Green fluorescent protein (GFP): applications in cell-based assays for drug discovery

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Green fluorescent protein (GFP) is a powerful tool for cell-based assays owing to the intrinsic fluorescence of this protein that allows real-time analysis of molecular events in living cells. A number of GFP variants have been developed with optimal properties for both high-throughput screening and high-content screening. The author discusses advances in basic GFP technology, including the discovery of fluorescent proteins from divergent bioluminescent species, as well as the development of various GFP biosensors suited to the drug discovery process.

GFP and its variants

In the bioluminescent jellyfish *Aequorea victoria* (Fig. 1), light is produced when energy is transferred from the Ca^{2+}-activated photoprotein aequorin to green fluorescent protein (GFP)[1–3]. This process occurs in specialized photogenic cells located at the base of the jellyfish umbrella and is thought to provide either a communication function or a defense mechanism to the organism. The cloning of the wild-type GFP gene (*wt GFP*)[4,5] and its subsequent expression in heterologous systems[6–8] has established GFP as a powerful reporter for the analysis of gene expression and protein localization in a wide variety of experimental systems, including drug discovery applications. When the gene is expressed in either eukaryotic or prokaryotic cells and illuminated by excitatory light, GFP emits a bright green fluorescence that is easily detected by a variety of instrument platforms. Light-stimulated GFP fluorescence is species-independent and does not require additional cofactors, substrates or gene products from *A. victoria*. Additionally, detection of GFP can be performed in living samples, and is amenable to real-time analysis of molecular events.

In addition to its expression alone, GFP has also been used extensively to express GFP fusions with a variety of other proteins and targeting sequences. In the majority of cases, chimeric genes encoding either N- or C-terminal fusions to GFP retain the normal biological activity of the heterologous partner, as well as maintaining fluorescent properties similar to those of native GFP (Refs 7,9–11). The use of GFP and its variants in this capacity provides a 'fluorescent tag' on the protein, which allows *in vivo* localization of the fusion protein. GFP fusions can provide enhanced sensitivity and resolution compared with standard antibody staining techniques[7], and the GFP tag eliminates the need for the fixation, cell permeabilization and antibody incubation steps that are normally required when using antibodies tagged with chemical fluorophores. Lastly, use of the GFP tag permits real-time kinetic studies of protein localization and trafficking[7,9,12–14].

The GFP chromophore

One of the remarkable features of GFP is that it is a naturally fluorescent protein, encoding the light-emitting chromophore within its primary amino acid sequence. The GFP chromophore consists of a cyclic tripeptide derived from Ser-Tyr-Gly at positions 65–67 in the protein[15] and is only fluorescent when embedded within the fully folded, complete GFP molecule. The crystal structure of GFP reveals a tightly packed β-can structure which encloses a

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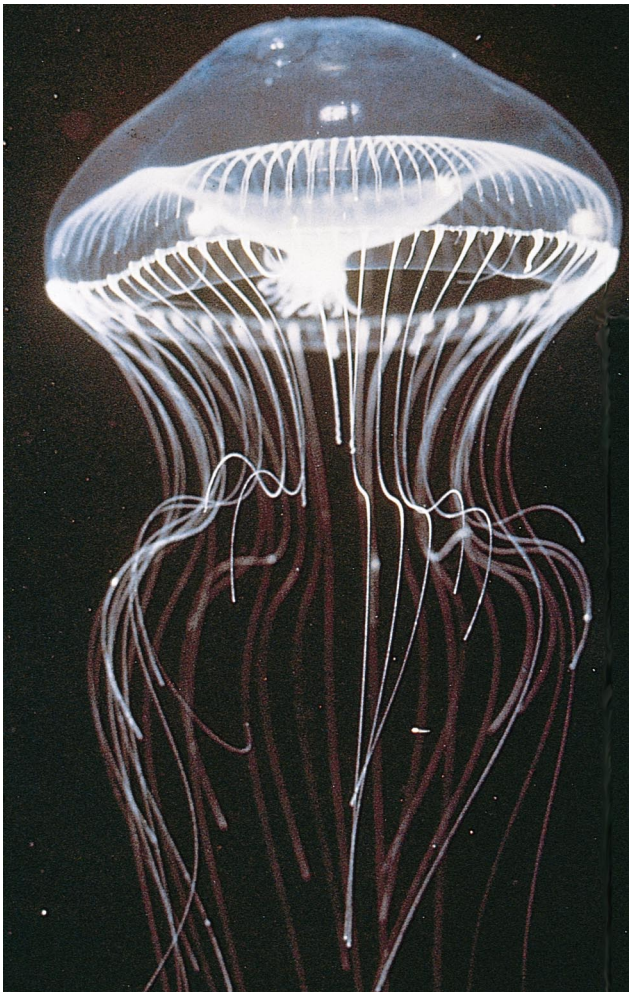

Figure 1. *Photograph of the jellyfish* Aequorea victoria *which produces the green fluorescent protein (GFP). (Photograph courtesy of Claudia Mills at the Friday Harbor Research Laboratories, WA, USA.)*

central α-helix containing the chromophore[16,17]. This structure provides the proper environment for the chromophore to fluoresce by excluding solvent and oxygen. Nascent GFP is not fluorescent, since chromophore formation occurs post-translationally[18]. The chromophore is formed by a cyclization reaction and an oxidation step at Tyr66 that requires molecular oxygen [Ref. 19; Davis, D.F., Ward, W.W. and Cutler, M.W. (1995) *Proceedings of the 8th International Symposium on Bioluminescence and Chemiluminescence*]. These steps are either autocatalytic or use factors that are ubiquitous, since fluorescent GFP forms in a broad range of cells and organisms. Chromophore formation might be the rate-limiting step in generating the fluorescent protein, especially if oxygen is limiting[1,19].

Red-shifted GFP variants

A number of 'red-shifted' variants of *wt GFP* have been described by several investigators, most of which contain one or more amino acid substitutions in the chromophore region of the protein[20,21]. The red-shifted terminology refers to the position of the major fluorescence excitation peak, which is shifted for each of these variants towards the red, from 395 nm in *wt GFP* to 488–490 nm (see Table 1). The emission spectra for such variants are largely unaffected, and these mutants still produce green light with a wavelength maximum of approximately 507–511 nm. The major excitation peak of the red-shifted variants encompasses the excitation wavelength of commonly used fluorescence filter sets (such as those used for fluorescein), thus the resulting signal is much brighter relative to *wt GFP*. Similarly, the argon ion laser used in most flow cytometers, confocal scanning laser microscopes and microplate instruments such as the FLIPR™ (Molecular Devices Inc., CA, USA) has a major laser line at 488 nm, thus excitation of the red-shifted GFP variants is much more efficient than excitation of *wt GFP* under these conditions. In practical terms, this means that the detection limits are considerably lower with the red-shifted variants on most instrument platforms.

The two most commonly used red-shifted GFP variants are Ser65Thr (Refs 18,22), which contains a Ser65 to Thr substitution in the chromophore, and GFPmut1 or enhanced green fluorescent protein (EGFP)[23,24], which contains the same Ser65Thr change plus a Phe64Leu mutation. GFPmut1 and EGFP have identical amino acid sequences, but the EGFP coding sequence has been further modified with 190 silent base changes to contain codons that are preferentially found in highly expressed human proteins[25]. The 'humanized' backbone used in EGFP contributes to efficient expression of this variant in mammalian cells and subsequently very bright fluorescence signals. The detection limit for EGFP in HeLa cells is equivalent to ~10,000 molecules per cell in the cytoplasm, or ~2000 molecules per cell on the surface[26]. Advantages of EGFP for applications in mammalian cells include improved sensitivity of detection, improved solubility, more efficient protein folding, faster chromophore oxidation to form the fluorescent form of the protein and reduced rates of photobleaching[27].

Blue, cyan and yellow fluorescent proteins

The properties of red-shifted variants such as EGFP largely overcome the limitations of *wt GFP* for single reporter applications. However, one important feature shared by *wt GFP* and each of these variants remains the same – they

Table 1. Fluorescence properties of enhanced green fluorescent protein (GFP) variants

Variant	Excitation max. (nm)	Emission max. (nm)	E_m	QY
Wild-type	395 (470)	509	21,000 (7150)	0.77
EGFP	488	597	55,000	0.70
EBFP	380	440	31,000	0.20
EYFP	513 (498)	527	36,500	0.63
ECFP	433 (453)	475 (501)	25,000	0.24

Abbreviations: EBFP, enhanced blue fluorescent protein; ECFP, enhanced cyan fluorescent protein; EGFP, enhanced green fluorescent protein; E_m, molar extinction coefficient in units of cm^{-1} M^{-1} determined with optimal excitation wavelengths for each variant; EYFP, enhanced yellow fluorescent protein, QY, fluorescence quantum yield.

all produce green light. With the availability of several EGFP colour variants (see Table 1), drug discovery scientists are now able to develop multicolour live cell assays to monitor different cellular events simultaneously. The enhanced blue fluorescent protein (EBFP), enhanced yellow fluorescent protein (EYFP) and enhanced cyan fluorescent protein (ECFP) variants listed in Table 1 produce fluorescence signals that are blue, yellow–green and cyan, respectively. Each of these emission variants provides good signal intensity in mammalian cells by virtue of elevated molar extinction coefficients (E_m) and improved expression levels by virtue of the same humanized backbone as that found in EGFP. Moreover, because of the relative excitation and emission spectra for these variants, it is possible to design filter sets that are compatible with multicolour reporter applications. The combination of these distinct spectral variants opens up a wide range of applications, such as the simultaneous analysis of multiple gene expression cascades and intracellular localization of different proteins. For example, the results shown in Fig. 2 illustrate detection of ECFP and EYFP fluorescence in the same living cell when these reporter proteins are targeted to the mitochondria and nucleus, respectively. Protein targeting vectors of this type can be useful in engineering cell lines to report on various intracellular translocation events. Detection of such events for high-throughput is best done with automated imaging systems such as those available from SEQ Ltd (NJ, USA) and Cellomics Inc. (PA, USA), as described below. In addition to visual detection methods, it has been shown that various combinations of GFP spectral variants can be used to quantify mixed cell populations by flow cytometry[28,29]. Lastly, the appropriate combination of GFP colour variants can be used to develop cell-based assays for molecular proximity based on the technique of fluorescence resonance energy transfer[8,18] (FRET; see Table 2 for examples).

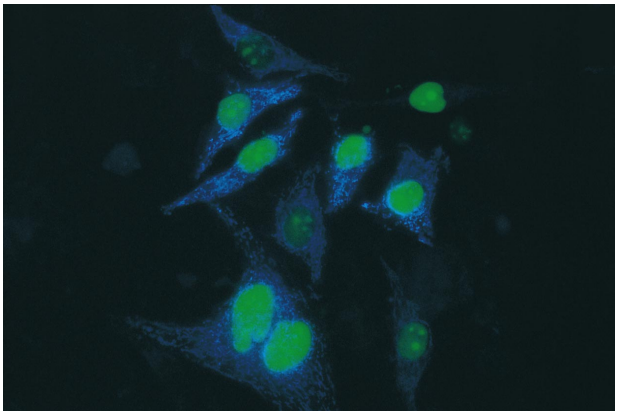

Figure 2. *Dual-colour detection of enhanced yellow fluorescent protein (EYFP) and enhanced cyan fluorescent protein (ECFP) by fluorescence microscopy. The expression vectors pEYFP–Nuc and pECFP–Mito (CLONTECH Laboratories Inc., CA, USA) were transiently transfected into HeLa cells using a liposome transfection reagent. At 48 h post-transfection, the cells were fixed with 3.7% formaldehyde in phosphate-buffered saline, pH 7.4, mounted in ProLong Antifade (Molecular Probes Inc., OR, USA), and visualized using a Zeiss Axioscope Model 50 microscope fitted with filter sets XF104 (EYFP) and XF114 (ECFP) from Omega Optical (CT, USA). Photographs were taken with a 40× oil objective.*

Use of GFP as a transcription reporter

The compact β-can structure of GFP renders this protein extremely stable and resistant to degradation by most cellular proteases[8,15]. In fact, the half-life of EGFP expressed in the cytoplasm of mammalian cells has been estimated to be greater than 24 h (Ref. 30). This level of stability is an advantage for many applications for which a stable fluorescence signal from GFP is desirable, but a serious disadvantage in applications to monitor changes in gene expression. In transcription reporter assays, changes in the level of the reporter protein are presumed to reflect changes in mRNA levels resulting from either induction or repression of *cis*-acting control elements linked to the reporter gene. The stability of the reporter protein, which must be considered in this regard as the reporter half-life, can have a profound impact on the induction window of the assay. For example, upon induction following addition of an effector (e.g. a drug candidate), the reporter protein

Table 2. Live-cell GFP biosensors

Biosensor construct	GFP type	Fluoresence measurement	Event detected	Refs
Transcription reporter biosensors				
NF-κB–dEGFP	d2EGFP	Intensity change	TNF or IL -1 signalling	41,CT
AP-1–dEGFP	d2EGFP	Intensity change	GF stimulation or AP-1 activation	CT
SRE–dEGFP	d2EGFP	Intensity change	Serum stimulation or SRF activation	CT
GRE–dEGFP	d2EGFP	Intensity change	Glucocorticoid stimulation	CT
CRE–dEGFP	d2EGFP	Intensity change	cAMP stimulation or PKA activation	CT
NFAT–dEGFP	d2EGFP	Intensity change	Ca^{2+} stimulation or NFAT activation	CT
HRE–dEGFP	d2EGFP	Intensity change	Stress or heat stimulation	CT
p53–dEGFP	d2EGFP	Intensity change	DNA damage or tumour suppressor activation	CT
Myc–dEGFP	d2EGFP	Intensity change	Cell growth	CT
HIV-1 LTR–GFP	EGFP	Intensity change	HIV infection	42
Protein localization and translocation biosensors				
ECFP/EYFP–Tub	ECFP/EYFP	Intensity localization	Microtubule dynamics	CT
ECFP/EYFP–Actin	ECFP/EYFP	Intensity localization	Actin myofilament dynamics	CT
ECFP/EYFP–Mito	ECFP/EYFP	Intensity localization	Mitochondrial localization and targeting	CT
ECFP/EYFP–Nuc	ECFP/EYFP	Intensity localization	Nuclear localization and targeting	CT
ECFP/EYFP–PM	ECFP/EYFP	Intensity localization	Plasma membrane localization and targeting	CT
ECFP/EYFP–ER	ECFP/EYFP	Intensity localization	Endoplasmic reticulum localization and targeting	CT
ECFP/EYFP–Golgi	ECFP/EYFP	Intensity localization	Golgi apparatus localization and targeting	CT
Annexin V–EGFP	EGFP	Intensity localization (plasma membrane)	Early detection of apoptosis	43,44,CT
Cytochrome *c*–GFP	EGFP	Intensity redistribution (mitochondria)	Early detection of apoptosis	45
PKC-γ-$C1_2$–GFP	S65T	Intensity redistribution	Diacylglycerol and phorbol ester indicator	46
PKC-γ-C2–GFP	S65T	Intensity redistribution	Intracellular Ca^{2+} indicator	46
βarr_2–GFP	S65T	Intensity redistribution	Activation of G protein-coupled receptors	47,48
GFP-MR	EGFP	Intensity redistribution	Mineralocorticoid receptor trafficking	49
β_2AR/S65T–GFP	S65T	Intensity redistribution	β_2-Adrenoceptor trafficking	50
hGR–GFP	S65T	Intensity redistribution	Glucocorticoid receptor trafficking	37,39
Shaker K^+–GFP	GFP-deltaC	Intensity change	Changes in membrane potential	51
Organelle-targeted EGFP	EGFP	Intensity change	Cytoplasmic and organelle pH indicators	52,53
FRET biosensors				
Cameleons	CFP, YFP	FRET	Cytoplasmic and organelle Ca^{2+} indicators	18,54,55
FIP–CB_{SM}	BFP, GFP	FRET	Intracellular Ca^{2+} indicator	56
GFP–Bax, BFP–Bcl-2	BFP, GFP	FRET	Apoptosis detection (protein–protein interaction)	45
EGFP–DEVD–EBFP	EBFP, EGFP	FRET	Apoptosis detection (caspase-3 protease activation)	57
Protein degradation biosensors				
IκB–EGFP	EGFP	Intensity change	TNF/IL -1 signalling and IκB degradation	CT
p53–EGFP	EGFP	Intensity change	Cell cycle analysis and p53 degradation	CT

Abbreviations: AP-1, activator protein-1; AR, adrenoceptor; βarr, β-arrestin; BFP, blue fluorescent protein; CFP, cyan fluorescent protein; CRE, cAMP-response element; CT, available from CLONTECH Laboratories, Inc.; DEVD, Asp-Glu-Val-Asp; ECFP, enhanced cyan fluorescent protein; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; EYFP, enhanced yellow fluorescent protein; FIP–CB_{sm}, fluorescence indicator protein–calmodulin binding domain; FRET, fluorescence resonance energy transfer; GRE, glucocorticoid-response element; hGR, human glucocorticoid receptor; HIV, human immunodeficiency virus; HRE, hypoxia response element; IκB, inhibitor of nuclear factor κ-binding; IL -1, interleukin-1; LTR, long terminally repeated unit; Mito, mitochondrion; MR, mineralocorticoid receptor; NFAT, nuclear factor of activated T cells; NF-κB, nuclear binding κ-binding; Nuc, nucleus; PKC-γ, protein kinase C-γ; PM, plasma membrane; S65T, serine 65 to threonine; SRE, serum-response element; SRF, serum-response factor; TNF, tumour necrosis factor; Tub, microtubule.

level might increase to a lesser extent than the observed fold increase because of the high basal level of reporter protein that has already accumulated in the cell. A recent review has further suggested that the long half-life of GFP could lead to high backgrounds when used in a high-throughput screening (HTS) environment[31]. Such limitations of GFP as a transcription reporter led to the development of a short half-life form of EGFP for use in HTS which was termed destabilized EGFP (dEGFP).

Destabilized EGFP and dEGFP biosensors

Creation of the dEGFP variant was achieved by fusing a region of the mouse ornithine decarboxylase (ODC) gene encoding residues 422–461 to the C-terminus of EGFP. This domain of ODC contains the amino acid sequence, PEST[32,33], which renders the protein susceptible to rapid proteolysis by a 26S proteosome when expressed in mammalian cells. As shown in Fig. 3, the EGFP–$ODC_{422–461}$ fusion (abbreviated d2EGFP) has an apparent half-life of 2 h in the presence of cycloheximide. Western blot analysis using anti-EGFP antibodies indicated that this decline in fluorescence following inhibition of protein synthesis is correlated with a similar decrease in d2EGFP protein levels[30]. Moreover, by selective mutagenesis of key amino acids within the PEST sequence, it was possible to identify two additional mutants having fluorescence half-lives of 1 and 4 h. These variants were termed d1EGFP and d4EGFP, respectively (Fig. 3).

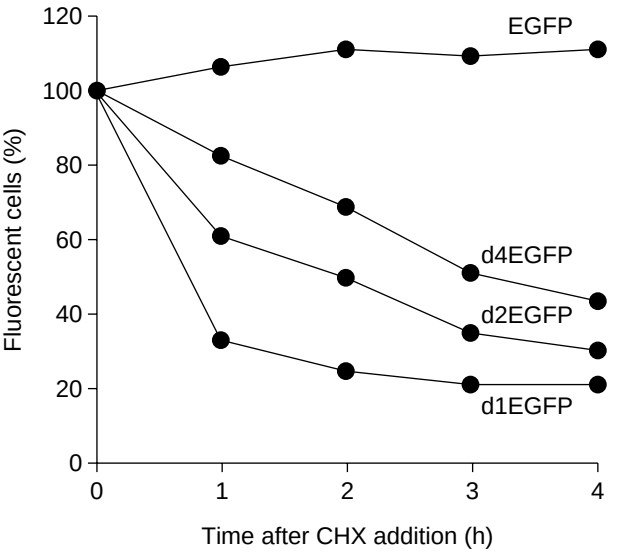

Figure 3. *Fluorescence stabilities of enhanced green fluorescent protein (dEGFP) variants. The expression vectors tetracyclin-responsive element EGFP (pTRE–EGFP), pTRE–d1EGFP, pTRE–d2EGFP and pTRE–d4EGFP (CLONTECH Laboratories Inc., CA, USA) were separately expressed in CHO-K1 Tet-off cells. At 24 h post-transfection, the cells were treated with 100 μg ml^{-1} cycloheximide (CHX) to inhibit new protein synthesis, and collected at the indicated time points for analysis by flow cytometry using a FACScalibur cytometer (Becton Dickinson, NJ, USA). The percentage of fluorescent cells was determined and plotted at each time point.*

To investigate the utility of dEGFP as a transcription reporter, different HEK293 cell lines were constructed expressing nuclear factor κ-binding (NF-κB) reporter vectors employing either d2EGFP or EGFP, as well as two more conventional transcription reporters – firefly luciferase and secreted alkaline phosphatase[34] (SEAP). The NF-κB signalling pathway is known to be activated by different inflammatory cytokines such as tumour necrosis factor (TNF) and interleukin-1, leading to the induction of more than 50 genes that are thought to participate in the inflammatory response[35,36]. As shown in Fig. 4, the reporters luciferase, SEAP and d2EGFP each gave a similar kinetic response to stimulation by TNF. Although the kinetic response provided by each reporter was similar, it is clear from the maximal fold induction that the enzymatic reporters SEAP and luciferase provide greater overall sensitivity relative to d2EGFP (4.5-fold versus >10-fold) using the detection hardware employed in this study. Cells expressing the NF-κB–EGFP reporter responded slowly to TNF stimulation and did not achieve as high a maximal signal (Fig. 4c). Similar results were obtained with this cell line, as well as with CHO cells transfected with CRE–dEGFP and GRE–dEGFP reporter-constructs using the FLIPR microplate instrument to detect the fluorescence from dEGFP.

As similar results were achieved with d2EGFP and the enzymatic reporters SEAP and luciferase, it would appear advantageous to employ this fluorescent reporter system in screening paradigms based on the detection of altered gene expression. Indeed, as listed in Table 2, the use of dEGFP variants has been extended to produce a variety of live-cell dEGFP biosensors that detect a diversity of signal transduction pathways. It is also worth noting that, because the dEGFP variants are created by fusion of ODC sequences to the C-terminus of the reporter, it is feasible to create a similar series of destabilized EBFP, EYFP and ECFP variants. Such a collection of short half-life spectral variants will allow analysis of multiple signal-transduction pathways in the same engineered cell line. Investigators should be cautioned that the post-translational maturation of the GFP

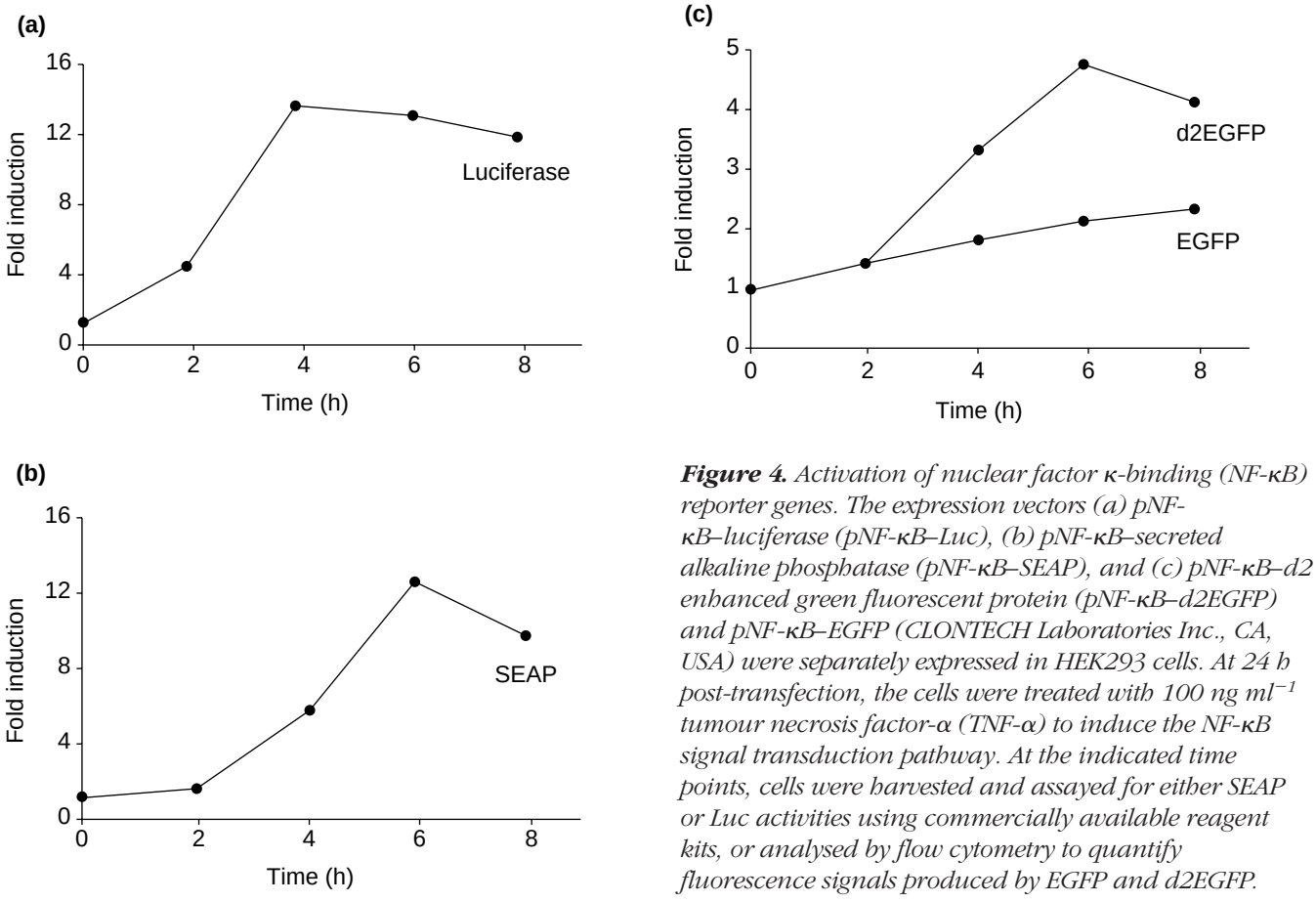

Figure 4. *Activation of nuclear factor κ-binding (NF-κB) reporter genes. The expression vectors (a) pNF-κB–luciferase (pNF-κB–Luc), (b) pNF-κB–secreted alkaline phosphatase (pNF-κB–SEAP), and (c) pNF-κB–d2 enhanced green fluorescent protein (pNF-κB–d2EGFP) and pNF-κB–EGFP (CLONTECH Laboratories Inc., CA, USA) were separately expressed in HEK293 cells. At 24 h post-transfection, the cells were treated with 100 ng ml^{-1} tumour necrosis factor-α (TNF-α) to induce the NF-κB signal transduction pathway. At the indicated time points, cells were harvested and assayed for either SEAP or Luc activities using commercially available reagent kits, or analysed by flow cytometry to quantify fluorescence signals produced by EGFP and d2EGFP.*

chromophore imposes an intrinsic lag time between protein expression and the detection of fluorescence. For this reason, the use of dEGFP and destabilized colour variants could be problematic for monitoring extremely rapid or transient changes in gene expression. Lastly, because screening assays based on dEGFP-engineered cell lines are inexpensive (no substrate addition or wash steps), are compatible with a variety of fluorescence instrument platforms, and allow for real-time analysis in living cells, it can be argued that dEGFP and related variants significantly expand the utility of transcription reporter assays for HTS.

Use of GFP to monitor protein localization

As mentioned above, in addition to applications of GFP for detecting changes in gene expression, the second major class of assays suited to this reporter system are protein localization studies. The specific biosensors employed in such assays are numerous, and include genetic fusions to monitor events such as cell surface receptor internalization, transcription factor trafficking, organelle and cytoskeletal dynamics during cell division, and protein kinase translocation (see Table 2). In each case, as the fluorescence properties of the associated GFP are generally not altered, the assay monitors changes in the location of the GFP fusion. By selective use of GFP colour variants, multicolour assays can be designed for screening, lead optimization and target validation to monitor the localization of several different targets in the same cell. Furthermore, because of the potential for detection of GFP fluorescence in real-time, such localization assays can provide dynamic information on both the spatial and temporal distribution of GFP fusion biosensors.

High-content screening

The most advanced system currently available for the detection of GFP fusion biosensors is the ArrayScan™ system from Cellomics. This fluorescence-imaging platform, together with a collection of high-content screening (HCS) assay sys-

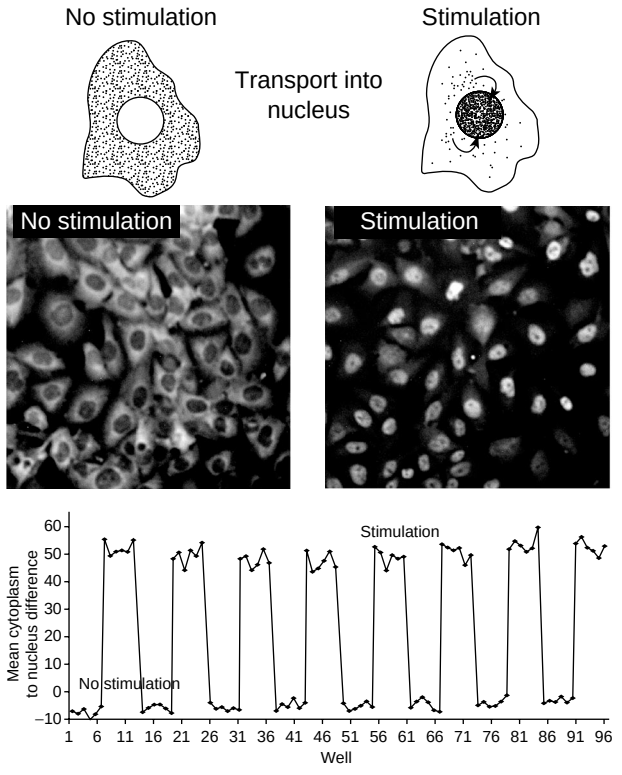

Figure 5. *Detection of hGR–GFP nuclear translocation using the ArrayScan™ system. HeLa cells were transfected with an expression vector encoding a chimeric protein consisting of the human glucocorticoid receptor fused to green fluorescent protein (hGR–GFP). Two channels of fluorescence were used to define the cytoplasmic and nuclear distribution of the hGR–GFP within each cell. Upper panel: localization of hGR–GFP within the cell before (left) and after (right) stimulation with dexamethasone. Centre panel: fluorescence micrographs showing the dynamic redistribution of hGR–GFP in fields of untreated and treated cells. High-content screening is performed on wells containing hundreds to thousands of transfected cells and the translocation is quantified for each cell in the field exhibiting GFP fluorescence. Lower panel: ArrayScan analysis of a 96-well plate containing hGR–GFP expressing cells. (Data kindly provided by Kenneth A. Giuliano and D. Lansing Taylor of Cellomics Inc., PA, USA.)*

tems, is suited to exploit fully the utility of GFP technology for the protein localization assays used in drug discovery. High-content screens have been developed to address the need for more detailed information about the temporal–spatial dynamics of cell constituents and processes. HCS automates the extraction of multicolour fluorescence information derived from specific fluorescence-based reagents such as GFP emission mutants[37,38]. The concept of HCS is to treat each cell as a 'well' that has spatial and temporal information on the activities of the labelled constituents.

Because there is a large and growing list of known biochemical and molecular events in cells that involve translocations of specific molecules and organelles, these dynamic processes are well suited as targets for HCS. For example, the binding of ligands to their specific receptors usually results in the translocation of the receptor–ligand complex from one cellular compartment to another where it regulates a physiological response (e.g. transcription). In general, hormone receptors are excellent drug targets because their activity lies at the summit of key intracellular signalling pathways. For example, human glucocorticoid receptor (hGR)–ligand complexes formed in the cytoplasm of mammalian cells translocate into the nucleus upon binding to a ligand[39]. Therefore, a high-content screen of hGR translocation in which target activity is measured within the context of the living cell has a distinct advantage over *in vitro* ligand–receptor binding assays. Moreover, the availability of up to two more fluorescence channels in the ArrayScan instrument permits the high-content screen to monitor two additional parameters in parallel, such as other receptors, other distinct targets or other cellular processes.

The design of a high-content screen for drug-induced hGR translocation using an hGR–GFP biosensor is as follows. (1) Indicator cells are cultured in a 96-well plate and transiently transfected with a plasmid coding for an hGR–GFP chimeric protein; alternatively, a cell line stably expressing the hGR–GFP construct can be used. (2) The indicator cells are then treated with lead compounds and the translocation of hGR–GFP into the nucleus is quantified over time or at a fixed time point. (3) Additional channels are then also monitored in assays employing other fluorescent probes.

To execute the screen, the ArrayScan system scans each well of the plate, images a population of cells and analyses the cells individually. As indicated in Fig. 5, two fluorescence channels are used to define the cytoplasmic and nuclear distribution of the hGR–GFP within each cell before and after stimulation with the glucocorticoid hormone dexamethasone. HCS is performed on wells containing hundreds to thousands of transfected cells and the translocation is quantified for each cell in the field exhibiting GFP fluorescence. As a second example of HCS, the results shown in Fig. 6 indicate how biosensor cells become a reagent for HCS by producing a chimera containing a mutant GFP and a protein that interacts specifically with intracellular microtubules.

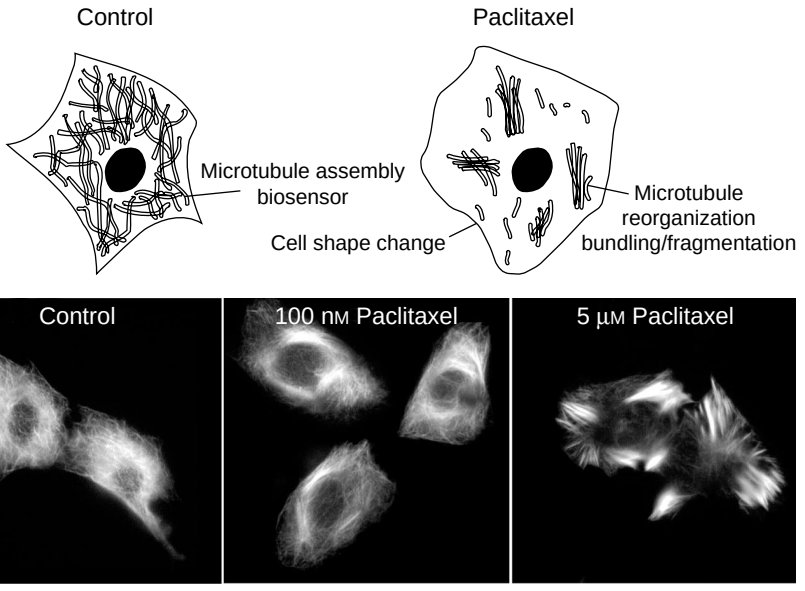

Figure 6. *A green fluorescent protein (GFP)-based fluorescent protein biosensor for high-content screening of microtubule stability in living cells. Upper panel: intracellular localization of a GFP-based probe for microtubules before (left) and after (right) drug treatment. Lower panel: fluorescence micrographs showing the dynamic redistribution of the microtubule probe in fields of untreated cells (left) and cells treated with 100 nM and 5 μM of paclitaxel (centre and right, respectively). The drug-induced reorganization of the microtubule cytoskeleton is measured using ArrayScan. (Data kindly provided by Kenneth A. Giuliano and D. Lansing Taylor of Cellonics Inc.)*

The coupling of molecular fluorophores such as GFP and its variants with other fluorescent protein biosensors using HCS is a unique approach to drug discovery. Drug targets normally screened for activity *in vitro* (e.g. purified tubulin) can now be screened directly at the site of drug action – the living cell. In the future, new reagents based on GFP fusions and other biosensors (Table 2), algorithms, screens and bioinformatics, will increase the power of the HCS approach to drug discovery.

Future prospects: there is more than one GFP in the sea!

Probably the greatest challenge to the broad application of GFP technology in drug discovery is that of sensitivity. Because GFP and its variants are naturally fluorescent proteins, there is no enzymatic amplification of the signal: one mole of GFP yields one mole of photons. For cell-based assays based on fluorescence intensity, the sensitivity provided by GFP biosensors is therefore largely dependent on the cellular concentration of the biosensor and the nature of the detection platform. As the power and versatility of GFP-based assays for drug discovery continue to be appreciated, instrument providers are certain to follow with concerted efforts to develop compatible detection platforms. But answers could also lie in the discovery of additional GFPs in bioluminescent species other than *A. victoria*.

Until recently, the only GFP reporters available were based on the coding sequence of *A. victoria* GFP, originally cloned by Prasher and coworkers[4]. However, evidence from various groups suggests that there are at least ten additional GFP-like proteins found in other marine invertebrates that produce green fluorescence[40]. Most of these other GFPs have been poorly characterized and little is known about the potential utility of the fluorescent proteins as genetic reporters. CLONTECH Laboratories, in collaboration with scientists working in Russia, have recently cloned three additional GFPs from soft sea corals in the phylum *Cnidaria*. Each protein shares approximately 30% sequence homology with GFP from *A. victoria* and contains a similar chromophore tripeptide. Although the findings are preliminary, one of the GFPs appears to yield fluorescence signals equivalent to those of EGFP when expressed in mammalian cells or *Xenopus* embryos. Efforts are under way to produce humanized forms of these new GFP genes, which should greatly enhance the signal intensity in mammalian systems. The effects of chromophore mutations on these GFPs are also being investigated in an attempt to produce various excitation or emission mutants, or both suited to multicolour analysis as described above.

In conclusion, with the advent of GFP mutants such as dEGFP and the different colour variants, continued development of fluorescence instruments for both cellular imaging and microwell detection, and the exciting discovery of new GFPs from other species, the future certainly seems bright for the use of GFP technology in the drug discovery process. This technology permits screening, lead optimization and target validation to be performed in an efficient and cost-effective manner in an environment rich with information – the living cell.

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In short…

The Irish national biotechnology agency, **BioResearch Ireland** (BRI; Dublin, Ireland), has signed a licensing and research agreement with the US biopharmaceutical company, **Inhibitex Inc.** (GA, USA) for research into the prevention and treatment of infections of the skin and wounds by the commonly hospital-acquired bacterium, *Staphylococcus aureus*. Because of the development of resistance of this organism to current antibiotics, these infections can cause life-threatening septicaemia and account for 40% of infection-induced hospital deaths.

Under the agreement, Inhibitex is to fund research for two years at the BRI's Pharmaceutical Biotechnology Centre in Dublin, followed by a further two years on achievement of the technological goals. In return, The BRI has given all licensing and sub-licensing rights of any proteins that are discovered as a result of the collaboration to the biopharmaceutical company.