. Valdivia, and S. Falkow, *Gene* **173**, 33 (1996).

⁸ M. Chalfie, "Green Fluorescent Protein: Properties, Applications, and Protocols." John Wiley & Sons, New York, 1998.

⁹ A. Cramer, E. A. Whitehorn, E. Tate, and W. P. Stemmer, *Nat. Biotechnol.* **14**, 315 (1996).

¹⁰ R. H. Valdivia and S. Falkow, *Science* **277**, 2007 (1997).

TABLE I
SPECTRAL CHARACTERISTICS OF SELECTED GREEN FLUORESCENT PROTEIN VARIANTS AND dsRED^a

Name	Excitation maximum ^a (nm)	Emission maximum (nm)	Estimated fluorescence intensity relative to wild type	Mutation	Reference ^b
GFP	395(475) [#]	508(503)	1		(1,2)
Wild type S65T	489	511	6	S65T	(2)
GFPmut1 (= EGFP)	488	507	35	F64L, S65T	(3)
GFPmut2	481	507	19	S65L, V68L, S72A	(3)
GFPmut3	501	511	21	S65G, S72A	(3)
GFPuv	396(476)	508	42	F100S, M154T, V164A	(4)
GFP5	(396)476 ^c	508	111	V163A, I167T, S175G	(5)
GFP ⁺	491	512	130	F64L, S65T, F99S, M153T, V163A	(6)
BFP	382	448	0.6	Y66H	(7)
	384	448	3	Y66H, V163A, S175G	(5)
CFP	433	475	3	Y66H, N146I, M153T, V163A, N212K	(8)
	432	480	1.5	Y66W, I123V, Y145H, H148R, M153T, V163A, N212K	(8)
YFP	513	527	6	S65G, V68L, S72A, T203Y	(9)
DsRED (= drFP583)	558	583	n.a. ^d	n.a. ^d	(10)

^a The value in parentheses is a minor peak.

^b Key to references: (1) M. Chalfie, Y. Tu, G. Euskirchen, W. W. Ward, and D. C. Prasher, *Science* **263**, 802 (1994); (2) R. Heim, D. C. Prasher, and R. Y. Tsien, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 12501 (1994); (3) B. P. Cormack, R. H. Valdivia, and S. Falkow, *Gene* **173**, 33 (1996); (4) A. Cramer, E. A. Whitehorn, E. Tate, and W. P. Stemmer, *Nat. Biotechnol.* **14**, 315 (1996); (5) K. R. Siemering, R. Golbik, R. Sever, and J. Haseloff, *Curr. Biol.* **6**, 1653 (1996); (6) O. Scholz, A. Thiel, W. Hillen, and M. Niederweis, *Eur. J. Biochem.* **267**, 1565 (2000); (7) R. Heim, A. B. Cubitt, and R. Y. Tsien, *Nature* **373**, 663 (1995); (8) R. Heim and R. Y. Tsien, *Curr. Biol.* **6**, 178 (1996); (9) M. Ormo, A. B. Cubitt, K. Kallio, L. A. Gross, R. Y. Tsien, and S. J. Remington, *Science* **273**, 1392 (1996); (10) M. A. Wall, M. Socolich, and R. Ranganathan, *Nat. Struct. Biol.* **7**, 1133 (2000).

^c Excitation intensity is similar at both wavelengths.

^d n.a., not applicable.

are very important for improving sensitivity. An overview of GFP variants is shown in Table I.

Conventional reporter proteins are enzymes [e.g., β -galactosidase (LacZ), chloramphenicol acetyltransferase (Cat), and luciferase (Lux)] whose signal amplification is derived from multiple substrate cleavage by one molecule of reporter

protein. However, activity of these reporters is also dependent on substrate levels and/or energy reserves within cells, whereas GFP needs no exogenous substrates or cofactors.^{4,11} Moreover, the use of luciferase is complicated by high background in bacterial populations, making it unsuitable for reporting expression in individual bacterial cells.¹² The value of fluorogenic β -galactosidase substrates is reduced by the need to load these substrates into organisms by cell permeabilization or osmotic shock.¹³ Several interesting reviews describe these types of reporter systems.^{14–16}

The fluorescence signal of posttranslationally modified GFP depends largely on the rate of biosynthesis of functional protein and the rate of dilution as the cell divides.¹² Wild-type GFP is very stable with a half-life of at least 24 hr in *E. coli*.¹⁷ Importantly, GFP fluorescence is stable under stress conditions, such as starvation,¹⁸ allowing determination of gene expression *in vivo*. However, care must be taken when performing acid stress experiments since GFP fluorescence is reduced at low pH.^{19,20} The availability of unstable GFP variants with half-lives of 40, 60, and 110 min enables the measurement of fast changes in expression patterns.¹⁷ There is an excellent book on the use of GFP which is essential reading.⁸ The development of new GFP variants is ongoing, leading to fluorescent proteins that are brighter, more stable, or have other improved characteristics.²¹ An interesting phenomenon that might be exploited in the future is photoactivation of GFP with blue light in a low oxygen environment, resulting in red-emitting GFP.⁵ In addition, the use of autofluorescent proteins such as red fluorescent drFP583 [commercially available as dsRED (Clontech, Palo Alto, CA)] and derivatives attracts growing attention.^{22–24}

¹¹ C. E. Nwoguh, C. R. Harwood, and M. R. Barer, *Mol. Microbiol.* **17**, 545 (1995).

¹² R. Tombolini and J. K. Jansson, *Methods Mol. Biol.* **102**, 285 (1998).

¹³ R. H. Valdivia and S. Falkow, *Curr. Opin. Microbiol.* **1**, 359 (1998).

¹⁴ B. B. Christensen, C. Sternberg, J. B. Andersen, R. J. Palmer, Jr., A. T. Nielsen, M. Givskov, and S. Molin, *Methods Enzymol.* **310**, 20 (1999).

¹⁵ C. Prigent-Combaret and P. Lejeune, *Methods Enzymol.* **310**, 56 (1999).

¹⁶ I. Hautefort and J. C. D. Hinton, *Phil. Trans. R. Soc. Lond. B Biol. Sci.* **355**, 601 (2000).

¹⁷ J. B. Andersen, C. Sternberg, L. K. Poulsen, S. P. Bjorn, M. Givskov, S. Molin, L. Molina, C. Ramos, M. C. Ronchel, and J. L. Ramos, *Appl. Environ. Microbiol.* **64**, 2240 (1998).

¹⁸ R. Tombolini, A. Unge, M. E. Davey, F. J. deBruijn, and J. K. Jansson, *FEMS Microbiol. Ecol.* **22**, 17 (1997).

¹⁹ G. H. Patterson, S. M. Knobel, W. D. Sharif, S. R. Kain, and D. W. Piston, *Biophys. J.* **73**, 2782 (1997).

²⁰ M. Kneen, J. Farinas, Y. Li, and A. S. Verkman, *Biophys. J.* **74**, 1591 (1998).

²¹ O. Scholz, A. Thiel, W. Hillen, and M. Niederweis, *Eur. J. Biochem.* **267**, 1565 (2000).

²² M. V. Matz, A. F. Fradkov, Y. A. Labas, A. P. Savitsky, A. G. Zaraisky, M. L. Markelov, and S. A. Lukyanov, *Nat. Biotechnol.* **17**, 969 (1999).

²³ M. A. Wall, M. Socolich, and R. Ranganathan, *Nat. Struct. Biol.* **7**, 1133 (2000).

²⁴ A. Terskikh, A. Fradkov, G. Ermakova, A. Zaraisky, P. Tan, A. V. Kajava, X. Zhao, S. Lukyanov, M. Matz, S. Kim, I. Weissman, and P. Siebert, *Science* **290**, 1585 (2000).

In summary, GFP variants are ideal for the study of development, cell biology, and bacterial pathogenesis; conventional reporter genes are limited by their inability to measure single-cell expression accurately in living bacterial cells.

Practical Considerations

The utility of GFP for a particular application depends very much on the type of bacteria and the precise research requirements. GFP can be used to localize bacteria in a particular environment^{25,18} or to visualize several bacterial populations at the same time, by using combinations of different GFP variants.^{26,27} Specific GFP-protein fusions can be localized in bacterial cells,²⁸ and GFP can also be used as an effective reporter of bacterial gene expression.²⁹ For each application it is important to decide whether to study live bacteria, or whether they should be fixed. Fixation allows permeabilization of cells (which provides a better access for antibodies), prevents antigen leakage, maintains cell structure, and stops all biological functions of cells, giving an instant image of what was occurring in the cell at the moment of fixation. A clear description of all types of fixatives and their advantages and disadvantages is available.³⁰ Fixation not only is critical from a safety point of view when working with pathogens, but also assists the study of temporal gene expression patterns through the study of sequential samples. Before choosing a fixative it is important to decide whether a sample will be analyzed directly, or whether additional staining will be necessary, which could affect GFP fluorescence.

Many studies have been performed using plasmid-borne GFP transcriptional fusions, as they provide a bright fluorescence signal and are simple to construct. However, care must be taken when studying *in vivo* expression with high copy number systems in animal models, as accurate measurements will rely on plasmid stability. We have found that selective pressure due to increased toxic GFP levels results in plasmid loss during *in vivo* studies (I. Hautefort, unpublished data, 2000). To avoid problems with plasmid stability in animal models and cultivated cell lines, we favor the use of single-copy stable integrated chromosomal GFP fusions that allow accurate quantification of GFP expression in individual cells. However, visualization of GFP expressed from a single copy requires a bright autofluorescent reporter molecule and very sensitive detection equipment.

²⁵ C. R. Beuzon, S. Meresse, K. E. Unsworth, J. Ruiz-Albert, S. Garvis, S. R. Waterman, T. A. Ryder, E. Boucrot, and D. W. Holden, *EMBO J.* **19**, 3235 (2000).

²⁶ G. V. Bloemberg, A. H. Wijfjes, G. E. Lamers, N. Stuurman, and B. J. Lugtenberg, *Mol. Plant Microbe Interact.* **13**, 1170 (2000).

²⁷ N. Stuurman, C. P. Bras, H. R. Schlaman, A. H. Wijfjes, G. Bloemberg, and H. P. Spaink, *Mol. Plant Microbe Interact.* **13**, 1163 (2000).

²⁸ A. Feucht and P. J. Lewis, *Gene* **264**, 289 (2001).

²⁹ R. H. Valdivia and S. Falkow, *Mol. Microbiol.* **22**, 367 (1996).

³⁰ E. Harlow and D. Lane, "Using Antibodies: A Laboratory Manual." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1999.

Methods to Detect or Measure Green Fluorescent Protein Expression

Several methods have been presented for assessing GFP fluorescence.⁴ In this article we describe how to visualize individual GFP-expressing bacteria by epifluorescence microscopy, fluorometry, flow cytometric analysis, and cell sorting.

Epifluorescence Microscopy and Image Analysis of Bacterial Cells

A number of studies have involved the visualization of GFP, either to localize GFP–protein fusions within bacterial cells or to monitor GFP-expressing bacteria in their environment.^{25,31} To capture images of infected tissues, cultivated cell lines or GFP-expressing biofilms, a very sensitive camera and image-grabbing system is required. Fluorescence and confocal microscopes are generally fitted with highly sensitive cooled charge-coupled device (CCD) cameras controlled by powerful analysis software. For quantitative imaging of fluorescence with a microscope, equipment requirements are even more specific, and accurate measurements of GFP fluorescence intensity *in vivo* is very difficult.³²

Fading of fluorescent molecules is a common problem in microscopy. Each fluorochrome has a limited capacity for excitation and emission and the emitted light tends to decline over time. One can limit fading by minimizing the exposure time to the source of excitation light, and by including an antifading agent in the medium used to mount the sample. Different antifading reagents are available, most of which act by scavenging free radicals liberated by excitation of the fluorochromes. The free radicals attack unexcited fluorochromes and damage them, thus producing exponential fading. The most commonly used antifade compound is triethylenediamine (Dabco) because of its solubility and chemical stability, although *p*-phenylenediamine is a useful alternative.^{33,34}

Protocols for Microscopy

We describe below a number of protocols that can be used to visualize GFP expression. They can also be used as a starting point for the development of specific protocols, as each application can require different conditions to obtain an optimal GFP signal.

MATERIALS

The equipment used in our laboratory to visualize low-level GFP expression is shown in parentheses.

³¹ V. Sourjik and H. C. Berg, *Mol. Microbiol.* **37**, 740 (2000).

³² D. W. Piston, G. H. Patterson, and S. M. Knobel, *Methods Cell Biol.* **58**, 31 (1999).

³³ G. D. Johnson and G. M. Nogueira Araujo, *J. Immunol. Methods* **43**, 349 (1981).

³⁴ G. D. Johnson, R. S. Davidson, K. C. McNamee, G. Russell, D. Goodwin, and E. J. Holborow, *J. Immunol. Methods* **55**, 231 (1982).

Epifluorescence microscope (e.g., Olympus BX51)
GFP (FITC) filter set (e.g., Excitation BP470–490nm, Emission DM500 dichroic beam splitter + BA515 barrier filters, Chroma)
Cooled CCD Camera [e.g., F-view (Norfolk Analytical, Hilgay, UK)]
Image analysis software [e.g., Analysis (SIS, Münster, Germany)]
Microscope slides and coverslips
Coating for slides [e.g., 1% (v/v) polyethyleneimine (PEI; Sigma St. Louis, MO)] or 0.01% (w/v) poly-(L-lysine)(Sigma)
Mounting medium [Mowiol 9% (w/v), Calbiochem, La Jolla, CA].

Note: For confocal microscopy Mowiol mounting medium is not appropriate since its refractive index is not well established.

Antifading agent, such as triethylenediamine (Dabco) or phenylenediamine (1 mg/ml, Sigma)
Cryostat (e.g., Reichert Jung Cryocut E, Leica)
Bunsen burner
Immersion oil
Phosphate-buffered saline (PBS) pH 7.4
Saponin (Calbiochem)
2-Methylbutane (isopentane, BDH, Poole, UK)
Liquid nitrogen
Freezing medium (OCT compound, Leica)
Supporting cork pieces to mount sample

IMAGING BACTERIA IN SUSPENSION

Method 1

1. Place uncoated slide on heating block at 45°.
2. Rapidly mix equal volumes of bacterial sample and low melting agarose in PBS (pH 7.4) at 45°, place quickly on slide, and apply coverslip.
3. Take slide directly from the block and cool at room temperature for 5 min in the dark.
4. Use phase contrast on an epifluorescence microscope to focus on bacteria, and a GFP filter set to detect GFP-expressing cells.
5. Capture images with a CCD camera using image analysis software and use for further applications.

Method 2

This method is only suitable for imaging bacteria that express high levels of GFP.

1. Coat slides for 2 min in 1% polyethyleneimine or for 1 hr at room temperature in 0.01% poly (L-lysine).

2. Wash slides for 30 min in deionized water or PBS and dry by centrifugation at 600g for 5 min at room temperature.
3. Apply 10 μ l of bacterial sample on the slide and air dry.
4. Fix bacterial cells by passing quickly through flame of Bunsen burner. *Note:* Drying samples can reduce the amount of GFP fluorescence dramatically.
5. Wash 30 min in deionized water and air dry.
6. Add mounting medium on the sample and place coverslip on top.
7. Examine as described above in Method 1, steps 4 and 5.

IMAGING OF INFECTED ANIMAL TISSUE SECTIONS OR OF CULTIVATED CELL LINES GROWN ON COVERSGLIPS

An increasing number of experiments are designed for localizing bacterial gene expression in particular infection models. The next two methods concern preparation of slides for sections of host infected tissue and observing bacterially infected mammalian cells.

Method 3: Tissue sample preparation, sectioning, and imaging

1. Fix tissue samples immediately after dissection of the bacterially infected animal host by incubating in glass or polypropylene tubes for 1 hr at room temperature in freshly prepared 4% paraformaldehyde (pH 7.4).
2. Wash thoroughly in PBS.
3. Incubate fixed tissue in 20% sucrose solution (in PBS) overnight to protect the tissue against alteration during cryopreservation. *Note:* Add 0.1% sodium azide if the incubation period at 4° extends 16 hr to prevent microbial growth.
4. Place an aluminum beaker containing 2-methylbutane into liquid nitrogen to reduce its temperature rapidly to -40° to -60°.
5. Stick the tissue sample to a piece of cork with freezing medium (OCT compound), and snap freeze in cooled 2-methylbutane.
6. Once frozen, store samples at -80° until further processing. *Note:* Storage at -80° for several months does not cause significant loss of GFP fluorescence.
7. Cut frozen sample with cryostat, and collect thin sections (4-6 μ m) directly onto freshly coated microscope slides [0.01% poly (L-lysine) (Sigma); see method 2]. Alternatively, thick cryosections (>20 μ m) are collected by floating in PBS.
8. If necessary, stain sections or cells with antibodies using 0.03% (w/v) saponin as permeabilizing agent. Staining of thick sections floating in PBS allows better access of antibody to target epitopes.
9. Mount coverslips on slides in medium containing an antifading agent (1 mg/ml phenylenediamine)
10. Examine as described above in method 1, steps 4 and 5.

Method 4: Imaging of bacteria in mammalian cells grown on coverslips

1. Grow mammalian cells in tissue culture on 0.01% poly(L-lysine)-coated coverslips and infect with the pathogen of interest.
2. If necessary, stain sections or cells with antibodies using 0.03% (w/v) saponin as permeabilizing agent and proceed as described in method 3, steps 9 and 10.

Fluorometric Analysis

Fluorometry is an easy, fast, and commonly available technique for detecting and measuring fluorescence intensity. Initially used in biochemistry for determining the luminescence properties of tryptophan in proteins or as a monitor for protein conformation,^{35,36} it has evolved into a useful tool for measuring levels of GFP expression and other fluorescent reporter systems in bacterial populations.

Fluorometry generally uses a quartz halogen lamp as light source and appropriate excitation (ex) and emission (em) filters, e.g., 485_{ex} nm/538_{em} nm for most GFP variants. Sample fluorescence should be measured in nonfluorescent cuvettes or microtiter plates using several dilutions of the samples, and compared with a recombinant GFP standard. Linear regression of the values obtained for defined concentrations of recombinant GFP allows the accurate quantification of fluorescing protein produced by a bacterial population at a particular time. Temperature-control and/or shaking options present on modern equipment allow the measurement of the kinetics of gene induction in living bacteria. Fluorometry can therefore be a fast and easy tool to screen for bacterial gene induction under various environments such as acidic pH or high osmolarity, on fixed samples or in real time, and lends itself to high throughput analysis. The detection limit of intracellular GFP in bacteria is $\sim 10^3$ molecules per bacterial cell, as described in the following example of a fluorometric analysis to investigate the variable effects of specific fixatives on GFP.

Protocols for Fluorometric Analysis

An increasing number of applications require the monitoring of temporal bacterial gene expression. Commonly, this is achieved by stopping the expression of *gfp* fusions at appropriate times by fixation. Similarly, localization of bacterial gene expression by microscopic observations of infected tissue sections or invaded cell lines usually requires fixation of the samples. Various fixatives are commonly used in microscopy or flow cytometry but can reduce GFP fluorescence. We compared the effect of several commonly used fixatives on GFPmut1 fluorescence in *Salmonella enterica* serovar *typhimurium*.

³⁵ B. R. Pattnaik, S. Ghosh, and M. R. Rajeswari, *Biochem. Mol. Biol. Int.* **42**, 173 (1997).

³⁶ E. G. Strambini and G. B. Strambini, *Biosens. Bioelectron.* **15**, 483 (2000).

TABLE II
FIXATION/PERMEABILIZATION PROTOCOLS^a

Fixative/permeabilizing agents	Incubation time (min)	Incubation temperature
100% Acetone	10	Room temperature
100% Methanol	15	4°
50% Acetone / 50% methanol	1	Room temperature
48% Acetone / 48% methanol / 4% formalin	1.5	Room temperature
Buffered formol acetone (pH 6.6)	0.5	Room temperature
4 ml 12.4 mM Na ₂ HPO ₄ , 1.22 mM KH ₂ PO ₄ 16.6 ml 4% (v/v) formalin 30 ml 100% acetone 20 ml H ₂ O		
2.5% (v/v) Glutaraldehyde	60	Room temperature
3% (w/v) Paraformaldehyde	30	Room temperature
4% (v/v) Formalin	1	Room temperature

^a Fixatives should be freshly prepared. See Fig. 1.

MATERIALS

Fluorometer with appropriate GFP filterset (e.g., excitation filter, 485 nm; HBW, 14 ± 2 nm; and emission filter, 538 nm HBW 25 ± 3 nm; Molecular Devices, Sunnyvale, CA).

Nonfluorescent flat bottom 96-well UV plate (Costar, Corning, NY).

GFP protein standard [e.g., purified recombinant enhanced green fluorescent protein (EGFP) (Clontech)].

PBS

0.22-μm membrane filter

METHOD TO ASSESS THE EFFECT OF FIXATIVES ON GFP FLUORESCENCE

1. Bacterial strains [*S. typhimurium* NCTC 12023 wild type [(identical to ATCC 14028s³⁷)] and JH2031, a 12023 derivative that contains a single chromosomal transcriptional *ssrA::gfpmut1* fusion) are grown in Luria–Bertani (LB) broth (10 g tryptone, 5 g yeast extract, 5 g NaCl per liter distilled, deionized water, pH 7.0) overnight at 37° in a shaking incubator at 250 rpm.

2. One ml of bacterial culture is harvested by centrifugation at room temperature in a microfuge at 6,000g for 5 min. *Salmonella* cells are then washed twice with 0.22 μm filtered PBS to remove the autofluorescent LB media. The number of bacteria is determined by optical density measurement (OD_{600nm}), adjusted to the same number for both strains and centrifuged again.

³⁷ J. Deiwick, T. Nikolaus, J. E. Shea, C. Gleeson, D. W. Holden, and M. Hensel, *J. Bacteriol.* **180**, 4775 (1998).

3. Bacterial pellets are resuspended in 1 ml of the fixative agent and treated as described in Table II.

4. Samples are subsequently washed three times, resuspended in PBS, and analyzed as follows: PBS is used as blank. Samples are twofold serially diluted in PBS. Duplicates of (diluted) samples are placed in individual wells of a 96-well microtiter plate. The highest concentration of bacteria was 1.2×10^8 cells per well. Duplicates of a series of recombinant EGFP dilutions (156 pg/ml to 5 ng/ml) are used to establish a standard curve.

5. The fluorescence of each well is determined using the GFP filter set on a fluorometer.

6. Quantitation of GFP fluorescence in terms of micrograms of recombinant EGFP protein is obtained by converting the relative fluorescence units (RFU) from the linear regression standard curve. The results are presented in Fig. 1. The amount of GFP fluorescence in the untreated JH2031 sample, corresponding to the fluorescence of 0.3 μ g of EGFP protein, is set as the 100% reference.

Figure 1 shows the effect of seven fixation procedures on GFP fluorescence. Acetone fixation results in a good preservation of GFP fluorescence, but as it permeabilizes the membrane, GFP will leak out of cells and complicate flow cytometric (see Fig. 4 on p. 61) and microscopic analysis. Methanol-based fixation has a dramatic effect on GFPmut1 fluorescence and is an unsuitable fixative for GFP. Glutaraldehyde increases the autofluorescence of the wild-type *Salmonella* strain considerably (220% brighter) and is therefore not appropriate for GFP-based studies. However, GFPmut1 fluorescence is largely unaffected by fixatives such as paraformaldehyde, formalin, and buffered formol acetone. The requirement of subsequent antibody labeling procedures will determine which of these three fixatives is most suitable.

Fluorometry provides a quick and useful way of measuring GFP fluorescence of a bacterial population, but has several limitations:

1. First, most of the media in which bacteria are grown is autofluorescent. Complex media such as LB often contain flavonoids, which emit light in the same wavelength range as GFP. To prevent this problem, bacterial samples need to be washed and resuspended in a nonfluorescent solution such as PBS. To perform kinetic measurements, in real time, the choice of a minimal growth medium with a lower level of autofluorescence is an option. Unfortunately, this limits the number of environmental signals that can be tested and restricts the detectable fluorescence intensity range, preventing the measurement of low levels of fluorescence.

2. A minimal number of bacteria expressing GFP are required to produce measurable fluorescence. GFP fluorescence detection occurs as soon as its intensity reaches at least 3 times or more the level of the negative control (here non-GFP expressing bacteria). For example on a Molecular Devices fMax fluorometer, 5×10^5

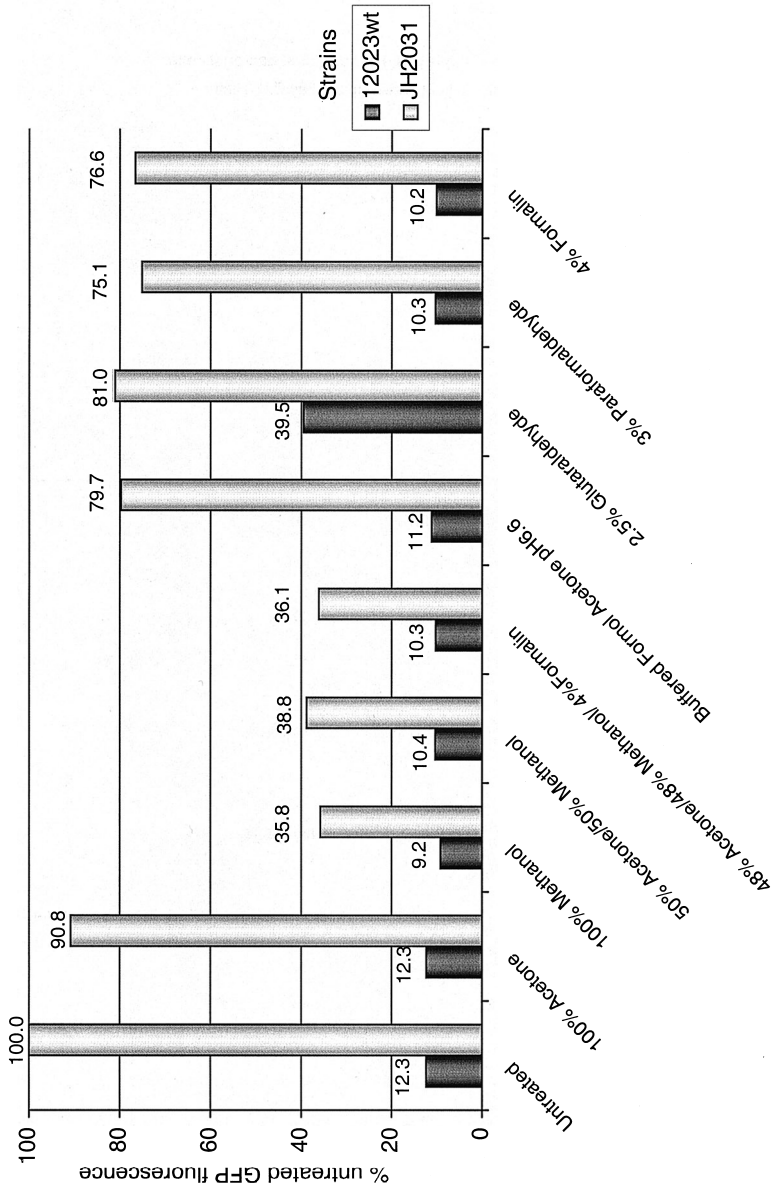


FIG. 1. Effect of various fixatives on GFP fluorescence. *Salmonella enterica* s.v. *typhimurium* 12023 derivative (JH2031, light bars) containing a single copy chromosomal *ssrA::gfpmut1* fusion was treated with various fixatives and compared with the wild-type 12023 strain (dark bars) as described in the text and Table II. Bacterial cells were subsequently washed three times in filtered PBS and green fluorescence was measured in microtiter plates in an iMax fluorometer (Molecular Devices). A purified recombinant EGFP protein standard (Clontech) was used.

Salmonella cells containing a chromosomal *ssrA::gfpmut1* fusion (Fig. 1) is the detection limit. This corresponds to $\sim 10^3$ molecules of EGFP per bacterial cell.

3. Fluorometry measures the total sum of fluorescence intensities of all bacterial cells in a population, which is not a good indicator of single-cell gene expression, as it cannot discriminate between cell-to-cell variations in gene expression. In contrast, new fluorescence-based technologies and improvements of existing techniques such as flow cytometry allow monitoring fluorescence of a more limited number of bacterial cells, even down to the level of single bacterial cells. However, this requires expensive equipment and experience to optimize and interpret parameters and data (see below).

Overall, fluorometry is a relatively inexpensive and rapid technique for measuring and comparing GFP fluorescence which does not require specific skills or experience. It can be very useful for high-throughput screening experiments using particular environmental inducing or repressing conditions. Kinetics of gene expression can also be measured with equipment offering temperature-control and/or orbital shaking options. However, the relatively low sensitivity of most systems has led researchers to explore technologies that allow monitoring GFP gene fusion expression at the single bacterial cell level, such as flow cytometry.

Flow Cytometry of Microorganisms

Flow cytometry is a powerful technique for studying individual mammalian or bacterial cells within a population. It has been used extensively for eukaryotic studies, such as immune cell maturation and cytokine production.³⁸ However, the relatively small size of microorganisms is close to the detection limit of flow cytometers, but the use of stains, autofluorescent proteins, and suitable antibodies make it possible to discriminate them from background noise. Flow cytometry can now be used for rapid analysis of individual bacterial cells, permitting the quantitative analysis of microbial heterogeneity.³⁹

Flow cytometry is a laser-based system capable of detecting many types of cells. Samples containing the cell population to be analyzed and/or sorted are carried in a fluidic stream to a specialized chamber where they are hit with a laser beam. Forward-scattered and 90° side-scattered light (respectively, FSC and SSC) is detected, and its intensity quantified and stored for each particle. Forward-scattered light gives information about the size of mammalian cells, but it is not accurate for small particles such as bacterial cells. Side-scattered light is a good indicator of mammalian cell granulometry and is useful for discriminating bacterial populations from other particles and electronic noise. When fluorescent stains are

³⁸ H. M. Shapiro, "Practical Flow Cytometry." Wiley-Liss, New York, 1995.

³⁹ G. Nebe-von-Caron, P. J. Stephens, C. J. Hewitt, J. R. Powell, and R. A. Badley, *J. Microbiol. Methods* **42**, 97 (2000).

used, the emitted fluorescence can also be detected by flow cytometry, amplified, and stored for each particle that passes through the laser beam.⁸ This approach allows information concerning DNA content, the amount of protein, cell cycle, and membrane integrity to be collected for each bacterial cell analyzed within an entire population.^{39,40}

The use of GFP in flow cytometry has facilitated major developments in eukaryotic and prokaryotic studies. In mammalian cells, GFP expression is often used as a selective marker for transfection experiments. In bacteria, the use of GFP has been based on the development of more soluble, brighter, blue- or red-shifted GFP mutants which were specifically selected for flow cytometric analysis.^{6,7,9,41} Because bacterial cells are at the lower detection limit of most commercially available flow cytometers, it is very difficult to discriminate between bacterial populations and electronic noise or dust particles in the carrier fluidic stream. Optimization procedures such as 0.1 μm filtration of the carrier fluid and fluorescent labeling of bacteria using dyes, fluorophore tagged antibodies or the use of bright GFP as a reporter system can largely overcome this. The ability of flow cytometry to distinguish between different levels of fluorescence intensity allows the monitoring of gene expression in individual bacteria, and the sorting of cells displaying distinct levels of GFP expression.⁴² The multiparameter information that is collected and stored for each bacterium or particle can be used to effectively display information on bacterial populations and on individual cells via histograms, dot plots, density plots, contour plots, and three-dimensional plots, depending on the type of information to be highlighted. Histograms depict one parameter with its intensity, whereas dot plots, density plots, and contour plots illustrate the distribution of particles within a population for two different parameters displayed (see Fig. 3). Three-dimensional plots allow separation of particle populations for more than two parameters at the same time. For each parameter detected, statistical analysis is possible at the level of each particle, allowing determination of gene induction in individual bacterial cells.

Protocol for GFP Analysis with a Benchtop Flow Cytometer

GFP detection in benchtop flow cytometers (e.g., Becton Dickinson FACScan or FACScalibur; Partec PASIII) can be performed with a standard argon ion laser, tuned at 488 nm for GFP excitation, with bandpass filters centered around 510–515 nm (e.g., 515/40 or 530/30). In the bigger and more complex flow cytometers (Coulter Epics or Altra, Becton Dickinson FACSvantage), tuneable lasers allow the efficient use of different GFP variants that possess excitation wavelengths distinct from 488 nm. The laser power must remain below 100 mW to avoid high noise levels on forward-scattered light detection.

⁴⁰ J. Vives-Rego, P. Lebaron, and G. Nebe-von-Caron, *FEMS Microbiol. Rev.* **24**, 429 (2000).

⁴¹ K. R. Siemering, R. Gollbik, R. Sever, and J. Haseloff, *Curr. Biol.* **6**, 1653 (1996).

⁴² R. H. Valdivia and L. Ramakrishnan, *Methods Enzymol.* **326**, 47 (2000).

MATERIALS

Benchtop flow cytometer (e.g., FACScalibur, Becton Dickinson)
Suitable software (e.g., Cell Quest, Becton Dickinson)
PBS, pH 7.4, 0.22 μM filtered
Vortex
Sonicating water bath
Vacuum system
FACS (fluorescence activated cell sorter) tubes
Dulbecco's modified Eagle's medium (DMEM; GIBCO)
Fetal bovine serum (FBS; GIBCO)
24-Well tissue culture plates
1% (v/v) Triton X-100 (Sigma)
4% Formalin (Sigma)

SETUP

1. Switch on flow cytometer and computer according to manufacturers' description and acquire data from the bacterial sample to set up parameters for the amplification voltages. As detection of bacteria requires the collection of information with logarithmic amplifiers for both light scattering and fluorescence, the dynamic range of detection is increased at the expense of higher background noise. *Note:* Using 0.1 μM filtered PBS and sheath fluid can reduce background noise.

2. The voltages of the FSC photodiode and SSC photomultiplier (PMT) detectors are gradually increased until the bacterial population is detected and clearly visible.

3. The voltage of the fluorescence PMT detector is gradually increased to set the detection borders of the lowest and highest intensity. This requires the use of fluorescent and nonfluorescent bacterial cells. The voltage is usually increased until the nonfluorescing population remains within the first log decade of the plot scale. If several fluorescent labels are used, compensation is set up to adjust spectral overlap. Unlabeled bacteria, bacteria labeled with each fluorescent stain separately, and bacteria labeled with both stains are required to set up compensation. It is important that compensation for each color be set using the brightest stained population. *Note:* Once set up, compensation and PMT voltage should remain unmodified throughout acquisition.

4. The size of the sample will vary depending on the type of experiment. For eukaryotic samples a concentration of around 10^6 cells/ml is used. If the aim of the work is to look at the predominant bacterial population, fluorescence analysis of 5×10^3 individual bacterial cells (around 10^6 cells/ml using the lowest flow rate, $12 \mu\text{l} \pm 3 \mu\text{l}/\text{min}$) usually gives reliable quantification of gene expression. A greater number of bacteria is necessary to look at a specific subpopulation. This

number can easily be increased if the subpopulation of interest represents less than 1% of the total population.⁸

Optimization of Bacterial Flow Cytometry. Aggregation and clumping of bacterial cells often occurs during flow cytometric analysis of certain bacterial species, such as mycobacteria. To overcome this problem, detergents can be used and samples sonicated prior to analysis.¹³ However, it is important to avoid reagents that affect GFP fluorescence.

The generation of aerosols during flow cytometric analysis of bacteria gives rise to safety concerns. The risk is particularly significant with droplet-forming flow cytometers (e.g., Coulter Epics or Altra, Becton Dickinson FACSVantage). On these machines, droplets of carrier fluid can be formed which only contain one particle. Working with pathogens on droplet-forming flow cytometers requires the machine to be located in an appropriate containment facility and an effective decontamination procedure to be used. A long rinse followed by 10% (v/v) bleach solution is usually sufficient, or the use of 0.5% sodium dodecyl sulfate (SDS) followed by 95% (v/v) ethanol. However, the use of ethanol risks DNA precipitation in the tubing of the fluidic system. An attractive alternative is to avoid the use of live bacterial cells by treating with fixative prior to analysis, which is appropriate for many applications.

Detection of GFP fluorescence as a reporter of gene expression requires the gene promoter of interest to be transcribed at a reasonable level. To ensure signal detection, most studies have used plasmid-borne transcriptional GFP fusions as reporter of gene activity. Such multicopy GFP expression allows optimal separation of green fluorescing bacteria from background noise. Figure 2 illustrates the expression of an *ssrA::gfpmut1* transcriptional fusion in *S. typhimurium*, either carried on a plasmid (pJSG110) or as a single copy integrated on the chromosome (strain JH2031). We favor a merodiploid approach, where the *gfp* gene fusion of interest is integrated at a specific site on the chromosome which does not affect virulence.

GFP expression of the plasmid-borne *ssrA::gfpmut1* fusion is easily detectable above the autofluorescence from the wild-type strain, as the presence of multiple *gfp* copies results in increased GFP fluorescence. However, such high levels of GFP protein can be toxic for the cells, resulting in partial or complete loss of the plasmid in animal models or cultivated cell lines (I. Hautefort, unpublished, 2000). Such toxicity could give rise to variable plasmid copy number between cells, preventing the accurate measurement of gene expression. However, *Salmonella* cells expressing the chromosomal *ssrA::gfpmut1* fusion only produce fivefold more GFP fluorescence than the autofluorescence of the negative control, illustrating the challenge of measuring low levels of gene expression. Importantly, chromosomal integrated fusions have the advantage of genetic stability and are present at the same copy number as the wild-type gene of interest, making them more reliable than plasmid-borne fusions.

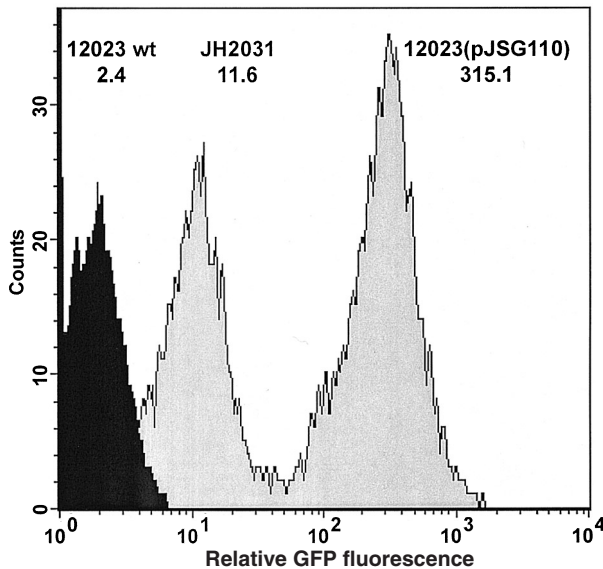


FIG. 2. Flow cytometric analysis of *S. typhimurium* 12023-derived strains expressing a *ssrA::gfpmut1* transcriptional fusion either from a plasmid (12023(pJSG110)), or as a single copy integrated on the chromosome (JH2031) compared with wild type 12023 (in black) that does not harbor any *gfp* gene. Strains were grown overnight in LB broth, fixed in 4% formalin solution for 1 min, and washed in PBS prior to their analysis by flow cytometry in a FACScalibur (Becton Dickinson). Relative GFP fluorescence of each strain is indicated.

Specific fluorescent antibodies that recognize the pathogen under study offer a useful method for discriminating bacteria from background noise. This approach allows the detector of the flow cytometer to be triggered first on the fluorescent antibody signal [for example, phycoerythrin (PE) that is measurable by a different detector than is used for GFP]. This PE-labeled population can be selected by drawing a region around it, a procedure called gating (see Fig. 5B). Only the GFP fluorescence of the gated population is subsequently measured, avoiding problems with background fluorescence. Figure 3 shows the flow cytometric separation of a mixture of GFP-expressing *Salmonella* and the corresponding wild-type strain.

Antibody labeling allows the discrimination between the bacterial population of interest and background noise, to generate robust experimental data (Fig. 3). However, suitable antibodies are not always available and the stability of some surface epitopes can be modified by fixation, preventing the use of antibodies. Intensity of the chosen fluorophore and its sensitivity to photobleaching also need to be considered. The use of live bacteria limits antibody use, and the stability of the antibody-antigen interaction can be lost on sorting. Although the use of antibodies allows a better distinction between the bacterial population and noise,

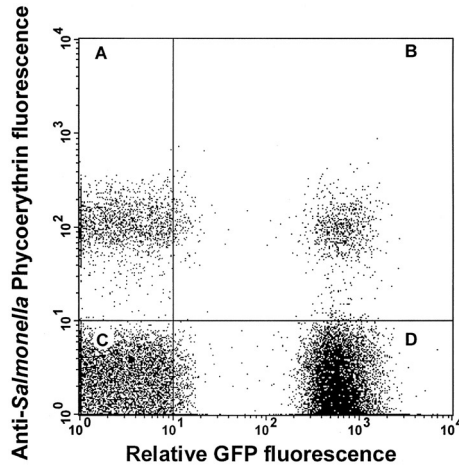


FIG. 3. Flow cytometric separation of wild-type and GFP-expressing *S. typhimurium* populations, either unlabeled or labeled with a phycoerythrin-conjugated anti-*Salmonella* antibody. Quadrants A and B show phycoerythrin-labeled SL1344 wild type and SL1344 expressing a plasmid-borne *rpsM::gfpmut3* fusion pFPV25.1 (29), respectively. Quadrants C and D show the same populations without label.

optimizing specific labeling procedures for the bacterial species of interest can be required.

The visualization of *gfp* fusions that are expressed at low levels requires a substantial increase in GFP brightness. This enables discrimination of the GFP-expressing bacteria from non-expressing cells and background noise. A variant of GFP, called GFP⁺, has shown 130-fold brighter fluorescence than the wild-type protein (21). GFP⁺ contains the GFP_{uv} and GFPmut1 mutations, which improve the folding and enhance brightness of the protein, respectively (Table I). GFP⁺ exhibits great promise for monitoring gene expression in bacterial cells. We tested the effect of acetone fixation on GFP⁺ fluorescence (Table II) by flow cytometry. As shown in Figs. 4A and 4B, acetone changes the light scattering properties of the GFP-expressing *E. coli* cells that carry a plasmid-borne *rpsM::gfp⁺* fusion. First, acetone treatment reduces the intensities of side- and forward-scattered light, suggesting modification of the bacterial cell surface. Second, the bacterial population is tightly clustered before treatment (Fig. 4A) while appearance of cell debris is visible in Fig. 4B. An important advantage of flow cytometry compared with fluorometry is shown in Fig. 4C. Although the 10% reduction in overall GFP⁺ fluorescence is for both methods the same (Figs. 1 and 4C), flow cytometry discriminates two distinct populations after acetone treatment. GFP⁺ fluorescence of the minor population, 25% of the bacteria, is reduced by 98%, likely due to GFP leakage. Therefore, acetone fixation cannot be trusted for flow cytometric analysis of GFP⁺ fluorescence in bacteria.

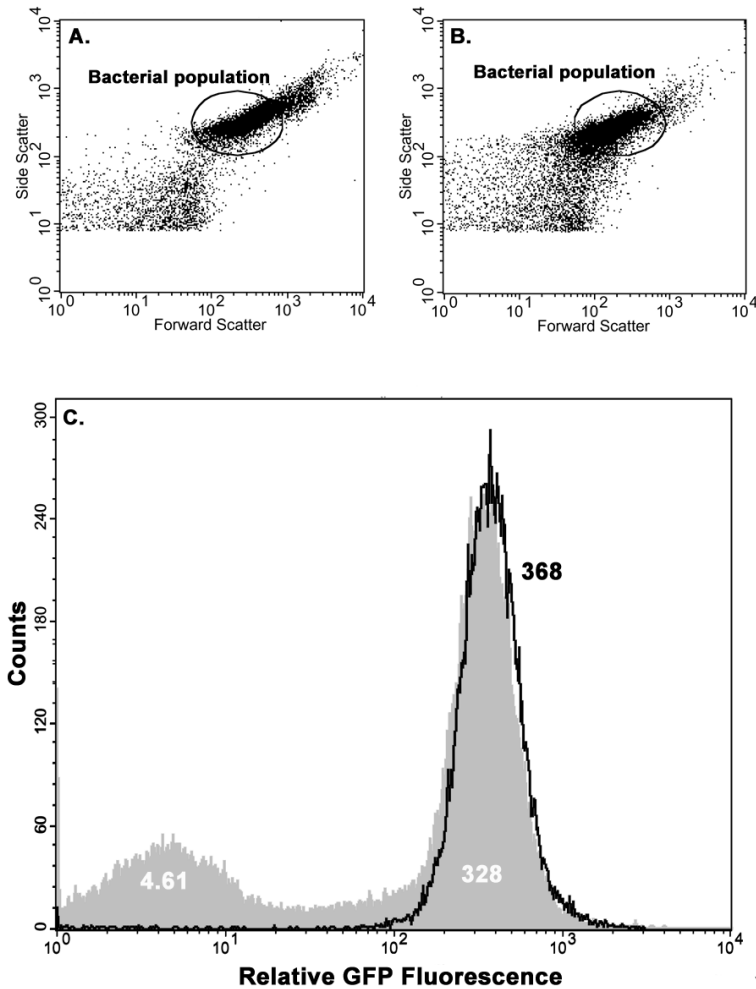


FIG. 4. The effect of acetone on GFP fluorescence in *E. coli* harboring a plasmid-borne *rpsM::gfp⁺* fusion measured by flow cytometry. (A) and (B) shows the light scattering properties of the bacterial cells before (A) and after (B) acetone treatment. GFP⁺ fluorescence was determined in the selected bacterial gated population. (C) compares GFP⁺ fluorescence before (solid line) and after (gray shaded line) acetone treatment. Numbers correspond to the relative GFP fluorescence intensities.

Fluorescence Activating Cell Sorting (FACS) of Bacteria. One of the most powerful features of flow cytometers is the ability to sort cells. Sorting of particles can be done on flow cytometers equipped with a droplet-forming flow-in-air sorting system (e.g., Coulter Epics or Altra, Becton Dickinson FACS Vantage) or electromechanically on a benchtop flow cytometer (e.g., Becton Dickinson FACS Calibur; Partec PASIII). Cell sorting has proved immensely important for

eukaryotic research and is gaining popularity in microbiology. The use of stains, autofluorescent proteins, and suitable antibodies makes it possible to discriminate bacteria from background noise and facilitates microbial cell sorting. Although commercial droplet-forming flow cytometers are able to sort single cells rapidly, they are costly, mechanically complex, and require highly trained personnel for operation and maintenance. Cell sorting is suited to a wide range of applications and produces valuable multiparameter information at a single bacterial cell level. Publications have demonstrated the possibilities of this technique.^{39,40}

Cell sorting is also possible on benchtop flow cytometers, although the mechanism of sorting does not enable the fast, accurate single-cell sorting in small volumes that can be achieved with flow-in-air machines. Benchtop flow cytometers lack the ability to sort bacteria and deposit individual cells onto agar plates, microscope slides, or microtiter plates. For further reading an excellent article describing protocols and application of GFP in bacteria and FACS in a benchtop flow cytometer is recommended.⁴² An interesting development is the production of a disposable microfabricated fluorescence-activated cell sorter, which sorts by means of electroosmotic flow and has been used for enrichment of living GFP-expressing *E. coli* cells.⁴³

Conditional Gene Expression during Invasion

Cultivated cell lines offer a simple model for the study of bacterial virulence gene expression. For pathogens such as *Salmonella typhi*, which lack an animal model, cultivated cell lines play an important role. Intracellular bacterial gene expression has been studied within various types of eukaryotic cells. Inoculum doses and incubation time necessary for the invasion of mammalian cells by the bacterial strain vary depending on the pathogen of interest. For example, *S. typhimurium* invades cultivated cells mainly within the first 45 min after infection.⁴⁴

Protocol for Bacterial Invasion of Mammalian Cells

The protocol presented here is an example of how to measure and compare gene expression levels of a *gfp* transcriptional fusion in intracellular and extracellular bacteria, to determine the level of induction of gene expression during invasion of the host cell.

1. Mammalian cells used for bacterial invasion are grown to confluent monolayers in 24-well plates in tissue culture medium such as Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. In parallel, the pathogen containing the *gfp* gene fusion to be tested is grown in appropriate medium for inducing invasion gene expression. For instance, *S. typhimurium*

⁴³ A. Y. Fu, C. Spence, A. Scherer, F. H. Arnold, and S. R. Quake, *Nat. Biotechnol.* **17**, 1109 (1999).

⁴⁴ B. D. Jones, C. A. Lee, and S. Falkow, *Infect. Immun.* **60**, 2475 (1992).

invasion genes are induced when the bacteria are grown overnight under high osmolarity and low oxygen.^{45,46}

2. Infection is generally preceded by at least two washing steps of the cell monolayer in prewarmed DMEM. The multiplicity of infection (MOI) with bacteria can vary from 5 : 1 to 100 : 1 for the same pathogen depending on the study. Bacteria can be gently centrifuged at room temperature (5 min, 2000g) onto the monolayer prior to incubation in order to maximize the contact between pathogen and eukaryotic cells. Infected cells are incubated at 37° in 5% (v/v) CO₂ atmosphere, or in normal atmosphere when the tissue culture medium contains sodium bicarbonate.

3. At the end of the incubation the monolayer is washed at least twice with prewarmed DMEM. To compare bacterial gene expression inside and outside mammalian cells after incubation, the traditional gentamicin protection approach is not required. Prewarmed DMEM is added and incubation is carried on for as long as appropriate for the study (usually 2 to 5 hr). At the end of the experiment, the supernatant containing extracellular bacteria is removed, and bacteria are fixed and analyzed by flow cytometry.

4. The monolayer is washed three times with ice-cold PBS. Subsequently, 200 μ l of 1% (v/v) Triton X-100 is added per well and incubated for 10 min on ice, while carefully pipetting the solution up and down to avoid foaming. Then 800 μ l PBS is added per well and lysed cells are scraped from the bottom of the well with a pipette tip. Intracellular and extracellular bacteria are fixed in 4% formalin for 1 min at room temperature, washed three times in PBS, and analyzed by flow cytometry.

Figure 5C shows induction of *ssaG::gfpmut3* plasmid-borne fusion (*mig10*) in *S. typhimurium*, 6 hr after invasion of epithelial HEp-2 cells. We observed 400-fold *ssaG* induction, as previously observed during invasion of macrophages, HEp-2, or dendritic cells.¹⁰ Figure 5A illustrates the use of a specific anti-*Salmonella* antibody to separate bacterial cells from HEp-2 cell debris. Without such labeling, it is very difficult to detect a small GFP-expressing bacterial population, because of the large amount of epithelial cell debris that causes the majority of acquired events.

*Differential Fluorescence Induction (DFI): A Powerful Technique to Identify *ivi* Genes*

Differential fluorescence induction (DFI) was developed to identify *ivi* genes and to measure *in vivo* gene expression levels.¹⁰ It is an alternative and complementary technique to the IVET promoter-trap approach or to the STM negative

⁴⁵ J. E. Galan, K. Nakayama, and R. D. Curtiss, *Gene* **94**, 29 (1990).

⁴⁶ V. Bajaj, R. L. Lucas, C. Hwang, and C. A. Lee, *Mol. Microbiol.* **22**, 703 (1996).

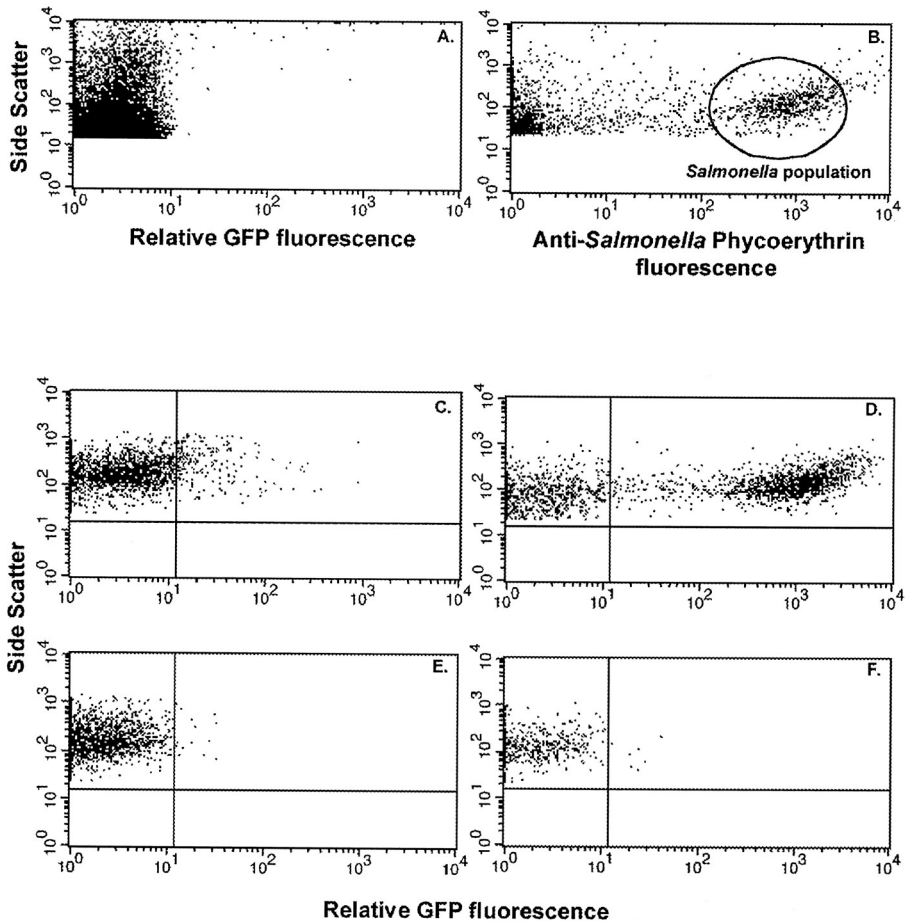


FIG. 5. Induction of *Salmonella ssaG* gene expression during invasion of epithelial HEp-2 cells. *S. typhimurium* SL1344 strain harbouring a plasmid-borne *ssaG::gfpmut3* fusion 10 (C,D) was used to infect HEp-2 cell monolayers in comparison with the wild-type SL1344 strain (E,F). Six hours after infection detergent was used to release intracellular bacteria which were analyzed by flow cytometry. To facilitate identification of the bacterial population among the green fluorescent HEp-2 cell debris (A), released bacteria were stained with a specific PE-conjugated anti-*Salmonella* antibody (B). Intracellular bacterial fluorescence was measured on the selected *Salmonella* gated population (D,F) in comparison with extracellular bacteria that remained outside the eukaryotic cells (C,E).

selection. DFI is based on the insertion of random chromosomal DNA fragments of the pathogen of interest into a plasmid vector upstream of a promoterless *gfp* gene and is described in Fig. 6. Bacteria containing *gfp* gene fusions are pooled and either used to infect cultured mammalian cells or exposed to various environmental stimuli *in vitro*, such as low pH. Bacteria with induced *gfp* expression are analyzed by

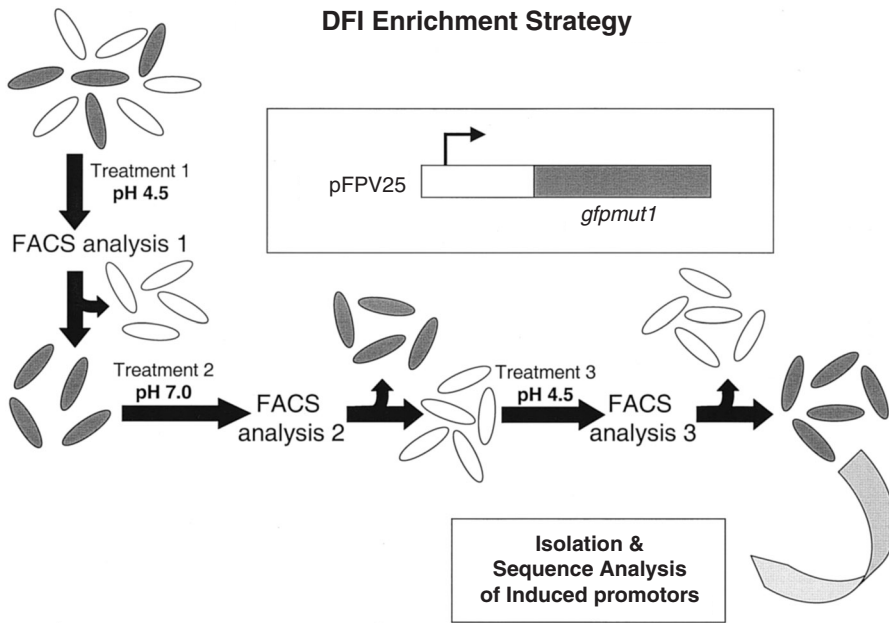


FIG. 6. Enrichment strategy of differential fluorescence induction. Redrawn from R. H. Valdivia and S. Falkow, *Mol. Microbiol.* **22**, 367 (1996).

flow cytometry and sorted on the basis of increased GFP fluorescence. The sorted bacterial population is subsequently grown in normal laboratory culture conditions and reanalyzed by flow cytometry. Only the bacteria which do not express GFP in these conditions are sorted, to eliminate constitutively expressed genes. A second selective round is performed and followed by flow cytometric analysis and sorting to ensure selection of genes that are induced only either during invasion or after exposure to a specific environmental signal. DNA fragments inserted upstream of the promoterless *gfp* gene that were responsible for GFP expression under the selective conditions are subsequently sequenced and identified.

DFI has identified genes of *S. typhimurium* that were induced on invasion of macrophages or dendritic or epithelial cells,¹⁰ or under acidic conditions.²⁹ DFI has also been applied to other bacterial pathogens such as mycobacteria⁴⁷⁻⁴⁹ and appears to be a promising approach for studying virulence gene expression during

⁴⁷ L. Kremer, A. Baulard, J. Estaquier, O. Poulain-Godefroy, and C. Locht, *Mol. Microbiol.* **17**, 913 (1995).

⁴⁸ S. Dhandayuthapani, L. E. Via, C. A. Thomas, P. M. Horowitz, D. Deretic, and V. Deretic, *Mol. Microbiol.* **17**, 901 (1995).

⁴⁹ L. Ramakrishnan, N. A. Federspiel, and S. Falkow, *Science* **288**, 1436 (2000).

infection by several pathogens. The potential of DFI has been enhanced by use of optical trapping, resulting in the identification of environmentally induced genes in single bacterial cells.⁵⁰

However, DFI possesses several limitations; like IVET, it will not identify genes that already exhibit GFP expression *in vitro*, but are essential for successful infection of a host by the pathogen. More importantly, DFI will only identify genes that are switched from OFF to ON *in vivo*; it does not give information about the role of *ivi* genes during infection.⁵¹

Summary

To date, the majority of studies of bacterial gene expression have been carried out on large communities, as techniques for analysis of expression in individual cells have not been available. Recent developments now allow us to use reporter genes to monitor gene expression in individual bacterial cells. Conventional reporters are not suitable for studies of living single cells. However, variants of GFP have proved to be ideal for the study of development, cell biology, and pathogenesis and are now the reporters of choice for microbial studies. In combination with techniques such as DFI and IVET and the use of flow cytometry and advanced fluorescence microscopy, the latest generation of GFP reporters allows the investigation of gene expression in individual bacterial cells within particular environments. These studies promise to bring a new level of understanding to the fields of bacterial pathogenesis and environmental microbiology.

Acknowledgments

We are grateful to Reginald Boone, Kamal Ivory, Margaret Jones, Douglas Kell, Gerhard Nebevon-Caron, Jonathan Porter, Howard Shapiro, and Raphael Valdivia for sharing expertise, and we thank Maria José Proenca for technical assistance. I.H. is supported by a Training and Mobility of Researchers fellowship from the European Union (contract number ERBFMRXCT9). R.J.M.B. and J.C.D.H. are supported by the BBSRC.

⁵⁰ D. Allaway, N. A. Schofield, M. E. Leonard, L. Gilardoni, T. M. Finan, and P. S. Poole, *Environ. Microbiol.* **3**, 397 (2001).

⁵¹ I. Hautefort and J. C. D. Hinton, *Methods Microbiol.* **31**, 55 (2002).