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# Fluorescent Proteins and Engineered Cell Lines

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## Summary

Green fluorescent protein and other fluorescent proteins provide powerful tools for high content analysis of cellular processes. Engineering fluorescent protein sensors for expression in cellular assays requires consideration of a wide range of design factors to produce fusion proteins capable of generating informative and biologically relevant data while meeting the rigorous demands of high content screening. The target protein, fluorescent protein, host cell line, construct components and orientation, expression level, and other factors all contribute to the performance of the sensor. This chapter reviews the process of selecting an optimum combination of these design elements for engineering cells for high content analysis.


**Key Words:** Automated microscopy; cellular sensors; fluorescent protein; GFP; high content analysis; high content screening.

## 1. Evolution and Engineering of Fluorescent Proteins

Since the cloning of *Aequorea victoria* green fluorescent protein (GFP) ***(1)***, and the subsequent first use of GFP as a biological reporter ***(2,3)***, 10 or more years ago fluorescent proteins have become one of the most powerful and versatile tools in the cell biologist's armory. For an early historical perspective of the potential of fluorescent proteins as viewed at this time *see* **refs. *4–7***.

Mutations of GFP were rapidly introduced yielding color variants and importantly, increased brightness and improved compatibility with fluorescence excitation sources commonly used for microscopy and flow-cytometry, including the key spectral S65T mutation ***(8)*** used in virtually all GFP variants today. Early work with GFP in mammalian cells was hindered by inefficient chromophore formation of the poikilothermic protein at 37°C. Introduction of the F64L mutation ***(9)*** resulted in improved folding of GFP at elevated temperatures and, when used in conjunction with the improved spectral characteristics provided by the S65T mutation and optimization of codon usage ***(10)*** yielded an enhanced GFP (EGFP) ***(11)*** that is 100-fold brighter in mammalian cells than wild-type GFP. The application of these and other mutations to *A. victoria* GFP and to fluorescent proteins and chromoproteins from other species ***(12–17)*** has produced the rainbow of fluorescent proteins now available for investigating biological systems. Key characteristics of fluorescent proteins are summarized in **Table 1** and three-dimensional structures for GFP and tetrameric DsRed are shown in **Fig. 1**. For reviews of the diversity of the fluorescent proteins available today *see* **refs. *18–22***.

The engineering of novel variants of fluorescent proteins continues apace with the recent development of proteins and mutants, which have switchable ***(23,24)*** and temporal ***(25)***

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**Table 1**
**Characteristics of Fluorescent Proteins**

| Protein | Excitation max (nm) | Emission max (nm) | Structure in cells[a] | Brightness (% EGFP) |
|---|---|---|---|---|
| *Blue* | | | | |
| EBFP | 383 | 445 | λ | 27 |
| Sapphire | 399 | 511 | λ | 55 |
| T-Sapphire | 399 | 511 | λ | 79 |
| *Cyan* | | | | |
| AmCyan1 | 458 | 489 | λλλλ | 31 |
| ECFP | 439 | 476 | λ | 39 |
| Cerulean | 433 | 475 | λ | 79 |
| CoralHue Cyan | 472 | 495 | λλ | 73 |
| *Green* | | | | |
| GFP | 395/475 | 509 | λ | 48 |
| EGFP | 484 | 510 | λ | 100 |
| Emerald | 487 | 509 | λ | 116 |
| Azami Green | 492 | 505 | λ | 121 |
| AcGFP | 480 | 505 | λ | 82 |
| ZsGreen | 493 | 505 | λλλλ | 117 |
| *Yellow* | | | | |
| EYFP | 514 | 527 | λ | 151 |
| PhiYFP | 525 | 537 | λ | 155 |
| Citrine | 516 | 529 | λ | 174 |
| Venus | 515 | 528 | λ | 156 |
| ZsYellow1 | 529 | 539 | λλλλ | 25 |
| *Orange* | | | | |
| CoralH Orange | 548 | 559 | λ | 92 |
| mOrange | 548 | 562 | λ | 146 |
| *Red* | | | | |
| DsRed | 558 | 583 | λλλλ | 176 |
| DsRed2 | 563 | 582 | λλλλ | 72 |
| DsRed-Express | 555 | 584 | λλλλ | 58 |
| mTangerine | 568 | 585 | λ | 34 |
| mStrawberry | 574 | 596 | λ | 78 |
| AsRed2 | 576 | 592 | λλλλ | 10 |
| mRFP1 | 584 | 607 | λ | 37 |
| JRed | 584 | 610 | λ | 27 |
| mCherry | 587 | 610 | λ | 47 |
| HcRed1 | 588 | 618 | λλ | 1 |
| mRaspberry | 598 | 625 | λ | 38 |
| HcRed-Tandem | 590 | 637 | λ | 19 |
| mPlum | 590 | 649 | λ | 12 |

[a]Monomer, λ; dimer, λλ; tetramerλλλλ.

properties, which further extend the capabilities of protein sensors for monitoring the dynamics of cellular processes. The range and diversity of fluorescent proteins available today allow complex investigations to be applied to isolated cells and whole organisms right across the biological spectrum from viruses ***(26)*** and bacteria ***(27)*** through yeast ***(28)***, invertebrates ***(29)***, insects ***(30)***, fish ***(31)***, reptiles ***(32)***, mice ***(33)***, pigs ***(34)***, and cattle ***(35)*** to primates ***(36)***.

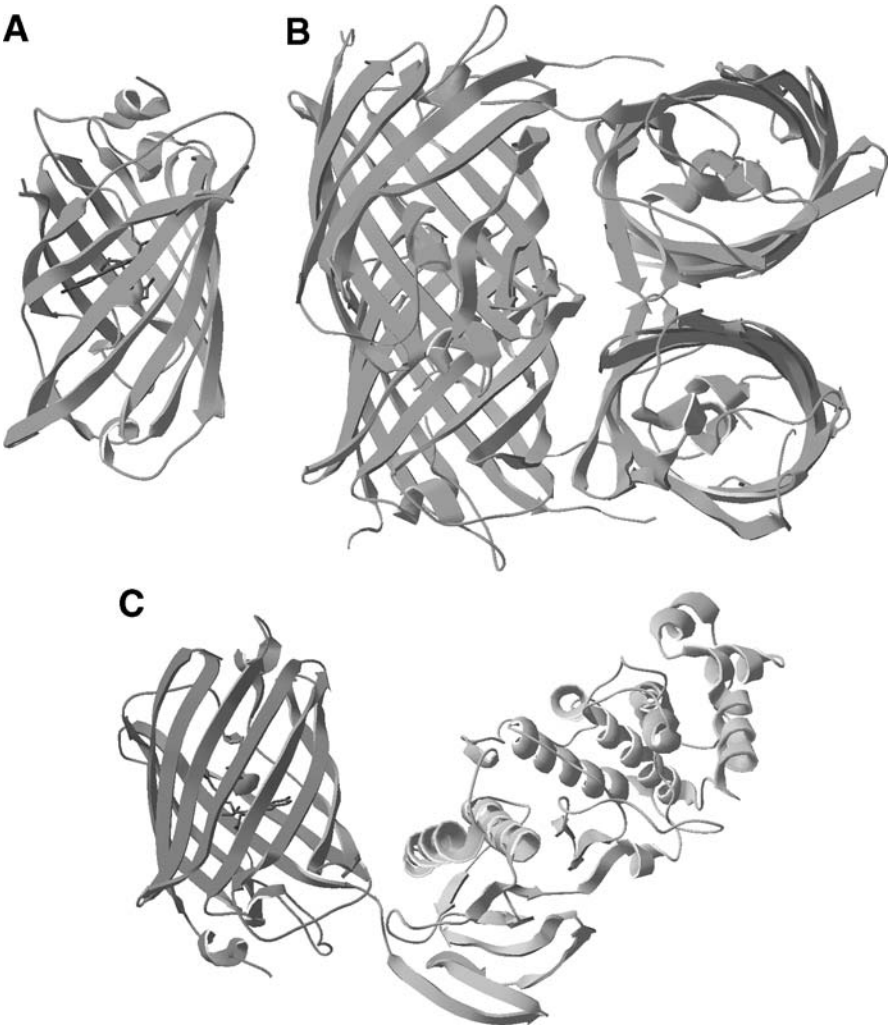


Fig. 1. Fluorescent protein structures. 3D models of **(A)** *A. victoria* GFP, **(B)** DsRed, and **(C)** GFP-p38 MAPK were visualized using Deepview/Swiss PdbViewer (www.expasy.org/spdbv) from coordinates obtained from the RSCB protein data bank *(122)* (www.rscb.org/pdb).

## 2. Evolution of High Content Screening Using Engineered Cells

The development of GFP and other fluorescent proteins suitable for expression in mammalian cells occurred in parallel with the emergence of the high throughput automated imaging and image analysis techniques *(37,38)*, which came to be known as high content screening (HCS). Although to a certain extent this was a chance coincidence in the emergence of two technologies, it was a very fortuitous alignment. The development of fluorescent proteins provided the means to tag proteins in living cells to directly and dynamically visualize biological processes that previously could only inferred from other techniques such as reporter gene assays. HCS instrumentation and analysis software provided the means to perform such experiments in sufficient number to make them applicable to the increasingly industrialized environment of drug discovery.

Initial HCS efforts focused on fixed cell assays using antibodies *(39,40)* but it was only a very short time between the first reports of cytoplasmic to nuclear protein translocation being visualized using glucocorticoid receptor-GFP fusion proteins *(41,42)* that HCS data were reported for HeLa cells transiently expressing glucocorticoid receptor-GFP fusion proteins *(43)*. Shortly thereafter use of a stable HEK293 cell line expressing a PTHR-GFP for HCS of G protein coupled receptor (GPCR) activation was reported *(44)*. From this point on engineered cell lines expressing GFP fusion proteins were rapidly adopted as a key tool for imaging gene expression and protein localization and redistribution *(45,46)* in drug development. This increase in the use of GFP fusion proteins continues to be accompanied by the mutually beneficial development of increasingly sophisticated high throughput fluorescence instrumentation capable of imaging and analyzing cellular events in live cells in real time *(47–49)*.

Since the development of optimum techniques for tagging proteins in mammalian cells with fluorescent proteins stable cells expressing GFP fusion proteins have been applied in studying a wide range of cellular molecules and processes including GPCR signaling *(50,51)*, cytoskeletal dynamics *(52)*, second messenger signaling *(53)*, protein kinase activity and localization *(54,55)*, chromatin structuring *(56)*, and protein trafficking *(57)*.

Although a number of publications present proof of principle data from HCS experiments for drug development *(58–63)*, for proprietary and other reasons publications describing the use of fluorescent proteins in full-scale screening are limited. One disclosed screen of 950,000 compounds *(64)* run on a GE Healthcare (Giles, UK) IN Cell Analyzer 3000 examined GPCR desensitization and internalization in a U2OS cell line stably expressing GFP-β-arrestin. Monitoring of GPCR activation through internalization of β-arrestin was initially reported *(65)* early in the development of fluorescent protein tagging, and was subsequently developed and industrialized for drug screening by Norak Biosciences (now Xsira Pharmaceuticals, Morrisville, NC) *(66)*. Recent developments in methods for gene knockdown with RNAi have combined with engineered cell lines expressing sophisticated fluorescent protein sensors and HCS to provide extremely powerful tools for elucidating gene function using RNAi screens (**Fig. 2**).

## 3. Designing Engineered Cells

Building a stable cell line that will withstand the rigors of HCS is a complex and often time-consuming business. In an ideal world all stable cell lines for HCS would be easy to engineer, select and maintain, brightly fluorescent and easy to image, free from any regulatory and patent constraints, an accurate model of a biological process, and provide robust and statistically valid data. In the real world this is rarely the case, and for most cell lines some compromises between opposing design factors inevitably have to be made to arrive at a cell line fit for the purpose for which it is intended. Some of the key design considerations involved in designing and engineering cells to express fluorescent fusion proteins are listed in **Table 2**. These elements are discussed in detail later with the aim of highlighting some of the key decision (or compromise) points involved in cell line design. Although many of the required engineering decisions can be made based on logic, preceding knowledge, or published data, it is inevitable, given the complex interrelationships between design options, that in many cases it is impossible to arrive at a single complete definitive design for a cell line. In these situations there is no alternative to empirical optimization; you have to get in the lab and figure out what works and what does not.

### *3.1. Target Protein Selection*

The first choice to be made in designing a cell line to report on a cellular signaling pathway or process is to identify a suitable protein for fusion to a fluorescent protein. In some cases this will be a simple choice; if a signaling pathway of interest has been well characterized and reported in the

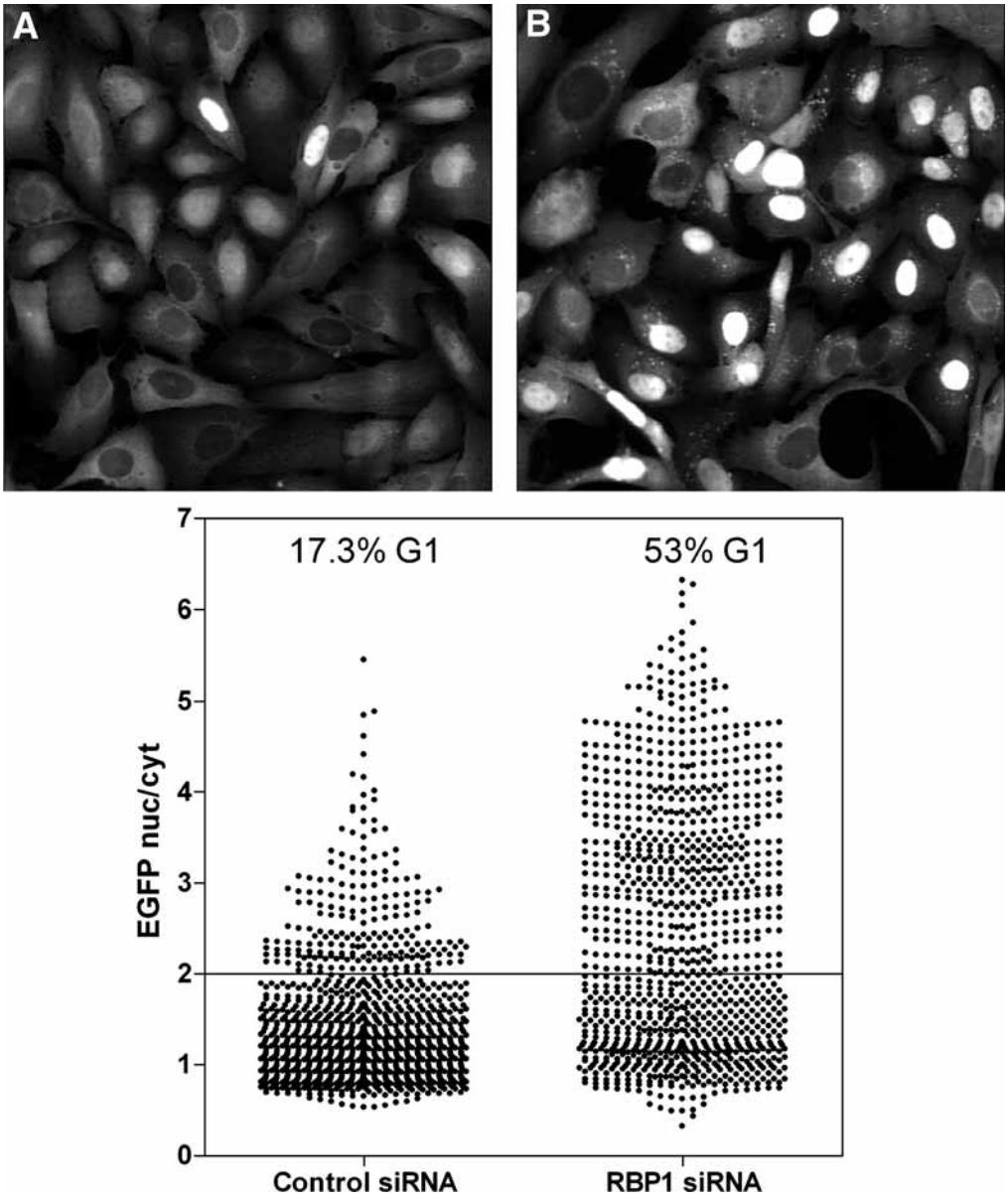


Fig. 2. HCS of siRNA gene knockdown screen in an engineered cell line. A stable U2OS cell line expressing an EGFP-helicase PSLD G1/S transition sensor was screened against a library of siRNAs for effects on G1/S transition. In G1 cells the EGFP-helicase PSLD is retained in the nucleus by a nuclear localization sequence. On transition to S-phase phosphorylation within the PSLD by Cyclin E/CDK2 unmasks a dominant nuclear export sequence leading to export of the sensor into the cytoplasm. Imaging and analysis of the nuclear/cytoplasmic distribution of the fusion protein allows quantitation of the G1/S block induced by siRNA knockdown of retinol binding protein 1 (RPB1) **(B)** relative to cells treated with a control siRNA **(A)**. RBP1 controls cellular levels of retinol and retinol and its metabolites, including retinoic acid, are known to affect levels and activities of cell cycle control proteins. Retinoic acid reduces transcription of cyclins D and E and increases the activity of the CDK2 inhibitors p21 and p27. We hypothesize that in cells with reduced RBP1 these combined effects significantly reduce the ability of CyclinE-CDK2 and CyclinD-CDK4 to phosphorylate retinoblastoma protein and progress cells past the G1/S checkpoint.

**Table 2**
**Design Elements for Engineered Cell Lines**

| Design element | Factors influencing choice |
|---|---|
| Target protein | Relevance to signaling pathway or process |
| | Reporting mechanism |
| | Timing of readout |
| | Specificity of readout |
| | Method of data abstraction |
| Host cell line | Ease of transfection |
| | Imaging quality |
| | Expression of pathway components |
| | Biological relevance |
| | Growth rate |
| | Biological containment category |
| | Tolerance and stability of fusion protein expression |
| Fluorescent protein | Suitable excitation and emission wavelengths for imaging instrumentation |
| | Compatibility with fusion partner folding and activity |
| | Sufficient brightness for imaging |
| | Color compatibility with cell staining |
| | Color compatibility with multiplexing |
| Construct order (FP-protein or protein-FP) | Protein terminus required for localization |
| | Exposed domain required for fusion protein function, interaction or processing |
| | Whole protein or domain |
| | Folding requirements of FP and fusion partner |
| Linker | Folding requirements of FP and fusion partner |
| Expression vector | Ease of cloning |
| | GMO containment category |
| | Selection marker |
| Promoter | Homologous or heterologous |
| | Level of expression compatible with biological relevance |
| | Level of expression required for imaging |
| Expression level | Interference or toxicity from over-expressed fusion protein |
| | Level of expression compatible with biological relevance |
| | Level of expression required for imaging |

literature, perhaps with data from antibody staining showing changes in the localization of a key protein, engineering can proceed based on a fairly sound foundation. If on the other hand no imaging-based data is available for the pathway then it will be necessary to make some informed choices regarding one or more target proteins based on whatever background information is available.

At this stage in the design process a target can be any protein that undergoes a change in some characteristic, which is detectable by subcellular imaging, for example, the protein appears, or disappears or moves. The specificity of the readouts from candidate target proteins now has to be considered and weighed against the ease of obtaining quantitative data. In some cases a trade-off between specificity and analysis complexity might be required in order to arrive at a robust HCS assay. For example, choosing a target protein that undergoes a dramatic cytoplasmic to nuclear translocation on stimulation, such as nuclear factor (NF)-κB p65 *(67)*, will simplify the task of acquiring data by image analysis over the choice of a more subtle redistribution of

fluorescence such as Rac movement to membrane ruffles *(68)*. However, this approach might not yield data of sufficient specificity if, like NF-κB p65, the target protein is involved in several cellular responses to external stimuli.

Similar considerations might have to be taken into account in choosing a target protein that undergoes a detectable change in intensity or location in a time-scale compatible with imaging. A protein that responds to a stimulus very quickly, for example, the rapid internalization of a membrane receptor *(69)*, might not allow sequential imaging of large numbers of tests and require more complex assay protocols requiring rapid imaging following stimulus.

In summary, the task at this point in the design process is to pick a target protein (or proteins) that will report a cellular response with the required specificity, with manageable kinetics for imaging, and with a change in fluorescence distribution and/or intensity that can be analyzed with available image analysis software.

### *3.2. Selecting a Host Cell Line*

Picking the right cell type to engineer can be as important as picking the right protein; there is no point in making a detailed and informed selection of an assay target protein and then expressing it in a cell line that lacks a vital part of a signaling pathway, or that has morphology or growth characteristics that make it difficult or impossible to acquire images of sufficient quality for image analysis.

Historically cell lines for high-throughput screening using macro-imaging to measure luminescent reporter gene *(70)*, calcium flux *(71)*, and other low resolution cellular assays have been chosen principally on the basis of their ability to express large amounts of a drug target, typically a membrane receptor. In these assays the cell line is essentially a conveniently packaged collection of reagents *(72)* configured to allow drug activity at a massively overexpressed target to dominate over all other read-outs from the cell. This sledge–hammer approach to cell engineering has its place in primary drug screening; biological subtlety is not necessarily required or desirable when faced with weeding out hits from a million or so compounds.

However, moving from high-throughput screening to HCS (or indeed employing HCS in high-throughput screening) is generally motivated by the desire to acquire higher quality, more accurate, precise and informative, data on the effect of a drug, or other perturbation on a biological system. Obtaining higher quality information from a model system requires a proportionally higher level and quality of design.

Increasing development of stable cell lines for HCS has been accompanied by a move away from protein expression factories like HEK 293 and CHO to cells which are compatible with high throughput subcellular imaging such as U2OS *(73)*. Recent improvements in chemical transfection methods *(74)* and the use of retroviral *(75)* and baculovirus *(76)* vectors have largely removed transfection efficiency as a limitation on cell choice and allow many different cell lineages to be considered for engineering stable cell lines for HCS. If large numbers of cells are to be grown and screened consideration should be given to additional factors including the cell growth rate, the biological containment category for the parental cell and any restrictions on the use of genetically modified cells, all of which might impact on resources required to culture, manipulate and image the engineered cells.

The chosen host cell type should be tolerant of fusion protein expression both in the short term, for example, the target protein or domain should not be inherently toxic, and in the long term, for example, fusion expression should remain at a constant level over a useable number of passages. Insertion of transgenes into certain cell types is known to result in gradual loss of expression *(77)* either through transcriptional silencing or loss of the integrated transgene. If long-term stable expression is a key requirement *(78)* it might be necessary to test a number of cell types or clones for genetic stability and susceptibility to chromatin modification *(79,80)*. For discussion of cellular tolerance of fluorescent proteins and GFP in particular see the following section.

### 3.3. Selecting a Fluorescent Protein

Once the target protein and a suitable cell type have been identified for engineering the decision has to be made regarding which of the variety of available fluorescent proteins would be most suitable. The first and obvious choice is based on color, on the grounds that there's no point in making a fusion protein that's not compatible with one's HCS instrument setup. This explains the continued predominance of green fluorescent proteins; the widespread availability of instrumentation with laser or lamp illumination at fluorescein wavelengths makes these fluors an obvious first choice, and the availability of a red nuclear stain *(81)* allows rapid imaging in a single pass on multicamera systems.

Although instrument compatibility is obviously important, in some cases biological compatibility might have to be taken into account. If detection sensitivity is a key issue, either because the process being monitored requires it, or because it is desirable to minimize expression of the fusion protein to prevent overloading a cellular process, then it might be advantageous to select a fluorescent protein of a different color and increased brightness, for example, using enhanced yellow fluorescent protein (EYFP) instead of EGFP. Of course this selection only makes sense if any nominal gain in fluorescent protein brightness is not negated by physical factors, for example, use of suboptimal imaging filters, or biological factors, for example, less efficient protein folding.

All of the above assumes that choice of fluorescent protein color is not dictated by other assay design constraints. If the cell line is to be used in a multiplexed assay, or if particular fluorescent markers are required for image analysis, the colors of other probes or stains might influence the choice of fluorescent protein. In these cases all parameters should be evaluated; it might be that changing the color of another component might allow a preferred fluorescent protein to be used to maximize sensitivity and biological compatibility, for example, changing the fluor on a second antibody from fluorescein to Cy5 would allow EGFP to be used instead of a less sensitive red fluorescent protein.

### 3.4. Construct and Vector Design

Key factors here are whether a whole protein is required for fusion protein function and the orientation of the fusion protein construct, that is, whether the fluorescent protein is fused through the amino or carboxyl terminus. The optimum design for a fusion protein will depend on the properties of the fusion partner, for example, a particular terminus might need to be preserved to retain protein function and/or to ensure correct localization. However, retaining full protein function is not always possible, necessary, or desirable, for a functional assay. In some cases retaining full protein function, for example, enzymic activity and stimulus responsive translocation, might produce a poorer assay than a fusion that does not retain catalytic activity because of cellular perturbation resulting from overexpression of the enzyme. In such cases engineering the construct to mask the enzyme domain, or removal of the domain from the construct might produce a better fusion protein.

The decision of whether to use an intact protein or to remove unwanted activities from the fusion protein by using only part of the target protein will depend on the availability of published data. In some cases for well-characterized proteins it might be known that functional domains are portable, that is, they can be abstracted and appended to fusion proteins and retain partial or full functionality.

The two cell cycle phase marker constructs shown in **Fig. 3** use portable protein domains from well-characterized proteins. The key CRS and D-box domains *(82,83)* in the amino terminal region of Cyclin B1 used in engineering the G2/M CCPM provide all the necessary functionality to allow the EGFP fusion protein to shadow the localization and destruction of endogenous Cyclin B1 and, hence, report cell cycle position and progression. In this case use of domains from the target protein rather than the entire protein is imperative to allow engineering of a

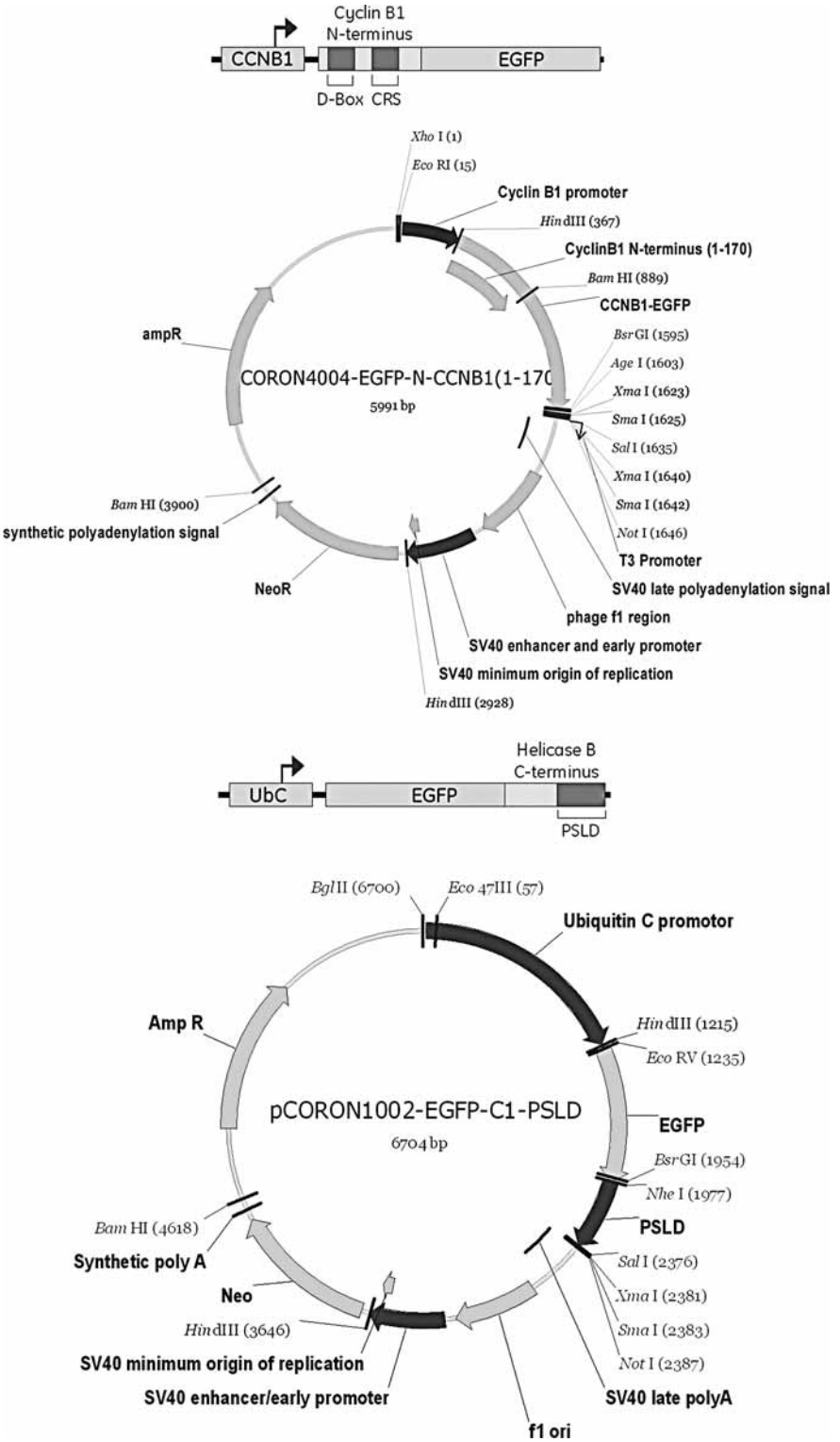


Fig. 3. EGFP cell cycle phase markers. Construct schematics (top) and vector maps (bottom) for G2/M (left) and G1/S (right) cell cycle sensors.

stable cell line, because stable expression of a full Cyclin B1 fusion protein interferes with cell cycle progression through interaction with Cyclin dependent kinases *(84)*. Similarly, the carboxyl terminus region of DNA helicase B used to engineer a stable G1/S CCPM cell line has been shown to contain a phosphorylation dependent subcellular localization domain (PSLD) *(85)*, which localizes helicase B to the nucleus in G1 cells. As for the Cyclin B1 construct described above, use of the minimal functional domain allows stable expression of an EGFP fusion protein without the toxicity associated with overexpression of helicase B.

In the absence of preceding knowledge of functionally portable domains the logical approach is to use the intact fusion protein as a fusion partner and evaluate the construct by transient expression. If the fusion is functional but gives problems with toxicity, deletion mutants might be worth investigating, but only after other construct design options have been exhausted.

Choosing which orientation to use to join the target and fluorescent proteins is the next decision point. In some cases, like the two constructs discussed earlier, the choice is obvious; the functional domains are appended to EGFP in the same orientation as in the native protein. If no published information exists on fusion of your target protein it might be possible to deduce a favored coupling strategy from any published data on the role of a particular terminus or domain in localization or function. In some cases only a minimal amount of structural information might be required to make an informed judgment. For example, in the case of membrane receptors, such as seven transmembrane receptors, it is fairly intuitive assumption that appending a fluorescent protein to the intracellular terminus is more likely to produce a correctly inserted membrane protein than adding a bulky fusion partner to the extracellular domain, and indeed this is the case for GPCRs *(86)*. If three-dimensional structural data are available for the target protein, computer modeling of the protein linked to the chosen fluorescent protein might give some guidance toward building an optimum construct (**Fig. 1C**). As a last resort when no information is available to guide a decision on fusion protein orientation then making both constructs and testing by transient expression is the only option (**Fig. 4**).

Fusion design must also take the structure of the fluorescent protein into account. *A. victoria* GFP has a flexible carboxyl terminal tail of approx 10 amino acids *(87)*, which makes its fusion to the amino terminus of other proteins possible without the addition of a linker (*see* **Fig. 1C**). Conversely, tetrameric DsRed is more amenable to fusion to the carboxyl terminus of proteins, as the amino termini project fully from the fluorescent complex.

If the nature of the fusion partner precludes coupling to the flexible terminus of the chosen fluorescent protein, introduction of a synthetic linker sequence or an additional protein stuffer sequence will permit efficient folding and maturation to yield both fluorescence and function. The length and composition of the linker should be optimized for each fusion protein, in many cases steric or folding interference can occur between the fusion partners if the linker is not sufficiently long and flexible. Glycine confers the most flexibility to a peptide chain, and the most widely used linker designs have sequences that primarily consist of glycine and serine oligomers, serine being interspersed to improve the solubility relative to a glycine homopolymer. Variations in linker design can significantly effect both expression and efficiency of protein folding and a number of variants might need to be constructed and tested (*see* **Fig. 5**).

Having designed the fusion protein the next task is to determine the promoter to use to control expression, and a number of strategies and choices are available. The aim in selecting a promoter is to get sufficient fluorescent protein expression for robust imaging and analysis without swamping the cellular process under study or producing toxic levels of expression; just enough and no more should be the target. For constitutive expression the heterologous CMV promoter *(88)* is most commonly used, giving high levels of expression though expression levels will vary with cell type. When maximal expression is not required or desirable, alternative constitutive promoters such as Ubiquitin C *(89)* might be used. In our hands the Ubiquitin C promoter

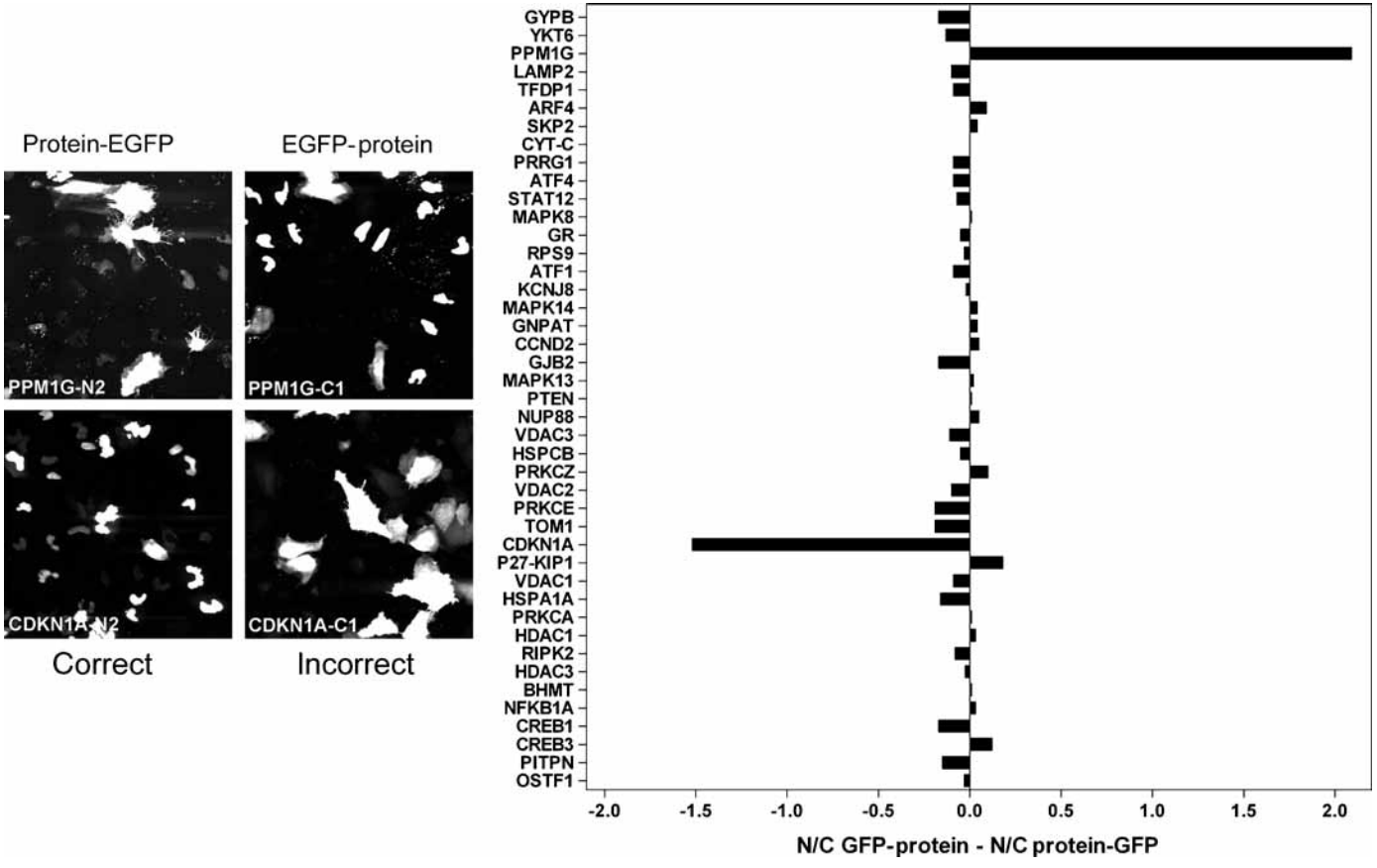


Fig. 4. Screening EGFP construct orientation for correct subcellular localization. N-terminal and C-terminal fusions for 43 cDNAs were transiently expressed in HeLa cells and nuclear and cytoplasmic expression determined by image analysis. Two proteins, protein phosphatase 1G (PPM1G) and p21 cyclin dependent kinase inhibitor (CDKN1A), showed differential expression between the oppositely orientated constructs with coupling of EGFP to the amino termini of the fusion partner giving the correct localization in both cases.

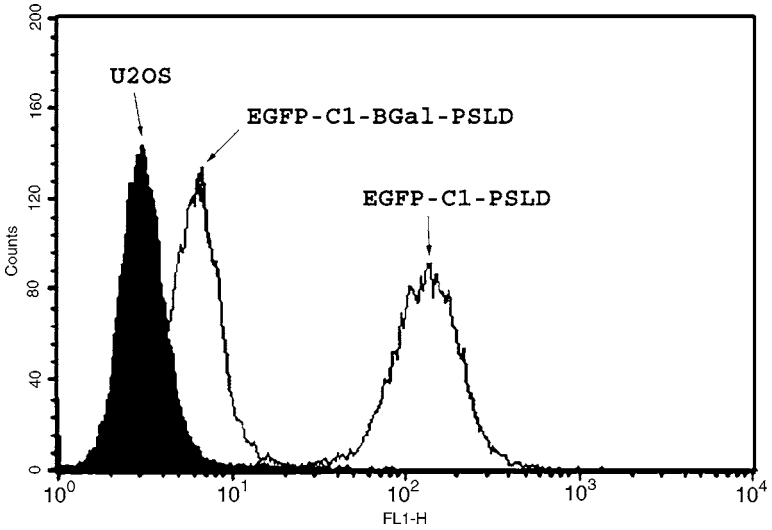


Fig. 5. Effect of construct and linker size on fusion protein expression. Two variants of the G1/S cell cycle phase marker shown in **Fig. 3** were transiently expressed in U2OS cells and EGFP fluorescence determined by flow cytometry. Inclusion of β-galactosidase as a linker (EGFP-C1-BGal-PSLD) to limit passive nuclear-cytoplasmic diffusion of the fusion protein yielded markedly lower expression than a construct containing a minimal linker sequence (EGFP-C1-PSLD).

yields expression levels around one-third of that of the CMV promoter, depending on the parental cell type. If the fusion protein is toxic in long-term culture, or if variable control of expression level is required it will be necessary to employ an inducible expression system such as those controlled by ecdysone ***(90)*** or tetracyline ***(91)***. In some cases in which the target protein is subject to variation in expression it might be desirable to employ the native promoter, as in the Cyclin B1-EGFP fusion described in the following section.

Remaining design factors such as choosing the expression plasmid and the selection marker are common with those for expression of any protein in a mammalian cell. For general methods relating to cloning and expression of heterologous proteins in mammalian cells *see* **refs.** ***92*** and ***93***. Representative vector maps for plasmids containing EGFP fusions under the control of a heterologous constitutive promoter and a homologous inducible promoter are shown in **Fig. 3**. Once engineering of constructs is completed they can be functionally tested using transient transfection ***(94)***. These preliminary optimization and validation experiments allow the localization and response of the fusion protein to stimuli to be evaluated (**Fig. 6**), and can provide information on the effects of expression level on function, which can be a valuable aid during subsequent selection of a stable cell line.

## 4. Selection and Validation of Engineered Cells

Once the construct is designed and transfected into the chosen cells now comes arguably the most important and sometimes protracted part of producing an engineered cell for HCS; selection and validation. The techniques and efforts required to select a cell clone will depend on both the facilities available and the nature of the assay to be performed with the cells. The overall aim is to produce a cell line of a quality which is fit for purpose. In some cases, particularly when the cell line is to be used for primary screening, where stable and robust assay performance over many thousands of assays is critical, this might require the cell line to produce data to a statistical metric, such as *Z*-factor ***(95)***, but have little regard to the effect of overexpression of the fusion protein on cellular biology. In contrast a cell line engineered for detailed analysis of activity

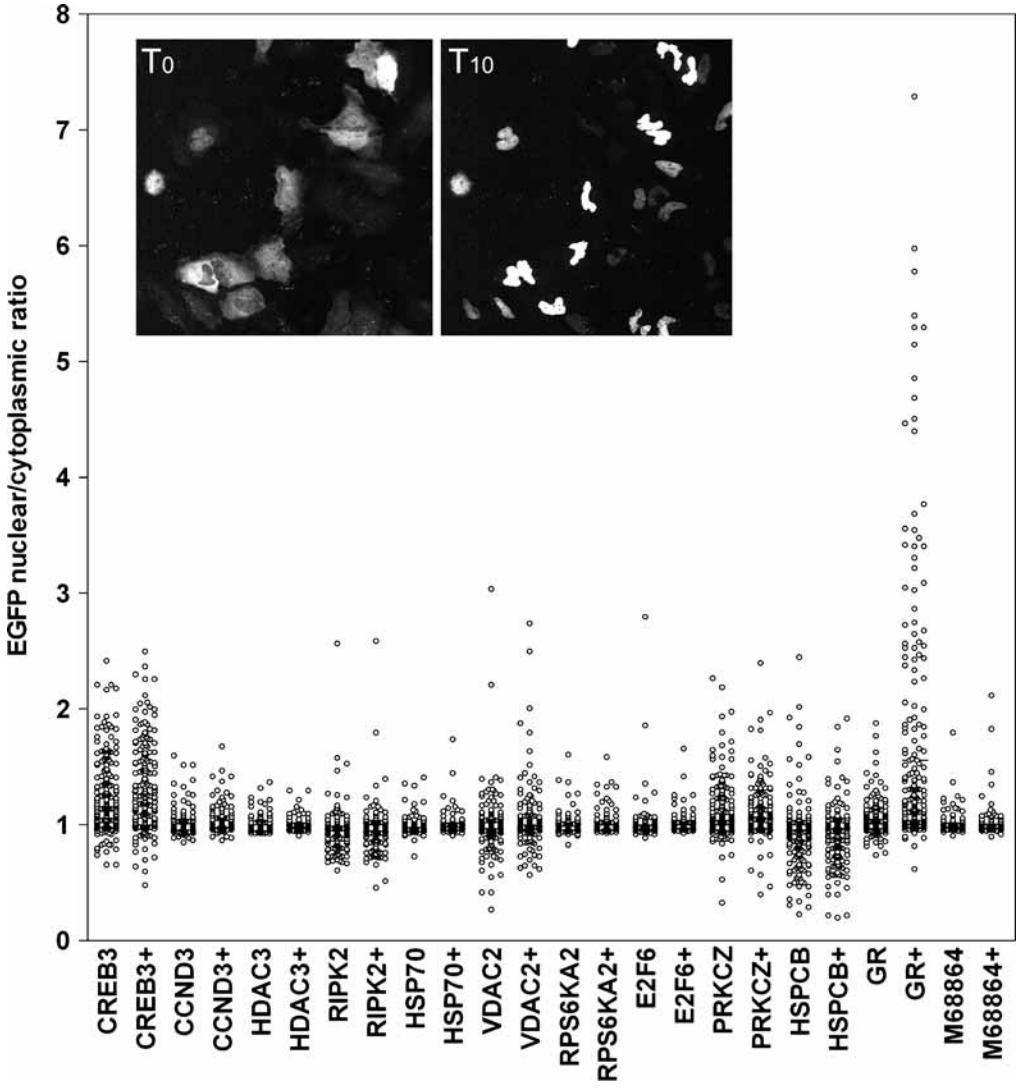


Fig. 6. Functional validation of EGFP fusions by screening transient expression. EGFP-cDNA fusion proteins previously screened for specific sub-cellular localization (**Fig. 4**) were evaluated for response to stimuli. HeLa cells expressing constructs were imaged before and after exposure to 10 μ*M* dexamethasone and fusion protein translocation determined for individual cells by image analysis. Under these conditions an EGFP-glucocorticoid receptor fusion (GR) showed translocation from cytoplasm ($T_0$) to nucleus ($T_{10}$) in response to dexamethasone with significant increases in the ratio of nuclear/cytoplasmic EGFP fluorescence (GR+).

against a cellular signaling pathway for secondary screening or target validation will require extensive selection and validation to derive a cell line in which the biology under study is not perturbed by expression of the sensor.

### *4.1. Selection of Cells*

Given that the primary task in cell selection is isolation of fluorescing cells from nonfluorescing cells it is not surprising that flow cytometry ***(96)*** is the method of choice for cell selection. Using fluorescence-activated cell sorting (FACS) to separate pools and individual cells with

different levels of expression allows an efficient dual selection strategy to be used to combine some of the elements of assay validation with selection. In this approach cells are FACS sorted and pooled into populations with different levels of fluorescent protein expression, for example, high, medium, and low. Samples from these populations can then be used for cloning by limiting dilution into wells of a 96-well plate and the remaining cell populations expanded to test under assay conditions. If a FACS with single-cell separation capability is available cells can be directly isolated into wells of 96-well plates in a second run using the same gate settings.

Although cell clones are expanding the mixed populations can be tested under HCS conditions to determine the optimum level of fluorescent protein expression for the desired cellular response. This period also allows a certain amount of assay development work to be carried out to investigate different assay and analysis parameters, including devising a working image analysis strategy for abstracting data. Once cell clones are expanded, cells with suitable expression levels can be screened individually to select a cell line for HCS use. The final cell line should be reanalyzed by flow cytometry to check for uniform expression and if necessary FACS sorted before expansion and freezing down stock cells.

### *4.2. Validation of Stable Cells*

Procedures used for validation of cells for HCS will vary widely depending on the nature of the fusion protein, the intended use of the cell line and the complexity of the biology involved, but generally involves two levels of testing; internal validation and external validation. First level internal validation tests the functionality of the engineered cell toward the assay it was designed to fulfill. This typically requires testing response to a series of known agonists/antagonists and establishing $EC_{50}$/$IC_{50}$ values and order of potency, reflecting the use of the cell line in HCS. Data should be compared with published data available, but equivalence of $EC_{50}$/$IC_{50}$ values should be interpreted with care, as these can vary considerably with assay procedure.

Second level external validation involves comparison of assay data generated using the fluorescent protein sensor and high content analysis against an independent assay measurement. This process can take a variety of forms, which may differ in their degree of independency depending on the analysis techniques available. The most stringent form of validation is to use a completely independent analysis process, for example, using propidium iodide staining of GFP expressing cells and flow cytometry to measure cell cycle distribution, which was the process used to validate the cell cycle sensors shown in **Fig. 3**. If a completely independent analysis procedure on a different instrument platform is not available, correlation of data from the fluorescent protein with data from an independent read-out in the same cells is a viable option, for example, correlation of translocation of the fusion protein with translocation of the endogenous target protein detected using antibody staining. For validation of the G1/S cell cycle sensor we used incorporation of bromodeoxyuridine as an independent marker for S-phase cells (**Fig. 7**). Further higher level biological validation will be needed in cell lines where it is required that introducing the fluorescent protein sensor does not perturb the very process it is designed to measure. Such studies will include establishing that the engineered cells display the same phenotype and growth characteristics as the parental cells (**Fig. 8**), and monitoring the effect of fluorescent protein expression on the biology of the engineered cells (*see* **Subheading 5.**).

## 5. Effect of Expressing Fluorescent Proteins on Cellular Biology

Expression of any recombinant protein in a cultured cell has the potential to perturb the biology of the cells, and might invalidate or obviate the purpose for which the engineered cell was created. Putting an additional load on cells by forcing them to express proteins fused to a large heterologous protein tag might disrupt or overload protein synthesis, trafficking, or proteolysis, leading to toxic effects. Despite the widespread use of fluorescent protein expressing cell lines, little work has been published on characterizing the effects of expressing tagged fusion proteins on the biology of the host cell.

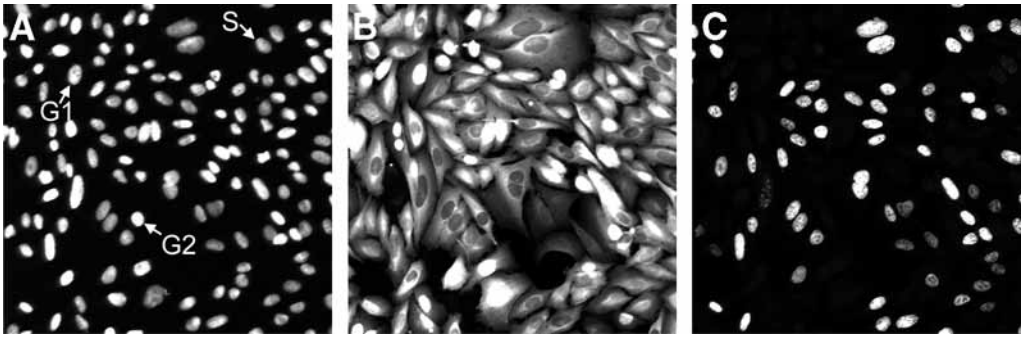


Fig. 7. Validation of EGFP fusion protein function against an independent sensor. U2OS cells expressing the G1/S cell cycle phase marker (**Fig. 3**) were pulse-labeled for 1 h with bromo-deoxyuridine (BrdU) and BrdU incorporation detected using a monoclonal anti-BrdU/nuclease and a Cy5 labeled second antibody. Three color imaging of **(A)** Hoechst stained DNA, **(B)** EGFP and **(C)** Cy5 allowed confirmation that the EGFP sensor was functioning as designed with intense nuclear EGFP fluorescence in nuclei of G1 cells (G1 arrowed), nuclear and cytoplasmic EGFP in BrdU positive S-phase cells (S arrowed), and predominantly cytoplasmic EGFP in G2 cells (G2 arrowed).

There is some historical evidence that in some circumstances very high levels of GFP expression might be toxic to mammalian cells ***(97)***, and early work with novel fluorescent proteins such as DsRed revealed abnormal localization of the DsRed fusion proteins with aggregation and toxicity in some cases ***(98,99)***.

However, it is now generally assumed that low to moderate expression of GFP or other monomeric fluorescent proteins is minimally perturbing to the host cell ***(100)***. One study that examined the effect of stable expression of a GFP-α tubulin fusion in LLCPK-1 cells ***(101)*** showed that the mitotic index and doubling time were unchanged for cells expressing GFP-α tubulin as 17% of total tubulin, although endogenous tubulin expression was reduced compared to parental cells. A further study ***(102)*** examined the effects of expression of a GFP-estrogen receptor fusion in MCF-7 cells and demonstrated that GFP-estrogen receptor expression does not alter cell doubling time or cell cycle distribution and does not interfere with the induction of estrogen receptor responsive genes.

To investigate the cellular consequences of GFP expression we have examined the effect of GFP expression by analyzing cell cycle progression and gene expression in a GFP stable cell line and in parental U2OS cells ***(103)***. The stable cell line ***(104,105)*** expresses a fusion of amino acids 1–170 from the amino terminus of Cyclin B1 coupled to EGFP, with expression under the control of the Cyclin B1 promoter. The EGFP fusion protein is consequently expressed and degraded in concert with endogenous Cyclin B1, but as the fusion protein lacks the C-terminal sequences necessary for Cyclin B1-CDK interaction, the EGFP fusion protein acts as a stealth sensor, shadowing endogenous Cyclin B1 expression and degradation without disturbing the cell cycle of cells in which it is expressed. To ensure minimal perturbation of the cell cycle the stable U2OS cell line was derived by screening a large number of clones and selecting a single clone with the minimal level of EGFP expression compatible with determination of cell cycle status by microscopy and image analysis. Measurement of EGFP fusion protein mRNA by quantitative RT-PCR showed the expression of the EGFP sensor to be equivalent to endogenous Cyclin B1 (7000 copies/cell in G2).

Analysis of cell cycle duration and cell cycle phase distribution by cell growth assays and flow cytometry revealed that the two cell lines had identical doubling times and cell cycle distributions. Microarray analysis showed a 0.9% (>twofold at $p < 0.001$ across 20,000 genes) difference in gene expression levels between parental and EGFP expressing U2OS cells, with no significant

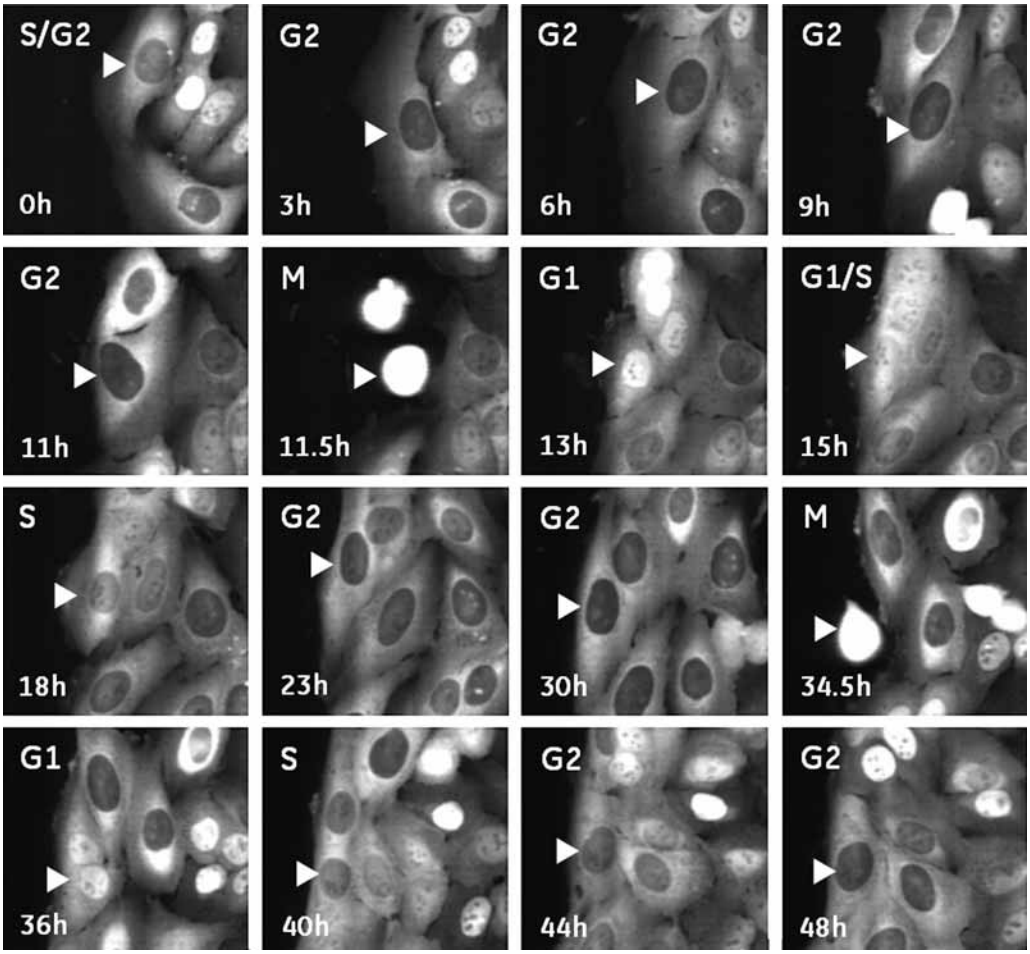


Fig. 8. Phenotype validation of EGFP expressing cells. U2OS cells expressing the G1/S cell cycle phase marker were imaged in culture over a 48 h period at intervals of 30 min. Examination of time lapse images showed a mitosis to mitosis time of 23 h (arrowed cell in frame 6 to frame 12 of those shown), an equivalent cell cycle time to parental U2OS cells, indicating that fusion protein expression does not prolong cell cycle transition.

differences in expression of Cyclins, CDKs, or CDK inhibitors between the two cell types. We conclude that engineering stable cell lines for low expression of EGFP fusion proteins is minimally perturbing to cellular biology.

## 6. Alternative Labels to Fluorescent Proteins for Engineering Cells for HCS

In recent years a number of genetically encoded labels have been developed for tagging proteins in living cells, which offer an alternative to fluorescent proteins. One method engineers recombinant proteins to insert four cysteines residues in an α-helix, which are subsequently labeled with arsenical fluorescein derivative *(106)*. The FlAsH label is cell permeable and non-fluorescent until it binds with high affinity and specificity to the tetracysteine domain. Although this protein tagging method adds less mass to the recombinant protein than the equivalent protein expressed as a GFP fusion, low affinity of binding requiring high expression of the target motif and high concentrations of the label with possible cellular toxicity limited adoption of the method. Recent developments of the technique *(107)* have increased the utility of the method by

improving labeling affinity using a modified hairpin-helix binding site and provision of a red fluorescing resorufin label (ReAsH). Use of the improved method has been reported for examining gap junction formation *(108)*, AMPA receptor trafficking in neuronal cells *(109)* and HIV-1 Gag protein localization in a range of cell types *(110)*.

A second method, termed HaloTag *(111)*, uses a haloalkane dehalogenase from *Rhodococcus rhodochrous* (mDhaA), which has been mutated to form a stable covalent bond when it binds a chlorinated substrate analog. Fusion of the 33-kDa mDhaA to a cellular target protein allows tagging with cell-permeable rhodamine and fluorescein ligands. Although this method slightly increases the size of the tagged fusion protein relative to an equivalent GFP fusion, the ability to use different fluorescent labels with the same engineered cells might be advantageous in some circumstances. However, because in contrast to the FlAsH and ReAsH labels previously described mDhaA ligands are fluorescent in free solution cells must be washed to remove unbound ligand preceding to imaging.

A third recently developed method for labeling recombinant proteins in living cells SNAP-tag *(112)*, labels fusion proteins of a mutated form of human alkylguanine-DNA alkyltransferase (AGT) with fluorescent ligands. Labeling is based on the irreversible and specific reaction of AGT with $O^6$-benzylguanine derivatives, leading to the transfer of the label to a reactive cysteine residue within the active site of AGT. Fusion proteins produced for labeling with this method are slightly smaller than fusions with GFP or other fluorescent proteins; however, as with HaloTag labels, the fluorescein and rhodamine derivatives used to label AGT are fluorescent at all times and must be removed before imaging. Additionally, as the method uses a mutant form of a human enzyme, some labeling of endogenous AGT might occur in some cell types.

Although the labeling methods described above offer alternative approaches to visualizing proteins in living cells, and allow the flexibility of changing fluorescence wavelengths without the need to engineer new fusion proteins, they are as yet unproven in long-term culture and in stable cell lines. Moreover, this flexibility comes at the price of more complex experimental protocols with additional labeling and washing regimes. Finally, and perhaps most importantly, there is the issue of the compatibility of these synthetic labels with the dynamics of cellular processes. All cellular proteins go through a life cycle of expression, action and destruction, and all cells have the necessary machinery to accomplish this. The chromophore of fluorescent proteins is integral to the protein structure; therefore, provided that cells are not overloaded with recombinant protein, the fluorescence of the fusion protein will follow the natural life cycle of the protein. This is not the case for fusion proteins labeled with synthetic fluors; proteolysis of the fusion protein will not necessarily destroy the fluorescent label. This might be particularly problematic for proteins which are rapidly turned over. In such cases the population of specifically labeled proteins will be quickly depleted although the background fluorescence from liberated label increases, giving a limited period following labeling in which to carry out any analysis. This feature of synthetic labels might preclude their use in many areas of cell biology, for example, the cell cycle, where key proteins are constantly degraded and replenished. Synthetically, tagged proteins represent a finite and rapidly depleted cellular resource, fluorescent proteins are a constantly renewable resource.

## 7. Future Perspectives

Although to date the majority of HCS assays using fluorescent proteins have used stable cell lines, recent developments in viral vector systems are opening the way for use of transient expression as a flexible means of engineering cells for HCS.

Expression of fluorescent fusion proteins using adenoviral *(113–116)* and baculoviral *(117,118)* expression systems allows rapid development of HCS assays without the time delays inherent in producing stable cell lines, and permits assays to be run in a variety of different cell types. Our laboratory is currently generating a large panel of adenovirally encoded sensors using

fluorescent fusion proteins and other sensors *(119)* for use in target validation and lead profiling. These constructs open the way to developing HCS assays in cell types, including primary cells, which offer a more physiologically relevant background for analysis of gene and drug function than standard transformed laboratory cell lines.

Ongoing research using stem cell lines engineered with GFP fusions *(120,121)* has the potential in the near future to generate a variety of differentiated cells expressing sensors for HCS in a cellular background, which closely matches the phenotype and physiology of diseased and normal tissue. Coupling of these new approaches to cellular engineering with ongoing advances in image analysis, which allow multiparameter phenotypic and morphological analysis will continue to advance the power of HCS to analyze complex cellular systems.

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