High Resolution Fluorescence Microscopy of PtK2 Cells Undergoing Mitosis in Microplates

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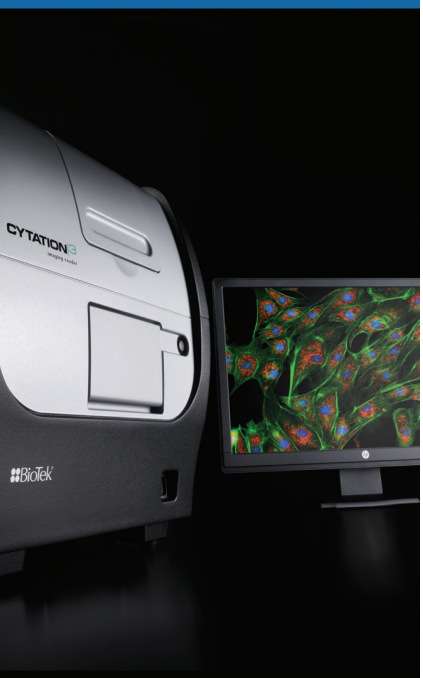

The use of fluorescent dyes in combination with cell fixation allows the visualization of cellular processes that have been preserved in time. The PtK2 cell line is commonly used to visualize the various stages of mitosis given their unique physical characteristics. Microplates are now available that allow for high resolution fluorescence microscopy to visualize mitotic cellular processes.

Key Words:

Montage

Image Stitching

Kidney Section

Widefield Microscopy

Immunostaining

Epifluorescence

Mitosis

Cell Cycle

Introduction

Cellular mitosis, when a cell duplicates into two identical daughter cells, is a fast and highly complex process in the cell cycle of all multi-cellular eukaryotic organisms. Mitosis can occur at several important junctures during the evolution of an organism such as within the growth and development phase or during cell replacement (skin cells, RBCs, etc.), as well as regeneration and asexual reproduction in some organisms. Mitosis remains an area of high interest given the central role played in cell division and reproduction. Defects in mitosis have been linked to unregulated cell division associated with many types of cancer. Furthermore, it has been shown that cancer cells exhibiting unregulated cell division contain mutations at genetic loci associated with regulation of mitotic checkpoints[1].

Mitosis, taken together with cytokinesis, defines the mitotic or M phase which accounts for approximately 20% of the cell cycle. While the mitotic process varies between different organisms, in general chromosomes in the cell nucleus are separated into two identical sets, each within a separate nucleus. In most cases, cytokinesis immediately follows mitosis resulting in a division of the cytoplasm, organelles and cell membrane resulting in two genetically identical daughter cells of roughly equal size.

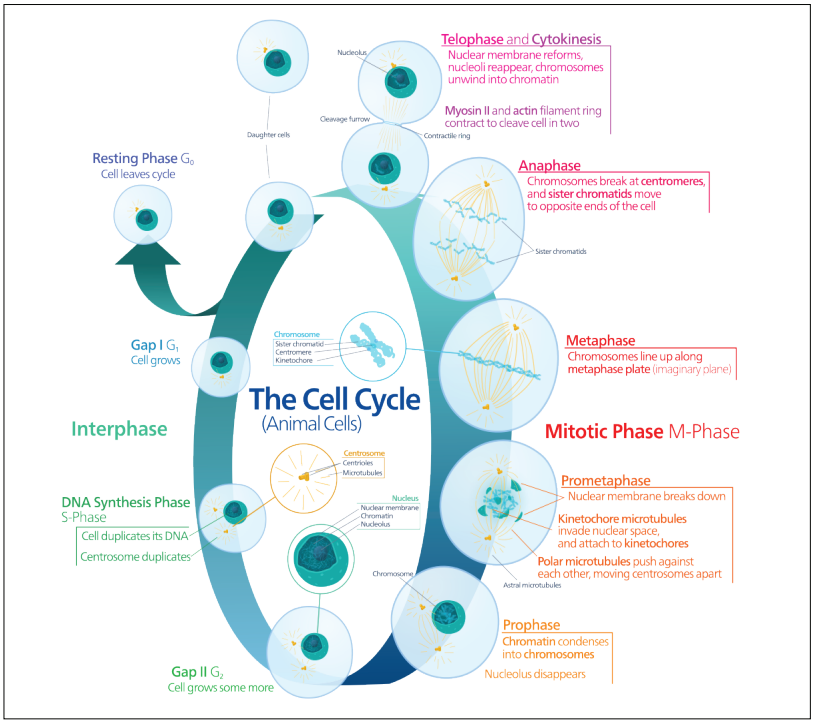

Figure 1. **Mitotic Phase (M-phase).** The stages of mitosis include: prophase, prometaphase, metaphase, anaphase, and telophase and the process of cytokinesis. During the process chromatin condenses to form sister chromatids that are separated and divided into two genetically matched daughter cells of equal size.

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The process of mitosis is divided into five identifiable stages: prophase, prometaphase, metaphase, anaphase and telophase (Figure 1).

Prophase: the nuclear membrane begins to dissolve and chromatin begins to condense to from chromosome pairs connected by a centromere. Sister chromatids, resulting from duplication of each chromosome pair during stationary or S phase, condense and attach to microtubule fibers coordinated by centrosomes. Each centrosome consists of a pair of centrioles and actin composed of microtubule fragments.

Prometaphase: follows with disintegration of the nuclear membrane and invasion of microtubules into the nuclear space. Late in prometaphase the microtubules attach to the kinetochores located at the center of each sister chromatid at the centromere. Molecular motors are thought to exist at each kinetochore and work in concert with microtubule polymerization and depolymerization to segregate and later separate the sister chromatids. The microtubules provide the framework for the formation of the mitotic spindle which will eventually draw the chromatids apart.

Metaphase: begins after attachment of the microtubules to the kinetochores. The centrosomes begin to pull the chromosomes to opposite poles of the cell. As this process begins, the chromosomes are seen to line-up between the two centrosomes at the metaphase plate or equatorial plane.

Anaphase: begins with the cleavage of attachment proteins that bind sister chromatids together. As the chromatids travel to opposite poles they take on a classic V-shaped appearance as they are tugged at the centromere with chromatids trailing. Once the chromosomes have reached opposite ends of the cell the kinetochore and associated microtubules degrade.

Telephase: is the final phase at which time the polar microtubules continue to polymerize forcing cell elongation. The nuclear membrane begins to form around each set of daughter chromosomes, the nucleoli reappear, and decondensation to chromatin begins to occur.

Cytokinesis: While telophase signifies the end of mitosis, cell division requires the process of cytokinesis. In animal cells, a cleavage furrow, formed by a contractile ring, develops at the former site of the metaphase plate. Eventually the ring contracts dividing the cell and separating the two newly formed nuclei. Plant cells form a cell plate at the center of the cell that will eventually become a cell wall, again separating the two nuclei.

The PtK2 mammalian line cell was derived from male rat-kangaroo (Potorous tridactylis) epithelial kidney cells by Wallen and Brown in 1962 to aid in the study of chromosomes[1]. The cells are particularly good for the study of mitosis as they have only a few, large chromosomes suitable for microscopic analysis. Additionally, the cells are relatively large and tend to remain flat throughout the cell cycle when grown as an adherent monolayer. The cells typically undergo mitosis on average every 2 to 3 hours allowing the possibility to capture cells at various stages of mitosis. A large repertoire of fluorescent probes currently exists to aid in the visualization of mitotic events at high resolution. Here we demonstrate the ability to capture images depicting several stages of the cell cycle, including mitotic events, occurring in PtK2 cells following cell fixation and staining in 96-well microplates.

Materials and Methods

Materials

PtK2 (NBL-5), kidney, Potoroo (Potorous tridactylis) cells (No.CCL-56 FZ) were purchased from ATCC (Manassas, Virginia, USA). Minimal essential media (MEM) (No.10370-021), fetal bovine serum (FBS) (No.10437-028), penicillin-streptomycin-glutamine, anti-α-tubulin (bovine), mouse IgG1, monoclonal (No.A11126), Alexa Fluor 488 F9ab)2 fragment of goat anti-mouse IgG (H+L) (No.A11017) and DAPI (No.D1306) were purchased from Life Technologies (Grand Island, NY). Greiner Bio-One SCREENSTAR 96-well microplates (No.6558660) was a gift from Greiner Bio-One North America, Inc. (Monroe, NC, USA).

Methods

Cell Culture and Microplate Seeding

Cell cultures were maintained at 37 °C, 5% CO_2 in a humidified incubator. Cultures were routinely trypsinized (0.05% Trypsin-EDTA) at 80% confluency. For experiments, cells were plated as a 6-point, 1:2 serial dilution with a top cell density of 10,000 cells per well in SCREENSTAR 96-well microplates. The cells were incubated and allowed to adhere overnight prior to fixation and staining.

Fixation and Staining

Cell fixation and staining was performed as previously described with the following modifications[1]. Cells were fixed by washing once with 200 µL of PBS followed by the addition of 200 µL 4% p-formaldehyde (PFA) and incubation for 10 minutes at room temperature (RT). The cells were then washed and permeabilized by washing 2 X 200 µL PBS followed by the addition of 200 µL PBS supplemented with 0.1% Triton X-100 and incubation for 5 minutes at RT. The cells were then washed with 200 µL PBS + 0.1% Triton X-100.

Primary antibody, 50 µL per well, mouse anti-alpha tubulin was added at a 1:750 dilution in DPBS supplemented with 30 mg/mL BSA and 0.1% Triton X-100 followed by incubation for 30 minutes at RT. Cells were washed with 200 µL DPBS, 0.1% Triton X-100. Secondary antibody, 50 µL per well, Alexa 488 labeled goat anti-mouse was added at a 1:500 dilution in DPBS supplemented with 30 mg/mL BSA, 0.1% Triton X-100 and allowed to incubate for 30 minutes at RT. The cells were washed 2 X 200 µL with PBS, 0.1% Triton X-100. Cells were stained simultaneously with Texas Red (TR) phalloidin and DAPI at 30 nM and 30 µM working concentrations, respectively, in DPBS, 0.1 % Triton-X 100 for 10 minutes at RT. The cells were washed with 200 µL PBS followed by the addition of 100 µL PBS and sealed with a TopSeal™-A clear adhesive film (No.6050195, Perkin Elmer, Waltham, MA, USA) prior to imaging.

Imaging

Cell were imaged using a Cytation 3 Cell Imaging Multi-Mode Reader (BioTek Instruments, Inc., Winooski, VT) configured with DAPI, GFP and Texas Red light cubes. The microscope uses a combination of LED light sources in conjunction with band pass filters to provide the appropriate wavelength of light. The DAPI light cube is configured with a 357/44 excitation filter and a 447/60 emission filter; the GFP light cube uses a 470/22 excitation filter and a 510/42 emission filter; while the Texas Red light cube uses a 585/29 excitation and 624/40 emission filters. Installed objectives included a combination of 10x, 20x, 40x and 60x magnifications.

Images were captured and stored in TIF format using the Cytation 3 and GEN5 data analysis software (BioTek Instruments, Inc., Winooski, VT).

Results

The use of fluorescent dyes for cell staining has led to significant advances in the field of microscopy in ways that are still being realized. The ability to visualize cellular process and morphological changes at a structural level has been one of the most insightful uses of such dyes. It is common practice to fix cells, essentially preserving their existing state for analysis at a later time. A primary benefit of combining cell fixation with the fluorescent staining is the ability to investigate a larger cell population than possible with a dynamic, live-cell culture given the sometimes lengthy image capture process. The higher density SCREENSTAR microplates used for this study were specifically designed for cell culture and to meet the demands of advanced microscopy in drug discovery. Plate densities of 96- and 384-well were used and analyzed to identify cells at various stages of cell growth and phases of mitosis.

As demonstrated in Figure 2, PtK2 cells can be fixed with 4% PFA and stained by use of a secondary antibody-Alexa 488 conjugate against a monoclonal antibody targeting the cytoskeletal protein tubulin, TR phalloidin targeting F-actin filaments and DAPI targeting nuclei. Once fixed and stained a large population of cells can be investigated to capture images representative of the various stages of the cell cycle. It is important to sample a large population of cells given the rapid nature of the progression of mitosis; on the order of 2-3 hours. Many stages of the cell cycle were identified including several high resolution images of the various phases of mitosis (Figure 2).

Clearly identifiable in all images are nuclear structures including nuclei containing the less dense, diffuse chromatin and/or condensed chromosomal structures stained with DAPI (blue). Figure 2a and 2c show several cells with large, diffuse nuclei while condensed chromatin (blue) and the beginning of spindle formation (bright green) is clearly visible in the central cell in Figure 2b. Figure 2d depicts a higher resolution example of chromosomes following condensation during the Prophase of mitosis following the dissolution of the nucleolus and nuclear membranes. Several images provide excellent examples of spindle formation and development of microtubule structures contributing to the alignment, separation and distribution of sister chromatid and migration of chromosomes to opposite sides of the cell during Metaphase through Anaphase (Figures 2d-h).

Telophase and Cytokinesis are the final events of cell division following separation of the chromosomes and the cytoplasm equally between the two daughter cells. A contractile ring forms creating a deep furrow between the two new cells. The nuclear membrane reforms and the nucleoli reappear. With the unwinding of chromosomes the nuclei now appear once again as diffusely stained structures (Figure 2i). The cells will now enter Interphase, a growth period, prior to subsequent division cycles. Alternatively, a cell may enter a prolonged period of growth and maintenance and may or may not undergo cell division at a later time or may enter a state of senescence (biological aging).

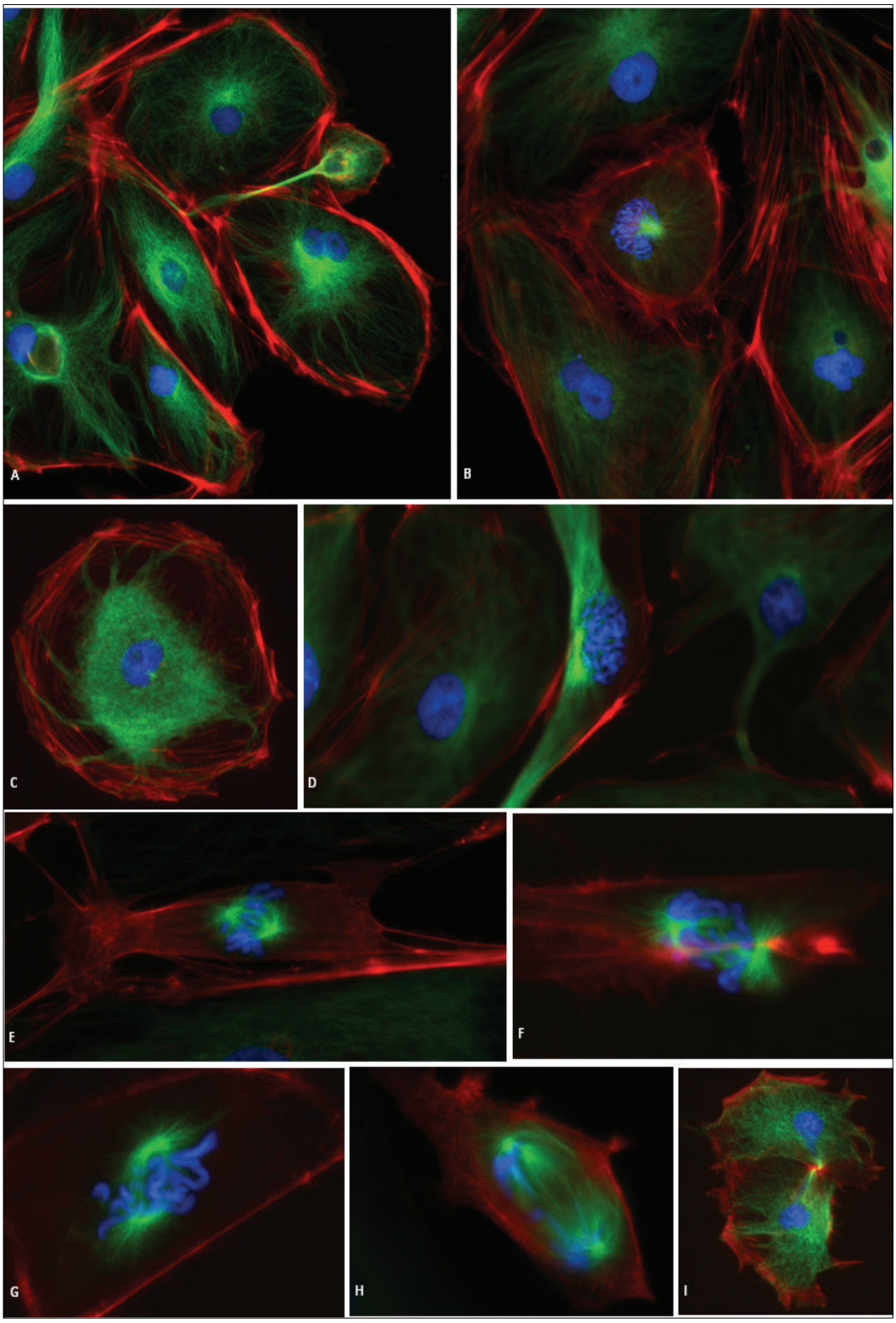

Figure 2. **PtK2 cells fixed and stained.** PtK2 cells fixed and stained with fluorescent dyes for tubulin (green), actin (red) and nuclei (blue). Various stages of mitosis are easily distinguished under magnifications ranging from 10x-60x. **A)** PtK2 cells imaged at 20x magnification, **B)** a nearly confluent cell monolayer depicting a cell in **Late Prophase** of mitosis as indicated by condensation of chromatin at the center of the image (20x), **C)** an individual PtK2 cell during **Interphase**, a growth period (20x), **D)** cell during **Late Prophase** highlighting spindle formation (green) and condensed chromatin (blue) at the center of image (40x) **E)** individual PtK2 cell during **Metaphase** with chromosomes lined up at metaphase plate (60x), **F)** visualization of PtK2 cell during **Anaphase** depicted by migration of sister chromatids to opposite poles in the cell (60x) **G) Late Anaphase** marked by the migration of chromosomes toward polar regions of the cell prior to cell division (60x) **H)** as cells transition between **Anaphase** and **Telophase** the chromosomes begin to unwind to chromatin and the contractile ring will begin to form prior to cytokinesis and **I) Cytokinesis** marks the end of mitosis marked by the formation of the nuclear membrane and nucleolus in each daughter cell (10x).

Conclusion

The ability to visualize cellular structures and processes with the aid of fluorescent dyes and proteins has allowed new insights into diverse areas of cell biology. The ability to fix and stain cells using immunostaining techniques targeting distinct structures allows a snapshot of biologically relevant events to be investigated in a large population of cells minimizing complex analysis due to dynamic processes. Examining a static population of PtK2 allowed numerous stages of the cell cycle and mitosis to be visualized and imaged using the Cytation™ 3 Cell Imaging Multi-Mode Reader.

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