Real-time imaging of gene expression in single living cells

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Recent advances in reporter gene technologies are now allowing us to image gene transcription at the single cell level, using either fluorescence or luminescence microscopy. Here, the basis of these techniques is outlined and their advantages and disadvantages in various biological systems are discussed.

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The development of reporter gene technology during the 1980s provided the impetus for a dramatic increase in our knowledge about transcriptional regulation. More recent improvements in these reporter genes, coupled with significant advances in detector technologies, are now allowing us to image gene expression non-invasively at the level of the single living cell. Such techniques are providing remarkable insights into the dynamics of gene expression during complex processes, such as the cell cycle and the stimulation of cells by hormones, growth factors and nutrients. Furthermore, these techniques can be applied to situations as diverse as mammalian cells in culture and *Drosophila* adults or embryos. Indeed, these techniques are now being used to monitor the dynamics of gene expression in living organisms.

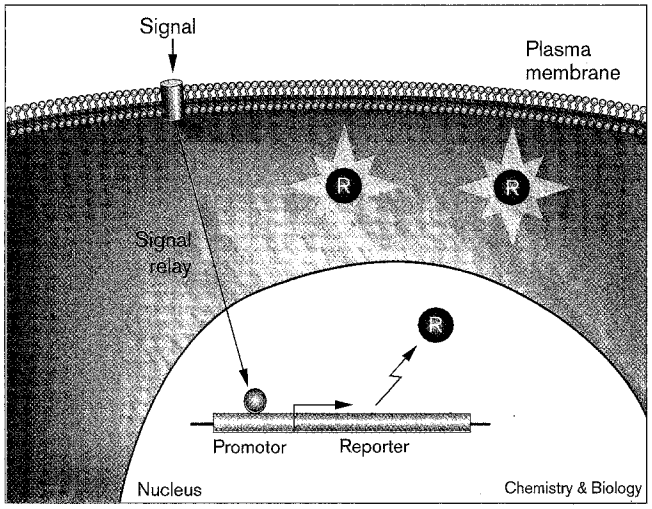
In this article, we will discuss the available imaging techniques that allow gene expression to be examined and analysed at the single cell level, with a particular emphasis on those techniques that allow gene expression to be followed dynamically and in 'real time'. The basics of reporter gene technology are illustrated in (Figure 1). We will discuss very recent advances that lead to the availability of a fluorescent read-out, including green fluorescent protein (GFP) from the jellyfish *Aequoria victoria* and bacterial β-lactamase. These methods will be compared with those that generate a bioluminescent signal, including the luciferases from the firefly *Photinus pyralis* and from the sea pansy *Renilla reniformis*. In particular, we will examine the pros and cons of each technique so that the reader can, we hope, assess the suitability of each method for their own particular application.

Systems that generate a fluorescent read-out

Green fluorescent protein

GFP is a 27 kDa protein that has a cyclised tripeptide (Ser65–Tyr66–Gly67) fluorophore buried deep within a β barrel (the structure of GFP has been solved to 1.9 Å resolution [1]). The wild-type protein exhibits two excitation peaks, at 395 and 475 nm, and a single emission peak at 508 nm [2,3]. A wide range of mutant forms of GFP have been developed that have altered spectral properties (e.g. Tyr66→His or 'blue fluorescent protein', BFP; λ_{ex} = 383 nm, λ_{em} = 447 nm), considerably increased brightness (e.g. Ser65→Thr; λ_{ex} = 489 nm, λ_{em} = 511 nm) or increased folding and stability (e.g. Tyr145→Phe) in mammalian cells. These proteins, which can be expressed readily in mammalian cells, bacteria, plant cells and yeast, to name but a few systems, have resulted in a considerable amount of interest in

Figure 1



The principles of reporter gene detection in living cells. All genes have an upstream promoter sequence that can be isolated and placed in front of a heterologous reporter gene (R; e.g. β-lactamase, green fluorescent protein, GFP, or luciferase). In the example shown, the activity of the promoter might be regulated by a signalling pathway emanating from a receptor in the plasma membrane such that the expression level of the reporter gene in question varies depending on the signal strength. This type of promoter–reporter construct can be introduced into cells by transfection and the activity of the reporter gene monitored in the living cell by fluorescence or luminescence microscopy. When using GFP, the fluorescence of the protein product can be measured directly. In the case of β-lactamase, measuring the change in fluorescence of an added cephalosporin substrate (CCF2) is required. For luciferase, measuring the luminescence generated by the oxidation of luciferin is required (see text for more details).

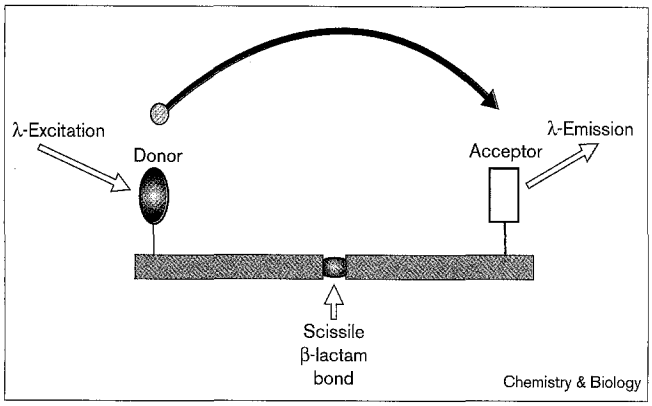
their use as reporter genes, as well as for fluorescent tagging of intracellular proteins that can be expressed and monitored *in vivo* [4–8].

In theory, therefore, using relatively inexpensive equipment (a standard epifluorescence microscope and a cooled charge coupled device [CCD] camera) it should be possible to use GFP as a reporter for studying gene expression in single living cells. Unfortunately, the physicochemical properties of GFP mean that it is not suitable for monitoring rapid changes in gene expression (over the minute to hour range). This is largely because, when it is expressed in cells, maturation of GFP (i.e., the folding and formation of the fluorescent protein) requires a finite time, generally 2–4 h, although this problem is less acute with mutant GFP variants [9]. Furthermore, although the expression of GFP can readily be detected when driven by strong (usually viral) promoters, the utility of GFP for studying the activity of weaker mammalian promoters is considerably more limited [10]. One exception is the insulin promoter, which is highly active in pancreatic islet β cells and whose activity has now been monitored using a GFP reporter gene [11,12].

Another problem, which has been suggested as a drawback to the use of GFP, is the stability of the protein. Because purified recombinant GFP is remarkably stable *in vitro*, Clontech (http://gfp.clontech.com/) have produced a mutant GFP that has decreased stability. Nevertheless we have observed that the half-life of wild-type GFP in living cells is of the order of 2–4 h. Theoretically, this would be short enough to allow observation of oscillatory changes in the activity of a promoter (S. Dobson, G.A.R. and J.M.T., unpublished observations). A more serious problem is that fluorescence measurements pose problems for quantitation, given day-to-day variation of the detection apparatus (e.g. the performance of the lamps, detecting camera, etc.); the lack of linearity between protein concentration and fluorescence intensity; and interference from cellular autofluorescence.

Theoretically, however, real-time simultaneous imaging of multiple reporter GFPs is feasible, although this has not yet been formally demonstrated using nonviral promoters. BFP and GFP[Ser65→Thr] have spectral properties that are sufficiently distinct that they may be separately detectable using suitable filter sets (or even UV and Kr/Ar lasers). BFP has a considerably lower quantum efficiency and extinction coefficient than GFP[Ser65→Thr], however, making it significantly less bright; it also suffers from a rapid bleaching rate. Other mutants (e.g. cyan fluorescent protein, CFP, λ_{ex} = 433 nm, λ_{em} = 480 nm; and yellow fluorescent protein, YFP, λ_{ex} = 480 nm, λ_{em} = 535 nm) are more stable to photo-bleaching and are brighter than BFP, but have greater spectral overlap than BFP and GFP[Ser65→Thr] [13].

Figure 2



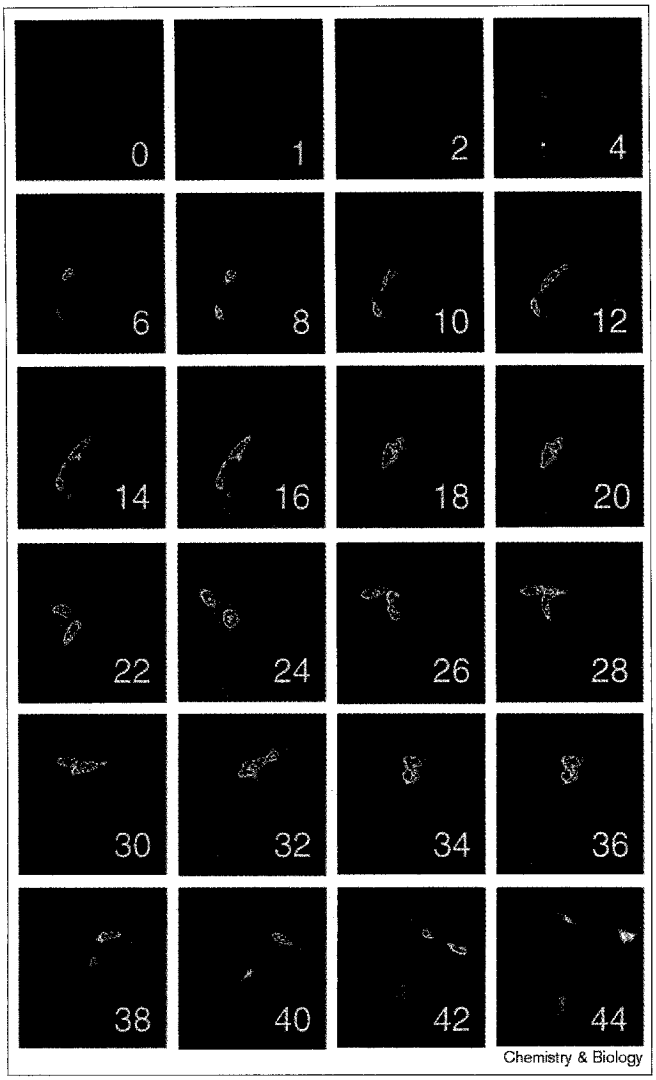
The principles of fluorescence resonance energy transfer (FRET) is illustrated. In the case of β-lactamase discussed in the text, the substrate cephalosporin derivative contains attached donor and acceptor fluorophores in close proximity. Excitation of the donor (at 409 nm in the case of CCF2) results in energy transfer to the acceptor such that a green emission (520 nm) occurs. When the scissile β-lactam bond is cleaved this energy transfer is prevented and a resultant blue emission (409 nm) from the donor is detected.

Fluorescence resonance energy transfer and β-lactamase

The ability of a chromophore to transfer energy to a near neighbour has been innovatively exploited by Zlokarnk and colleagues [14,15] to measure gene expression. The FRET technique (Figure 2) uses a cephalosporin molecule that has an attached fluorescent donor and an acceptor (termed CCF2). When excited by ultraviolet light, the donor (λ_{ex} = 409 nm, λ_{em} = 447 nm) transfers fluorescent energy to the acceptor (λ_{em} = 520 nm) such that emission of green light (λ = 520 nm) occurs. When CCF2 is cleaved by β-lactamase, the donor and acceptor are separated such that FRET no longer occurs efficiently and blue light (λ = 447 nm) is emitted. Indeed a 447:520 nm emission ratio provides a measure of the extent of substrate cleavage corrected for local changes in substrate concentration.

Remarkably, and given a sufficiently long incubation time with the substrate (up to 16 h), this method can allow detection of as few as 50 molecules of β-lactamase per cell [14]. As with GFP, however, this level of sensitivity largely depends on the level of cellular autofluorescence. The β-lactamase method has proved particularly well-suited for fluorescence-activated cell sorting (FACS), in which cells expressing a gene of interest can be isolated [14]. The fact that the substrate irreversibly accumulates and is trapped within the cells markedly limits the utility of this technique for measuring changes in gene expression dynamically: a decrease in the amount of the reporter enzyme will not be reported, and repeated measurements of reporter gene expression in the same cell are not possible. Furthermore, at the present time, and until the development of new FRET substrates with altered spectral properties, only a

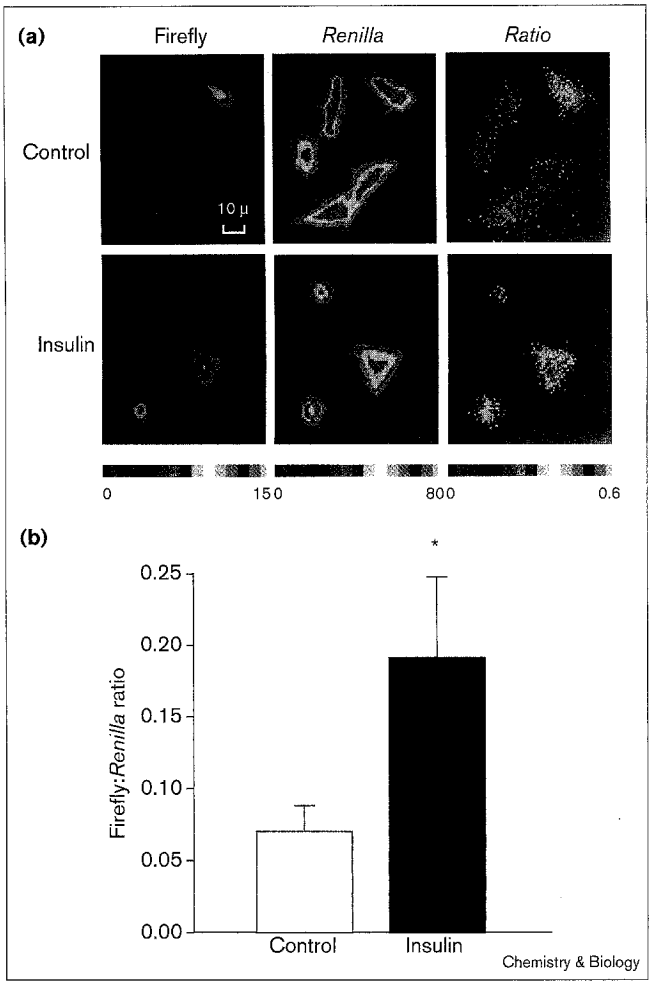
Figure 3



Extended measurements of gene expression during the cell cycle. Rama 27 rat mammary fibroblasts were cultured on coverslip dishes for 24 h without serum. The cells were microinjected with 0.1 μg/ml plasmid containing c-*fos*-luciferase and then maintained at 37°C on the microscope stage in an environment of 5% CO_2. After a 3–4 h recovery phase, images were acquired at 2 h intervals over a 44 h period in the presence of 5% serum. The cells were visualised using a Zeiss Axiovert 135TV microscope equipped with a 10 × 0.5 NA objective. As a result of the culture conditions on the microscope, it was possible to track the luciferase expression in these cells over a period that spanned the first cell division. Connect to our website (http://www.bch.bris.ac.uk/staff/tavare/ChemBiol/fig1.html) using Netscape Navigator (version 3.0 or better) or Microsoft Internet Explorer (version 4.0) to view the time-lapse sequence (also available as Supplementary material with the internet version of this paper).

single reporter enzyme can be monitored. Hence, normalization of the activity of a regulated promoter to that of a nonregulated promoter — which is essential to compensate for nonspecific alterations in the basal transcriptional, translational or metabolic status of a cell — cannot be achieved.

Figure 4



Ratiometric imaging of multiple luciferase reporters in single cells. **(a)** Chinese hamster ovary cells were microinjected with a mixture of c-*fos*–luciferase plasmid and pRL.CMV (Promega; encoding *Renilla reniformis* luciferase under cytomegalovirus promoter control; 150 μg/ml each). Photon counting (5 min) was performed, in the presence of 1 mM luciferin followed by 5 μM coelenterazine, using a Photek ICCD216 intensified camera, onboard an Olympus Axiovert IX–70 microscope (10 × 0.4 NA objective). The colour bar represents photons/5 min/pixel for firefly and *Renilla* luciferase activities, or the calculated ratio, as shown. **(b)** Summary of the effect of insulin on c-*fos* promoter activity. The calculated ratio of firefly : *Renilla* luciferase activities was 0.071 ± 0.017 (mean ± S.E.M, n = 19 cells; open bar) in the absence of insulin and 0.191 ± 0.057 (n = 18 cells, filled bar; $p < 0.01$ by unpaired Student's t test) in the presence of the hormone.

Systems that generate a luminescent read-out

The luciferases

Expression of the luminescent reporter genes (luciferases) from *Photinus pyralis* (firefly) [16,17] and *Renilla reniformis* [18] can be monitored independently in single living cells using extremely sensitive photon-counting digital-imaging cameras [19]. These can be either TV-rate photon-counting intensified cameras or integrating slow-scan cooled CCD cameras. For this ultra-low light imaging application,

two- or three-stage microchannel plate (PMT)-based intensified cameras have been widely used (e.g. as manufactured by Hamamatsu Photonics, http://www.hpk.co.jp/products/producte.htm, or Photek, http://www.photek.com/). Alternatively, one can use liquid-nitrogen-cooled back-thinned CCD cameras (supplied by Hamamatsu Photonics or Princeton Instruments, http://prinst.com/), as they have comparable performance and better resolution over longer integration times.

Generally, the luciferase method involves the introduction of the reporter plasmid by direct intranuclear microinjection or transfection (transient or stable). Cells are sufficiently permeable to the added cofactors luciferin and coelenterazine [20,21], that intracellular luciferase luminescence can be then immediately measured; under most circumstances the intracellular concentrations of the cofactors O_2 and ATP are not limiting, such that luminescence is directly proportional to the level of gene expression. To illustrate the utility of this method for studying the dynamics of gene expression, we microinjected into rat mammary fibroblasts a plasmid placing the firefly luciferase gene under the control of the promoter of the c-Fos transcription factor (Figure 3). The cells were stimulated with serum and cell luminescence was followed on the microscope stage using an intensified CCD camera. Luminescence was first detectable within 60 min of microinjection and increased progressively, but heterogeneously, over 44 h period. The cells exhibited apparently normal motility, as well as the capacity to pass through a full cell cycle.

In a study of the prolactin promoter in stably transfected cell lines, luciferase expression was tracked for up to 70 h [22]. Because luciferin is highly stable in culture medium for several days, but coelenterazine is not, only the firefly luciferase enzyme can be used for long-term non-invasive real-time imaging (M.R.H.W., unpublished observations). Importantly, however, as the camera is capable of discriminating individual photons, the method is both highly sensitive and quantitative, using either the firefly or *Renilla* luciferases.

The use of multiple luciferases [10,23] is demonstrated in the experiment shown in Figure 4. Chinese hamster ovary cells were injected with the c-*fos*–luciferase plasmid and pRL.CMV, which places the *Renilla reniformis* luciferase under the control of the constitutive cytomegalovirus promoter, and then treated with or without insulin before imaging. After imaging in the presence of luciferin, *Renilla reniformis* bioluminescence was monitored entirely independently, simply by adding the rapidly cell-permeant cofactor coelenterazine (Figure 4a). The ratio of the firefly : *Renilla* luciferase photon production rate was then obtained digitally to demonstrate the specific induction of the c-*fos* promoter by insulin (Figure 4b).

Although it is difficult to distinguish firefly and *Renilla* luciferases due to their spectral overlap, this problem can now be alleviated by the recent introduction of red-shifted (λ_{em} > 600 nm) variants of the firefly enzyme [24]. Thus soon it should be possible, using suitable filter sets, to simultaneously record the expression level of two independent luciferase reporter genes utilising the same cofactor.

The use of luminescence imaging for studying gene expression in living organisms has also been demonstrated. For example, transgenic *Drosophila* expressing luciferase under the control of the promoter for the circadian rhythmicity gene *per* in the pacemaker cells of the head exhibited circadian rhythms of luciferase bioluminescence for several days [25]. Finally, luciferase imaging can be used to monitor gene expression both

Table 1

Comparison of the fluorescent- and luminescent-based assays for monitoring gene expression in single cells.

	GFP and variants	β-Lactamase	Luciferase
Sensitivity (reporter molecules per cell)	10^5–10^6 [27]	50 [14]	10^4–10^5 [20]
Quantitation	+	+	+++
Dynamic measurements	↑(↓)	↑	↑↓
Dynamic range (x-fold)	10–100	10–100	10^5
Co-factor requirement	None	Cephalosporin analogue	Luciferin or coelenterazine
UV irradiation	No (unless using BFP)	Yes	No
Simultaneous imaging of multiple reporters	Yes*	No	Yes†
Half-life of reporter gene	2–4 h (GFP)	Irreversible	2–4 h (firefly); > 10 h (*Renilla*)‡
Suitable for HTS	Yes	Yes	Yes
Approximate cost of equipment	$70, 000	$70, 000	$110, 000

*Assumes the use of GFP variants with altered spectral properties that has yet to be formally proven; †using firefly and *Renilla* luciferases and luciferin and coelenterazine as co-factors, respectively; ‡M.R.H.W., unpublished observations; HTS, high-throughput screening.

superficially and in deep tissues of mice that are still alive [26].

Choosing a method

All of the methods we describe for studying gene expression at the single-cell level have advantages and disadvantages that we have attempted to summarise in Table 1. Only luciferase imaging currently allows rapid, quantitative dynamic imaging of gene expression and has been proven to allow simultaneous detection of multiple reporter genes in the same cell. Although the spectral variants of GFP might allow similar analyses, fluorescence imaging suffers from the inherent problem of background autofluorescence and is only semi-quantitative. Imaging with β-lactamase is sensitive if a long-term incubation with fluorigenic substrate can be tolerated (detection of as little as 50 molecules per cell over a 16 h incubation period) but probably has a similar sensitivity to that of luciferase imaging when using a 5 min incubation period (i.e., as compared to a 5 min integration period for collecting the luminescent image). GFP is, perhaps, the least sensitive of the three methods.

Of all the methods, GFP is the least invasive, although the cofactors for both β-lactamase and the luciferases do not appear to be toxic to cells. β-Lactamase imaging requires irradiation with UV light which can be toxic to cells if given over long periods of time. Ratiometric measurement of multiple reporter genes in the same living cell is a major advantage of luciferase imaging over fluorescent-based techniques, including the various mutant forms of GFP. Such measurement is crucial in certain situations where a correction must be made for transfection/microinjection efficiency or cell viability (e.g. during drug screening). All the methods are applicable to high-throughput screening, although only GFP and β-lactamase are suitable for use in fluorescent cell sorting. Luciferase and GFP, but not β-lactamase, allow dynamic imaging of both increases and decreases in gene expression due to their relatively short half-lives. This is precluded for β-lactamase because of the requirement for product accumulation. Only luciferase imaging allows the collection of repeated images of the same cells over many hours or days, however; this cannot be achieved with β-lactamase and would be extremely difficult using GFP because of phototoxicity.

In summary, if real-time imaging is a requirement, then, at present, luciferase outperforms the fluorescence-based methods in several important respects (sensitivity, quantitation and product lability). If real-time imaging is not a priority, however, and only a single snap-shot of the extent of activity of a promoter is required, then β-lactamase could be the system of choice because of its enhanced sensitivity. Thus with the recent introduction of the β-lactamase assay, there is now a growing list of non-invasive single cell assays available for academic and pharmaceutical research.

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