Mitosis Through the Microscope: Advances in Seeing Inside Live Dividing Cells

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Supporting Online Material

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REVIEW

Mitosis Through the Microscope: Advances in Seeing Inside Live Dividing Cells

Conly L. Rieder* and Alexey Khodjakov*

The most visually spectacular events in the life of a cell occur when it divides. This is especially true in higher eukaryotes, where the size and geometry of cells allow the division process to be followed through a microscope with considerable clarity. In these organisms, the membrane surrounding the nucleus breaks down after the replicated DNA has condensed to form discrete chromosomes. Several new structures are then assembled to separate the chromosomes and partition the cytoplasm into two separate cells.

The German anatomist Walther Flemming was one of the first to describe the cell division process (*1*). In 1882 he coined the term "mitosis" to characterize the formation of paired threads (Greek = mitos) during division of the cell nucleus (Fig. 1). These threads, which formed from a substance Flemming called chromatin, came to be known as the "chromosomes." The definition of mitosis has since been expanded to include "cytokinesis," the process by which the cell cytoplasm is partitioned at the end of nuclear division.

Until the late 1940s, research on mitosis was primarily restricted to an examination of cells that had been preserved in a lifelike state by chemicals (i.e., fixed) and then colored with dyes to generate contrast between their different components (*2*). These descriptions revealed that the division process is fundamentally the same in all somatic cells. In animals, mitosis is mediated by a bipolar, spindle-shaped apparatus that appears to be assembled in the cytoplasm from two radial arrays of fibers, known as "asters." These asters form in association with two separating "centrosomes" that define the spindle poles (Fig. 1, E and F). Early studies also noted that each chromosome possesses two small organelles on its surface that are positioned back-to-back and on opposite sides of the chromosome. As the spindle forms, these "kinetochores" acquire fibers that attach them to one of the spindle poles, so that opposing sister kinetochores are attached to opposite poles (Fig. 1J). Collectively, the spindle and its associated centrosomes, kinetochores, and chromosomes are referred to as the mitotic apparatus.

Flemming noted that the chromosomes, which are scattered throughout the cytoplasm after nuclear envelope breakdown (Fig. 1D), are collected by the spindle and positioned on a plane halfway between the two poles (Fig. 1F). After this "metaphase" alignment is completed, the two chromatids forming each chromosome disjoin, and each moves toward its respective pole in a process termed "anaphase" (Fig. 1, G and H). Once the two groups of chromosomes reach their respective poles, they coalesce to form the new daughter nuclei, after which cytokinesis pinches the cytoplasm into two new cells (Fig. 1I).

Considering that $\sim 2.5 \times 10^8$ cells are dividing in the human body at any given time (*3*), even if few errors occur, many genetically abnormal cells will be produced during the lifetime of an organism. Some of these will lose their ability to regulate the cell cycle, which is one of the attributes of cancer cells (*4*). An important goal of cancer research is, therefore, to define the molecular mechanisms that form the spindle and generate the forces to move the chromosomes. A more recent focus is to understand how the cell regulates progression through the division process. Surprisingly, these problems are intimately linked because chromosome motion and progression through mitosis are both governed by the formation of kinetochore fibers.

As the description of a cellular event becomes more accurate, the corresponding molecular model(s) become more meaningful. Because mitosis involves many concurrent visible events, advances in understanding the mechanisms involved are historically linked to technological advances in light microscopy. What follows is a brief and roughly chronological review of these advances, with selected examples of how they have progressively refined our understanding of mitosis in higher animal cells (*5*).

Observing the Behavior of the Spindle and Chromosomes in Living Cells

The development of cell culture methods in the 1920s set the stage for studies on how living vertebrate cells divide. Early observations were hampered, however, because cellular components are not naturally contrasted when viewed with traditional bright-field optics (Fig. 2A). This situation changed radically in the mid 1950s

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with the introduction of phase contrast (Fig. 2D), for which Zernike won the Nobel Prize in 1955, as well as polarization (Fig. 2B) and differential interference contrast (DIC) (Fig. 2C) microscopy. By coupling a cinè camera to a microscope equipped with these contrast-generating optics, stunning time-lapse movies were produced that illustrated the complex and dynamic nature of the division process. The polarization microscopy studies of Inoué and colleagues (*6*) revealed that the fibers within the spindle are real structures (Fig. 2B; movies S1 and S2), and that the spindle shrinks or grows in response to various treatments. At the same time, phase contrast and DIC movies revealed that the duration of mitosis is temperature dependent, that it varies widely among organisms, and that it takes ~1 hour to complete in mammals at 37°C. These studies began to detail the complex motions exhibited by chromosomes and other components during the division process (movie S3). Together they defined the events that underlie mitosis and set guidelines for how these behaviors were to be modeled (*7*, *8*).

A High-Resolution View of the Mitotic Apparatus

The introduction of the electron microscope (EM) in the 1960s allowed investigators to characterize the structure of the mitotic apparatus in fixed cells, with a resolution near the molecular level. From the earliest EM studies it was evident that the spindle birefringence seen by polarization light microscopy (Fig. 2B) arises from a dense array of roughly parallel, straw-shaped structures termed "microtubules" (MTs) (Fig. 2E). Within the metaphase spindle, one end of each MT is found near a pole, whereas the other is either free in the spindle or associated with a kinetochore (*9*) (Fig. 2F). In animals, kinetochores appear as dense, compact fibrous plaques that are closely associated with the surface of the chromosomes; in metaphase cells, each kinetochore binds 20 to 30 MT ends (Fig. 2E).

By the start of the 1970s, it was evident that spindle MTs grow and shorten and that this behavior is critical for spindle function. This conclusion helped spur research into how MTs are generated, how they become organized, and how they change length. To this end, methods were developed to study the behavior and formation of MTs both in the test tube and by EM. This work revealed that MTs are assembled from a polymerization of (tubulin) protein subunits and that during mitosis this polymerization process is normally initiated by the centrosomes. Concurrent studies revealed that each MT is polarized and that its growth occurs primarily at one end termed the "plus" end. Within the spindle, MT plus ends are positioned away from the centrosome whereas the "minus" ends are concentrated near the polar regions (Fig. 2F). By the end of the 1970s, it was clear that (i) the spindle normally forms from an interaction between two highly dynamic and polarized arrays of MTs; (ii) it is the fast-growing MT plus ends that terminate on kinetochores; and (iii) in the region where the arrays overlap, neighboring MTs are of opposite polarity.

Because cells examined by EM must first be killed (fixed), this technique provides no information on the dynamic behavior of the molecules and assemblies involved in division. To reveal the mechanisms behind the subprocesses that define mitosis, investigators needed to develop techniques for selectively following one or more proteins, in the living cell, with high temporal and spatial resolution. The first breakthrough in this area occurred in the early 1980s at the Marine Biology Laboratory (Woods Hole, Massachusetts, USA).

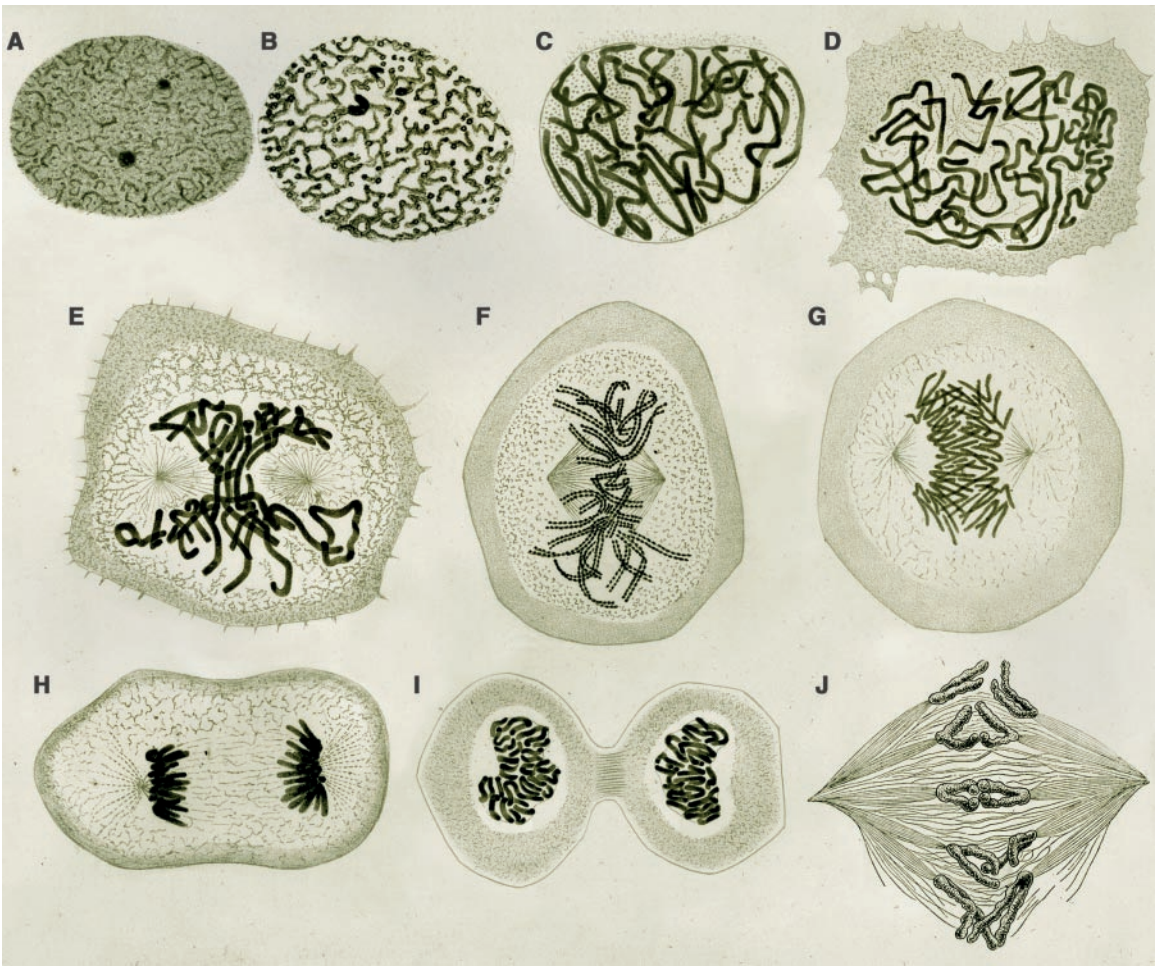

Fig. 1. Drawings of mitosis in newt cells found in Flemming's (*1*) book. (**A** to **J**) During prophase (A to C) the chromosomes form within the nucleus from a substance termed "chromatin" because of its affinity for dyes. After nuclear envelope breakdown (D), the chromosomes interact with the two separating "centrosomes" (E) to form a spindle-shaped structure (E and F). After the chromosomes attach to the spindle, they become positioned on its equator, halfway between the two poles (G). Once this "metaphase" stage is achieved, the two chromatids comprising each chromosome disjoin and move toward the opposing poles (G and H). During the final stages of mitosis, neighboring chromosomes within the two groups fuse to form the daughter nuclei (H and I), and the cell becomes constricted between them (I) by cytokinesis. (J) Drawing from Schrader's (*2*) book depicting conspicuous chromosomal (kinetochore) fibers during early anaphase in *Lilium*.

Seeing More in Live Dividing Cells: Video-Enhanced Light Microscopy

The development of video technology in the early 1980s revolutionized light microscopy. By mount-

ing a video camera on a microscope, time-lapse images of cells could be recorded onto magnetic storage media. Compared to film, video is less complex and expensive, with the bonus that the behavior of interest can be analyzed during an experiment. Moreover, video technology allows complexes to be visualized in living cells, even when their dimensions are more than an order of magnitude smaller than the resolution limit of the optics. This is because video cameras can detect contrast differences invisible to the human eye, and these differences can be electronically enhanced. Although all modes of light microscopy gain from video enhancement, DIC benefits the most and can detect individual MTs in live cells (*10*) (fig. S1).

One immediate impact of video-LM was that it formed the basis of a motility assay that led to the discovery of kinesin (*11*), the first cytoplasmic motor protein identified to move along MTs. Since then, a number of kinesin-like proteins have been discovered, most of which transport cargo toward the MT plus ends (i.e., away from the spindle poles). Some, like Eg5, are bifunctional and can cross-link and exert pushing forces between MTs of opposite polarity (*12*). By means of similar video-based methods, another motor, cytoplasmic dynein, was discovered that moves components toward the MT minus end (i.e., toward the spindle poles) (*13*). The discovery of dynein and the kinesins was important to understanding how mitosis works because the formation and integrity of the spindle were subsequently shown to be dependent on many of these motors (*14*). Also, motor proteins are located at the kinetochore, where they contribute forces for chromosome motion and regulate MT disassembly (*15*).

Video microscopy also provided a direct approach for studying how MT assembly and disassembly are controlled. In 1984 Mitchison and Kirschner (*16*), on the basis of EM data, hypothesized that MTs are "dynamically unstable" in that, at any given time, their plus end can be growing, shrinking, or in transition between these states. Shortly thereafter, this insightful prediction was proven in living cells by video microscopy (*17*, *18*). One remarkable outcome of this discovery is that it provides a simple explanation for how an interphase array of MTs can be rapidly replaced by a spindle-shaped structure during mitosis (*19*). One need only control the parameters involved in regulating the transitions between the shrinking and growing states to convert the long, relatively stable MTs of interphase cells into the shorter, highly dynamic, astral arrays from which the spindle is constructed.

The idea that kinetochores can attach to the forming spindle by capturing astral MTs was directly demonstrated by video microscopy (*17*). One consequence of this "search-and-capture" mechanism is that one kinetochore on a chromosome usually attaches to the spindle before its sister does. When this happens, the attaching kinetochore rapidly pulls the chromosome toward the pole to which it is attaching (movies S4 and S5). As a result, during spindle formation, a variable number of "mono-oriented" chromosomes that have only one attached kinetochore are seen near the poles (Figs. 1E, and 3, C and D). Subsequent videomicroscopy studies revealed that this kinetochore switches between two activity states: one that allows it to move poleward in response to a force, and another that allows it to be pushed (or pulled) away from its associated pole (*20*). This discovery, that kinetochores are "directionally unstable," currently forms a cornerstone for modeling how chromosomes become aligned on the spindle equator (*21*).

Video microscopy can be combined with other technologies to obtain information that is more than a description of events. For example, when high-energy pulses of laser light are focused through a microscope, individual organelles can be selectively destroyed in cultured cells (*22*). Using this microsurgery approach, we discovered that kinetochores do more during mitosis than attach the chromosomes to the spindle and generate forces. Until they attach to the spindle, they also produce a signal that delays anaphase, which forms the basis of a complex cell cycle checkpoint control (*23*, *24*). The pathway behind this checkpoint is so sensitive that just one unattached kinetochore prevents the chromatids from separating throughout the cell.

Finally, a combination of video microscopy and genetics can be used to define how specific proteins are involved in the division process. By comparing mitosis in live wild-type and mutant cells, it has been shown, e.g., that cytoplasmic dynein is required for positioning the spindle in yeast (*25*), as well as in moving chromosomes in *Drosophila* spermatocytes (*26*) (movies S6 and S7). Video microscopy is also useful for studying mutations in thicker specimens, e.g., the worm (*Caenorhabitis elegans*) embryo, especially mutations that affect spindle positioning and cytokinesis (*27*). These later studies have solidified the idea that cytokinesis entails several distinct stages that are reversible until the very end.

Video-enhanced microscopy is not without an Achilles' heel: Its superior sensitivity

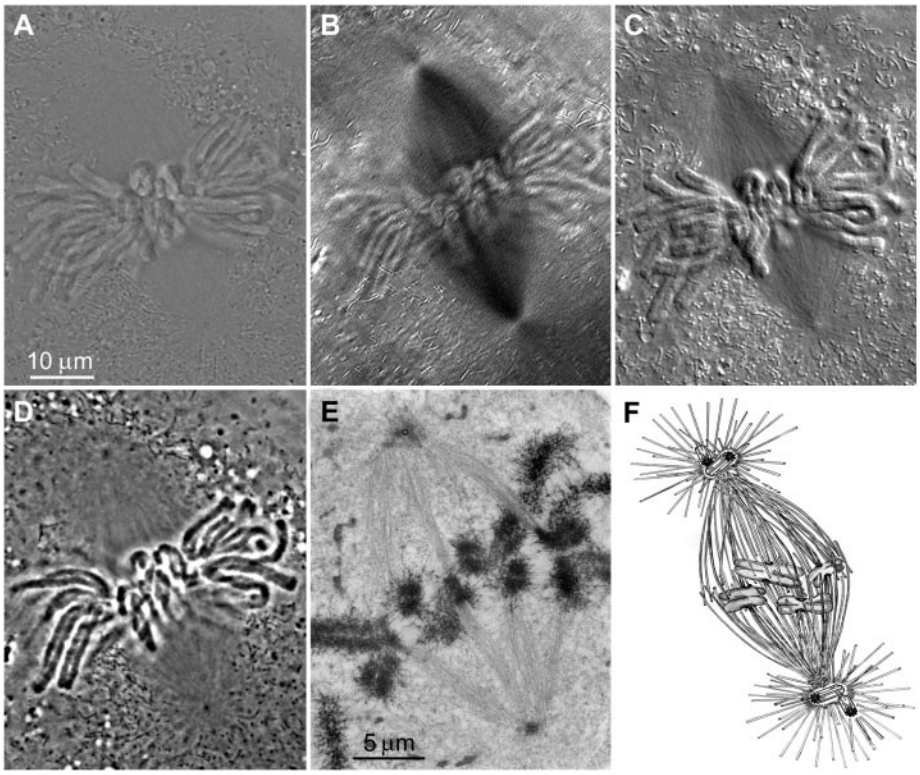

Fig. 2. Bright-field (**A**), polarized-light (**B**), differential interference contrast (**C**), and phase-contrast (**D**) micrographs of the same living newt cell in metaphase of mitosis. The introduction of contrast-producing optics in the 1950s allowed the division process to be studied in living cells. Electron microscopy in the 1960s revealed that the fibrous elements of the spindle consist primarily of MTs, some of which are organized into bundles that terminate on each kinetochore (**E**). In animals each spindle pole contains a centrosome, which contains a pair of centrioles and associated material. Kinetochores appear as diminutive, platelike structures that are positioned on opposite sides of each chromosome. A schematic representation of a metaphase spindle [adapted from (*48*)] is shown in (**F**).

makes live cells appear so crowded with minute moving components of unknown composition that it is difficult to follow what is going on. In many ways the technique provides too much information. To overcome this problem, a method for following only the structure(s) or molecule(s) of interest in an empty (unstained) background was needed (fig. S1). Work on this problem began in the 1940s, and its solution is now a reality.

Seeing Less Can Sometimes Reveal More: Fluorescence Microscopy

Dyes for generating contrast between cellular components were available early on (Fig. 1), but their specificity was not sufficient to localize individual proteins. This changed with the introduction of indirect immunofluorescence (IMF) light microscopy, a method that is based on the highly specific antigen-antibody interaction. In this procedure, cells are fixed and permeabilized to expose internal antigens, and then stained with an antibody to the protein of interest. Next, a second, fluorescently labeled, antibody is used to define the location of the first antibody, and hence the protein of interest (Fig. 3; fig. S1). This revolutionary approach, which allows researchers to locate any protein with a high spatial resolution, produced an explosion of information on the composition of the mitotic apparatus and how its chemistry changes during division. For example, since 1980 more than 20 proteins have been shown by IMF to permanently or transiently reside in the kinetochore region; some of these are structural, some are involved in attaching MTs to the kinetochore and in moving the chromosomes, some play a role in cytokinesis, and still others control progression through mitosis (*28*).

Fluorophores are inherently unstable, and when excited they emit photons and return to their original state; or they "break" and permanently lose their ability to fluoresce. This feature makes it possible to kill (i.e., photobleach) all of the fluorophores within a given area with a focused beam of light. In turn, this provides a method for determining how quickly a protein associated with an organelle turns over. The logic is that if proteins containing bleached fluorophores leave the structure, they will be replaced from the pool of nonbleached fluorescent molecules residing in cell. The resultant "fluorescence recovery after photobleaching" (FRAP) can be observed and its parameters measured (movie S8).

For a FRAP study, the cell must contain a functionally active fluorescently tagged protein. This was originally achieved by isolating and labeling tubulin and then microinjecting it back into a cell. Such studies led to the discovery that spindle MTs turn over very rapidly, even during metaphase, but that kinetochore fiber MTs turn over much more slowly (*29*, *30*). FRAP, and its modification photoactivation, also helped to establish that anaphase in vertebrates is driven primarily by an activity associated with the kinetochore (*31*, *32*). FRAP has also been used to study the behavior of kinetochore proteins, especially those involved in controlling the metaphase-anaphase transition (*33*, *34*).

Recently, another method to reveal movements of cellular proteins has been developed that involves loading cells with very low concentrations of fluorescent molecules (*35*). Under this condition, those few fluorophores that become incorporated into continuous structures, such as MTs and actin, form visible patches termed "speckles." Because speckles create internal reference marks within the structure, speckled microscopy effectively combines the benefits of fluorescence microscopy (FM) with fluorescence-marking techniques like FRAP. An added value is that, in speckled microscopy, movements of labeled molecules can be followed for long periods, whereas during FRAP the photobleached area gradually disappears as subunits exchange. Also, speckled microscopy eliminates the potentially harmful effects produced by the high-intensity light used for photobleaching. This technique has been used to show that the poleward movement of tubulin subunits along kinetochore fiber MTs (termed flux) is a characteristic feature of spindles formed in *Xenopus* oocyte extracts (*12*) (movie S9) and in *Drosophila* embryos (*36*). Importantly, the precision of speckled imaging enables the movement of two different components to be compared in the same sample. Using this feature, it has been found that the kinesin-like protein Eg5 remains stationary in bipolar spindles, whereas MT subunits continuously flux poleward (*12*). This suggests that Eg5 is bound to a spindle "matrix" material through which the spindle MTs pass.

Initially, vital fluorescence microscopy was applicable only to cells that could be microinjected. Also, because the method requires chemically binding a fluorophore to a protein, it was restricted to proteins like tubulin that can be isolated in bulk. The strictures have now been eliminated with the introduction of green fluorescent protein (GFP) (*37*). GFP can be used to "paint" almost any molecule simply by expressing DNA constructs, which are formed by fusing the gene for GFP with the gene for the protein of interest (Fig. 4). This technological breakthrough has led to the production of hundreds of GFP fusion proteins, which can be temporarily or permanently expressed not only in most cells, but also in many whole organisms (mice, monkeys, etc.).

The advantages of GFP technology are many. It can be used to study proteins present in organelles that cannot be easily isolated, such as centrosomes and kinetochores, and in which only a few copies of the protein are

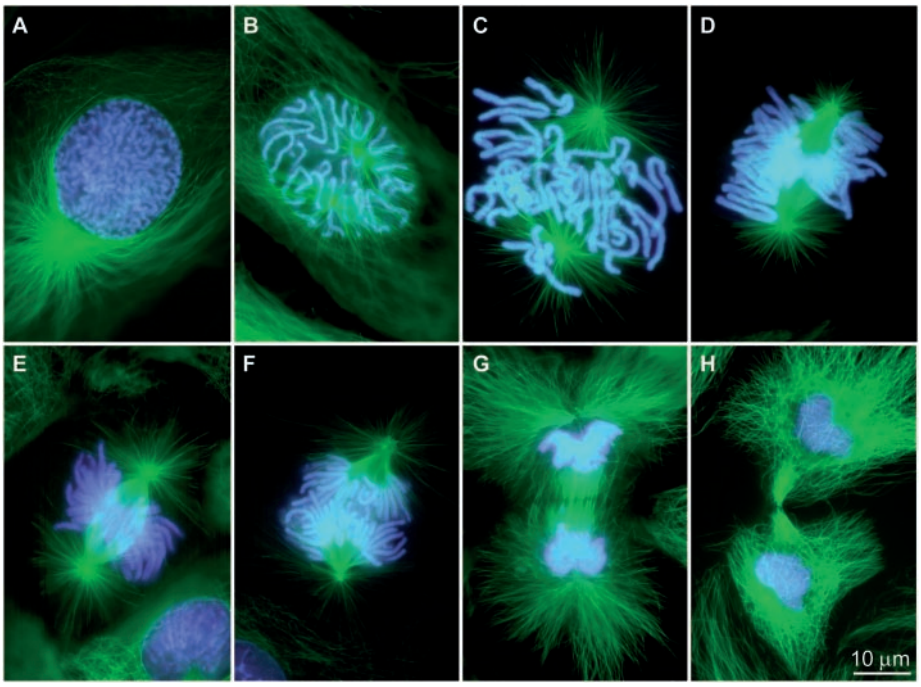

Fig. 3. (**A** to **H**) Fluorescence micrographs of mitosis in fixed newt lung cells stained with antibodies to reveal the microtubules (green), and with a dye (Hoechst 33342) to reveal the chromosomes (blue). The spindle forms as the separating astral MT arrays, associated with each centrosome (A to C), interact with the chromosomes. Once the chromosomes are segregated into daughter nuclei (F and G), new MT-based structures known as stem-bodies form between the new nuclei (G). These play a role in cytokinesis (H). Compare with Fig. 1.

present. For example, shortly after the introduction of GFP, several stable cells lines were generated in which the normally invisible centrosomes could be clearly seen because they were specifically labeled with γ-tubulin–GFP (*38*), centrin 1–GFP (*39*), or centrin 2–GFP (*40*). The ability to see and follow centrosomes in living cells rapidly led to several novel discoveries. It was found, e.g., that centrosomes exhibit previously unseen extensive motions within the cell, and that the mother and daughter centrioles can separate and move independently through the cytoplasm, only to rejoin and separate again (*39*).

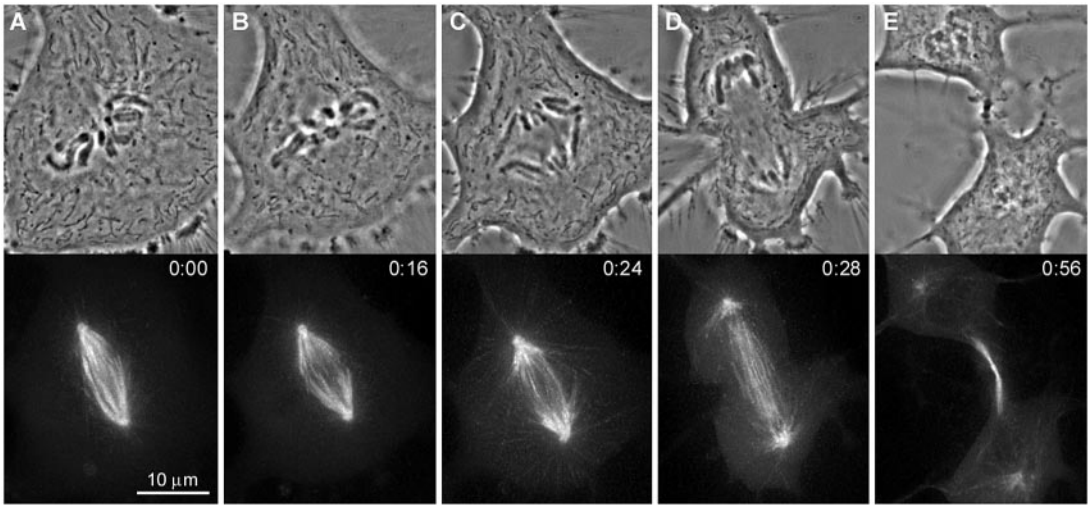

Fig. 4. (**A** to **E**) Multimode microscopy of a dividing rat-kangaroo cell expressing GFP–α-tubulin. Near-simultaneous phase-contrast (upper) and fluorescence (lower) imaging were used to follow the distribution of microtubules during anaphase and cytokinesis. Each fluorescence image represents a maximum-intensity projection of 15 optical sections collected at 0.5-μm steps, and then deblurred to remove out-of-focus fluorescence. During anaphase, the chromosomes move toward the poles (B and C), while the poles themselves move farther apart (B to D). During this elongation, stem-body microtubules are formed between the chromosome groups (D) that coalesce during cytokinesis to form a single bright microtubule bundle (the midbody) (E) Time (in hours:min) is indicated in the upper right corner of each frame.

Because GPF technology is based on FM, it can be used to quantify the number of molecules associated with an organelle. For example, in work with γ-tubulin–GFP, the amount of γ-tubulin in the centrosome was found to remain relatively constant until the onset of mitosis, at which time it rapidly increases fivefold (*38*). This maturation of the centrosome correlates with its enhanced ability to nucleate MTs. GFP imaging can also be combined with FRAP (see above) to show that centrosome-associated γ-tubulin is in constant exchange with a cytoplasmic pool (*38*). In another GFP-FRAP study, it was shown that the important checkpoint control protein Cdc20, which is concentrated in centrosomes and kinetochores, turns over rapidly at both of these organelles in a MT-independent manner (*34*). A similar approach has demonstrated that topoisomerase II is a dynamic component of the centromere-kinetochore complex and not simply an immobile structural protein of the chromosomal scaffold (*41*).

Budding yeast cells are a powerful system for defining and studying the molecules behind cell division. However, because the chromosomes in this organism do not condense during mitosis, it was not possible to compare their behavior with that of chromosomes in other cells. Yet, after the introduction of GFP, an elegant way to follow chromosome movement in yeast was developed. By incorporating Lac operator binding sites into defined regions of chromosomes and then expressing a GFP-labeled Lac repressor, the position and behavior of chromosomes could be followed in live cells (*42*). These studies revealed that although the chromosomes in budding yeast do not form a conventional metaphase plate, as in higher organisms, they do move toward the poles during anaphase.

An exciting new facet of FM imaging has been made possible by the development of different GFP isoforms (e.g., cyan and yellow GFP), as well as a true red fluorescent protein, that can be expressed in the same living cell. Not only does this allow for the simultaneous localization of more than one protein during mitosis (*43*), it also provides a method for studying protein-protein interactions by fluorescence resonance energy transfer (FRET). Most recently, FRET was used to prove the hypothesis, proposed several years before, that factors associated with chromosomes in *Xenopus* oocyte extracts generate a gradient of Ran-GTP (guanosine 5′-triphosphate) that biases MT polymerization so that it occurs only near chromatin (*44*).

Looking into the Future

Photoactivatable GFP isoforms are now available that exhibit very little fluorescence until activated with short pulses of violet light (*45*). No doubt these will become important tools to study how the spindle works. Furthermore, advances in FM will continue to parallel those in GFP technology. Over the past few years, tremendous improvements have been made in processing FM data sets. These include the development of special techniques to restore the true distribution of light sources within the specimen (*46*). Importantly, these mathematical methods can compensate (to an extent) for the information lost in the undersampled data sets acquired during live cell imaging. This means that such methods can improve the resolution of the original fluorescence image and even overcome resolution restrictions imposed by the diffraction limit. Indeed, even now it is possible in some instances to resolve details with twofold greater resolution than is possible with conventional FM (*47*). In the future, image restoration will no doubt become a standard procedure, and the limits of FM will be stretched even further.

Finally, hardware developments, particularly in the area of camera sensitivity, are making it possible to decrease the intensity of the excitation light used for fluorescence imaging. The ultimate goal is to make FM a truly noninvasive tool capable of following live cells through multiple generations. Even today, thousands of fluorescent images can be recorded of dividing cells without photodamage (movie S10). With this volume of data, it is possible to analyze complex spatiotemporal processes in live cells, including spindle formation and nuclear envelope re-formation (*43*).

In summary, without killing the cell, we can now see and study the complex molecular machinery responsible for the formation of new cells. This ability, combined with the power of genetics and novel methods for eliminating individual proteins (i.e., RNA interference) and organelles (i.e., laser microsurgery), will likely produce answers to many of the questions proposed by Flemming and his colleagues almost 125 years ago.

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REVIEW

Visualizing Signals Moving in Cells

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Cells display a highly complex spatiotemporal organization, required to exert a wide variety of different functions, for example, detection, processing, and propagation of nerve impulses by neurons; contraction and relaxation by muscle cells; movement by leukocytes; and adsorption and secretion of nutrients and metabolites by epithelial cells lining the gut. Successful execution of these complex processes requires highly dynamic information transfer between different regions and compartments within cells. Through the development of fluorescent sensors for intracellular signaling molecules coupled with improved microscopic imaging techniques, it has now become possible to investigate signal propagation in cells with high spatial and temporal resolution.

Advances in molecular genetics and biochemistry have led to the identification of many new signaling molecules and interactions between them, as documented in the elaborate signaling maps that are currently under development (*1*). These maps consist of boxes indicating molecules connected by arrows that delineate the possible flow of information (signals) between them to result in specific cellular actions such as gene expression, movement, cell division, etc. These maps, however, do not take into account the spatial and structural aspects of these signaling pathways, which in real cells are very important. Understanding these pathways and mechanisms of signal propagation in cells will require the measurement of many signaling reactions, with high spatial and temporal resolution. Most cells are small and the concentration of signaling molecules is generally low; therefore, these measurements require both considerable magnification and sensitivity. The most widely used detection methods are, therefore, based on fluorescent microscopic imaging techniques.

Microscopy and detection techniques have improved considerably in sensitivity over the last decades, and it is now possible to take fluorescence images in the μs to ms range. Through the use of confocal and deconvolution microscopy, it has also become possible to measure several fluorescence signals simultaneously in the same cell with high three-dimensional spatial and temporal resolution (*2*, *3*). Using total internal reflection microscopy it is now possible to image single fluorescent molecules in living cells (*4*). Data analysis requires the development of advanced visualization and analytical techniques. Furthermore, because many of the signaling reactions taking place in cells involve complex positive and negative nonlinear feedback as well as transport, their dynamics can give rise to a wide variety of nonintuitive behaviors. To interpret and understand these data it is becoming increasingly necessary to model and analyze them using qualitative and quantitative mathematical models (*5*–*7*).

Widely Different Mechanisms for Signal Movements

Cells respond to signals from the outside world. In many cases, these signals are detected by plasma membrane–bound receptors. Activation of a cell surface receptor typically triggers several intracellular signaling pathways, resulting in an information transfer between the membrane and other cellular locations and compartments, which involves the physical movement of signaling molecules through the cell. Examples are small molecule second messengers that may spread by diffusion or can actively propagate with the aid of the local regeneration of the messenger, as in the case of propagation of calcium waves within cells. However, much larger molecules such as proteins or even protein complexes can act as signals by moving among different cellular locations. Examples of

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