Application Brief

Cancer Biology

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Detailed Characterization of Mitosis Using Cell Population Analysis and Auto ROI Defined Confocal Imaging

Author

Paul Held, PhD

Agilent Technologies, Inc.

Introduction

In proliferating cells, the cell cycle consists of four phases (Figure 1). G1 is the interval between mitosis and DNA replication that is characterized by cell growth. Replication of DNA occurs during the synthesis (S) phase, which is followed by a second gap phase (G2) during which growth and preparation for cell division occurs. Mitosis and the production of two daughter cells occur in M phase.[1] Mitosis can be further divided into four stages: prophase, metaphase, anaphase, and telophase. Generally, most cells are quiescent and do not undergo division unless signaled to enter the active segments of the cell cycle. These nonproliferating cells are often referred to as being in G0.

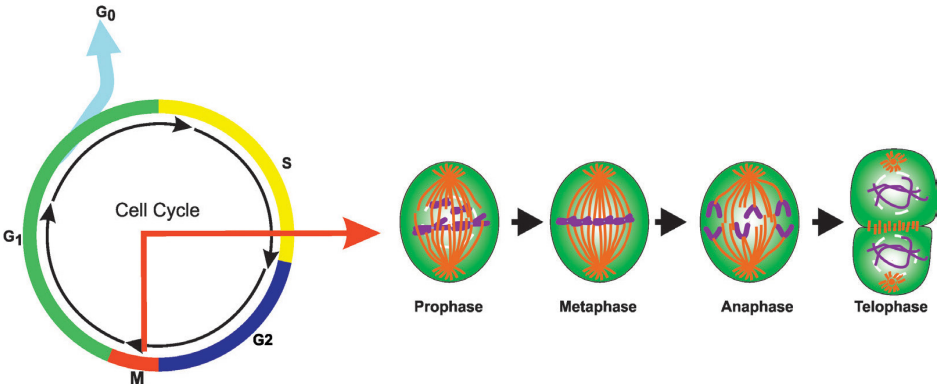

Figure 1. Stages of the cell cycle and mitosis.

As depicted in Figure 1, mitosis occupies only a small portion of the cell cycle, as it generally occurs quite rapidly once the decision to divide has been made. In an asynchronous population of cells the likelihood of a cell being in mitosis is relatively small, making them difficult to find. While mitotic cells can be identified at low magnification with antibodies specific to mitotic biomarkers, characterization of the different stages of mitosis requires higher magnification to visualize specific subcellular structures. Imaging rare cellular events at high magnification can present a number of issues. By their nature, high-magnification objectives magnify only a very small portion of the available viewing area of a microplate well. Manually searching for these cellular events can be time-consuming and subject to bias. Automation of the imaging area can relieve the investigator of the tedium, but will produce enormous amounts of data that will need to be sifted through to obtain the desired data. The region of interest (ROI) feature of the Agilent BioTek Gen5 microplate reader and imager software can be used to automate the process by using low magnification imaging to automatically find the desired rare events before imaging at high magnification.

This application brief demonstrates the use of the Auto ROI feature of Gen5 to identify mitotic cells for further characterization using confocal microscopy performed by the Agilent BioTek Cytation C10 confocal imaging reader. P-histone H3 is a biomarker for mitotic cells.[2] The detection and quantitation of mitotic cells use immunofluorescence imaging at low magnification from an asynchronous population of fixed and stained cells. These relatively rare cells (4 to 8%) can be identified with the Gen5 Auto ROI feature as a target for further characterization at higher magnification (60x). In addition, low magnification will be used to identify and quantitate mitotic nuclei in an asynchronous cell population in order to determine the mitotic index.

Materials and methods

Tissue culture

RPE1 human fibroblasts were cultured in Advanced DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin at 37 °C in 5% CO_2. Cultures were routinely trypsinized (0.05% trypsin-EDTA) at 80% confluence. Cells were plated into Nunc black-sided clear glass bottom 96-well microplates such that there were 2,000 total cells per well. After 24 hours to allow for attachment, the media was removed and replaced with fresh media containing various concentrations of FBS. Cells were incubated for 48 hours and then fixed with 4% paraformaldehyde.

Immunostaining

Cells were permeabilized (PBS, 0.1% triton X-100) for 15 minutes and blocked (PBS, 3% BSA, 0.1% triton X-100) for 60 minutes at RT. After blocking, mouse anti-tubulin, part number A-11126, from Life Technologies (Carlsbad, CA) and rabbit anti-phospho-histone H3 antibodies, part number 53348S, from Cell Signaling Technologies (Danvers, MA) were added (1:500 dilution in PBS, 3% BSA, 0.1% triton X-100) and incubated for 120 minutes at room temperature. The plate was washed 3x with PBS and the secondary Alexa Fluor 647 labeled goat anti-mouse IgG antibody, part number A21235, from Life Technologies (Carlsbad, CA) and AlexaFluor 555 goat anti-rabbit IgG, part number 4413, from Cell Signaling Technologies (Danvers, MA) each diluted 1:500 dilution in PBS, 1% BSA, 0.3% triton X-100) were added and incubated for 120 minutes at RT followed by 3x wash with PBS. Cells were counterstained for 15 minutes with Hoechst 33342 (10 µM) and AlexaFluor 488 phalloidin, part number 12379, from Life Technologies (Carlsbad, CA) followed by 2 washes with 200 µL of PBS. After aspiration, 250 µL of PBS were added to all wells and the plates sealed using an optically clear adhesive plate sealer.

Imaging

For mitotic index experiments, fixed cells were imaged using a Cytation C10 configured with a DAPI and TRITC LED cubes using a 4x objective. The DAPI cube is configured with a 377/50 excitation filter and a 447/60 emission filter. The TRITC cube is configured with a 556/20 excitation filter and a 600/37 emission filter. High-magnification analysis of mitotic cells used the ROI feature of Gen5 to identify mitotic nuclei. ROIs were defined in the discovery step using a 4x objective along with TRITC and DAPI widefield cubes. Threshold and size discrimination of the TRITC-labeled nuclei were used as a selection for higher-magnification analysis. The DAPI channel was used for selection integrity, ensuring that the TRITC signal localized to the nuclei. Subsequently, identified ROIs were imaged using the 60x objective with confocal DAPI, GFP, and CY5 laser cubes and a 60 µm spinning disks. For each ROI, a series of z-stack images spanning 14 µm in 1 µm slices was captured. Z-projections were developed using maximum signal intensity.

Results and discussion

Mitotic cells can easily be identified using immunofluorescence. Cells containing phosphorylated histone H3 (p-histone H3) can be identified with a specific monoclonal antibody. Subsequent staining with an AlexaFluor 555 labeled secondary antibody visualizes the positive cell

nuclei for image-based analysis (Figure 2). By using the nuclear stain Hoechst 33342, all cell nuclei can be identified using the DAPI channel. Subpopulation analysis with the TRITC channel further identifies cell nuclei that are also positive for mitosis.

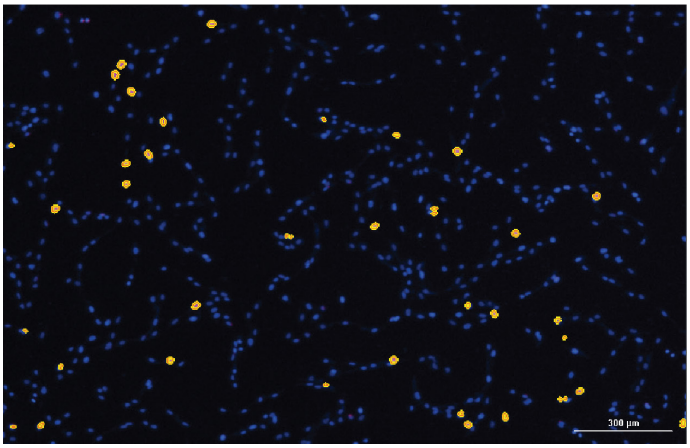

Figure 2. Low-magnification image of fixed and stained RPE1 cells. Mitotic cells were automatically identified and indicated in yellow.

Using these data, one can calculate the mitotic index of a cell population. Mitotic index, which is the percentage of cells undergoing mitosis at any time, is the most common marker used for asynchronous determination of cell cycle length. As demonstrated in Figure 3, the mitotic index increases with serum concentration. RPE1 cells exposed to serum concentrations of 0.1% or less for 48 hours have a mitotic index of 3.5%, whereas cells treated with 10% serum have an index of 7%.

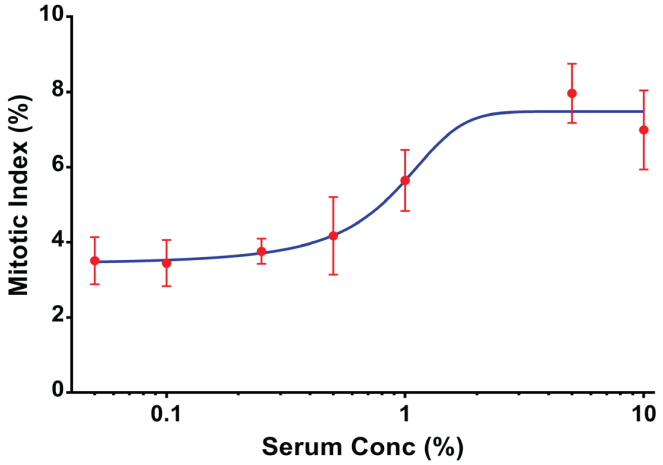

Figure 3. Mitotic index of RPE cells treated with various concentrations of serum.

Provided that the same sample is also stained with cytoskeletal dyes and/or antibodies, cell morphology of cells in various stages of mitosis can be imaged at higher resolution. Figure 4 provides some images of RPE1 cells in various stages of mitosis. These cells were identified using the ROI feature of Gen5. Once these regions of interest had been identified, a series of z-stack images was made using the DAPI, GFP, and CY5 channels with the 60x objective to identify nucleus, actin, and tubulin respectively.

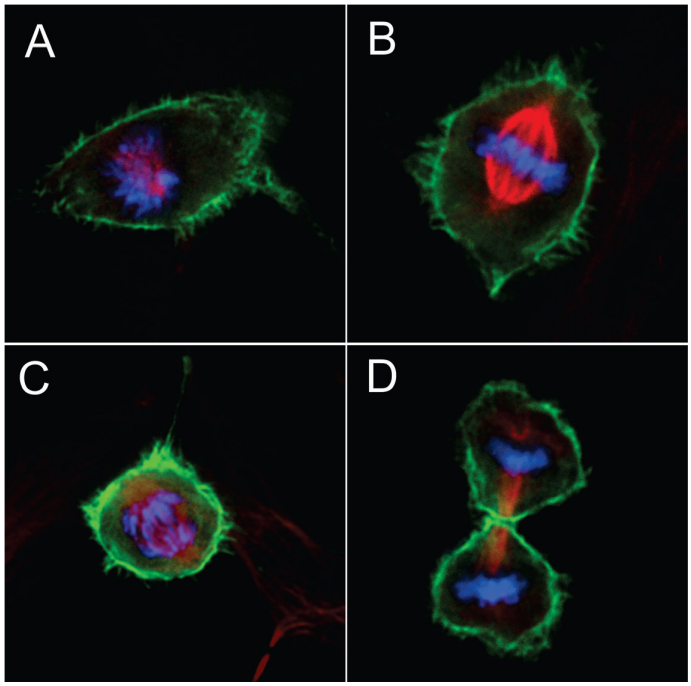

Figure 4. Immunofluorescent images of RPE1 cells in different stages of mitosis. Fixed RPE1 cells were stained for actin (green), tubulin (red) and DNA (blue). Confocal images representing (A) prophase; (B) anaphase; (C) metaphase; and (D) telophase stages of mitosis.

Because cancers are essentially uncontrolled cellular proliferation, numerous pharmacological agents affecting cell cycle progression have been used as treatments. While proliferating cells can be arrested in any stage of the cell cycle, mitosis is uniquely appropriate due to its relatively short length and the ability to target the mitotic checkpoint. Several drugs target the microtubule assembly of mitosis. For example, vinblastine binds to tubulin and is known to disrupt microtubule formation.[3] Interestingly, paclitaxel, which stabilizes microtubules, is also an effective antineoplastic agent.[3] Because mitosis is a relatively rare event in an asynchronous population of cells, it is often difficult to image many of these cells. Scanning large areas at high magnification requires large amounts of time and

considerable data storage. Alternatively, the process is accomplished by searching a large image area manually. Even when mitotic cells are identified with a distinct marker, this process is tedious and can result in bias. The ROI feature of Gen5 automatically scans an image area using a low-magnification screen, then imagines only areas of true interest. While only the TRITC signal was needed to identify mitotic cell nuclei, it was found to be advantageous to also image the cells in the DAPI and GFP channels concurrently. The DAPI channel provided a visual confirmation that the TRITC signals, seen as spots, colocalized with DAPI and were indeed nuclei rather than nonspecific binding. Further confirmation of the mitotic status of the cell was made with cell morphology, identified by the cytoskeletal staining of actin.

Conclusion

The Agilent BioTek Cytation C10 confocal imaging reader in conjunction with the Agilent BioTek Gen5 microplate reader and imager software are a unique combination of hardware and software packages that can provide both low-magnification high-throughput quantitative image analysis, as well as high-resolution content analysis.

References

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