Application Note

Cancer Biology

Agilent

Trusted Answers

Monitoring Cell Cycle Progression Using Microscopy

Fluorescence-based imaging to assess cell cycle progression

Authors

Paul Held, PhD and
Brad Larson
Agilent Technologies, Inc.

Abstract

The concept of the cell cycle is used to describe the repetition of events that lead to the duplication of cellular organelles during cellular replication. Compounds that affect cellular transition through the cell cycle have the potential to be effective anticancer agents. This application note describes the use of digital widefield fluorescence microscopy in conjunction with different fluorescence assays to monitor cell cycle progression in live and fixed human tissue culture cells.

Introduction

Cell cycle progression is a highly regulated process with a number of checkpoints. Two classes of regulatory molecules, cyclins and cyclin-dependent kinases (Cdks) are responsible for cell cycle progression control (Figure 1).

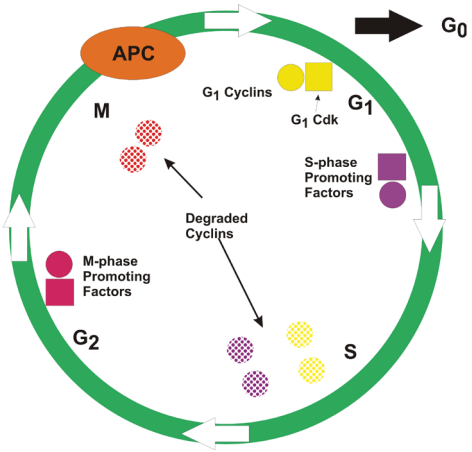

Figure 1. Schematic of cell cycle.

Cyclins and Cdks are genetically conserved proteins that work in concert as heterodimers with the cyclins serving as regulators, while Cdks serve as the catalytic units. Cyclins have no catalytic activity and Cdks are inactive in the absence of a partner cyclin. When activated by a bound cyclin, Cdks phosphorylate specific proteins, resulting in their activation or inactivation depending on the protein. The protein target is dependent on the combination of cyclin and Cdk. Cdks are constitutively expressed in cells whereas cyclins are synthesized at specific stages of the cell cycle, in response to various molecular signals.[1]

Two stages within the cell cycle are readily discernible. Mitosis (M-phase), the process of cell division, can be observed through microscopy (Figure 2). DNA synthesis (S-phase) is the process of deoxyribonucleic acid duplication within the nucleus, which can be identified by the incorporation of nucleotides, most notably thymidine.

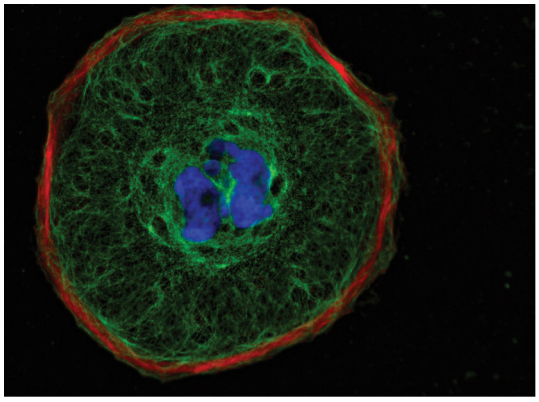

Figure 2. A rat kidney cell undergoing mitosis. PtK2 cells were fixed and stained with fluorescent dyes for tubulin (green), actin (red), and DNA (blue).

As DNA replication is a prerequisite for mitosis, proliferative capacity can be inferred from DNA synthesis through measurements of labeled nucleotide incorporation during the S-phase of the cell cycle. Proliferating cells incorporate four different nucleotides, (thymidine, guanine, cytosine, and adenine) into deoxyribonucleic acid (DNA) during S-phase. Because ribonucleic acid (RNA) synthesis substitutes uracil for thymidine, any incorporation of thymidine into nucleic acid polymers would be expected to be DNA only. Extra-nuclear DNA such as that found in the mitochondria is insignificant relative to the amount of DNA found in the nucleus and is usually ignored. This significant difference has been exploited to assess the timing and extent of DNA replication as a marker for cellular proliferation. While several methods have been used to identify proliferating cells in S-phase, the most accurate methods are based on the incorporation and measurement of nucleosides and their analogs in newly synthesized DNA. Initial studies used autoradiography of cells labeled with [^{3}H]-thymidine to investigate DNA synthesis and cell proliferation *in vivo*.[2] Labeling tissue-cultured cells with [^{3}H]-thymidine allows measurement of whole populations by scintillation counting[3], making this a rapid and accurate assessment of proliferation *in vitro*. With increasing costs for disposal and the inherent danger of radioactive materials, nonradioactive replacements for [^{3}H]-thymidine have been developed.

Click-iT EdU technology using fixed cells

The thymidine analog 5-bromo-2-deoxyuridine (BrdU) is a nonradioactive nucleoside that will incorporate into DNA and can be used to measure DNA synthesis by microscopy or flow cytometry.[4] Detection of incorporated BRdU is accomplished by identification of the BrdU moiety by specific monoclonal antibodies. Unfortunately, access to the epitope by the antibody requires partial denaturation by acid[4], heat[5], or nuclease treatment.[6] The harsh treatment can adversely affect sample integrity and quality, which makes costaining with other antibodies challenging.

A recently described chemical method of labeling DNA synthesis in cells and tissues uses the alkyne-substituted nucleoside 5-ethynyl-2-deoxyuridine (EdU) as a reactive substrate for azide-substituted fluorescent dyes. During the copper-catalyzed alkyne azide cycloaddition (CuAAC or "click") reaction, a covalent bond is formed between the alkyne base and the azide dye. In this application, the EdU contains the alkyne and the Alexa Fluor dye contains the azide (Figure 3). This reaction is rapid, specific, and requires minimal sample processing.[7] The milder treatment compared to BrdU labeling maintains cellular morphology and allows the use of antibodies specific to other targets within the cell. Cell nuclei undergoing DNA synthesis and incorporating EdU would exhibit a bright green fluorescence (Figure 4).

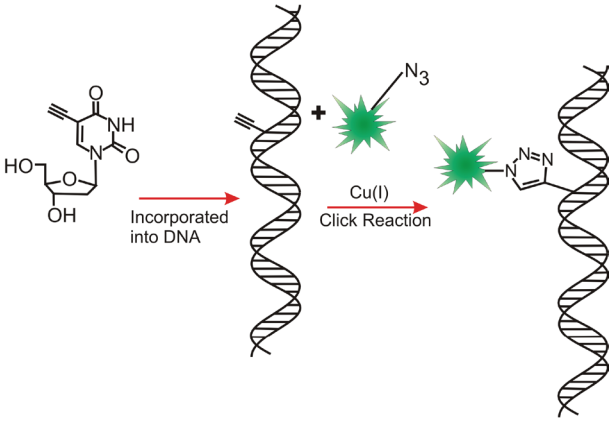

Figure 3. The Click-iT EdU reaction. The modified thymidine analogue EdU is efficiently incorporated into newly synthesized DNA and fluorescently labeled with Alexa Fluor 488 in a copper-catalyzed azide-alkyne cycloaddition reaction.

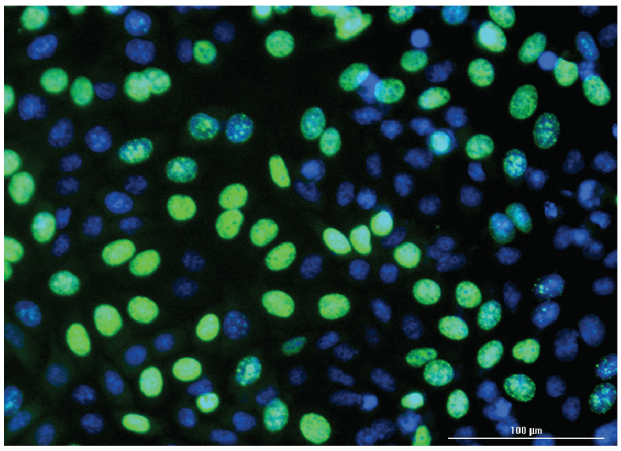

Figure 4. Click-iT EdU-treated HeLa cells. HeLa cells were serum-starved for 24 hours, after which fresh media with 10% serum was added. Four hours after the addition of fresh media, cells were pulse-chased with Edu for 30 minutes. Green nuclei indicate DNA synthesis.

FUCCI technology using live cells

Fluorescence ubiquitination cell cycle indicator (FUCCI) technology is based on two cell cycle-regulated proteins, geminin and Cdt1, fused to a green (emGFP) and red (TagRFP) fluorescent protein, respectively.[8] As Cdt1 and geminin are present only during specific phases of the cell cycle, the fluorescent protein chimeras are similarly cell-cycle dependent. Ubiquitination by specific ubiquitin E3 ligases target the chimeric constructs for degradation and display temporal regulation of activity, resulting in the cycling of geminin and Cdt1 levels during the cell cycle. Geminin-GFP is degraded in the G1 phase, while the presence of Cdt1-TagRFP is indicated by red fluorescence within nuclei. During the S, G2, and M-phases, Cdt1-TagRFP is degraded and only geminin-GFP remains, resulting in green-fluorescent nuclei. During the G1/S transition, when Cdt1 levels are decreasing and geminin levels are increasing, both proteins are present, giving a yellow-fluorescent nuclear signal. This cyclical color change (red to yellow to green) can be used to track progression through the cell cycle and division (Figure 5).

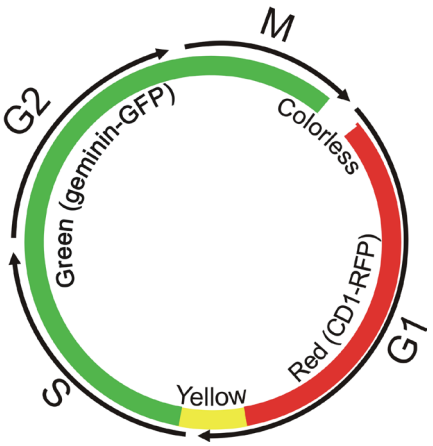

Figure 5. Dynamic color change of Premo FUCCI cell cycle sensor. FUCCI is a fluorescent, two-color sensor of cell cycle progression and division in live cells. Cells change from red in the G1 to yellow in the G1/S interphase and green in S, G2, and M-phases, as geminin and Cdt1, fused to one green and red fluorescent proteins respectively are expressed at specific points in the cell cycle.

Materials and methods

Cell culture

HCT116 cells were cultured in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum and penicillin streptomycin. HeLa cells were cultured in Advanced Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum and penicillin streptomycin. Both cultures were maintained at 37 °C in 5% CO_2 and routinely trypsinized (0.05% trypsin-EDTA) at 80% confluency. Cells were plated at the indicated cell density as required in 100 µL of growth medium.

Click-iT EdU

HeLa cells were plated at 2,000 cells per well in phenol red free F12/DMEM supplemented with 10% FBS and allowed to attach for four to six hours at 37 °C in 5% CO_2. After attachment, media was replaced with phenol red free F12/DMEM without FBS. Cells were serum-starved for 24 hours before release by adding fresh media containing 10% serum. Cells were pulse-chased with EdU solution for 30 minutes at different times after serum starvation release. At the end of 24 hours, cells were fixed, permeabilized, and reacted with AlexaFluor 488-azide as described by the assay kit instructions. Cells were then subsequently counterstained with Hoechst 33342 and imaged.

FUCCI

HCT116 plated at a density of 5,000 to 25,000 cells per well in 100 µL of growth medium were transfected with the FUCCI sensor using BacMam 2.0 virus constructs. Cellular transfections were optimized and performed at a working concentration of 40 particles per cell. After the addition of virus, cells were allowed to incubate overnight before drug treatment. The following day, transfected cells were treated with various concentrations of BI 2536, an inhibitor of Polo-like kinase (PLK1), for 24 hours.

Imaging

Experimental cultures were imaged using an Agilent BioTek Cytation 5 cell imaging multimode reader configured with DAPI, GFP, and RFP light cubes. LED light sources in conjunction with band pass filters and dichroic mirrors provided appropriate wavelength light. The DAPI light cube used a 337/50 excitation filter and a 447/60 emission filter, GFP light cube used a 469/35 excitation filter and a 525/39 emission filter, while the RFP light cube used a 531/40 excitation and 593/40 emission filter.

Image analysis

Digital image data were analyzed for mean fluorescence intensity and object cell counting using Agilent BioTek Gen5 microplate reader and imager software. Gen5 defines contiguous regions or areas that are outlined by a designated threshold intensity value, as well as minimum and maximum size limits. These regions are counted as "objects" or "cells".

Table 1. Object threshold values for positive subpopulation objects. Threshold value represents the mean object fluorescence.

Experiment	Color	Threshold
Serum Release	GFP	20,000
Mevinolin Treatment	GFP	10,000
BI256 Treatment	GFP	20,000

Results and discussion

Fixed cells: Click-iT EdU

Pulse chase labeling of nuclei can identify cells that are undergoing S-phase DNA synthesis. Serum-starved cells will pause in late G1 resulting in low levels of DNA synthesis. When stimulated by the addition of complete media containing 10% serum, they quickly enter into S-phase. As demonstrated in Figure 6, the percentage of HeLa cells

incorporating the thymidine analog EdU into chromatin increases as a result of the addition of serum-containing media to serum-starved cells. Subpopulation analysis of nuclei identified by Hoechst 33342 staining demonstrates an increase in the percentage of green positive nuclei that have been identified through Hoechst 33342 staining.

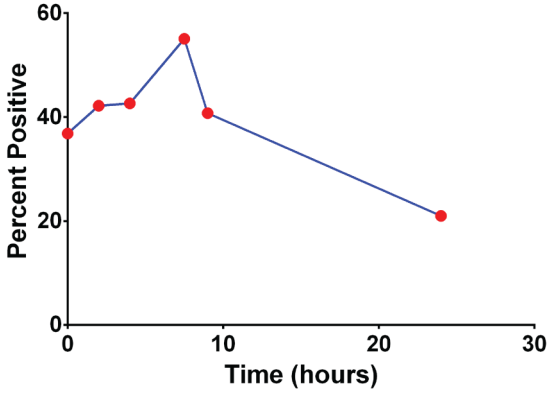

Figure 6. Percentage of S-phase cells with serum starvation release. HeLa cells released from serum starvation were pulse-chased with EdU for 30 minutes at periodic intervals. After 24 hours, cells were reacted with AlexaFluor 488-azide, fixed, stained with Hoechst 33342, and imaged. Image object analysis depicts the percentage of green positive nuclei as a percentage of total nuclei. A threshold value of 20,000 for mean green fluorescence was deemed positive for nuclei identified with Hoechst 33342 staining.

In addition, compounds that are known to stall cell cycle progression can be identified by decreases in the percentage of cells synthesizing DNA. As demonstrated in Figure 7, treatment of cells with mevinolin results in a dose-dependent decrease in the percentage of cells actively synthesizing DNA. This compound, which inhibits the enzyme HMG-Co A reductase, has been shown to stall cells in G1. The enzyme HMG Co A reductase (HMGR), the rate limiting step in the biosynthetic pathway for sterols, produces mevalonate, which serves as the precursor for a number of cellular compounds necessary for cellular growth.[9] The failure to completely eliminate S-phase is most likely the result of residual cellular stores of necessary sterol products and an increase in HMGR protein synthesis in response to the inhibitor.[9]

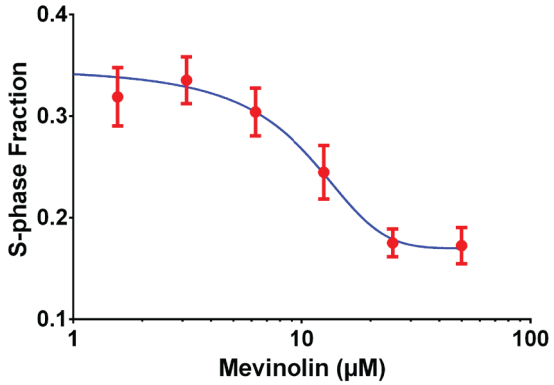

Figure 7. Effect of mevinolin on the percentage of cells in S-phase. HeLa cells were treated with various concentrations of mevinolin for 24 hours. After treatment, cells were pulse-chased with EdU for 30 minutes followed by fixation with 4% paraformaldehyde. Cells were reacted with AlexaFluor 488-azide, counter stained with Hoechst 33342, and imaged. Image object analysis depicts the percentage of green positive nuclei as a percentage of total nuclei. A threshold value of 10,000 for mean green fluorescence was deemed positive for nuclei identified with Hoechst 33342 staining.

Live cells: FUCCI

Cells transfected with the FUCCI sensor using BacMam 2.0 can also be monitored for cell cycle progression using fluorescence object subpopulation analysis. Cells in G1 express the CD1-RFP chimera and can be identified by red fluorescence, while those in G2 express geminin-GFP and will fluoresce green. By straining all nuclei with Hoechst 33342, subpopulation analysis can be used to determine G1 and G2/M cells based on red or green fluorescence. As demonstrated in the images from Figure 8, treatment with the compound B1 2536 results in an increase in the number of green positive cells.

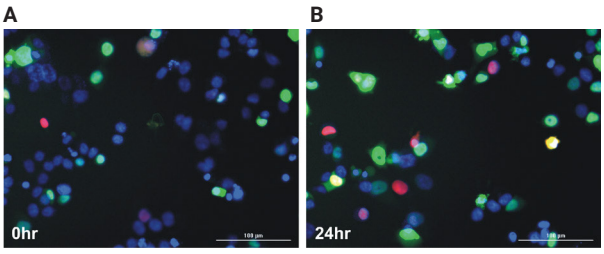

Figure 8. Effect of compound BI 2536 on G2/M cell cycle arrest in HCT116 cells. HCT116 cells transfected with the FUCCI sensor using BacMam 2.0 virus constructs were treated with BI 2536 for 24 hours. Cells were then stained with Hoechst 33342 and imaged using DAPI, GFP, and RFP channels. Image A depicts cells untreated, and image B are cells treated with 16 nM BI 2536.

Using object subpopulation analysis, the percentage of cells in G1 or G2/M can be calculated as a percentage of the total number. With increasing concentrations of BI 2536, a known G2 blocker, the percentage of cells in G2 increases (Figure 9).

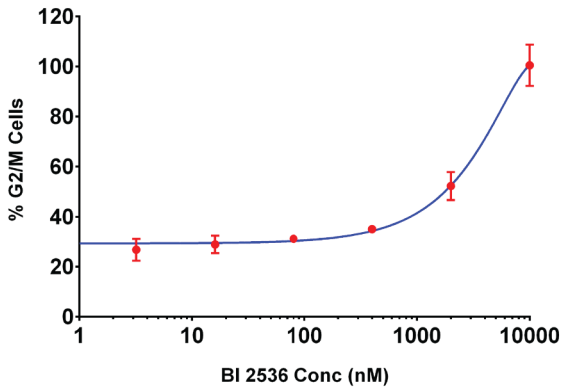

Figure 9. Effect of BI 2536 on cell cycle progression of HCT116 cells. Cell cycle sensors were incubated with 5,000 HCT116 cells for 24 hours before the addition of Hoechst 33342 dye and BI 2536 compound (0 to 10,000 nM). After 30 hours of drug exposure, cells were imaged using a 4x objective with DAPI, GFP, and RFP channels.

Conclusion

These data demonstrate the ability of fluorescent sensors to identify and quantitate cells at various points in the cell cycle. S-phase cells by nature of the DNA synthesis required for the duplication of genetic information can be identified by the incorporation of nucleoside analogs such as EdU. This modified nucleoside can be targeted chemically with reactive fluorescent derivatives, resulting in fluorescently labeled nuclear material. Those cells that exhibit fluorescence with pulse-chase experiments are identified as S-phase cells.

Dynamic changes in the temporal position of cells in the cell cycle can be observed with live cells. Using the FUCCI sensors, which exhibit color changes as a function of the cell cycle position, cells can be sorted into the various stages of the cell cycle by their fluorescence. Cells that exhibit red fluorescence are in G1, while those that fluoresce green are G2/M. The transition from G1 to S is marked by yellow fluorescence and the transition from M to G1 is marked by a loss of fluorescence.

Agilent BioTek Cytation cell imaging multimode readers are an ideal platform for live cell imaging. With up to four different color channels and multiple objectives, the Cytation enables both rapid screening and detailed high-magnification imaging of stained or inherently fluorescent cells. In addition to its imaging features, the Cytation readers offer a full complement of microplate reader capabilities including dual monochromator- and filter-based fluorescence detection. The readers have rigorous temperature control in the read chamber as a standard feature and can be configured with the Agilent BioTek gas controller module, which allows control of carbon dioxide and oxygen levels in the read chamber. This allows the researcher to run long-term kinetic measurements on microplates unattended without the worry of maintaining media pH.

In addition to reader control, Agilent BioTek Gen5 microplate reader and imager software is capable of performing quantitative image analysis. Objects are identified based on size and fluorescence threshold criteria. Population analysis provides information regarding object size, perimeter length, and circularity, along with fluorescence signal variance and intensity. Subpopulation analysis can further analyze identified objects based on more stringent criteria.

References

1. Vermeulen, K.; Van Bockstaele, D. R.; Berneman, Z. N. The Cell Cycle: a Review of Regulation, Deregulation and Therapeutic Targets in Cancer. *Cell Prolif.* **2003**, *36*, 131–149
2. Taylor, J. H.; Woods, P. S.; Hughes, W. L. The Organization and Duplication of Chromosomes as Revealed by Autoradiographic Studies Using Tritium-Labeled Thymidine. *Proc. Natl. Acad. Sci. USA* **1957**, *43*, 122–128.
3. Fallot, P. *et al.* Utilisation du compteur á scintillations en milieu liquid pour la mesure directe de la radioactivité du tritium incoporé dans des cellules de mammifères cultivée *in vitro*. *Int. J. Appl. Radiat. Isot.* **1965**,*16*, 349–356.
4. Gratzner, H. G.; Leif, R. C.; Ingram, D. J. The Use of Antibody Specific for Bromodeoxyuridine for the Immunofluorescent Determination of DNA Replication in Single Cells and Chromosomes. *Exp. Cell Res.* **1975**, *95*, 88–94.

5. Tang, X. *et al.* Antigenretrieval Procedure for Bromodeoxyuridine Immunolabeling with Concurrent Labeling of Nuclear DNA and Antigens Damaged by HCl Pretreatment. *J. Neurosci.* **2007**, *27*, 5837–5844.
6. Gonchoroff, N. J. *et al.* S-Phase Detection with an Antibody to Bromodeoxyuridine. Role of DNase Pretreatment. *J. Immunol. Methods* **1986**, *93*, 97–101.
7. Salic, A.; Mitchison, T. J. A Chemical Method for Fast and Sensitive Detection of DNA Synthesis *In Vivo*. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 2415–2420.
8. Sakaue-Sawano, A. *et al.* Visualizing Spatiotemporal Dynamics of Multicellular Cell-Cycle Progression. *Cell* **2008** Feb 8, *132(3)*, 487–98. PubMed PMID: 18267078
9. Held, P. Thesis: Cell Cycle Regulation of HMG-Co A Reductase, Albany Medical College, Albany, NY; **1990**.

www.agilent.com/lifesciences/biotek

RA44412.401724537

Printed in the USA, May 12, 2022
5994-3385EN

Agilent
Trusted Answers