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Cell cycle staging of individual cells by fluorescence microscopy

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Abstract

Progression through the cell cycle is one of the most fundamental features of cells. Studies of the cell cycle have traditionally relied on the analysis of populations, and they often require specific markers or the use of genetically modified systems, making it difficult to determine the cell cycle stage of individual, unperturbed cells. We describe a protocol, suitable for use in high-resolution imaging approaches, for determining cell cycle staging of individual cells by measuring their DNA content by fluorescence microscopy. The approach is based on the accurate quantification by image analysis of the integrated nuclear intensity of cells stained with a DNA dye, and it can be used in combination with several histochemical methods. We describe and provide the algorithms for two automated image analysis pipelines and the derivation of cell cycle profiles with both commercial and open-source software. This 1–2-d protocol is applicable to adherent cells, and it is adaptable for use with several DNA dyes.

INTRODUCTION

The coordination between genome duplication and faithful chromosome segregation to daughter cells is an integral part of growth and reproduction, and it is essential to ensure genome stability and maintenance¹. Deregulation of cell cycle control promotes genome instability and has been implicated in developmental abnormalities and numerous diseases, particularly cancer^{2,3}. The accurate staging of cells in the cell cycle is pivotal for the elucidation of cell cycle regulatory mechanisms, but it is also frequently used in studies addressing differences in cellular behavior during different cell cycle stages.

Cell cycle status and progression has traditionally been measured using population-based methods such as flow cytometry⁴, which is generally not compatible with high-resolution cell biological techniques and does not allow tracking of individual cells over time. Recent

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approaches have resulted in the development of methods to accurately determine and track the cell cycle phase of individual cells and to combine this information with other cellular features assessed by imaging, such as localization of a protein or morphological changes of organelles and cells. Most of these methodologies involve selective labeling of replicating cells^{5–13}, staining with specific cell cycle markers^{14–18} or expression of cell cycle phase-specific reporters^{19–21}. Although these methods have proven useful for the study of key aspects of cell cycle regulation and coordination with other cellular functions such as DNA repair, senescence or apoptosis^{22,23}, they can only probe specific cell cycle stages, and thus combinatorial use of multiple methods is required to probe a given process comprehensively throughout the entire cell cycle. In addition, these methods are not often easily compatible with each other, as they require spectrally overlapping fluorophores or interfering detection conditions. Similarly, the use of multiple reporters is laborious, carries the risk of artifacts owing to the required engineering and selection procedures, and decreases the availability of nonoverlapping regions of the spectrum that can be used for the concomitant visualization of other cellular features by imaging-based techniques.

Overview of the procedure

We present here a protocol for determining the cell cycle stage of all individual cells in a population by measuring their DNA content by fluorescence microscopy (Fig. 1). The approach is based on the accurate, image analysis-based quantification of the integrated nuclear intensity of cells stained with the DNA-binding dye DAPI²⁴, and does not require genetic engineering to introduce markers or prior knowledge of cell cycle-specific markers. The protocol describes in detail the plating of cells (Step 1) and the fixation and staining of cells with DAPI (Step 2). We outline two fluorescence microscopy protocols to acquire images of stained nuclei using either a high-content confocal microscope system (Step 3A) or a standard wide-field microscope (Step 3B). Automated pipelines for image analysis and the derivation of DNA content histograms representing cell cycle staging data using dedicated scripts are described for both imaging modalities (Step 4 and Fig. 1). The population of cells within each cell cycle phase (G1, S, G2/M) can be defined by applying thresholds on the integrated DAPI intensity or by modeling of the produced histograms using cell cycle analysis software (Fig. 1). We have successfully used this protocol to analyze the effect of cell cycle stage on the formation of chromosome translocations in living cells²⁵.

Applications and limitations of the protocol

Assessment of the cell cycle status of individual cells has been the basis for the experimental evaluation of cell cycle regulation in response to intracellular and extracellular cues. For instance, the number of cycling cells and the rate of cell cycle progression provide important indices of cell growth and tumorigenicity^{3,5}. Moreover, many cellular functions, such as DNA replication, chromosome segregation or DNA repair, occur in a cell cycle-specific manner or are regulated in a cell cycle-specific manner²⁶. By offering an extremely fast and precise way to assess the cell cycle status of individual cells and to combine this information with multiparametric analysis, flow cytometry has in the past four decades substantially increased our analytical capabilities to understand cell cycle effects, and it has been used as the gold standard tool for cell cycle analysis^{4,27}. This method relies on cells being labeled

with a fluorochrome that stains DNA quantitatively and thus accurately reports the DNA content²⁸. The development of powerful laser-scanning cytometry instrumentation in the mid-1990s (ref. 29) has further improved flow cytometry resolution and allowed multiparametric DNA ploidy studies to be conducted in combination with cytogenetics, providing a tool that has been widely used in cytopathology and cyto- diagnostics^{30–32}. Despite their documented strengths, these methodologies require specific instrumentation and are limited in their ability to pair the cell cycle status of individual cells with subcellular morphological features that require detection by high-resolution imaging.

By using a rationale similar to that of laser-scanning cytology, the protocol described here outlines the steps for the quantitative derivation of the cell cycle status of single cells by measuring the integrated intensity of nuclear DNA staining by fluorescence imaging (Fig. 1). This strategy provides information about the stage of individual cells, and it can be used to assess the changes of population-based cell cycle profiles in response to various treatments and over time. By using a similar methodology, DNA content analysis by quantifying Hoechst nuclear staining has previously been used to assess the cell cycle perturbations induced by HSP90 inhibitors in various cancer cell lines³³. As an imaging-based method, it can be easily coupled with high-content and high-throughput data sets, and it can be used to generate quantitative, fully automated and unbiased cell cycle distributions. As an example, a high-content, high-throughput shRNA-based screen identified novel modulators of the DNA damage response (DDR) by assessing, among other parameters, the cell cycle distribution of the transduced cells as derived by the quantification of nuclear staining by Hoechst³⁴. Importantly, the ability of such imaging-based methods to probe the cell cycle phase of individual cells and to correlate it with the subcellular localization or the expression levels of multiple DNA loci or proteins tagged with different fluorescent markers, either by FISH or antibody detection, opens up new avenues for spatiotemporal modeling of cell cycle-regulated processes. In a cell-based system for visualizing chromosome damage and translocations, this approach was recently combined with antibody staining and high-throughput imaging to assess the pairing frequency of chromosome breaks within different cell cycle populations²⁵.

In the procedure described here, the cell line of interest is stained with the DNA-binding dye DAPI, and the acquired images are quantified by automated image analysis to calculate the integrated nuclear intensity of each cell to obtain DNA content histograms (Fig. 1). However, the protocol can be adapted to any other DNA staining dye that quantitatively binds DNA, such as the Hoechst dyes^{10,28} or DRAQ5 (ref. 35). As many dyes are cell-permeant, no permeabilization step is required. Moreover, the reported compatibility with living cells of some dyes, such as DRAQ5, makes this dye suitable for use of this method in live-cell experiments³⁶. However, as prolonged incubation with DRAQ5 has been shown to block cell cycle progression and to interfere with several chromatin-associated processes, this dye should be cautiously used only for short-duration live-cell experiments^{37,38}.

Limitations of the approach

The protocol relies on the accurate segmentation of nuclei for DNA content quantification. Therefore, this method is not applicable when nuclei segmentation is inaccurate, as, for

instance, has been shown for segmentation procedures in tissue sections stained with DNA dyes³⁹. Moreover, although cells in early stages of mitosis are accurately classified into the G2/M population, late-stage mitotic cells (e.g., during anaphase and telophase) are falsely included in the G1 peak, as they are recognized by the image analysis algorithm as separate objects. This leads to a generally smaller G2/M population in imaging based cell cycle analysis compared with traditional flow cytometry. In addition, owing to the dilution of the dye in dividing cells, the method is not compatible with live-cell experiments of cells transversing through mitosis. As a consequence, when analysis of the mitotic population is needed, the use of the DNA content analysis in combination with other mitotic morphological features or mitosis-specific markers⁴⁰ is highly recommended.

Comparison with other methods

Other imaging-based methods for assessing the cell cycle status of individual cells include metabolic labeling procedures that probe cells transversing S-phase^{5–13}, staining methods that use specific cell cycle markers such as cyclin A, proliferating cell nuclear antigen (PCNA) or Cdt1 (refs. 14–18), or they involve the generation of cellular systems that stably express various cell cycle phase-specific reporters^{19–21}. In contrast to the protocol described here, these methods are able to probe only specific stages of the cell cycle. For example, immunolabeling after incorporation of modified DNA precursors such as the nucleotide analog BrdU, chlorodeoxyuridine (CldU) or iododeoxyuridine (IdU)^{5,13,41}, or detection of incorporation of 5-ethynyl-2'-deoxyuridine using 'click chemistry' that does not require fixation or denaturation^{7,8}, allows the precise detection of S-phase cells only. Similarly, cell cycle can be visualized by immunostaining for cyclin A^{43,44} or the cell cycle protein geminin⁴⁵, whereas immunofluorescence for the licensing factor Cdt1 can be used to mark cells in G1 (refs. 15,46). Similarly, S-phase cells can also be identified by high expression of DNA polymerase or PCNA⁴², whereas mitotic cells can be detected by immunostaining with the mitotic marker phospho-histone H3 (ref. 40). Cells in S, G2 and M phases of the cell cycle reporters can be integrated to generate stable cell lines or transgenic animals expressing cell cycle-specific fluorescent markers. The widely used fluorescence ubiquitination cell cycle indicator (FUCCI) system, based on the expression of the cell cycle oscillators Cdt1 and geminin tagged with different fluorescent proteins, marks cells in G1 or S/G2/M phases, respectively^{19,47}, whereas visualization of cell division can be achieved by the marked changes in cell morphology or the changes in the localization of the scaffolding protein anillin²⁰. However, none of these immunostaining or reporter-based methods is sufficient to determine the cell cycle stages of all individual cells in a population, and therefore combinatorial use of these tools is required. This approach is technically complex and requires the use of multiple spectral imaging channels, reducing the capability for concomitant visualization of other cellular features, such as GFP- labeled cellular structures. In contrast, the strategy we describe here uses a single and inexpensive compound, the commonly used DNA binding dye DAPI, to quantify the cellular DNA content by imaging, thereby allowing cell cycle staging of individual cells in combination with other cell biological methodologies such as immunofluorescence, FISH or live-cell imaging. This method has been validated by observing the expected enrichment of cell populations in specific cell cycle stages after perturbation with cell cycle inhibitors or DNA damage agents and by direct comparison with DNA content analysis by flow cytometry (Figs. 2 and 3).

Experimental design

Plating cells, fixation and staining with DAPI.—The protocol outlined here describes the cell cycle staging of individual NIH3T3 mouse cells on the basis of their DNA content quantified by fluorescence microscopy. Depending on whether high-throughput microscopy or wide-field microscopy is chosen for fluorescence imaging, the cells of interest are either plated in 384-well imaging plates or on coverslips, respectively. Regardless of the imaging modality, cells are fixed with 4% (vol/vol) paraformaldehyde (PFA) and permeabilized with 0.3% (vol/vol) Triton X-100 to allow the uptake of the cell-impermeable DNA labeling dye DAPI. In addition to DAPI, other cell-permeant DNA counterstains such as Hoechst dyes¹⁰ or DRAQ5 (ref. 35) can be used without the need for permeabilization after fixation. Plating at subconfluency is required for optimal fluorescence microscopy; for NIH3T3 cells²⁵, the optimal density is ~3,000 cells per well of a 384-well plate. As precision in quantifying the DNA content of individual cells is crucial to the success of the protocol, optimization of nuclei staining conditions, so as to obtain robust but not saturated staining signals, using the DNA dye of choice in the desired cell type is highly recommended.

Fluorescence microscopy and image analysis.—Image acquisition by fluorescence microscopy of the stained nuclei, and the subsequent quantification of their DNA content by automated image analysis, are integral parts of this protocol. To achieve this in both high-throughput and low-throughput settings, we developed two protocols to acquire and quantify images of the DAPI-stained cells with either a high-throughput confocal imaging system or with a standard fluorescence microscope. The first protocol uses a Nipkow spinning-disk confocal Opera QEHS imaging system (PerkinElmer), which is equipped with a 405-nm laser for the excitation of the fluorescent dye and using a 20× 0.7-numerical aperture (NA) water-objective lens²⁵. Owing to the system's confocality and the high NA of the lens, and as mitotic cells are commonly found further away from the coverslip or dish bottom compared with interphase cells (Supplementary Fig. 1a,b), the acquisition of multiple images in different planes along the *z* axis (stacks) is required (Supplementary Fig. 1). The optimal number and spacing of the imaging planes can vary according to the NA of the objective lens and according to the cell line in use. As described in this protocol, when a 20× 0.7-NA water objective is used, three imaging planes 7 μm apart are sufficient to capture the fluorescence signals from all cycling cells (including cells in mitosis; Fig. 2 and Supplementary Fig. 1), whereas a higher number of nonoverlapping imaging planes is required when higher-NA and higher-magnification objectives are used. For example, we have successfully implemented this protocol with a 40× NA 0.9 water-immersion objective and seven optical sections each separated by 2 μm in the *z* axis²⁵.

As a second option, we outline a protocol for a standard wide-field fluorescence microscope (e.g., Deltavision, Applied Precision) equipped with a UV light source for the excitation of the DNA dye and a 10× 0.4-NA air objective. This configuration allows the quantification of DAPI-stained cells and the derivation of cell cycle profiles by acquiring single DAPI staining images without the need for the image acquisition of *z* stacks (Fig. 3). Moreover, other confocal microscopes or epifluorescence microscopes are entirely appropriate to achieve similar results^{33,34}. To obtain cell cycle profiles with discrete populations of G1, S and G2/M cells, the quantification of the DNA content of a minimum of 500 cells is

necessary (Supplementary Fig. 2). This number of cells can easily be obtained by imaging ~10 fields of view using a 20× objective lens. Higher numbers of images and cells yield smoother, more accurate cell cycle profiles (Supplementary Fig. 2). As our method requires the accurate quantification of a cell's DNA content, it is crucial to obtain images in which the emitted fluorescence falls within the dynamic range of the detection, and thus parameters related to optimal staining, light-source intensity and exposure are important.

To calculate the DNA content of individual nuclei, the integrated intensity of the nuclear DAPI signal is measured in both approaches by image analysis. For the high-throughput-microscopy pipeline, we provide an automated image analysis script written for the PerkinElmer 'Acapella' high-content image analysis software. The script first gathers a stack of images in the same channel and field of view and generates an average projection in *z* of all the images in the stack. As a second step, nuclei are segmented on the basis of the projected DAPI image. In addition, nuclei touching the image border are filtered out. Finally, the script calculates the DAPI integrated intensity in the nucleus on a single-cell basis (Fig. 2a). The image analysis script is fast (ten fields in <5 s) and the nuclei detection parameters can be easily adjusted for optimal detection in different cell lines. The cell level data are then exported as multiple text files (.txt; one for each analyzed microplate well) in which the averaged integrated nuclear DAPI intensity of every individual imaged cell per well is provided in a single column within the file (Supplementary Fig. 3, attribute 'Z_IntegratedDapiInt').

For the wide-field microscopy option, we provide two image analysis pipelines for the open-source image analysis software CellProfiler⁴⁸. The first CellProfiler pipeline uses all the images in the DAPI channel to generate a single illumination function, which is then used to correct for systematic illumination biases specific to the microscope used for image acquisition. The second CellProfiler pipeline segments the DAPI-stained nuclei, excludes the cells at the border of the images and calculates the DAPI integrated intensity at the single-cell level (Attribute 'Intensity_IntegratedIntensity_DAPICorr'). By using our computing platform (see Equipment Setup), this image analysis pipeline is able to analyze 50 images in ~1.5 min. The illumination correction step before the actual nuclei segmentation and DAPI fluorescence intensity quantification is highly recommended, as inconsistencies owing to illumination variability across the image fields may affect accuracy in the intensity measurements that is essential for the correct determination of the cell cycle status⁴⁸.

Both image analysis routines (Acapella and CellProfiler) offer the option to save image overlays of the DAPI integrated intensity values together with the nuclear segmentation border of each individual cell (Figs 2a and 3a, and Step 4A(iv) and 4B(xi), for options A and B, respectively). On the basis of the DAPI integrated intensity values that define distinct cell cycle phases, as obtained by setting gates on the cell cycle histograms in the following protocol steps, these overlays can be used to track back the cell cycle status of each individual cell. In addition, the single-cell values for the DAPI integrated intensity attribute are stored in the .txt result files. Once the DAPI integrated intensity values for cell cycle gating have been empirically set, a particular cell can be assigned to the appropriate phase of the cell cycle according to its DAPI integrated intensity value. Although this protocol obviously cannot provide a strategy for every possible high-content image analysis, the

approach suggested above can be used as a starting point and guideline to correlate other image analysis attributes (e.g., fluorescence intensity of a marker, cell size, cell shape and so on) with the cell cycle status at the single-cell level.

Generation of cell cycle profiles and gating of cells within different cell cycle phases.—When the frequency distributions of the integrated DAPI intensity of all individual nuclei per sample are calculated and plotted, cell cycle profiles with discrete G1, S or G2/M cell cycle populations emerge (Figs. 2b and 3b). These histograms can be produced manually using any spreadsheet software, such as Excel, or they can be automatically derived using statistics software. To this end, we provide two similar, but independent, scripts written in R⁴⁹. These scripts read the single-cell data generated either by Acapella or by CellProfiler and automatically derive the histogram plots of the DAPI integrated intensity for each experimental condition and save them as .png files (Figs. 1, 2b and 3b). In addition, the cell cycle profile in the untreated control histogram plot can then be used to calculate the percentages of cells belonging to each phase of the cell cycle (G1, S and G2/M) by manually setting gates for the DAPI integrated intensity attribute (Fig. 4). The appropriate thresholds for these gates can be determined by the user and set in the R script, which will then calculate the percentages on a per treatment basis and save them in a .txt file table. Alternatively, the percentages of cells within distinct cell cycle phases can be derived by using cell cycle modeling features of image cytometry software. As an example, we use the cell cycle modeling features of FCS Express 5 Pro (De Novo software) to directly compare the cell cycle distributions derived by the method described here with those obtained by the traditional DNA content analysis by flow cytometry. This comparison revealed similar populations of cells within different cell cycle phases (Supplementary Fig. 4). This protocol can be performed in 1–2 d in parallel with established cell biological methodologies such as immunofluorescence, FISH or short-term live-cell imaging.

MATERIALS

REAGENTS

- Appropriate cell line: NIH323 mouse cells, the U2OS human osteosarcoma cell line and human immortalized skin fibroblasts have been successfully used by following this protocol, e.g., NIH3T3 mouse cells
- DAPI (Sigma-Aldrich, cat. no. D9542)
- PBS (Gibco, cat. no. 14190–144)
- Paraformaldehyde (PFA), 16% (vol/vol) solution (EM grade, cat. no. 19208, Electron Microscopy Sciences) ! **CAUTION** PFA is a known human carcinogen. It is toxic on inhalation.
- Triton X-100 (Sigma-Aldrich, cat. no. T9284)
- DMEM with 4.5 grams per liter D-glucose (Gibco, cat. no. 11960) supplemented with 10% (vol/vol) FBS and 2 mM L-glutamine (Gibco, cat. no. 25030), and 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, cat. no. 15140)
- Trypsin-EDTA, 0.25% (wt/vol) (1x; Gibco, cat. no. 25200–072)

- Aphidicolin (Sigma-Aldrich, cat. no. A4487) ! **CAUTION** Aphidicolin is toxic upon swallowing and on contact with the skin.
- Etoposide (Sigma-Aldrich, cat. no. E1383) ! **CAUTION** Etoposide is a known carcinogen. It is toxic upon swallowing and on contact with the skin.
- Camptothecin (Sigma-Aldrich, cat. no. C9911) ! **CAUTION** Camptothecin is a carcinogen. It is toxic if swallowed.

EQUIPMENT

- Tissue culture incubator at 37 °C, 5% CO₂
- Biosafety cabinet for tissue culture
- Tissue culture dishes, 150 × 25 mm (BD Falcon, cat. no. 353025)
- Plates, 384 well (Cell Carrier, PerkinElmer)
- Conical tubes, 15 and 50 ml (NEST Biotechnology)
- Disposable plastic serological pipettes (individually wrapped; 5, 10 and 25 ml, Corning)
- Micropipettes P1000, P200, P20, P10 and P2 (Gilson)
- Microcentrifuge tubes, 1.5 ml (Eppendorf)
- Hemocytometer, 0.0025 mm² (Labor Optik, Neubauer)
- Opera QEHS high-content screening microscopy system (PerkinElmer) or Deltavision microscope (Applied Precision)
- Acapella high-content imaging and analysis software (PerkinElmer) or CellProfiler (<http://www.cellprofiler.org/>)
- R (<http://cran.r-project.org/>)
- RStudio (<http://Rstudio.com/>)
- Excel (Microsoft Office)

REAGENT SETUP

Triton X-100, 0.3% (vol/vol) in PBS—Mix 0.75 ml of Triton X-100 with 249.25 ml of PBS to prepare 0.3% (vol/vol) Triton X-100 in PBS. Store it at room temperature (RT; 20–25 °C) for up to 1 month.

PFA fixation solution—Dilute 10 ml of 16% (vol/vol) PFA solution into 30 ml of PBS to prepare a 4% (vol/vol) PFA fixation solution, or dilute 10 ml of 16% (vol/vol) PFA solution into 10 ml of PBS to prepare an 8% (vol/vol) PFA fixation solution. ▲ **CRITICAL** Freshly make up the solution on the day of the experiment. Alternatively, the solution can be aliquotted and frozen (–20 °C) for up to 1 year, but after thawing it should be used only once.

DAPI stock—Dissolve 5 mg of DAPI in 1 ml of Milli-Q water to a stock concentration of 5 mg/ml and store it at 4 °C for up to 1 year.

DAPI staining solution—Mix 1 µl of DAPI stock with 2.5 ml of PBS to obtain a 2.5-ml solution of DAPI with a final concentration of 2 µg/ml. Store the solution at 4 °C for 1 week.

Aphidicolin—This is a ready-made solution supplied as a 1 mg/ml solution in DMSO. ▲ **CRITICAL** Aphidicolin is light-sensitive. Store it at −20 °C for up to 5 years and protect it from light.

Etoposide—Dilute etoposide in DMSO at a final concentration of 10 mM and store it at −20 °C for up to 2 years.

Camptothecin—Dilute camptothecin in DMSO at a final concentration of 10 mM and store it at 4 °C for up to 12 months.

EQUIPMENT SETUP

▲ **CRITICAL** In principle, any fluorescence microscope with a stable illumination source that is able to acquire images of cells stained with DAPI can be used for this protocol. Here we have used a high-throughput confocal imaging system (Opera, PerkinElmer) or a standard wide-field fluorescence microscope (Deltavision, Applied Precision), as described below.

Opera high-content screening system—Each PerkinElmer Opera high-content imaging system is configured by the manufacturer to suit specific customer requirements, and it is operated by the Opera Evoshell 1.8.1 software. The system used in developing these protocols is equipped with four diode lasers for sample illumination (405, 488, 561 and 640 nm) and with four high-quantum-efficiency cooled CCD cameras for simultaneous acquisition of multiple fluorescence channels. These cameras are located in the light path behind the Nipkow spinning-disk unit, and they provide confocality for the captured digital image data. A ‘flat field’ illumination correction process that takes advantage of an Opera Adjustment plate provided by PerkinElmer is implemented in Acapella and applied to all images acquired by the Opera system. This is essential for accurate ‘per cell intensity measurements. If another type of high-throughput-imaging instrument is used, the illumination uniformity across the individual fields should be carefully evaluated. A dedicated high-speed laser autofocus system determines the physical position of the bottom of the microplate before capture of each image field on the Opera. Inconsistencies in autofocus performance could also cause issues with the accuracy of ‘per cell measurements. Therefore, autofocus reproducibility should be experimentally confirmed.

Deltavision microscope (Applied Precision)—In Step 3B, stained cells were imaged using a microscope (IX70; Olympus) controlled by a Deltavision System (Applied Precision) with SoftWoRx 3.5.1 software (Applied Precision) and fitted with a CCD camera (CoolSnap; Photometrics). We used a 10× 0.4-NA air objective, and images were acquired at a 1,024 × 1,024 pixel resolution, with a pixel size equivalent to 0.647 µm in .x and y.

Computer used for image analysis—Image analysis using Acapella (version 2.0, PerkinElmer) was performed on a custom-built ‘workstation- class system, consisting of an Intel 5400 chipset motherboard with dual LGA 771 CPU sockets housing two Xeon processors (each processor unit containing four physical computational cores). The system used a 32-bit Windows XP operating system, resulting in a maximal addressable system memory of ~3.2 GB (DDR2, 800 MHz frequency). The workstation was linked to institutional remote data storage hard drive arrays via gigabit networking infrastructure.

CellProfiler for image analysis—CellProfiler (version 2.1, version 0c7fb94) was run on a 27-inch Apple iMac (3.5 GHz Intel Core i7, 32 GB 1,600 MHz DDR3 RAM, Nvidia GeForce GTX 780M 4,096 MB graphics card) running Mac OS X 10.9.3. The CellProfiler ‘IlluminationCorrection.proj’ and ‘CellCycleAnalysis.proj’ pipelines are available as Supplementary Data. The test data set of .dv image files used for the analysis is also available as Supplementary Data.

R for cell cycle profile histograms generation and analysis—R (version 3.1.0 (2014-04-10)- ‘Spring Dance’ and above; <http://cran.r-project.org/>) was run on the same platform used for the CellProfiler analysis by using RStudio (version 0.98.978 and above, RStudio <http://Rstudio.com/>), an interactive development environment software for R. For the required installation and setup of R, please consult their online documentation. The ‘CellCycle Histograms_Acapella.R’ and ‘CellCycleHistograms_CP2.R’ R scripts are provided as Supplementary Data. The following R packages are required: ‘ggplot2’ (version 1.0.0 and above)⁵⁰, ‘data.table’ (version 1.9.2 and above) data.table: extension of data.frame; <http://CRAN.R-project.org/package=data.table>), ‘stringr’ stringr: Make it easier to work with strings. R package version 0.6.2; <http://CRAN.R-project.org/package=stringr>) and ‘plyr’ (version 1.8.1 and above)⁵¹. If these packages are not already present in the local R library, they must be installed by running the `install.packages()` function before the first use of the provided R scripts.

PROCEDURE

Plating of cells ● TIMING 1 d

1| For high-throughput confocal microscopy, plate cells in multiwell imaging plates, as described in Step 1A. For wide-field microscopy, follow Step 1B to grow cells on coverslips.

A. Plating cells in multiwell imaging plates

- i. *Day 1.* Trypsinize logarithmically growing cells using trypsin-EDTA and count the cells using a hemocytometer.
- ii. Resuspend 1.2×10^5 cells in a total volume of 2 ml of DMEM warmed to 37 °C (~60 cells per microliter of medium) and manually pipette 50 μ l of cell suspension in each well of a 384-well plate for a minimum of four wells (3×10^3 cells per well).

▲ **CRITICAL STEP** The number of plated cells needs to be optimized for different cell lines or primary cells. It is crucial

that plated cells be attached, forming a monolayer. The indicated number is optimal for plating NIH3T3 mouse cells.

▲ CRITICAL STEP A minimum of 500 cells is required for image acquisition (which corresponds to approximately ten imaging fields acquired in a single well), but using higher numbers of cells (e.g. 5×10^3 to 5×10^4 cells per histogram) results in smoother histograms (Supplementary Fig. 2).

▲ CRITICAL STEP When plating in the imaging plate, it is recommended to leave the plate at RT for at least a half-hour after seeding to avoid swirls and deposition of cells at the edge of the well.

- iii. Incubate the plate at 37 °C, 5% CO₂ to allow the cells to attach (the minimum time to attach may vary between different cell lines; make sure that the cells have attached completely). If desired, after the cells have attached, perturb the cell cycle distribution by incubating the cells at 37 °C, 5% CO₂ in the presence of 5 μM aphidicolin, 1 μM etoposide or 1 μM camptothecin for 22 h.

B. Plating cells on coverslips

- i. *Day 1.* Trypsinize logarithmically growing cells using trypsin-EDTA and count the cells using a hemocytometer.
- ii. Autoclave the circular coverslips and place one coverslip into each of four wells of a 24-well plate in the biosafety cabinet.
- iii. Plate $\sim 1 \times 10^5$ cells in each well.

▲ CRITICAL STEP The number of plated cells needs to be optimized for different cell lines or primary cells. It is crucial that plated cells be attached, forming a monolayer.

- C. Incubate the plate at 37 °C, 5% CO₂ to allow the cells to attach (the minimum time to attach may vary between different cell lines; make sure that the cells have attached completely). If desired, perturb the cell cycle distribution by incubating the cells at 37 °C, 5% CO₂, after they have attached, in the presence of 5 μM aphidicolin, 1 μM etoposide or 1 μM camptothecin for 22 h.

Fixing cells and staining with DAPI ● TIMING 1 h

2| If the cells have been grown in multiwell plates for high-throughput confocal microscopy (Step 1A), fix and stain the cells as described in Step 2A. If the cells have been grown on coverslips for wide-field microscopy (Step 1B), follow Step 2B to fix and stain the cells.

A. Fixing and staining cells for high-throughput confocal microscopy

- i. *Day 2.* To fix the cells plated in 384-well imaging plates, pipette 50 μ l of 8% PFA per well into medium to give a final 4% PFA concentration, and incubate it for 20 min at RT (20–25 °C).

▲ **CRITICAL STEP** Fixation in the presence of medium is used to avoid cell detachment before fixation.

- ii. Wash the cells twice with 1x PBS.

■ **PAUSE POINT** Fixed cells can be stored in 1x PBS at 4 °C for up to a week. Seal the imaging plate using Parafilm and replenish with 1x PBS if evaporation occurs.

- iii. Permeabilize cells by dispensing 50 μ l of 0.3% (vol/vol) Triton X-100 in each well, and incubate the plate at RT for 8 min.

▲ **CRITICAL STEP** Permeabilization of the cells is not absolutely required for staining, as DAPI will eventually enter the cells. However, this step will permit staining within minutes of incubation.

- iv. Wash the cells twice with 1x PBS.

- v. Dispense 30 μ l of the DAPI staining solution in each well and incubate for 20 min.

▲ **CRITICAL STEP** Optimization of the DAPI staining solution concentration and incubation time may be required for different cell lines.

- vi. Wash the cells twice with 1x PBS. Dispense 100 μ l of 1x PBS in each well and proceed to Step 3A.

▲ **CRITICAL STEP** DAPI is pH-sensitive, so storage in PBS or experimental treatments may affect the staining process.

B. Fixing and staining cells for wide-field microscopy

- i. *Day 2.* Wash the cells twice with 1x PBS.

- ii. Fix the cells by dispensing 0.5 ml of 4% (vol/vol) PFA, and incubate them at RT for 10 min.

- iii. Wash the cells twice with 1x PBS.

■ **PAUSE POINT** Fixed cells can be stored in 1x PBS at 4 °C for up to a week. Seal the plate using Parafilm and replenish with 1x PBS if evaporation occurs.

- iv. Permeabilize the cells by dispensing 0.5 ml of 0.3% (vol/vol) Triton X-100 and incubate them at RT for 5 min.

▲ **CRITICAL STEP** Although permeabilization of the cells is not absolutely required for staining, as DAPI will eventually

enter the cells, this step will permit staining within minutes of incubation.

- v. Wash the cells twice with 1x PBS.
- vi. Dispense 0.5 ml of the DAPI staining solution in each well, and incubate the plate for 20 min.
- vii. Wash the cells twice with 1x PBS.
- viii. Dispense 10 μ l of mounting medium for fluorescence on the top of a microscope slide. Use forceps to place each coverslip on top of the mounting medium in such a way that the cells are in contact with the mounting medium and the microscope slide, without introducing air bubbles. Seal the edges of the coverslips on the microscope slides by applying clear nail polish or any mounting medium. Allow each slide to dry for at least 1 h, protected from light.

▲ **CRITICAL STEP** DAPI is pH-sensitive, so storage in PBS or changes in pH by experimental treatments may affect the results of the measurements.

▲ **CRITICAL STEP** Level the coverslip on the slide by applying gentle force by using the forceps to ensure that all cells are in focus when acquiring images during Step 3B.

Microscopy ● TIMING 1–2 h

3| Follow Step 3A to perform high-throughput confocal microscopy on cells prepared as described in Step 2A. Follow Step 3B to perform wide-field microscopy on cells prepared as described in Step 2B.

A. High-throughput fluorescence microscopy TIMING 1 h

- i. Switch on the Opera high-content screening system.
- ii. Start the Opera software.
- iii. Open the CONFIGURATION tab and select from the plate type menu the Opera adjustment plate and the appropriate objective, typically the 20 $\times$ 0.7-NA water-objective lens.
- iv. Activate the 405-nm laser. Press the ‘Liquid ON’ button to prime the water-immersion system.
- v. Open the MICROSCOPE tab and click TABLE IN/OUT, insert the Opera adjustment plate and take reference images to perform optics corrections according to the manufacturer’s instructions.
- vi. In the CONFIGURATION tab, change to the appropriate 384-well plate type. Click on TABLE IN/OUT and load the 384-well plate containing the cells. In the MICROSCOPE tab, select one of the

wells containing cells and define the optimal exposure parameters: select a single exposure and use the check-box to select the 405-nm laser, the camera 1 and set for 2x binning. Select the appropriate filters for the optical path (camera 1: 475/50, Detect_dichro: 510, Primary_dichro: 405/488/561/640). Press the exposure button to look at the stained cells. Check whether the cells are forming a monolayer and whether they are properly stained.

- vii. Select the optimal focus height and click the 'take height' button.

▲ **CRITICAL STEP** Selecting the optimal focus is crucial for the success of the protocol. Select the focus height corresponding to the middle plane of the cells that typically exhibits the highest average intensity, as displayed in the 'brightness statistics' on the top and left of the image display (see also Step 3A(x) and Supplementary Fig. 1).

- viii. Determine the optimal laser intensity by clicking EXPOSE, and then adjust the laser power and exposure time as desired. Save the exposure parameters.

▲ **CRITICAL STEP** Avoid saturated or underexposed cells by monitoring the 'brightness statistics' values at the top and left of the image display.

? TROUBLESHOOTING

- ix. Create the plate layout marking the desired wells that you want to image. Open the NAVIGATE tree, right-click on the experimental folder and select CREATE LAYOUT. Follow the 'layout wizard' to select the desired wells and click FINISH. Similarly, create the well sublayout by selecting CREATE SUBLAYOUT in the NAVIGATE tree and select the number and locations of the fields per well that will be acquired. For a typical experiment, imaging of a minimum of ten fields (~500 cells) is required, but a higher number of cells will provide smoother cell cycle profiles and is recommended (Supplementary Fig. 2).

- x. Click on CREATE STACK in the NAVIGATE tree to define the number and the interval of the acquired z-stacks. Choose three z-stacks that are 7 μm apart (+7 μm from the middle plane, as defined in Step 3A(vii), 0 μm from the middle plane and -7 μm from the middle plane); see also Supplementary Figure 1.

▲ **CRITICAL STEP** During the z-stack definition, ensure that cells in mitosis, which are typically visualized in the upper focal plane, are also included (Supplementary Fig. 1).

- xi.** Under the EXPERIMENTAL DEFINITION tab, drag and drop all the experimental conditions (optics correction files, exposure parameters, plate layout, sublayout and stack definition), defined in Step 3A(viii-x), and save them by right-clicking on the experimental folder and selecting 'save current: Experiment'
- xii.** In the AUTOMATIC EXPERIMENT tab, drag and drop the saved experiment file in the 'Experiment' marked yellow area. In the Barcode area, include the name of the current experiment.
- xiii.** Press 'start' to initiate the imaging process. The OPERA imaging system saves acquired images in the '.flex' format. Save these files in a directory accessible to the Acapella software.

B. Wide-field microscopy ● TIMING 2 h

- i.** Initiate the SoftWoRx software.
- ii.** Under 'File' press 'Acquire-Resolve 3D', and at the opening 'Restore 3D' window press the lamp sign to initiate the Xenon bulb.
- iii.** Select excitation = DAPI, emission = DAPI, image size = 1,024 × 1,024, lens = 10x and binning = 1.
- iv.** On the microscope, select the 10x lens. Place the slide and focus to find DAPI-stained cells.
- v.** At 'Resolve 3D' window, select the optimal exposure. Avoid exposure settings that acquire images with saturated pixels. Press 'acquire' and check whether the cells form a monolayer.

? TROUBLESHOOTING

- vi.** At 'Resolve 3D' window, press 'Experiment,' and in the pop-up window under the 'Sectioning' tab unclick 'Z sections'. Under the 'Channel' tab, press refresh exposure.
- vii.** Press 'Run experiment' and select a name for the saved .dv file.

▲ **CRITICAL STEP** The file names should follow this format:

'DAPI_<TreatmentName><ImagingFieldNumber>_R3D.dv '.
<TreatmentName> should not contain either numbers or special characters. <ImagingFieldNumber> should contain only numbers.

- viii.** On the microscope, find a different field and press 'Run' to acquire new images. Repeat this step at least ten times per condition.

▲ **CRITICAL STEP** When imaging different fields, keep the exposure settings and the focal plane constant.

▲ CRITICAL STEP Under these acquisition conditions, approximately ten images are required to image ~3,000 cells to obtain the cell cycle profile, as shown in Figure 3b. When higher magnifications are used, a higher number of images is required.

Image analysis to calculate the integrated intensity of the DAPI staining ● TIMING 10–30 min

4| Follow Step 4A to analyze data obtained in Step 3A. Follow Step 4B to analyze data obtained in Step 3B.

A. Image analysis of data obtained using high-throughput confocal microscopy TIMING 10 min

- i. Open the Acapella software. In the ‘Acapella player’ tab, drag and drop the image analysis script: ‘averageDAPIstaining3D.msr’.
- ii. Select the ‘Acapella Editor’ tab, and on lines 23–28 manually change the number of planes used in z, the last and first plane in z and the first and last field acquired per well. For example, for three stacks in z and nine different fields per well imaged; save the script after editing it:

```
set(NumberOfPlanes=3)
```

```
set(lastzplane=3)
```

```
set(firstzplane=1)
```

```
set(StartField=1)
```

```
set(EndField=9)
```

- iii. Select the ‘Acapella Player’ tab and drag and drop the script containing the parameters: ‘Script_Parameters.mpr’.

▲ CRITICAL STEP The provided nuclei detection parameters have been optimized for NIH3T3 mouse cells. Optimize the nuclei detection parameters for the cell line of interest by changing the parameters on the ‘Acapella player window’ and by visually inspecting the detection outcome.

- iv. Under the ‘Acapella Player’ window, check the ‘Store Nuclear Intensity overlay image’ box if you wish to save all the analyzed images (under the directory: C:\temp) with overlays of the integrated intensity values with the nuclear segmentation of each individual cell (Fig. 2a, right panel). These overlays can then be used to track back the cell cycle status of each individual cell, on the basis of the values of the integrated DAPI intensity that define distinct cell cycle phases (as will be obtained by setting gates on the cell cycle histograms in Step 5A(ix)).

- v. Under the 'Path' window, select the directory containing the .flex image files, and under 'Wells' select the individual wells to be analyzed. Press 'Run Script' to initiate the analysis. The image analysis script produces single-cell level data that are saved in the C:\temp folder as one text file per each well. The file names follow this pattern: '<BarcodeName>_RXXCXX.txt', where RXX corresponds to the row number and CXX corresponds to the column number of the originating well.

? TROUBLESHOOTING

- vi. (Optional) The 'Z_IntegratedDapiInt' attribute column (column F in Excel, Supplementary Fig. 3a) in each text file contains the integrated nuclear intensity values of all individual nuclei detected in the images from this well. If desired, obtain the cell cycle profiles by creating histograms of the data contained in this column by using the 'data analysis' tool and the 'histogram' function in Excel manually. Alternatively, continue with Step 4A(vii) to automatically generate histogram plots using R.
 - vii. Create a working directory (folder) for the 'CellCycleHistograms_Acapella.R' script. The working directory must contain two subfolders: one subfolder must be named 'Experimental_Metadata' and must contain the 'GlobalLayout.txt' file that maps the experimental treatments to the appropriate well positions by row and column numbers (Supplementary Fig. 3b). The other subfolder must be named 'Acapella_Output'. Copy the single-cell results .txt files generated by Acapella (Step 4A(v)) from the C:\temp folder into 'Acapella_Output' folder.
- B.** Image analysis of data obtained using wide-field microscopy TIMING 30 min

▲ CRITICAL STEP The protocol assumes previous basic knowledge of CellProfiler. For the complete online documentation on how to download and install CellProfiler, go to <http://www.cellprofiler.org/>. The CellProfiler website also provides extensive documentation and tutorials. Be sure to download CellProfiler Version 2.1 or above so that the the CellProfiler.proj files work.

- i. Launch CellProfiler2 and open the 'IlluminationCorrection.cproj' analysis pipeline. The '?' button at the bottom left corner of the main CellProfiler window and in proximity to most of the parameter settings provides direct access to the documentation from the software.
- ii. Select the 'Images' input module in the 'Input modules' window. Right-click in the 'File list' window and select 'Clear File List' to eliminate previously loaded images. Right-click in the same

window and select 'Browse Images', and browse and select the images in .dv format to be analyzed. Skip the 'Metadata' module. Select the 'Names and Types' module and press the 'update' button on the 'Module Settings' window to verify that all the images are loaded properly. Skip the 'Groups' module.

▲ CRITICAL STEP The 'IlluminationCorrection.cpproj' pipeline assumes the presence of one channel (DAPI) per .dv image. For multichannel images, or for images in other formats, the parameters of the Metadata 'Names and Types' and 'Groups' modules will need to be set according to the file format, number and identity of channels and so on.

For details please refer to the official CellProfiler documentation.

- iii. Select the 'CorrectIlluminationCalculate' module in the 'Analysis modules' window. The saved parameters for this module in the provided 'IlluminationCorrection.cpproj' were chosen on the basis of the test image set provided as Supplementary Data. The parameters might need to be changed if nuclei in the images to be analyzed diverge from the test data set (e.g., the use of different magnification objectives and/or different nucleus morphology and density). This module will produce a single illumination correction function that will represent the background for every pixel of the image calculated over the entire image data set.

▲ CRITICAL STEP As 'Background' is selected as the correction method, it is essential to not rescale the illumination function. Please refer to the original CellProfiler documentation for an extensive explanation of the 'CorrectIlluminationCalculate' module and of its parameters. Skip the 'SaveImages' module.

- iv. Click on the 'View output settings' button in the 'Output' window and select an appropriate 'Default output folder' where the illumination function 'DAPI_Ill_correction.mat' file will be saved.
- v. Press 'Analyze Images'. A progress bar in the lower left corner of the main CellProfiler window will indicate an estimated time of completion for the analysis.
- vi. Open the 'CellCycleAnalysis.cpproj' analysis pipeline in CellProfiler2.
- vii. Select the 'Images' input module. Right-click in the 'File list' window and select 'Clear File List' to eliminate previously loaded images. Right-click in the same window and select 'Browse Images', and browse and select the images in .dv format to be analyzed.

▲ **CRITICAL STEP** In the ‘File list’ window, make sure to also select the ‘DAPI_III_correction.mat’ illumination correction file generated by the previous pipeline (Step 4B(i-iv)).

- viii. In the ‘Metadata’ module, press the ‘Update metadata’ button under ‘Module settings’ to verify that CellProfiler is properly extracting the appropriate metadata from the image file headers. In the ‘NamesAndTypes’ ‘Module settings’ window, select the proper ‘Single image location’ for the ‘DAPI_III_correction.mat’ generated by the ‘IlluminationCorrection.cpproj’ pipeline (Step 4B(i-v)). Press ‘update’ to verify successful setting of the parameters for this module. Skip the ‘Groups’ module. Select the ‘Names and Types’ module and press the ‘update’ button on the ‘Module Settings’ window to verify that all images are loaded properly.

▲ **CRITICAL STEP** The ‘CellCycleAnalysis.cpproj’ pipeline assumes the presence of one channel (DAPI) per .dv image. In case of multichannel images, or of images in other formats, the parameters of the Metadata ‘Names and Types’ and ‘Groups’ modules will need to be set according to the file format, number and identity of channels and so on. For details please refer to the official CellProfiler documentation.

- ix. Select ‘CorrectIlluminationApply’ under the ‘Analysis Module’ window.

▲ **CRITICAL STEP** If ‘Background’ was selected as the correction method in the ‘IlluminationCorrection.cpproj’ pipeline, make sure that the illumination function is applied using the ‘Subtract’ method. For details on the ‘CorrectIlluminationApply’ module parameters choices, please refer to the original CellProfiler documentation.

- x. Select the ‘IdentifyPrimaryObjects’ module that is used to segment nuclei using the DAPI image. The original parameters in this module were chosen on the basis of the test image data set provided as Supplementary Data and, if needed, represent a starting point for further optimization. Please change the ‘IdentifyPrimaryObjects’ parameters to suit the DAPI images to be analyzed. For details, please refer to the CellProfiler documentation.

? TROUBLESHOOTING

- xi. Ensure that the ‘MeasureObjectIntensity’, ‘MeasureObjectSizeShape’, ‘OverlayOutlines’, ‘DisplayDataOnImage’ and ‘SaveImages’ are checked. These

modules will measure the DAPI intensity and nuclear morphological parameters. In addition, they will generate overlay images of both the nucleus border outline and of the single-cell integrated DAPI intensity values (Fig. 3a). These overlays can then be used to track back the cell cycle status of each individual cell on the basis of the values of the integrated DAPI intensity that define distinct cell cycle phases (as obtained by setting gates on the cell cycle histograms in Step 5B(x)). These images can also be useful during postanalysis to verify the nuclear segmentation process on several imaging fields. If the image data set is too large, or if there is no need to save the nuclear border overlay images, uncheck the box by 'SaveImages' in the 'Analysis modules' menu.

- xii. In 'View output settings', select the output directory as the previously created 'CP2_CellCycle_Output' folder.
- xiii. Press 'Analyze Images'. At the end of the run, the 'CP2_CellCycle_Output' folder will contain, among other files, a 'Nuclei.txt' file containing the single-cell results from the CP2 analysis. Each row of the table included in the 'Nuclei.txt' file corresponds to a single nucleus, and each column represents either categorical or numerical attributes generated by the CP2 analysis. The column named 'Metadata_FileLocation' includes the full path and name of the image from which the analyzed nucleus comes from, and the column named 'Intensity_IntegratedIntensity_DAPICorr' includes the values of the corrected DAPI integrated intensity for each cell.

Generation of cell cycle profiles and gating of cells into different phases of the cell cycle

TIMING 10 min

5| If image analysis was performed using Step 4A, continue data analysis as described in Step 5A. If Step 4B was used for image analysis, continue with Step 5B.

A. For data obtained from high-throughput confocal microscopy

- i. Launch RStudio and set the working directory (i.e., the folder containing 'Experimental_Metadata' and 'Acapella_Output', see Step 4A(vii)) using the `setwd()` function at the R prompt in the console window.

▲ **CRITICAL STEP** The secondary analysis strategy of the single-cell results described in this protocol assumes previous installation and setup of R and RStudio, as well as some previous basic knowledge of the R language.

▲ **CRITICAL STEP** Remember to enclose the working directory path in inverted commas (' ... '). In addition, if you

are working on a Windows environment, make sure to use forward slashes and not backslashes in the directory path.

- ii. In RStudio, open the 'CellCycleHistograms_Acapella.R' script.
- iii. In line 48 of the script, set the maximum value for the DAPI integrated intensity attribute to be displayed on the x axis of the histogram plot (for example, `DAPI.max <- 8e5`, i.e., 8×10^5).
- iv. In line 51, set the bin width of the histogram (for example, `DAPI.binwidth <- 1e4`, i.e., 1×10^4).
- v. In lines 54 and 55, change the values assigned to `DAPI.width` and/or `DAPI.height`. These two variables control the width and height in inches of the histogram plot .png files generated in Step 5A(vii).
- vi. On line 62, input the exact names (case sensitive) assigned to the treatment conditions. Each name must be surrounded by inverted commas and separated from each other by a comma. These names must exactly match the ones used in the 'GlobalLayout.txt' file (Step 4A(vii)).
- vii. Select lines 38–130 and paste them at the R prompt in the console window. Press 'return'. The script will read, append and experimentally annotate all the separate .txt Acapella results files into a single data table. In addition, it will automatically save one histogram plot per treatment for the DAPI integrated intensity as a .png file ('DAPI_Histogram_<treatmentname>.png') in a newly created 'R_Output' folder inside the working directory.

? TROUBLESHOOTING

- viii. Examine the plots generated by the R script and go back to Step 5A(iii-vi) if graphical parameters of the histogram plots need to be modified. Save the modified script. Once the parameters have been modified, repeat Step 5A(vii).
- ix. Once the desired graphical parameters of the histogram plot have been defined, decide on and set the threshold values of DAPI integrated intensity used to calculate the percentages of cells belonging to the G1, S or G2/M phases of the cell cycle in lines 134–136 of the script. Appropriate values for these thresholds must be empirically found by examining the generated cell cycle profiles. The first value in the parentheses in each line represents the lower value for the DAPI integrated intensity gate, whereas the second one represents the upper value. Save the script once more when appropriate combinations of values have been found.
- x. Copy lines 133–161 of the script, and then paste them at the R prompt in the console window. Press 'enter'. The script will

produce a table of the percentages of cells belonging to the different phases of the cell cycle based on their DAPI Integrated Intensity values ('CellCycle_Percentages_table.txt'). This table, together with an additional table containing the binned values for DAPI integrated intensity that can be used to generate plots with other graphical or statistical analysis software ('CellCycle_BinnedFrequencies_table.txt'), is saved as a .txt file in the 'R_Output' folder.

B. For data obtained from wide-field microscopy

- i. Launch RStudio and set the appropriate working directory by using the R function `setwd()` at the prompt in the console window.

▲ **CRITICAL STEP** The secondary analysis strategy of the single-cell results described in this protocol assumes previous installation and setup of R and RStudio, as well as some previous basic knowledge of the R language.

▲ **CRITICAL STEP** Remember to enclose the working directory path in inverted commas (' ... '). In addition, if you are working on a Windows environment, make sure to use forward slashes and not backslashes in the directory path.

▲ **CRITICAL STEP** The working directory should contain the 'CP2_CellCycle_Output' subfolder containing the CellProfiler analysis results ('Nuclei.txt', Step 4B(xiii)).

- ii. Open the 'CellCycleHistograms_CP2.R' script in RStudio. This script reads the single-cell data present in the Nuclei.txt file, experimentally annotates each single-cell measurement by extracting the treatment name from the original image name, and then generates histogram plots of the DAPI integrated intensity attribute and a table containing the calculated cell cycle phase percentages based on user-defined thresholds.
- iii. On lines 36 and 39, change the set value for the maximum DAPI integrated intensity (DAPI.max) that is displayed in the histogram and the bin width (DAPI.binwidth), respectively.
- iv. On lines 42–43, set the width and height dimensions (in inches) for the .png files generated by the R script (Step 5B(viii)).
- v. On line 50, insert the names of the different experimental treatments used in the experiments (e.g., 'untreated', 'aphidicolin' and so on).

▲ **CRITICAL STEP** Each treatment name string must be surrounded by inverted commas and separated from other strings by a comma. These names must be identical to the

ones extracted from the filenames by using the regular expression pattern defined on line 61 (Step 5B(vi)).

- vi. In Line 61 of the script, set the regular expression pattern to extract the treatment information from the original .dv file names contained in the 'Metadata_FileLocation' column of the 'Nuclei.txt' results file. The script expects the original .dv file names to contain a string specifying the experimental treatment that they refer to (e.g., 'untreated', 'aphidicolin' and so on). The .dv file names should follow this format: 'DAPI_<TreatmentName><ImagingFieldNumber>_R3D.dv'. <TreatmentName> should not contain either numbers or special characters. <ImagingFieldNumber> should contain only numbers.

▲ CRITICAL STEP The pattern defined in the provided script will work with the treatments and file names used in the test data set provided with this article. Users will need to modify the pattern according to the actual .dv file names they have previously defined (Step 3B(vii)). For details on regular expression patterns, consult the official R documentation.

- vii. Save the edited R script. Copy lines 27–99 of the script and paste them at the prompt in the Rstudio console window.
- viii. Press 'enter'. The R code that was pasted should run without giving errors, and it should generate an 'R_output' folder containing .png files for histogram plots for the DAPI Integrated Intensity on a per-treatment basis ('DAPI_Histogram_<treatmentname>.png').

? TROUBLESHOOTING

- ix. Visually inspect the histograms and, if needed, go back and repeat Step 5B(iii,iv) by changing the histogram plot parameters (Maximum DAPI value, bin width and .png file dimensions) and rerun (Step 5B(vii,viii)). Iterate until suitable results are obtained.
- x. On the basis of visual inspection of the generated DAPI integrated intensity histogram profiles (Step 5B(viii)), set, on lines 103–105, the minimum (first value between the parentheses) and the maximum (second value between the parentheses) integrated DAPI intensity values that define the gates for classification of nuclei in the G1, S or G2/M phases of the cell cycle.
- xi. Save the R script. Copy lines 100–131 of the script and paste them at the prompt in the RStudio console window.
- xii. Press 'enter'. The R code should not give errors, and it should generate a file named 'CellCycle_Percentages_table.txt' that contains the calculated percentages of nuclei belonging to the G1,

S or G2/M of the cell cycle, on a per-treatment basis. The script also generates an additional table ('CellCycle_BinnedFrequencies_table.txt') that contains bin labels and bin counts to be optionally used to generate histogram plots with alternative statistical software packages. Both the .txt results files can be found in the 'R_Output' subdirectory.

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

● TIMING

For high-throughput confocal microscopy

Step 1A, plating of cells in multiwell imaging plates: 1 d Step 2A, fixing and staining cells with DAPI: 1 h Step 3A, high-throughput fluorescence microscopy: 1 h

Step 4A, image analysis to calculate the integrated intensity of the DAPI staining: 10 min

Step 5A, generation of cell cycle profiles and gating of cells into different phases of the cell cycle: 10 min

For wide-field microscopy

Step 1B, plating of cells on coverslips: 1 d

Step 2B, fixing and staining cells with DAPI: 1 h

Step 3B, wide-field fluorescence microscopy: 2 h

Step 4B, image analysis to calculate the integrated intensity of the DAPI staining: 30 min

Step 5B, generation of cell cycle profiles and gating of cells into different phases of the cell cycle: 10 min

ANTICIPATED RESULTS

By using this protocol, the cell cycle stage of individual cells can be determined by fluorescence microscopy. This method is based on the calculation of the DNA content by the routinely used nuclear counterstain DAPI, and thus it can be combined with other imaging-based methods of interest without additional modifications. It is anticipated that DNA content histograms, as routinely obtained by flow cytometry (Figs. 2 and 3), are derived and used to define the population of cells within G1, S or G2/M cell cycle phases (Fig. 4 and Supplementary Fig. 4). The stage of the cell cycle of individual cells can be then be determined and correlated with other cell biological features of the same cells derived by a plethora of imaging-based methods such as immunofluorescence, FISH or live-cell imaging.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1 |.

Overview of the protocol. Cells of interest are plated in imaging plates or on coverslips compatible for high-throughput confocal or standard wide-field fluorescence microscopy, and the cells are fixed and nuclei are stained with DAPI. When high-throughput confocal microscopy is used (e.g., Opera, Steps 1A–5A), images of stained cells are acquired in 3D, and automated image analysis is used to calculate the averaged DAPI integrated intensity for each cell with single-cell resolution (Acapella software). When wide-field fluorescence microscopy is used (e.g., Deltavision, Steps 1B–5B), single images of the stained cells are acquired and automated image analysis measures the integrated DAPI staining intensity (CellProfiler). In both pipelines, the histograms of DNA content in the cell population are then calculated to generate cell cycle profiles (using R), and cells in the different cell cycle phases are identified by applying visually selected cutoffs. Alternatively, cell cycle modeling (using FCS Express 5 software) can be used to automatically calculate the percentages of cells within the different phases. This protocol allows staging of individual cells, as well as the generation of population-based cell cycle profiles.

Figure 2 |.

Cell cycle profiles derived by using high-throughput confocal fluorescence microscopy. **(a)** Images of cells stained with DAPI are acquired in 3D using a high-throughput confocal microscope (e.g., Opera), and automated image analysis (e.g., Acapella) is used to first generate an average image projection in the *z*-plane of the stack and then to segment the nuclei based on the projected DAPI image (left), to exclude identified nuclei at the image borders (middle) and to finally calculate the integrated nuclear intensity of DAPI per nucleus (right). Scale bar, 10 μ m. **(b)** Comparison of imaging and flow cytometry data. Cell cycle profiles obtained by traditional, population-based DNA content analysis by flow cytometry (propidium iodide staining, top) and single-cell confocal fluorescence microscopy (DAPI staining, bottom) of control cells or cells treated with 5 μ M aphidicolin, 1 μ M etoposide or 1 μ M camptothecin for 22 h.

Figure 3 |.

Cell cycle histograms obtained by using standard wide-field fluorescence microscopy. **(a)** A standard epifluorescence microscope (e.g., Deltavision) was used to acquire images of cells stained with DAPI and automated image analysis (e.g., CellProfiler) was used to detect all nuclei (left and middle), and to calculate the integrated nuclear intensity of all cells (right). Scale bar, 10 μ m. **(b)** Cell cycle profiles of the indicated treatments were obtained by DNA content analysis by flow cytometry (propidium iodide staining, top) or by using standard wide-field fluorescence microscopy (DAPI staining, bottom).

Figure 4 |.

Calculation of population of cells within distinct cell cycle phases. Untreated cells stained with DAPI were imaged using wide-field microscopy and analyzed as in option B of this protocol (Fig. 3b). Visually selected discrete cutoffs of the distinct cell cycle phases are used to calculate the percentages of cells within G1, S and G2/M phases using the R software (left). Alternatively, the single-cell DAPI integrated intensity values obtained with CellProfiler were loaded into FCS Express plus (version 5) for DNA cell cycle modeling via the Multicycle, AV plug-in (right). Data were modeled according to the default autofit settings to automatically calculate the percentages of cells within G1, S and G2/M phases, while taking into account cells transitioning between cell cycle phases

TABLE 1 |

Troubleshooting table.

Step	Problem	Possible reason	Solution
3A(viii), 3B(v)	Cells do not form a monolayer	Cells were plated too densely	Optimize the number of cells plated on the basis of the imaging plate or dish used (Step 1A(ii) and 1B(iii))
4A(v), 4B(x)	Nuclei detection masks do not accurately segment the stained nuclei	The nuclei detection algorithm has been optimized for mouse NIH3T3 cells	Cell lines with different morphology or size may require further optimization by choosing alternative nuclei detection algorithms or parameters and by visual inspection of the results
4A(v)	The projections of the stained nuclei, as they appear in the 'Acapella player' window when the script is running, show 'ghost' nuclei that seem to belong to different fields	The number of planes in z (z-stacks) and the acquired number of fields per well do not correspond to the numbers in the image analysis script (Step 4A(ii))	Insert the correct numbers of planes used in z, the last and first plane in z and the first and last field acquired per well, as shown in the example in Step 4A(ii)
5A(vii), 5B(viii)	The R script generates an error	Not all required R packages have been installed The working directory has not been set appropriately	Make sure that the R packages required by the 'CellCycleHistograms_Acapella.R' (Step 5A) or by the 'CellCycleHistograms_CP2.R' (Step 5B) scripts are part of the local R library on the computer that is being used to run the script. If this is not the case, run the command <code>install.packages(c('data.table', 'ggplot2', 'stringr', 'plyr'))</code> at the R prompt in the console window in RStudio only before the first time that the script is run. For details on how to install R packages, consult the official online R documentation (http://cran.r-project.org/) Set the working directory and make sure that it contains all the required folders and files (as detailed in Steps 4A(vii) and 5A(i) or 4B(xiii) and 5B(i), respectively).
	The produced histogram is either not centered in the plot or the cell cycle profile is not well resolved	The bin width and the maximum value of the produced histogram are not optimal	Step 5A: On line 48 of the R script, increase the maximum value of the produced histogram (Step 5A(iii)) and/or modify the bin width on line 51 (Step 5A(iv)) Step 5B: On lines 36 and 39, change the maximum DAPI value (DAPI.max) and the bin width (DAPI.binwidth) variables (Step 5B(iii))
		The selected exposure parameters are not optimal	Make sure that during Step 3A(viii) or 3B(v) no underexposed or saturated DAPI signals are acquired
		The nuclei detection algorithm does not accurately segment the stained nuclei	See Troubleshooting for Step 4A(v) and 4B(x)
		Nonoptimal selection of z planes (for option A, Step 3A(x))	Select z-stacks to cover the entire volume of the cells. Make sure that cells in mitosis, which are typically visualized within the upper planes in z, are covered (Step 3A(x))
		Cells do not form a monolayer and z-stack selection is impossible to adjust so as to include all cells (option A, Step 3A(vi) and option B, Step 3B(v)) or the mounted coverslip (option B) was not leveled properly (Step 2B(viii))	Re-plate the cells (Step 1A(ii) and 1B(iii)) or remount the coverslip (Step 2B(viii))