22 Moore, S.C. *et al.* (2002) The elusive structural role of ubiquitinated histones. *Biochem. Cell Biol.* 80, 311–319
23 Baarends, W.M. *et al.* (1999) Histone ubiquitination and chromatin remodeling in mouse spermatogenesis. *Dev. Biol.* 207, 322–333
24 Chen, H.Y. *et al.* (1998) Ubiquitination of histone H3 in elongating spermatids of rat testes. *J. Biol. Chem.* 273, 13165–13169
25 Tovich, P.R. and Oko, R.J. (2003) Somatic histones are components of the perinuclear theca in bovine spermatozoa. *J. Biol. Chem.* 278, 32431–32438
26 Jasinskiene, N. *et al.* (1995) Embryonic regulation of histone ubiquitination in the sea urchin. *Dev. Genet.* 16, 278–290

0167-7799/$ - see front matter
doi:10.1016/j.tibtech.2004.03.009

Realization of β-lactamase as a versatile fluorogenic reporter

Robert E. Campbell

Department of Chemistry, University of Alberta, Edmonton, AB T6G 2G2, Canada

β-Lactamase has emerged as the heir apparent to β-galactosidase as a catalytic reporter for imaging biological events in live mammalian cells. In recent years, several publications have demonstrated the advantages of β-lactamase as a reporter in applications ranging from monitoring of gene transcription to detection of protein–protein interactions. Now, Rao *et al.* have demonstrated that β-lactamase can also serve as a sensitive fluorogenic reporter for imaging *Tetrahymena* ribozyme activity in live cells. This assay should pave the way for the screening of large libraries of ribozyme mutants by flow cytometry and, therefore, the isolation of variants with improved splicing activity in the cytoplasm of mammalian cells.

For endeavors ranging from academic-scale fundamental cell-biology research [1] to applied industrial-scale high-throughput screening [2,3], live-cell fluorescence-based assays are among the most powerful tools at a biologist's disposal. The popularity and utility of these approaches can be largely attributed to the autofluorescent proteins (FPs) from *Aequorea* jellyfish and various species of coral, which have become the most important enabling reporter technology available [4,5]. Although FPs are nearly ideal labels for fluorescent tagging of recombinant proteins in live cells, as reporters of gene expression they are considerably less than perfect. An individual FP molecule harbors a single fluorophore, meaning that ~0.1–1 μM cytosolic FP is necessary for the reliable detection of gene expression in a live mammalian cell [6,7]. By contrast, a concentration of a catalytic reporter that is a thousand times less (~10^{-4} μM) can generate a robust fluorescent signal that is measurable by flow cytometry or fluorescence microscopy [8]. However, because catalytic reporter systems comprise a recombinant enzyme and an exogenous fluorogenic substrate, these systems are more complex to employ than FPs and present several additional considerations [8,9]. For example, the fluorogenic substrate must be membrane permeable, non-toxic and generate a highly polar-fluorescent product that does not leak from the cell owing to passive diffusion across the plasma membrane. The reporter-enzyme activity must not occur naturally in the host organism, and yet the introduction of this molecular alien must not have any adverse effect on normal cell function. It was an acute awareness of these considerations and dissatisfaction with traditional reporter genes, such as *lacZ* (the gene encoding β-galactosidase), that prompted Tsien *et al.* [8] to develop a new catalytic reporter system based on β-lactamase (the amp^R gene product) (Figure 1a). In recent years, this versatile reporter system has been used extensively as a sensitive reporter of gene expression, but it has also been modified to detect both protein–protein interactions [10–12] and the fusion of HIV-1 viral particles [13]. Most recently, the β-lactamase reporter system has been modified by Rao and coworkers for the detection of *Tetrahymena* ribozyme activity in live mammalian cells [14].

β-Galactosidase: the classic reporter system

The β-lactamase reporter system (Figure 1a) has had to prove its worth in the shadow of the traditionally favored β-galactosidase reporter system (Figure 1b). Although there are certain circumstances in which these two enzymes can work in conjunction (e.g. in vectors for blue–white cloning with ampicillin selection), they are strictly competitors when acting as reporters of gene expression in mammalian cells. The most sensitive fluorogenic substrate for detecting β-galactosidase in single live mammalian cells is fluorescein di-β-D-galactopyranoside (FDG) [15,16] (Figure 1b). However, the β-galactosidase–FDG reporter system has several drawbacks that reflect the genesis of a reporter system that was not designed, but rather appropriated, for use in live mammalian cells. As discussed, a live mammalian cell reporter system is subject to several considerations that are simply not relevant to β-galactosidase assays for most histochemical and routine applications in molecular biology. Some commonly cited drawbacks of β-galactosidase include its large size (the monomer is 116 kDa), obligate tetramerization and endogenous expression in some mammalian cell types [8,9,11,16]. FDG, as with most β-galactosidase substrates, is not readily membrane permeable (Figure 1b). However, the

Corresponding author: Robert E. Campbell (robert.e.campbell@ualberta.ca).

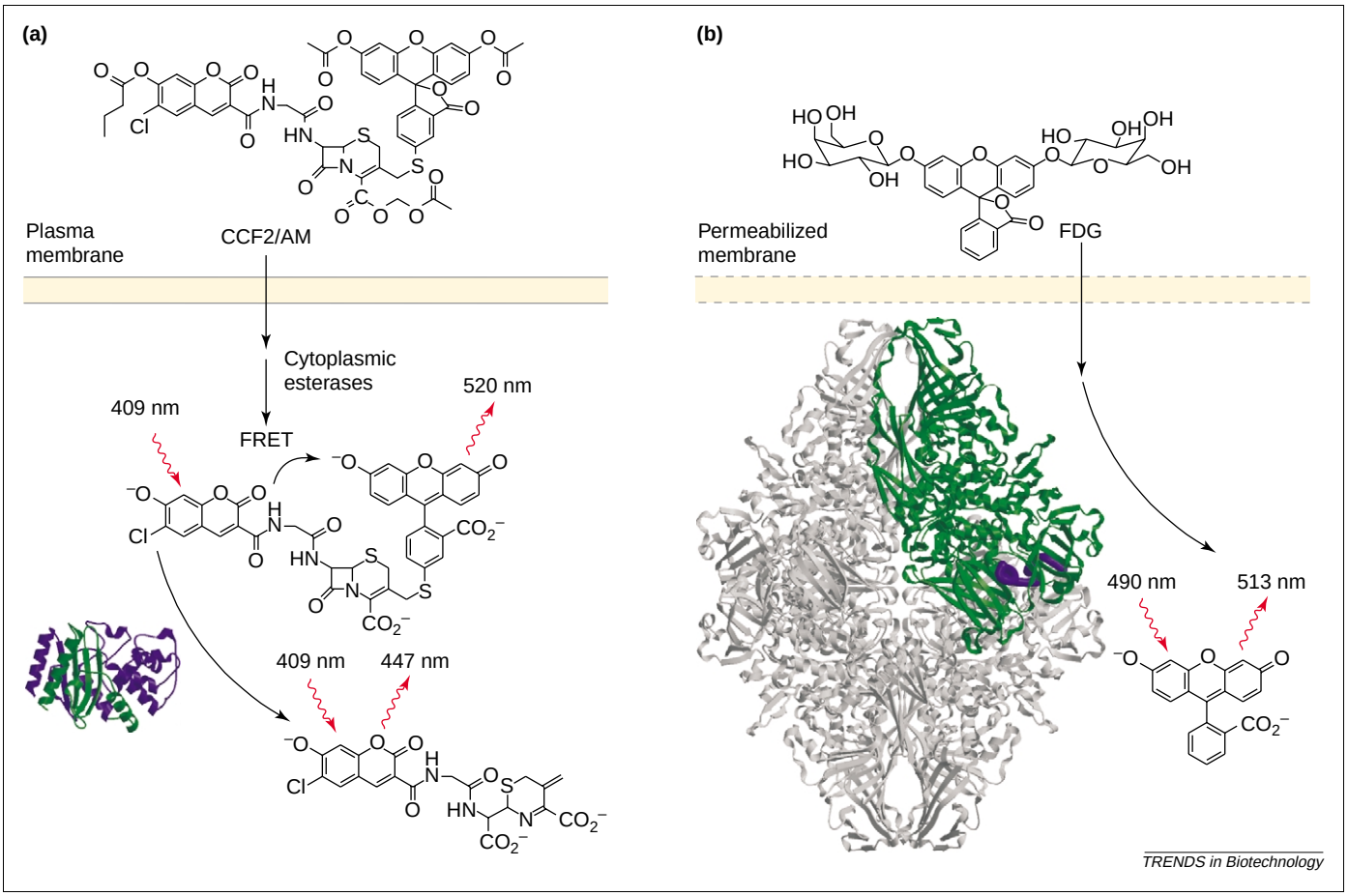

Figure 1. β-Lactamase–CCF2/AM and β-galactosidase–fluorescein di-β-D-galactopyranoside (FDG) are fluorogenic reporter systems for biological events in live mammalian cells. Translation of *amp*^{R} (the gene encoding β-lactamase) or *lacZ*-(the gene encoding β-galactosidase) results in production of the catalytically active reporters β-lactamase **(a)** and β-galactosidase **(b)**, respectively. Alternatively, these genes can be split or otherwise modified so that additional post-transcriptional or post-translational events are necessary to reconstitute the active reporter enzyme. The tetramer of β-galactosidase (PDB code: 1DP0 [28]) and the monomer of β-lactamase (PDB code: 1FQG [29]) are shown to scale. The two fragments of each enzyme (only a single polypeptide chain of the β-galactosidase homotetramer is colored) that associate in protein-fragment – complementation assays (PCA) are colored purple and green for the N- and C-terminal fragment, respectively. (a) CCF2/AM is the membrane permeable pro-substrate form of the fluorogenic β-lactamase substrate CCF2. Hydrolysis of CCF2 by β-lactamase results in disruption of intramolecular fluorescence resonance energy transfer (FRET) between the two fluorophores meaning the fluorescence emission changes from green to blue. (b) FDG is the most sensitive substrate for detection of β-galactosidase but cell permeabilization (e.g. by hypotonic shock) is required for efficient intracellular loading. Hydrolysis of the non-fluorescent FDG by β-galactosidase is a two-step process that releases two equivalents of galactose, and generates an equivalent of intensely fluorescent fluorescein.

fluorescein product of FDG hydrolysis is membrane permeable and, thus, leaks from cells at physiological temperatures [9,16]. Despite these numerous drawbacks, the β-galactosidase–FDG system is the reporter technology that has been used in many live-cell applications including the detection of protein–protein interactions by fragment complementation [17].

β-Lactamase: harnessing a dangerous enzyme

TEM β-lactamase is a relatively small (29 kDa) monomeric enzyme that cleaves penicillin and cephalosporin derivatives with high efficiency [8]. An unfortunate consequence of this is that, in the war against pathogenic bacteria that exhibit ever-greater resistance to β-lactam antibiotics, TEM β-lactamase is one of the most powerful weapons in the enemy's arsenal [18]. However, if we put aside the looming threat of antibiotic resistant 'superbugs', we might consider some other aspects of TEM β-lactamase that make it a nearly ideal catalyst for certain biological applications. Upon enzymatic cleavage of a cephalosporin derivative, the product undergoes a facile rearrangement that, by design, can result in the elimination of a portion of the molecule. This feature of β-lactamase-catalyzed hydrolysis of cephalosporins has potential applications in prodrug-based therapeutic approaches [19]. Latent cytotoxic agents can be coupled to the cephalosporin scaffold such that they are released in their active form only by the catalytic action of β-lactamase. If the β-lactamase can be targeted to the vicinity of a cancerous cell as an antibody conjugate, a systemically administered prodrug would only be converted to its cytotoxic form in the vicinity of the diseased cell and, thus, adverse affects on healthy tissue would be minimized.

The basic chemistry of β-lactamase prodrug activation is identical to the chemistry of fluorogenic β-lactamase substrates (Figure 1a). In fact, the technological breakthrough that first enabled the use of β-lactamase as a catalytic reporter was the chemical synthesis of a membrane-permeable fluorogenic cephalosporin-type substrate known as CCF2/AM [the acetoxymethyl (AM) ester of coumarin cephalosporin fluorescein (CCF2)] [8]. This relatively non-polar 'prosubstrate' passively diffuses through the plasma membrane and becomes trapped in the cytoplasm due to the action of nonspecific intracellular

esterases that cleave the labile esters that mask the four negative charges of the free substrate CCF2. If the cell is not expressing the β-lactamase reporter enzyme, CCF2 remains intact and fluoresces green under violet excitation owing to intramolecular fluorescence resonance energy transfer (FRET) between the coumarin donor and the fluorescein acceptor. If β-lactamase is present in the cytoplasm, CCF2 is rapidly hydrolyzed, resulting in the spontaneous elimination of the fluorescein acceptor and a dramatic increase in the blue coumarin fluorescence as a result of the loss of FRET. Real-time detection of the blue and green fluorescence affords a ratiometric signal that is minimally affected by variations in cell size and substrate uptake.

Battle of the beta's

In a recent article by Knapp *et al.* [9], the two rival reporter systems, β-lactamase–CCF2/AM and β-galactosidase–FDG, were evaluated and compared with respect to several indicators of performance including dynamic range, low reporter-level detection and rare-event detection by flow cytometry. It was conclusively demonstrated that the β-lactamase–CCF2/AM reporter system is superior in all three criteria. In this comparison, the properties of the actual enzymes ended up being a minor consideration, and it was actually the respective properties of CCF2/AM and FDG that were under scrutiny. CCF2/AM passively diffuses into cells and is well retained once inside (once it has been converted to CCF2), whereas FDG requires hypotonic shock for loading, and the fluorescent product (free fluorescein) passively diffuses out of the cell. CCF2 generates a ratiometric fluorescent signal that greatly improves signal-to-noise, but FDG generates fluorescence only at a single wavelength. These beneficial features provide sufficient sensitivity for the detection of one β-lactamase-positive cell in a population of 10^6 β-lactamase negative cells; a 100-fold improvement over the β-galactosidase–FDG system [9]. Owing to the nature of the reaction catalyzed by β-galactosidase, it is unlikely that a ratiometric substrate with beneficial properties similar to those of CCF2 could ever be designed. However, even if such a substrate were to be introduced, the properties of the enzymes themselves would still require consideration. On a per-polypeptide basis, the second-order rate constant for β-lactamase-catalyzed hydrolysis of CCF2 ($1.3\ \mu M^{-1}s^{-1}$) [8] is approximately six times faster than β-galactosidase-catalyzed hydrolysis of FDG ($0.2\ \mu M^{-1}s^{-1}$) [20]. In addition, a single polypeptide of β-galactosidase is approximately four times larger than a single copy of β-lactamase and, thus, β-lactamase provides ~24 times the catalytic activity for the same amount of reporter-protein synthesis.

It is important to note that the advantages of the β-lactamase–CCF2/AM system are relevant only to live mammalian cells. Until a β-lactamase equivalent of the popular β-galactosidase substrate 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (otherwise known as X-gal) becomes available, β-galactosidase will remain the reporter of choice for most histochemical and molecular-biology applications.

A one-in-a-million live-cell assay for improved ribozymes

The bottom line is that, although both β-lactamase and β-galactosidase are powerful live-cell-reporter technologies, there are several distinct advantages associated with the β-lactamase–CCF2/AM system. These advantages are significant enough that, by revisiting known systems in which β-galactosidase was used as reporter and modifying them to use β-lactamase instead, new vistas of opportunity are opening up. This point was nicely illustrated by a small flurry of papers during 2002 [10–12] in which live-cell protein-fragment complementation assays (PCA) based on β-lactamase–CCF2/AM (Figure 1a) were demonstrated to be superior to β-galactosidase PCA [17] (Figure 1b).

Moreover, the recent work from Rao and coworkers [14], in which they have constructed a β-lactamase–CCF2/AM-based reporter system for *Tetrahymena* group I intron ribozyme activity, stands on the shoulders of previously described live-cell assays that are based on β-galactosidase [21]. This new reporter construct consists of the *Tetrahymena* group I intron inserted into the gene that encodes β-lactamase. The self-excision of the intron results in the generation of the complete mRNA for β-lactamase that is in turn translated to form the active reporter enzyme. Thus, the ratio of green fluorescence to blue fluorescence for an individual CCF2/AM-treated cell is dependent on the activity of the ribozyme. Examining the treated cells either by fluorescence microscopy or flow cytometry enabled the researchers to assess ribozyme activity with single-cell resolution. Despite using a red-fluorescent co-transfection marker to normalize the observed ribozyme activity for variations in transfection efficency, a considerable amount of cell-to-cell heterogeneity in ribozyme activity was observed. Perhaps the most exciting application of this reporter system is fluorescence-activated cell-sorter (FACS) screening [22,23] of large pools of mammalian cells to isolate rare ribozyme variants with increased activity in live cells. Rao's group demonstrates the feasibility of this approach using flow cytometric analysis of a series of ribozyme variants with insertions of varying sizes in a loop adjacent to the splicing site. Despite the heterogeneity in each discrete, genetically identical population, they were able to identify a variant with fourfold greater activity in mammalian cells [14].

Concluding remarks

Based on recent trends in reporter technology, it seems likely that β-lactamase will continue to grow in popularity as it is adapted for use in an ever-greater variety of systems and new substrates become available [24]. In the quest for ribozymes with improved activity in mammalian cells, the next obvious step is to exploit the one-in-a-million sensitivity of the β-lactamase–CCF2/AM reporter system for FACS analysis of large pools of mammalian cells expressing ribozyme variants. Fluorescence screening of live cells (predominantly bacteria) was essential in the evolution of both green and red FPs from scientific curiosities to useful biochemical tools [25,26]. With cautious optimism, one can similarly hope that molecular evolution by fluorescence screening of live cells will result in ribozymes that are improved biochemical tools or

potential therapeutics capable of repairing errant mRNA transcripts that are associated with certain inherited diseases [27].

Acknowledgements
I thank Jin Zhang for a critical reading of the article.

References

1 Zhang, J. *et al.* (2002) Creating new fluorescent probes for cell biology. *Nat. Rev. Mol. Cell Biol.* 3, 906–918
2 Gribbon, P. and Sewing, A. (2003) Fluorescence readouts in HTS: no gain without pain? *Drug Discov Today* 8, 1035–1043
3 Abraham, V.C. *et al.* (2004) High content screening applied to large-scale cell biology. *Trends Biotechnol.* 22, 15–22
4 Tsien, R.Y. (1998) The green fluorescent protein. *Annu. Rev. Biochem.* 67, 509–544
5 Matz, M.V. *et al.* (2002) Family of the green fluorescent protein: journey to the end of the rainbow. *Bioessays* 24, 953–959
6 Niswender, K.D. *et al.* (1995) Quantitative imaging of green fluorescent protein in cultured cells: comparison of microscopic techniques, use in fusion proteins and detection limits. *J. Microsc.* 180, 109–116
7 Rutter, G.A. *et al.* (1998) Real-time imaging of gene expression in single living cells. *Chem. Biol.* 5, R285–R290
8 Zlokarnik, G. *et al.* (1998) Quantitation of transcription and clonal selection of single living cells with β-lactamase as reporter. *Science* 279, 84–88
9 Knapp, T. *et al.* (2003) Detection of β-lactamase reporter gene expression by flow cytometry. *Cytometry* 51A, 68–78
10 Galarneau, A. *et al.* (2002) β-Lactamase protein fragment complementation assays as *in vivo* and *in vitro* sensors of protein-protein interactions. *Nat. Biotechnol.* 20, 619–622
11 Wehrman, T. *et al.* (2002) Protein–protein interactions monitored in mammalian cells via complementation of β-lactamase enzyme fragments. *Proc. Natl. Acad. Sci. U. S. A.* 99, 3469–3474
12 Spotts, J.M. *et al.* (2002) Time-lapse imaging of a dynamic phosphorylation-dependent protein–protein interaction in mammalian cells. *Proc. Natl. Acad. Sci. U. S. A.* 99, 15142–15147
13 Cavrois, M. *et al.* (2002) A sensitive and specific enzyme-based assay detecting HIV-1 virion fusion in primary T lymphocytes. *Nat. Biotechnol.* 20, 1151–1154
14 Hasegawa, S. *et al.* (2003) Imaging *Tetrahymena* ribozyme splicing activity in single live mammalian cells. *Proc. Natl. Acad. Sci. U. S. A.* 100, 14892–14896
15 Rotman, B. *et al.* (1963) Fluorogenic substrates for β-D-galactosidases and phosphatases derived from fluorescein (3,6-dihydroxyfluoran) and its monomethylether. *Proc. Natl. Acad. Sci. U. S. A.* 50, 1–6
16 Nolan, G.P. *et al.* (1988) Fluorescence-activated cell analysis and sorting of viable mammalian cells based on β-D-galactosidase activity after transduction of *Escherichia coli* lacZ. *Proc. Natl. Acad. Sci. U. S. A.* 85, 2603–2607
17 Rossi, F.M. *et al.* (2000) Interaction blues: protein interactions monitored in live mammalian cells by β-galactosidase complementation. *Trends Cell Biol.* 10, 119–122
18 Bradford, P.A. (2001) Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clin. Microbiol. Rev.* 14, 933–951
19 Smyth, T.P. *et al.* (2000) β-Lactamase-dependent prodrugs – recent developments. *Tetrahedron* 56, 5699–5707
20 Huang, Z.J. (1991) Kinetic fluorescence measurement of fluorescein di-β-D-galactoside hydrolysis by β-galactosidase: intermediate channeling in stepwise catalysis by a free single enzyme. *Biochemistry* 30, 8535–8540
21 Jones, J.T. *et al.* (1996) Tagging ribozyme reaction sites to follow *trans*-splicing in mammalian cells. *Nat. Med.* 2, 643–648
22 Ibrahim, S.F. and van den Engh, G. (2003) High-speed cell sorting: fundamentals and recent advances. *Curr. Opin. Biotechnol.* 14, 5–12
23 Daugherty, P.S. *et al.* (2000) Flow cytometric screening of cell-based libraries. *J. Immunol. Methods* 243, 211–227
24 Gao, W.Z. *et al.* (2003) Novel fluorogenic substrates for imaging β-lactamase gene expression. *J. Am. Chem. Soc.* 125, 11146–11147
25 Cormack, B.P. *et al.* (1996) FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* 173, 33–38
26 Campbell, R.E. *et al.* (2002) A monomeric red fluorescent protein. *Proc. Natl. Acad. Sci. U. S. A.* 99, 7877–7882
27 Long, M.B. *et al.* (2003) Ribozyme-mediated revision of RNA and DNA. *J. Clin. Invest.* 112, 312–318
28 Juers, D.H. *et al.* (2000) High resolution refinement of β-galactosidase in a new crystal form reveals multiple metal-binding sites and provides a structural basis for α-complementation. *Protein Sci.* 9, 1685–1699
29 Strynadka, N.C. *et al.* (1992) Molecular structure of the acyl-enzyme intermediate in β-lactam hydrolysis at 1.7 Å resolution. *Nature* 359, 700–705

0167-7799/$ - see front matter
doi:10.1016/j.tibtech.2004.03.012

Letter

A reason to be optimistic about biodiesel: seed meal as a valuable soil amendment

Michael F. Cohen and Mark Mazzola

USDA-Agricultural Research Service, Tree Fruit Research Laboratory, Wenatchee, WA 98801, USA

In his Opinion article 'Confessions of a bioenergy advocate', H.R. Bungay splashes buckets of cold realism onto some of the extravagant claims and projections made regarding the potential of biomass refining [1]. However, for one aspect of this article that we are familiar with, omission of some facts might have led readers to an overly pessimistic view.

Specifically, Bungay correctly notes that the price of biodiesel is linked to the cost of alcohol (a reactant in the transesterification process) and the price obtained for glycerol, a by-product of the process. However, the importance of the price obtained for the seed meal by-product in determining the competitiveness of biodiesels derived from seed oils was not mentioned [2]. At present the cost of producing biodiesel from rapeseed (*Brassica napus*) is only slightly offset by returns from the

Corresponding author: Mark Mazzola (mazzola@tfrl.ars.usda.gov).