Stealth sensors: real-time monitoring of the cell cycle

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The cell cycle is of key importance to many areas of drug discovery. On the one hand this fundamental process provides the opportunity to discover new targets for anticancer agents and improved chemotherapeutics, but on the other hand drugs and targets in other therapeutic areas must be tested for undesirable effects on the cell cycle. Historically, a wide range of techniques have been developed to study the cell cycle both as a global biochemical process and at the molecular level. This article reviews a range of methods for analysis of the cell cycle and introduces a novel class of dynamic stealth sensors based on expression, localization and degradation of green fluorescent protein under the control of key components of cell cycle regulation. These sensors allow the precise status of the cell cycle to be determined in live cells by fluorescence imaging without perturbing cell cycle progression.

To increase the sophistication of technologies available to study this fundamental cellular process, we are developing a range of green fluorescent protein (GFP) cell cycle phase markers (CCPMs) that continuously report the status of the cell cycle in living cells.

What are the applications of CCPMs?
As CCPMs do not interfere with the cell cycle of the host cell, and allow non-destructive determination of cell cycle position, these sensors have many potential applications in cultured cells and more complex model systems. Perhaps most significantly, CCPM expression in stable cell lines allows continuous and individual monitoring of the status of the cell cycle in every cell in the culture and, consequently, removes the need to work with synchronized cells to identify cell-cycle-related phenomena.

Applications in drug discovery include screening for substances that directly or indirectly modify the cell cycle as potential anticancer agents and for treatment of other cell-proliferation-related processes such as fibrosis and angiogenesis, as well as screening drug candidates against other targets for unwanted effects on cell cycle progression. Multiplexing with a second analysis can be valuable where it is suspected that an agent is active or inactive towards cells in a particular phase of the cell cycle, or to control for cell-cycle-dependent or toxicity-related variations in an assay readout. The use of CCPMs in conjunction with other fluorescent probes for ligand binding, ion flux or other cellular processes will enable the cell cycle dependency of many signalling pathways used by drugs or other therapeutic regimes to be investigated.

CCPM expression in transgenic organisms and animals has significant potential for study of the cell cycle in normal and abnormal...
Box 1. The eukaryotic cell cycle

The cell cycle is an ordered set of events culminating in cell division into two daughter cells (Fig. I). Non-dividing cells exist outside the cell cycle in G0. The phases of the cell cycle are designated G1, S, G2 and M. In G1 (gap 1) cells increase in size, produce RNA and synthesize protein. In S (synthesis) DNA replication occurs to produce DNA ready for cell division. In G2 (gap 2) cells continue to grow and produce new proteins. In M (mitosis) cell growth and protein production stop, and each cell undergoes a complex and orderly division into two daughter cells.

The approximate duration (hours) of each cell cycle phase for a typical mammalian cell with a cell cycle time of 24 h is shown in Fig. II.

How do CCPMs work?
The progression of the cell cycle is tightly regulated by defined temporal and spatial expression, localization and destruction of several cell cycle regulators, which exhibit highly dynamic behaviour during the cell cycle. One of the most extensively characterized cell cycle regulators in human cells is cyclin B1, temporal and spatial expression and destruction of which controls cell transition from G2 to M and its exit from M. Cyclin B1 expression is driven by a cell-cycle-phase-specific promoter that initiates expression at the end of S phase and peaks during G2 [16]. Once expressed, cyclin B1 constantly shuttles between the nucleus and cytoplasm during G2, but is primarily cytoplasmic because the rate of its nuclear export is much greater than its import. At the start of mitosis, cyclin B1 rapidly accumulates in the nucleus in a phosphorylation-dependent manner [17,18]. Thus, the localization of cyclin B1 in the cell can be used to mark the transition from G2 to M. Once a cell reaches metaphase or, more accurately, when the spindle-assembly checkpoint is satisfied, cyclin B1 is very rapidly degraded [19].

We have exploited these exquisite spatial and temporal control mechanisms to develop cell cycle sensors based on growth and development when used in conjunction with other tools such as RNA interference or gene-knockout or -knock-in strategies. The recent discovery of a direct connection between neuronal cell cycle re-entry and oxidative stress in the ageing central nervous system using a mutant mouse model [10] highlights the potential of dynamic cell cycle sensors for studying disease and degenerative processes in vivo.

In cell biology research, multiplexing a CCPM with other fluorescent read-outs from organelle-specific markers, fusion proteins with other fluorescent proteins, or other probes gives the potential to correlate virtually any cellular event or process for which a probe is available with cell cycle position. Cell-cycle-dependent responses to cell stimulation have been reported previously including cell-cycle-dependent expression of endothelin receptors [11] with consequent variation in endothelin-induced apoptosis, variations in Ca2+ mobilization in response to vasopressin [12] owing to variations in signal transduction that use different G-proteins at separate phases of the cell cycle, and the cell-cycle-dependent response to the CCK-B/gastrin ligand CI-988 [13]. Cell cycle position can also significantly alter response to chemo- and radiotherapy. Wortmannin has been shown to radiosensitize cells in G1 preferentially [14] and similarly, the results of combined taxol and radiation treatment have been shown to vary with the cell cycle [15].
linking GFP expression to key components of the cell cycle. One of these constructs based on components of cyclin B1 dynamically reports cell progression through the G2/M section of the cell cycle. The GFP construct is expressed under the control of the cyclin B1 promoter, producing a fusion protein of GFP linked to the cytoplasmic-retention sequence (CRS) and degron sequence from cyclin B1. When transfected into a cell the appearance and disappearance of the GFP construct shadows the behaviour of the cell’s endogenous cyclin B1. As all cyclin–CDK interaction domains are absent, the construct operates as a stealth sensor and does not perturb the cell cycle of cells in which it is expressed.

Cells expressing the G2/M stealth sensor begin to show GFP fluorescence in late S phase with the intensity of fluorescence increasing through G2 (Fig. 1). As cells progress into prophase the GFP-containing fusion protein translocates to the cell nucleus and the subsequent transition to metaphase is marked by uniform cellular fluorescence accompanying nuclear breakdown. At the completion of mitosis, the transition to anaphase results in the rapid disappearance of GFP fluorescence as the sensor is subject to ubiquitination and proteasome degradation. Cells subsequently remain dark through G1 and early S phases before the process is repeated.

The status of the cell cycle can be determined by measuring the intensity of GFP fluorescence in individual cells by imaging or flow cytometry. For further precision, imaging at sub-cellular resolution can be used to determine both the intensity and distribution of GFP in individual cells (Fig. 2). The latter can be used to discriminate between cells whose cell cycle position is separated only by minutes, for example at the G2–prophase transition (Fig. 3), and combining these measurements with morphological parameters such as the shape and size of cells allows all phases of mitosis to be distinguished.

What distinguishes CCPMs from other available methods of cell cycle analysis?

CCPMs are the only easily automated method for determining the cell cycle status of individual living cells using non-destructive analysis. Although a wide range of other techniques are available to monitor the status and progression of the cell cycle (Table 1), these methods all produce either population-average data, require fixation or lysis, interfere with cell cycle progression or require complex morphological analysis from time-lapse video recordings.

The first distinction to be made in reviewing established methods of cell cycle analysis is to separate methods that produce data describing the proliferative activity of a cell
population from those that can be used to determine the cell cycle position of an individual cell.

**Methods for measuring cell proliferation**

Measuring the incorporation of [14C]- or [3H]-thymidine by scintillation counting was one of the earliest methods of determining cell proliferation, and is still widely used today. More recent developments [21] have allowed thymidine incorporation to be measured in a homogeneous microplate assay format.

Several non-radioactive alternatives to thymidine incorporation assays have been developed. These include enzyme-linked immunosorbent assay (ELISA) measurement of incorporation of the modified nucleotide bromo-deoxyuridine (BrdU) [22,23] into replicating DNA, and staining of proliferation-specific antigens such as Ki-67 [24].

### Table 1. Techniques and applications for cell cycle monitoring

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*Abbreviations: BrdU, bromo-deoxyuridine; CFSE, carboxy-fluorescein diacetate succinimidyl ester; ELISA, enzyme-linked immunosorbent assay; GFP, green fluorescent protein; PCNA, proliferating cell nuclear antigen; PI, propidium iodide.*
Colourimetric methods based on substrate conversion by mitochondrial and other cellular enzymes are also used to measure cell growth. Although these assays are often referred to as cell-proliferation assays, strictly speaking they are cell-mass assays. Unlike measuring thymidine or BrdU incorporation, these assays do not provide any inherent measure of cell cycle progression, and give only a measure of cell proliferation, that is, increase in cell number, relative to another population.

Other methods for measuring cell proliferation (i.e. increasing cell numbers) have been reported based on measuring electrical impedance [27], dissolved oxygen [28] and others. However, as for the colourimetric assays discussed above, these do not directly report cell cycle parameters and have not been widely adopted.

All of the above methods provide data on the overall proliferation within a cell population under examination, but do not identify the status of individual cells. Adaptation of these assays to imaging, for example by micro-autoradiography of [3H]- or [14C]-thymidine incorporation [29] or by immunocytochemical or immunofluorescence detection of BrdU [30] (Fig. 4) permits identification of cells that have traversed S phase, but does not yield information on the cell cycle position of other cells under analysis.

**Methods for analyzing cell cycle distribution**

To determine the cell cycle status of all cells in a population it is a prerequisite that the analytical technique can resolve at least to the level of a single cell. Of the two qualifying techniques available, flow cytometry and microscopy, flow cytometry has become firmly established as the standard method for analyzing cell cycle distribution.

The DNA content of cell nuclei varies through the cell cycle in a predictable fashion – cells in G2 or M have twice the DNA content of cells in G1, and cells undergoing DNA synthesis in S phase have an intermediate amount of DNA. Consequently, staining of cellular DNA with propidium iodide [31] (Fig. 5), or other fluorescent dyes [32] that are compatible with live cells, followed by flow cytometry permits measurement of the relative proportion of cells in G1, S and G2/M. However, analysis by propidium iodide staining and flow cytometry is necessarily destructive and hence requires multiple samples to study cell cycle progression, which can become rate limiting where many hundreds of samples are to be analyzed. In addition, flow cytometry does not yield fine resolution of cell cycle position in G2/M as the DNA content is the same in all cells.

A combination of DNA staining with pulsed BrdU incorporation [33] can be used to resolve the cell cycle position further. Dual-parameter analysis of DNA staining and/or BrdU incorporation can also be used with antibodies to cell-surface markers to profile cell cycle distribution in a defined sub-population of cells [34–36].

Although to date flow cytometry has remained the dominant method for analyzing the cell cycle, many of the above techniques have also been applied to microscopic analyses [37,38].
Measuring cell cycle progression

The techniques described above all provide information in various forms from a single point in time (e.g. propidium iodide staining for DNA content) or integrated over a period in time (e.g. thymidine or BrdU incorporation). One further technique, cell-division tracking [39,40], allows the replicative history of a cell population to be analyzed. In this method cells are loaded with a fluorescent dye such as carboxy-fluorescein diacetate succinimidyl ester (CFSE), which is partitioned between daughter cells at each successive round of cell division with a twofold reduction in fluorescence. Subsequent analysis of cell fluorescence by flow cytometry reveals the number of cell divisions undergone by each cell in the population. This technique has also been used in multi-parameter analyses combined with BrdU and proliferation-marker staining [41].

Real-time analysis of cell cycle events

The application of GFP and imaging techniques to cell cycle analysis has enabled significant advances to be made in understanding the timing of the molecular events that control the cell cycle. Fusing GFP with key cell-cycle-control proteins [42–46] and other cellular components [47–49] has provided very significant insights into the molecular organization behind the cell cycle. However, although these specialized approaches provide valuable data on the mechanisms and components involved, they are not generic methods for monitoring the cell cycle.

What are the strengths and weaknesses of CCPMs?

Stable expression of a CCPM in a living cell provides a non-perturbing, continuous, real-time read-out of cell cycle position as the cell progresses through the cell cycle. These properties permit stably expressing CCPM cell lines to be used to study cell cycle progression in unsynchronized cells, removing the inconvenience and possible side effects of cell synchronization regimes, and allow other live-cell assays to be correlated with cell cycle position in multiplexed analyses. As the CCPM sensors are genetically encoded, expression in model organisms and transgenic animals has considerable potential for studying the cell cycle and dependent processes in vivo.

Use of CCPM in cell cycle studies requires some preliminary work to establish stable expression of the sensor in the cell of interest, and to select cells in which the expression level does not perturb the cell cycle by overloading the ubiquitination and degradation machinery used to reset the sensor during each cell cycle. Consequently, using CCPMs will not be the method of choice for short-term studies, which require only the basic parameters for the cell cycle distribution of a cell population, where the speed and convenience of flow cytometry will yield the desired results. Similarly, if simple comparison of proliferative activity between cell cultures is required, any of the DNA-synthesis or cell-mass assays described previously will provide the necessary data from a quick and convenient procedure.

However, if a significant amount of cell cycle analysis is to be undertaken, for example, screening a large number of candidate drugs or analyzing the cell cycle dependency of a cellular event in a multiplexed format, use of CCPM-expressing cells allows these same gross population studies to be performed while also permitting much more detailed analysis of the cell cycle and factors and events influencing or related to cell cycle progression.

Who are considered to be the major competitors in the field?

Molecular probes (http://www.probes.com) provide a variety of reagents for profiling cell cycle distribution by flow cytometry including propidium iodide and SYTOX dyes. A more recent addition to DNA stains for flow cytometry, DRAQ5 from Biostatus (http://www.biostatus.co.uk), can be used to stain live cells but, in common with other DNA-binding flours, interferes with DNA synthesis and cell division. A variety of kits for measuring cell proliferation by MTT/XTT reduction and BrdU incorporation are available from Roche (http://www.roche-applied-science.com), Sigma (http://www.sigmaaldrich.com), Molecular Probes and other suppliers. Antibodies to cyclins, other cell cycle components...
and cell-proliferation antigens, such as Ki-67, suitable for immunostaining of fixed cells are available from Clontech (http://www.clontech.com), Abcam (http://www.abcam.com), Sigma, Molecular Probes and others. None of these reagents, kits or procedures permit real-time continuous monitoring of cell cycle position in living cells.

**What partnerships do you have to date?**
The CCPM technology was developed in collaboration with Dr Jon Pines, Wellcome/CR UK Institute of Cancer and Developmental Biology, Cambridge. We are currently working with Dr Elaine Sullivan, Director of Advanced Science and Technology, Astra Zeneca to apply CCPM and high-throughput imaging to drug screening.

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**References**

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