

Automated Cell Synchrony Determination Using Microscopic Imaging in Live Cells

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Abstract

The replication of cells is responsible for tissue, organ, and species growth and reproduction. Proliferating cells repeatedly transition between cellular duplication or interphase and cell division or mitosis. Progression through the cell cycle is a highly regulated process with a number of checkpoints. Likewise, the effect of compounds on cellular proliferation is a key element of the ADME/Tox drug discovery process. For these reasons the assessment of cell cycle progression under experimental conditions is highly desirable.

With nuclear staining, cells can be identified as being in G₁ or G₂/M based on the intensity of nuclear fluorescence. Cells in G₂/M having approximately 2X the fluorescence intensity as cells in G₁. Treatment of cells by compounds can result in cells being halted at various points of the cell cycle. For example, exposure to increasing concentrations of nocodazole results in a greater percentage of cells having a 2X nuclear content as measured by nuclear staining intensity. At high concentrations approximately 80% of the cell population have 2X nuclear content; whereas at low concentration or with untreated cell, the cell sub-population is about 15%. Vinblastine treatment of cultured cells has also been shown to stall cells in G₂ phase of the cell cycle. With increasing concentrations of vinblastine the percentage of cells identified as G₂/M by nuclear staining increases 3-fold, while cells identified as having G₁ content decreases accordingly. Conversely, hydroxyurea, which halts cells in S-phase, reduces the fraction of G₂ cells in NIH3T3 cells in a concentration dependant manner. Untreated NIH3T3 cells have approximately 15% of their numbers in G₂ phase of the cell cycle; with high concentrations of hydroxyurea this percentage falls to about 1% after a 24 hours exposure.

Cellular nuclear content has also been assessed using frequency histograms of fluorescence intensity. Untreated asynchronous HeLa cells grown in 10% serum demonstrate a major frequency peak that corresponds to cells with G₁ nuclear content, as well as a smaller less well defined peak that corresponds to G₂. The intermediate region of nuclear content between G₁ and G₂ cells represents cells in S-phase undergoing the process of DNA duplication. Cells treated with thymidine demonstrate a build-up of cells stalled in S phase.

Cells actively duplicating their DNA (S-phase) can be identified using incorporation of EdU into cellular DNA with subsequent labeling with fluorescent tags via Click-IT® chemistry. Cells in the presence of full serum or newly released from a G₁ cell cycle block exhibit marked increases in labeled nuclei as compared to cells serum starved.

Assay Process

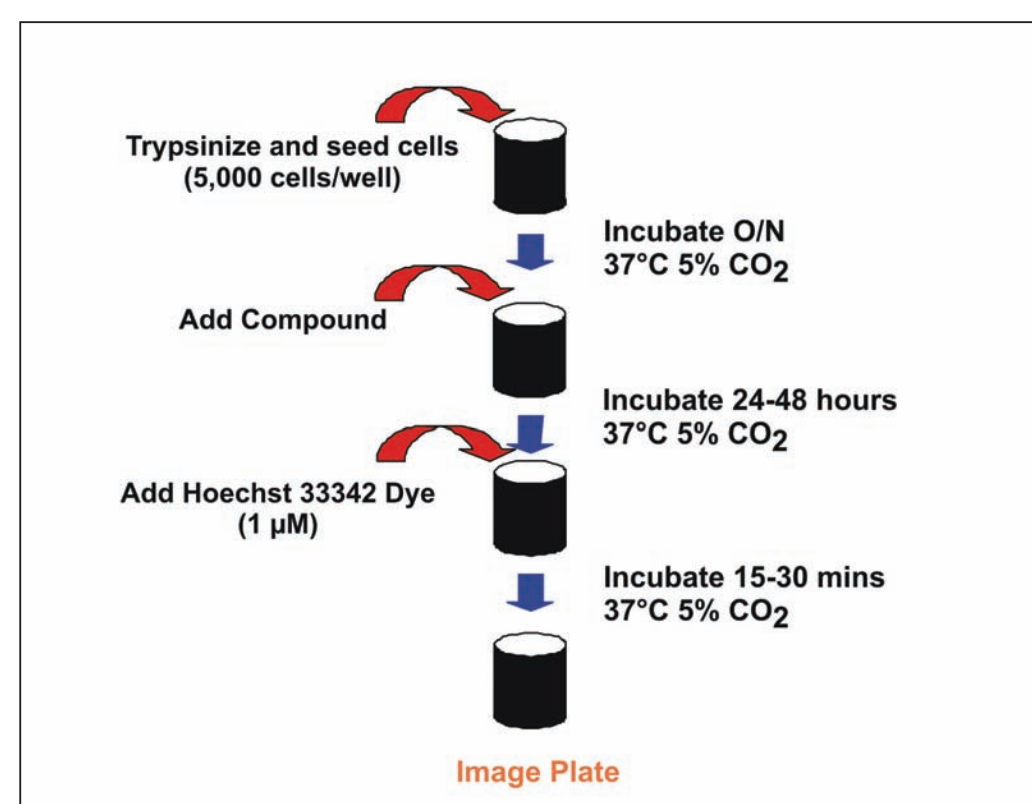


Figure 1. Assay Process For Cell Synchronization Analysis. Cells were trypsinized from stock cultures and seeded into 96-well microplates at a concentration of 5000 cells per well in a volume of 100 µL and allowed to attach overnight (16 hrs). The following day the intended drug treatment was added in 100 µL as a 2X stock diluted in full media. Cells were exposed to drug treatment for 24 hours then stained with Hoechst 33342 prior to imaging.

Instrumentation



Figure 2. Cytation 3 Cell Imaging Multi-Mode Reader

Materials and Methods

Cell Culture
NIH3T3, HCT116 and HeLa cells were cultured in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum and penicillin-streptomycin at 37 °C in 5% CO₂. Cultures were routinely trypsinized (0.05% Trypsin-EDTA) at 80% confluency. For cell transfection cells were plated at a density of 5,000 to 25,000 cells per well in 100 µL growth medium.

Imaging
Transfected cultures were imaged using a Cytation 3 Microplate Imager (BioTek Instruments, Winooski, VT) Configured with DAPI, GFP and RFP light cubes. The imager uses a combination of LED light sources in conjunction with band pass filters and dichroic mirrors to provide appropriate wavelength light. The DAPI light cube uses a 337/50 excitation filter and a 447/60 emission filter, GFP light cube uses a 469/35 excitation filter and a 525/39 emission filter, while the RFP light cube uses a 531/40 excitation and 593/40 emission filters.

Image Analysis
Digital Image data was analyzed for mean fluorescence intensity as well as object cell counting using Gen5 data analysis software. Gen5 defines contiguous regions or areas that are outlined by designated threshold intensity value, as well as minimum and maximum size limits. These regions are counted as "objects" or "cells".

Cell Cycle

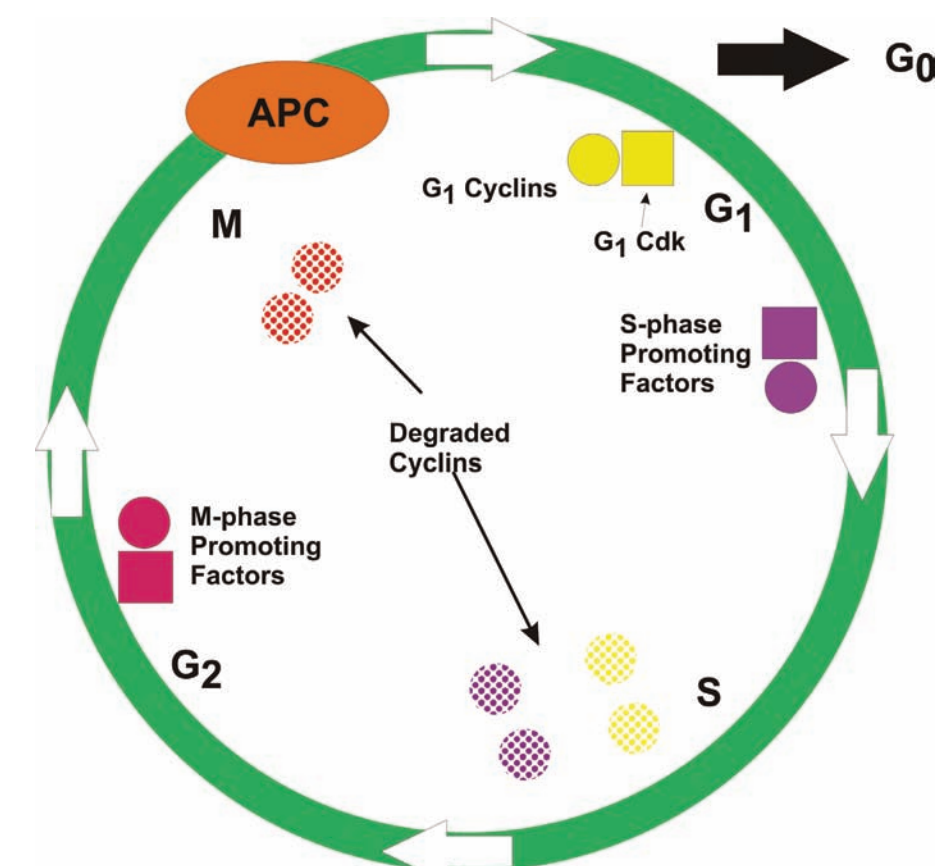


Figure 3. Schematic of the Cell Cycle. Proliferating cells repeatedly transition between cellular duplication or interphase and mitosis. The terminology (G₁, G₂, S, G₂, and M) places in temporal perspective two dichotomies; mitosis (M), and DNA synthesis (S). G₁, the post mitotic gap, G₂ the post-synthetic phase or pre-mitotic gap; and G₀, a phase in which non-dividing cells exist, are terms used for simplicity.

G2 Determination

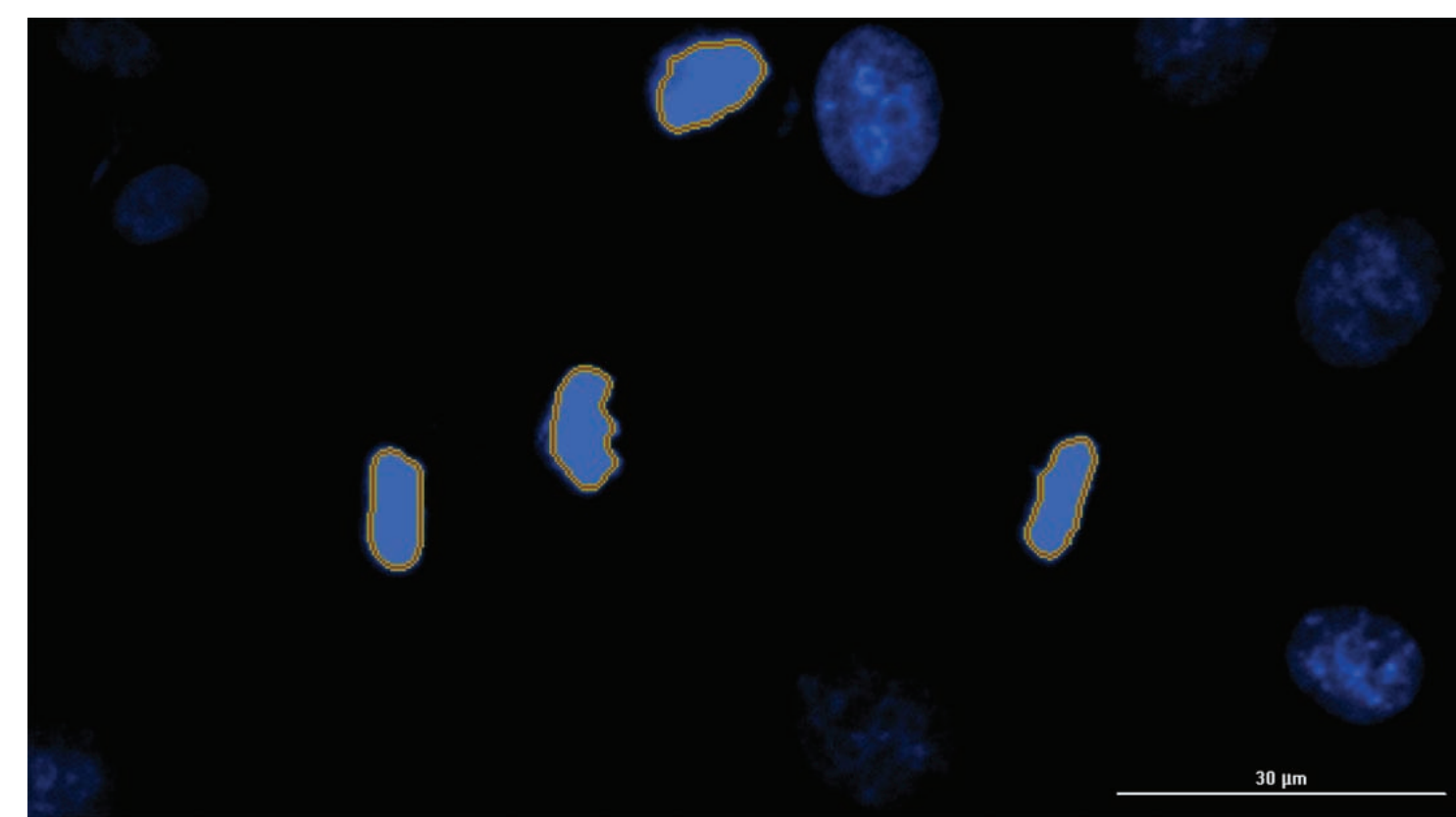


Figure 4. Hoechst 33342 stained HeLa cell nuclei. Vinblastine treated cells were stained with Hoechst 33342 and G2/M content nuclei identified (yellow trace) with object cellular analysis.

Vinblastine Treatment

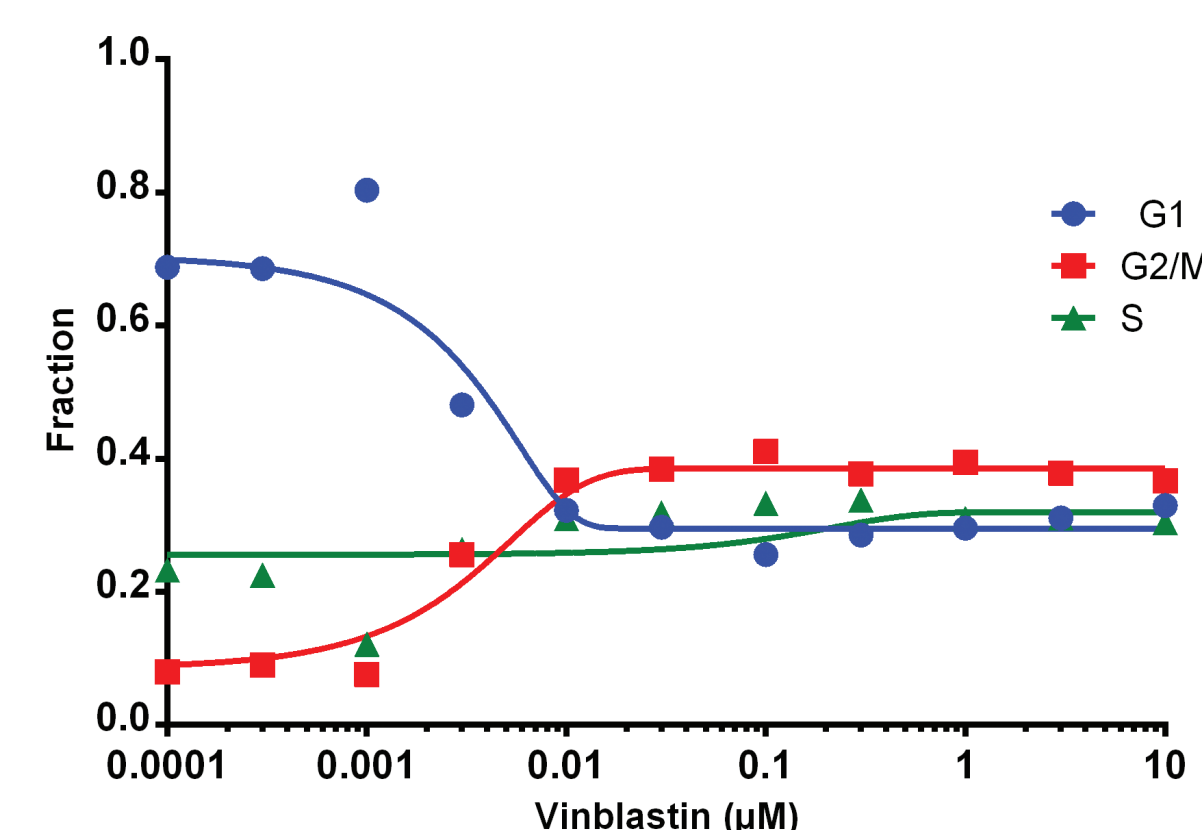


Figure 5. Vinblastine Treatment of HeLa Cells. Cells treated with vinblastine for 24 hours were stained with Hoechst 33342 and imaged using the DAPI channel and a 10X objective. Nuclear content was identified using Object fluorescent mean threshold analysis. Nuclei with a mean fluorescence <21,000 were considered G1 nuclei, while those >25,000 were deemed G2/M. The remainder identified as S-phase.

Nocodazole Treatment

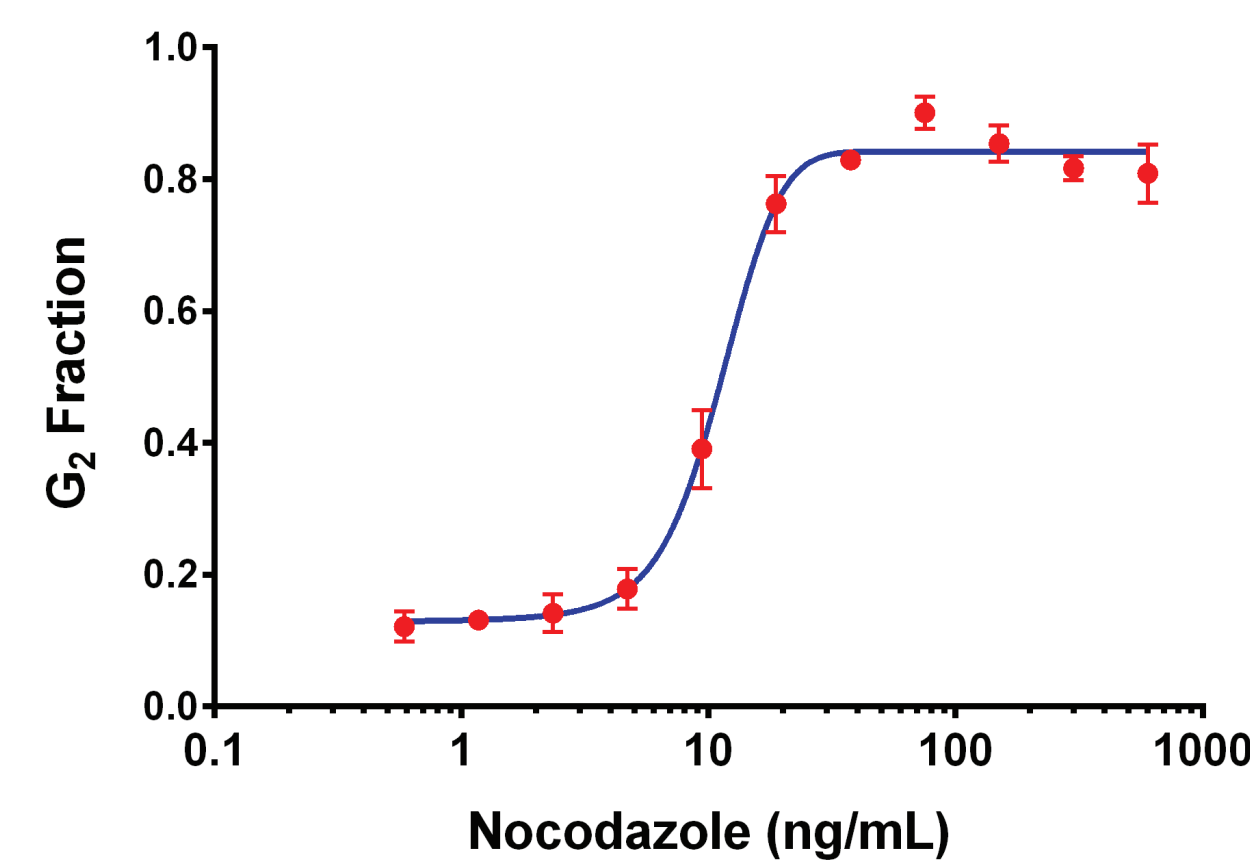


Figure 6. Nocodazole treatment of HeLa cells. Cells treated with Nocodazole for 24 hours were stained with Hoechst 33342 and imaged using the DAPI channel and a 4X objective. Nuclear content was identified using Object fluorescent mean threshold analysis. Nuclei with a mean fluorescence >25,000 were considered G2/M nuclei.

Hydroxyurea Treatment

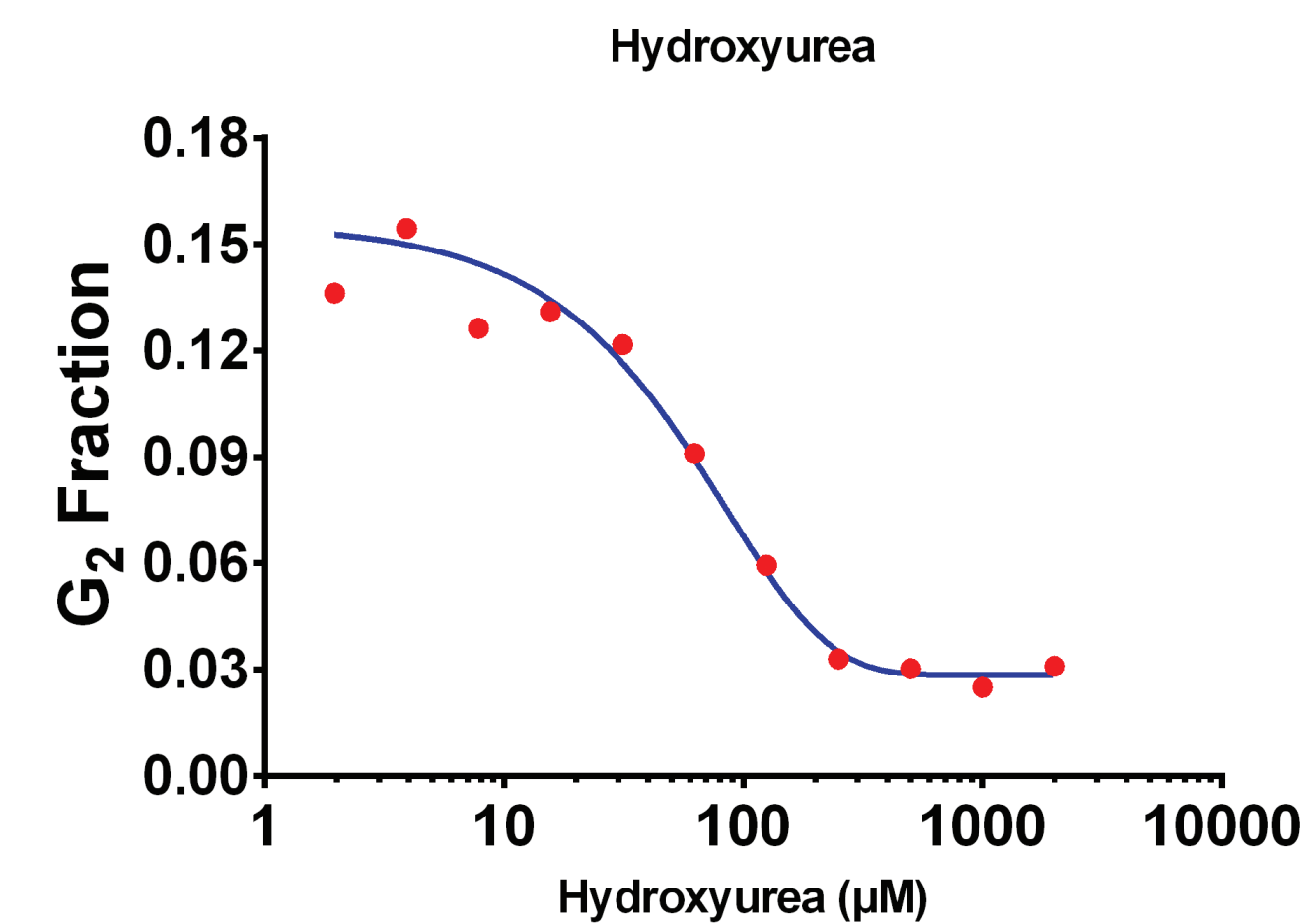


Figure 7. Hydroxyurea treatment of NIH3T3 cells. NIH3T3 cells were seeded into 96-well microplates (5,000 cells/well) and allowed to attach overnight. The following day cells were treated with various concentrations of hydroxyurea for 24 hours. After drug treatment, cells were stained with 1 µM Hoechst 33342 for 15 minutes and imaged using the DAPI channel.

Thymidine Treatment

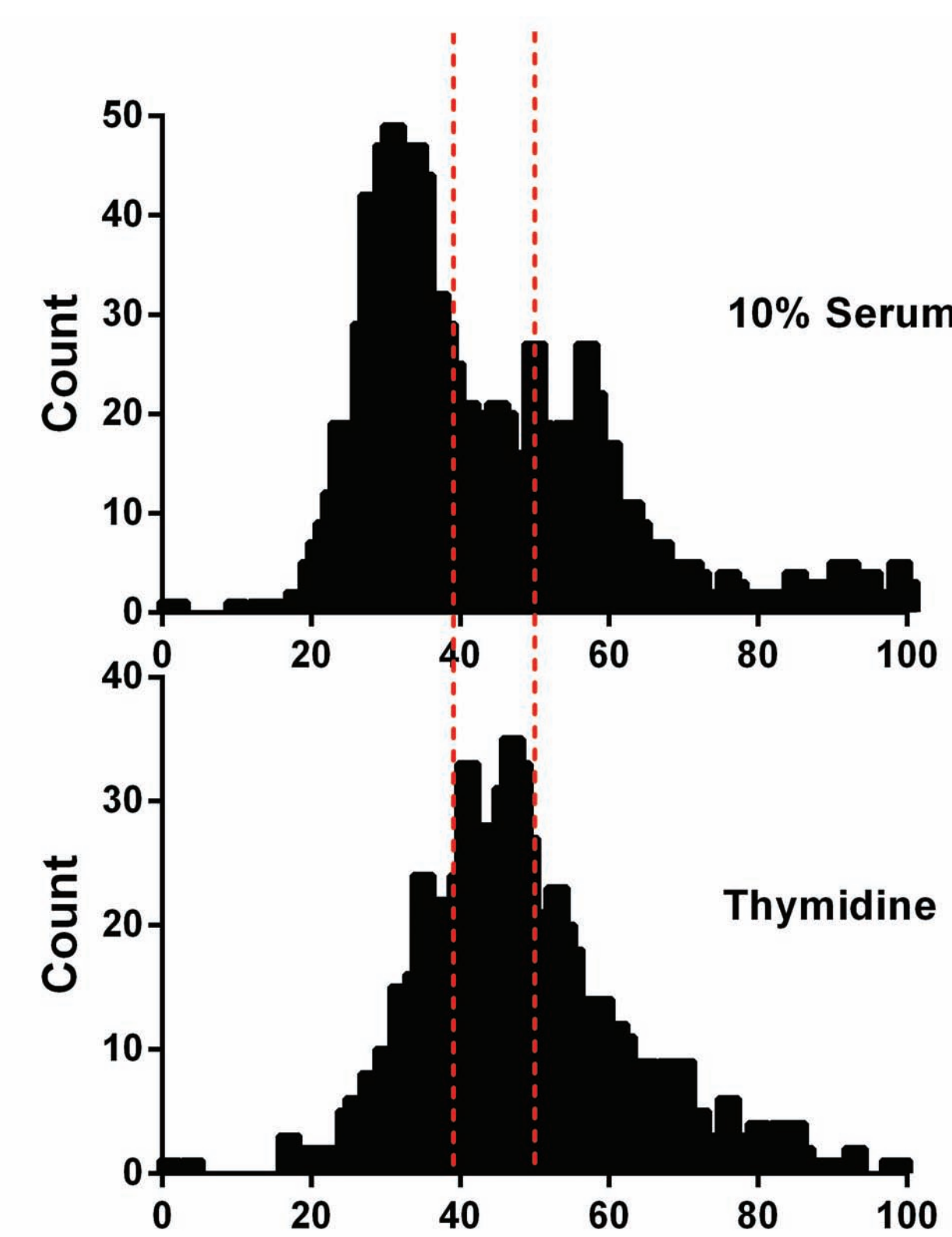


Figure 8. Nuclear content of thymidine and untreated HeLa cells. Histogram of control cells (10% serum) with no treatment show a two peak distribution from a heterogeneous population of G₁-phase and G₂/M-phase. Histogram of thymidine (2 mM) treated cells shows a shift in the distribution of a population of cells, predominately in the S-phase of cell cycle. Dashed lines indicate fluorescence intensity of S-phase nuclear content.

S-Phase Detection

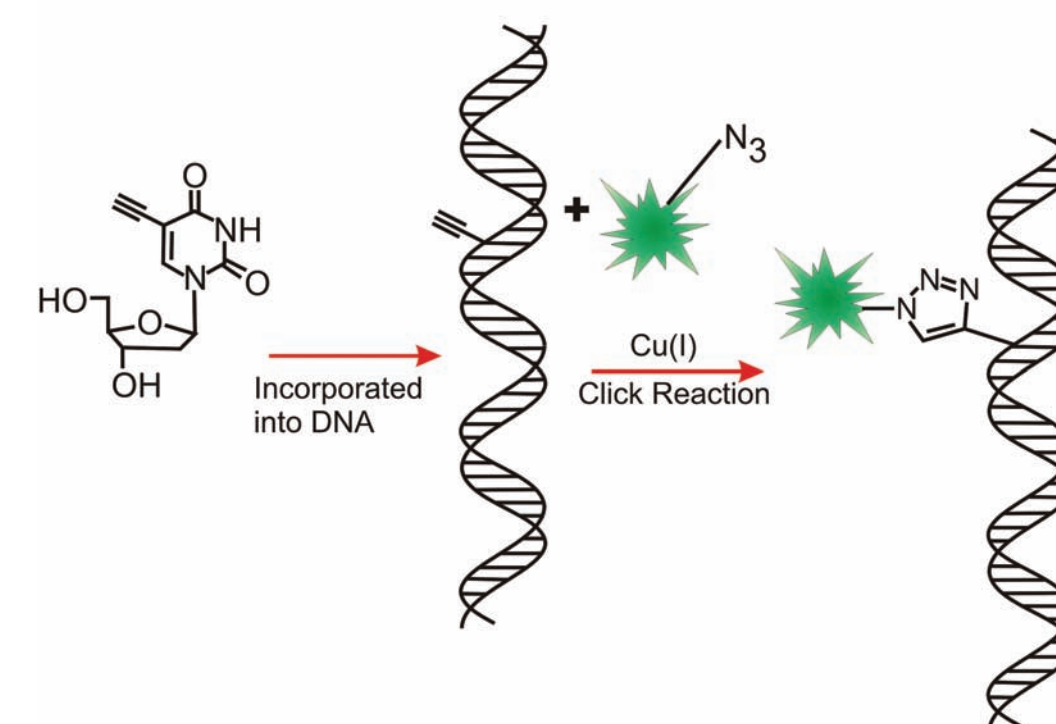


Figure 9. Click-IT® Edu Reaction. The modified thymidine analogue Edu is efficiently incorporated into newly synthesized DNA and fluorescently labeled with Alexa Fluor® 488 in a copper-catalyzed azide-alkyne cycloaddition reaction.

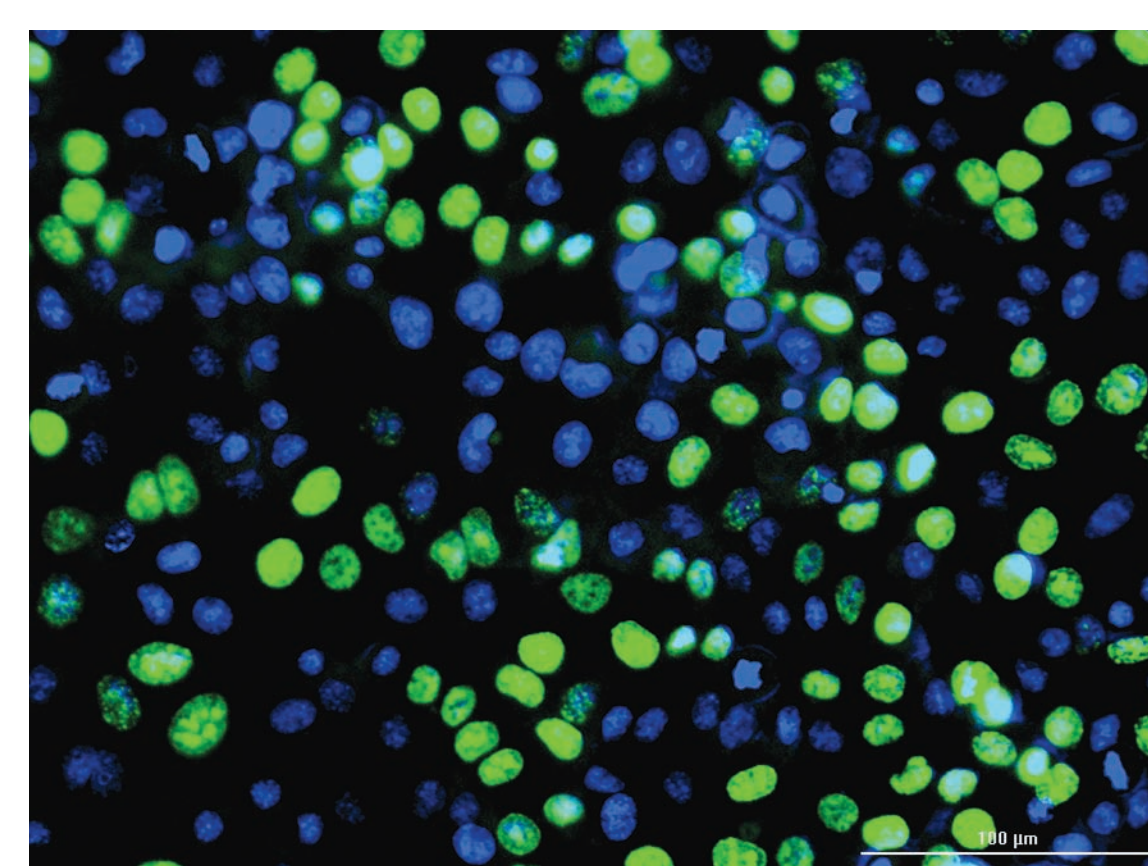


Figure 10. Click-IT® Edu Treated HeLa Cells. HeLa cells were serum starved for 24 hours, after which fresh media with 10% serum was added. Four hours after the addition of fresh media, cells were pulse-chased with Edu for 30 minutes. Green nuclei indicate DNA synthesis

Serum Starvation Release

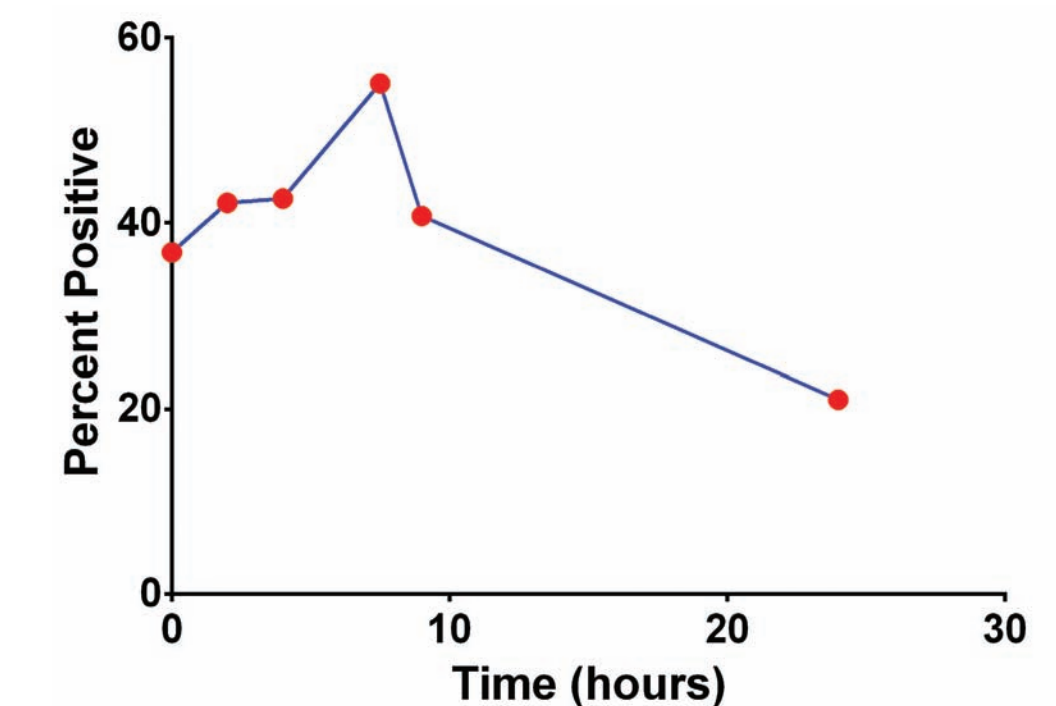


Figure 11. Percentage of S-phase cells with serum starvation release. Cells released from serum starvation were pulse-chased with Edu for 30 minutes at periodic intervals. After 24 hours cells were reacted with AlexaFluor 488-azide, fixed, stained with Hoechst 33342 and Imaged. Image object analysis depicts the percentage of Green positive nuclei as a percentage of total nuclei.

Premo™ FUCCI Cell Cycle Sensor

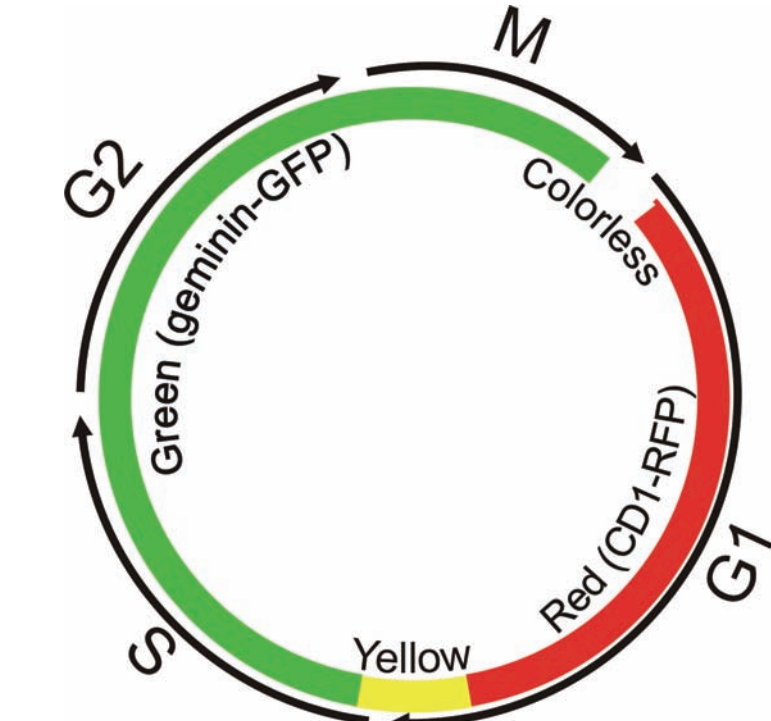


Figure 12. Dynamic Color change of Premo™ FUCCI Cell Cycle Sensor. FUCCI is a fluorescent, two-color sensor of cell cycle progression and division in live cells. Cells change from red in the G1 to yellow in the G1/S interphase and green in S, G2, and M phases, as geminin and Cdt1, fused to one green and red fluorescent proteins, respectively are expressed at specific points in the cell cycle.

G2/M Detection

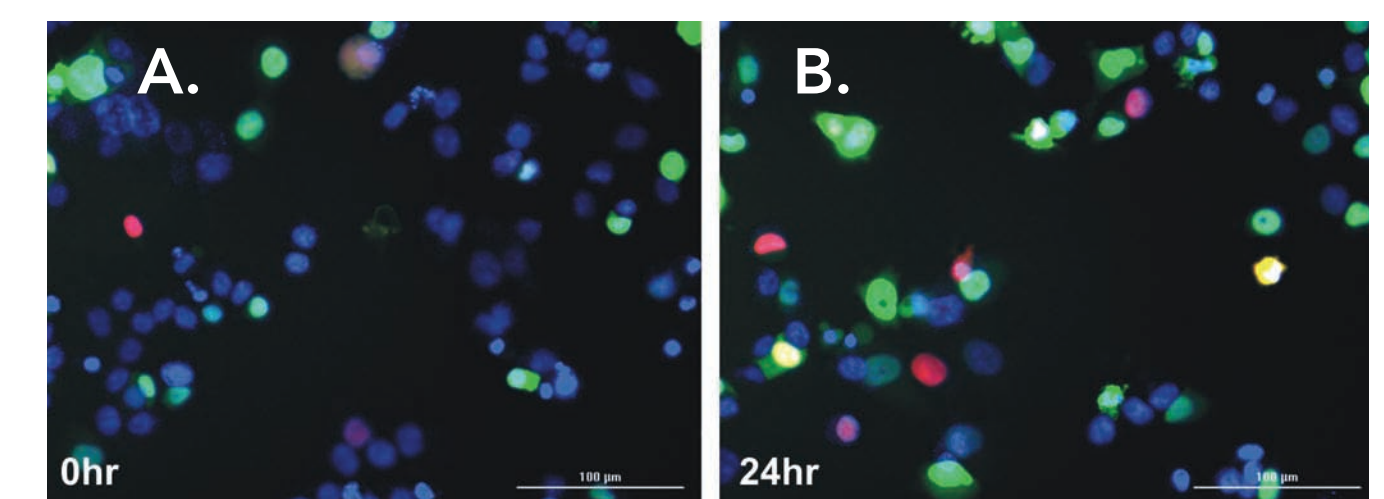


Figure 13. Effect of Compound BI 2536 on G2/M cell cycle arrest in HCT116 cells. HCT116 cells transfected with the FUCCI sensor using BacMam 2.0 virus constructs were treated with various concentrations of BI 2536, an inhibitor of Polo-like kinase (PLK1), for 24 hours. Cells were then stained with Hoechst 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.

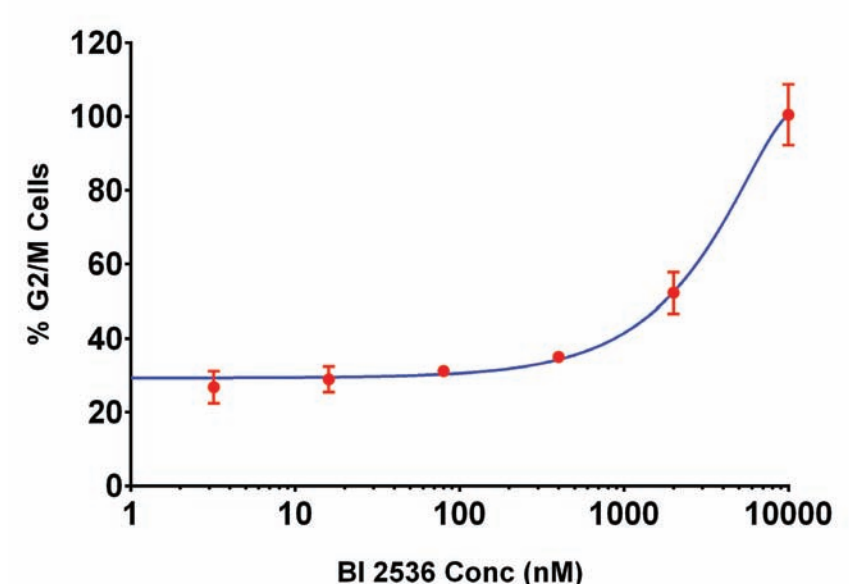


Figure 14. Effect of BI 2536 on Cell Cycle Progression of HCT116 cells. Cell cycle sensors were incubated with 5,000 HCT116 cells for 24 hours prior to the addition of Hoechst 33342 dye and BI 2536 compound (0 to 10,000 nM). After 30 hours of drug exposure, cells were imaged using a 4X objective with DAPI, GFP and RFP channels.

Conclusions

- Nuclear content can be assessed by live cell imaging
 - Membrane permeable dyes
 - G2/M vs. G1 and S-phase nuclei can be discriminated
 - Object Mean Intensity
- S-Phase cells can be identified and quantified
 - Modified nucleotide incorporation
 - Click-IT® labeling technology
 - Positive cells exhibit green fluorescence
- Dynamic changes in cell cycle can be observed and quantified
 - FUCCI Fluorescent Cell Cycle Sensor
 - BI 2536 stalls cells in G2
- Cytation 3 Imager has a number of features that enable live cell imaging
 - Auto-focus and auto-exposure
 - Multiple color imaging capabilities
 - Gas Controller and temperature control allow long term studies and time lapse videos
- Quantitative image analysis using Gen5 Software
 - Mean signal determination
 - Population analysis
 - Allows for easy assay development