Chapter 8

Imaging of Mitotic Cell Division and Apoptotic Intra-Nuclear Processes in Multicolor

Kenji Sugimoto and Shigenobu Tone

Abstract

To follow the cell division cycle in the living state, certain biological activity or morphological changes must be monitored keeping the cells intact. Mitotic events from prophase to telophase are well defined by morphology or movement of chromatin, nuclear envelope, centrosomes, and/or spindles. To paint or simultaneously visualize these mitotic subcellular structures, we have been using ECFP-histone H3 for chromatin and chromosomes, EGFP-Aurora-A for centrosomes and kinetochore spindles and DsRed-fused truncated peptide of importin alpha for the outer surface of nuclear envelope as living cell markers. Time-lapse images from prophase through to early G1 phase can be obtained by constructing a triple-fluorescent cell line (Sugimoto et al., *Cell Struct. Funct.* **27**, 457–467, 2002). Here, we describe the multicolor imaging of mitosis of a human breast cancer cell line, MDA435, and a further application to characterizing the apoptotic chromatin condensation process in isolated nuclei by simultaneously visualizing kinetochores with EGFP and chromatin with a fluorescent dye, SYTO 59.

Key words: Apoptosis, mitosis, kinetochore, time-lapse imaging, chromatin condensation.

1. Introduction

The mitotic cycle of mammalian cells consists of interphase and five mitotic stages, as illustrated in **Fig. 8.1a** (1). Centrosomes duplicate in G1/S phase and "prekinetochores" doubles in G2 phase (2). Chromatin condensation occurs and mitotic chromosomes are observed first in prophase, while the duplicated centrosomes move to the opposite poles and maturate into asters. After nuclear envelope breakdown, the asters capture the kinetochores of mitotic chromosomes in prometaphase and then form a typical mitotic spindle (3). Chromosomes are congressed to

D.B. Papkovsky (ed.), *Live Cell Imaging*, Methods in Molecular Biology 591,
DOI 10.1007/978-1-60761-404-3_8,

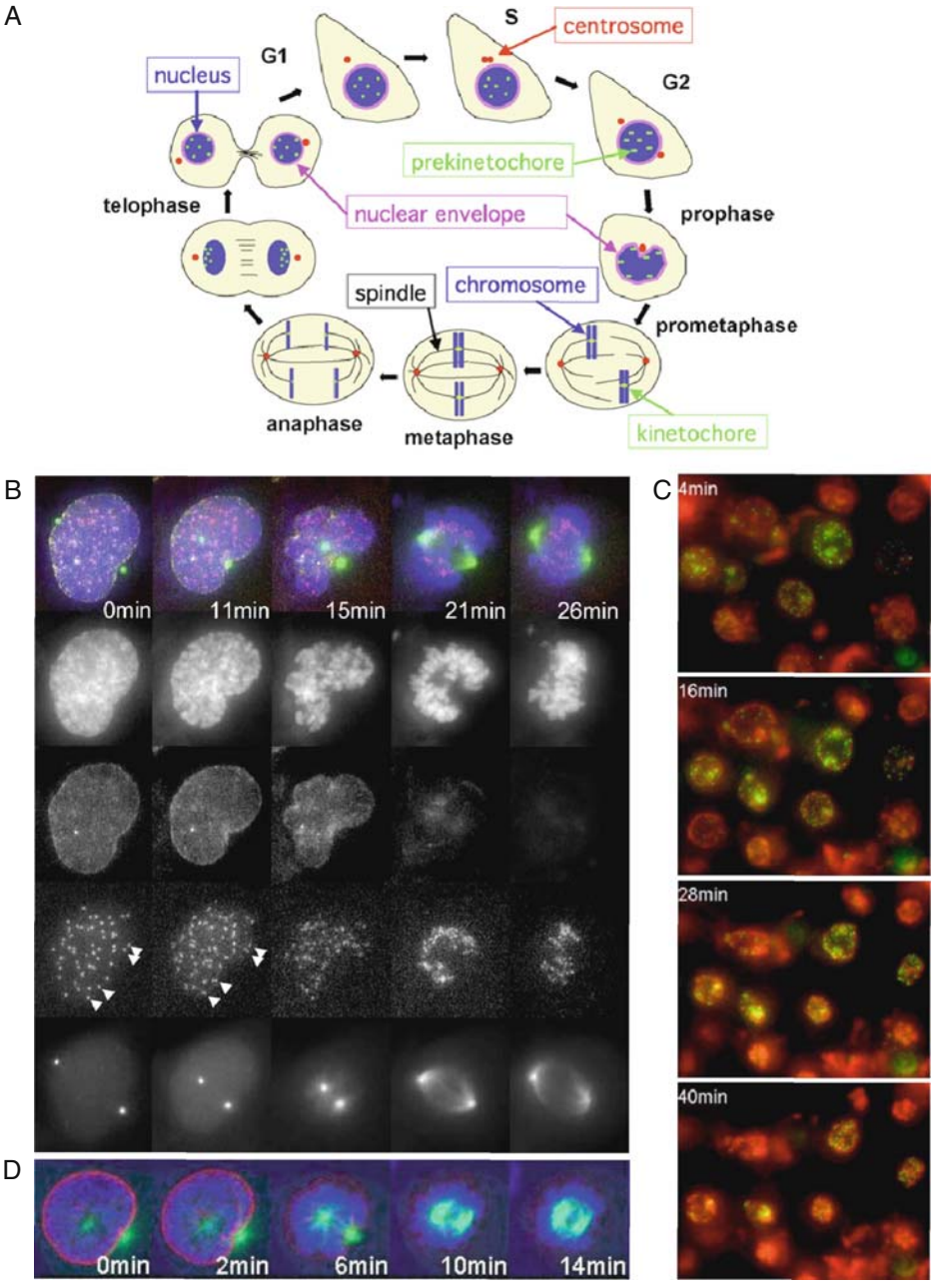

Fig. 8.1. Imaging of mitotic cell division and apoptotic intra-nuclear processes. (**a**) A schematic illustration of mitotic cycle of mammalian cells. (**b**) Live cell image of prophase-to-metaphase of a quadruple-fluorescent MDA435 cell line. ECFP-histone H3, EGFP-Aurora-A, mKO-CENP-A, and DsRed-importin-alpha were stably expressed to visualize chromatin (*blue*), centrosomes/asters/kinetochore spindles (*green*), kinetochores (*red*), and nuclear envelope (*yellow*). Time-lapse images were captured by a conventional wide-field microscope system (1). The separate images of ECFP, EGFP, mKO, and DsRed were also shown below. (**c**) Time-lapse imaging of cell-free apoptosis. MDA-AF8 nuclei were incubated with S/M extract and imaged by fluorescent microscopy (10). DNA was visualized using SYTO 59 (*red*) and kinetochores with EGFP-CENP-A (*green*). Representative images are shown. (**d**) Time-lapse images of triple-fluorescent MDA435 cell line captured by a laser confocal system (*see* **Fig. 8.3**). Fluorescent images are ECFP-histone H3 (*blue*), EGFP-alpha-tubulin (*green*), and DsRed-importin-alpha (r*ed*) (1). (Reproduced from (1) and (10) with permission from Elsevier.)

and aligned at the spindle equator in metaphase. Sister chromatid separation is a visual sign for anaphase onset and the following process is straightforward. The daughter chromosomes are segregated to the poles in anaphase and the nuclear envelope reforms around the sister chromatids in telophase. These events after anaphase onset occur within 10 min or so, followed by spindle deformation and chromosome decondensation in early G1 phase.

To accurately identify each mitotic stage, it would be very convenient if the mitotic apparatus has been differentially visualized or painted by a set of multiple fluorescent proteins with various fluorescent wavelengths from cyan to far-red (4). Recently, we constructed three quadruple-fluorescent MDA435 cell lines in which chromatin, kinetochores, nuclear envelope, and either the inner centromere, or microtubules, or centrosomes/spindles are simultaneously visualized with ECFP, EFGP, mKO, and DsRed (1). Each mitotic stage of the individual cells could be identified simply by capturing live cell images under a microscope without any requirement of cell-fixing or staining steps (**Fig. 8.1b**).

Dynamic changes in the compaction of nuclear chromatin are one of the characteristic phenomena of apoptotic execution (5). During apoptosis, however, the level of chromatin condensation is even greater than that observed in mitosis. The chromatin drastically undergoes a phase change from a heterogeneous, genetically active network to an inert, highly condensed form that is fragmented and packaged into "apoptotic bodies." Cell-free systems with isolated nuclei have been developed to study the molecular mechanisms of apoptotic execution (6–9). The protocol we described here was originally developed in Dr. Earnshaw's laboratory in Edinburgh (6, 7). We further applied the live cell imaging to a cell-free system to characterize the biochemical mechanism underlying apoptotic chromatin condensation (10, **Fig. 8.1c**). Three distinct stages of apoptotic nuclear condensation were revealed by the time-lapse images of isolated nuclei prepared from a kinetochore-fluorescent cell line, MDA-AF8 (11).

2. Materials

2.1. Cell Culture and Electroporation

1. Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical, Tokyo, Japan).
2. Trypsin (0.25%) (Gibco/BRL, Bethessa, MD).

3. Plasmid DNAs: pEGFP-AF8 for a kientochore marker, prepared with spin column such as a "Quantum Prep Plasmid Miniprep Kit" (Bio-Rad, Hercules, CA). Use pECFP-histone H3 for chromatin/chromosome, pEGFP-Aurora-A for centrosomes/kinetochore spindle, and pDsRed-importin-alpha for nuclear envelope (12). pTK-Hyg and pPur (Clontech, Palo Alto, CA) are for hygromycin B and puromycin, respectively.
4. K-PBS: 30 mM NaCl, 120 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 5 mM $MgCl_2$
5. Electroporation cuvette (4 mm width) (Bio-Rad).
6. Electroparation apparatus: Gene Pulser and capacitance extender (Bio-Rad) (*see* **Note 1**).

2.2. Screening of Fluorescent Cells

1. Geneticine (G418 sulfate) (Nakalai Tesque, Kyoto, Japan): dissolved with 0.5 M HEPES buffer, pH 7.2, to final concentration of 0.8–1.6 mg/mL and filtrated through 0.22 mm filter.
2. Hygromycin B (50 mg/mL, Roche Diagnostics GmbH, Nonnenwald, Penzberg, Germany).
3. Puromycin (Sigma): dissolved in PBS(–) to make a stock solution (0.5 mg/mL).
4. Cloning ring (7 mm in diameter) (Iwaki Glass Co. Ltd., Chiba, Japan).
5. Coverslip (12×12 mm) (Matsunami Glass Ind. Ltd., Osaka, Japan).
6. 4% paraformaldehyde (Nakalai Tesque): dissolved in PBS(–) at 60°C and neutralized to pH 7.4–7.6 with 2 N NaOH.
7. DAPI-containing glycerol: dissolve 0.1 g DABCO (Sigma) with 1 mL 0.2 M Tris–HCl (pH 7.5) and mix well with 9 mL glycerol (nonfluorescent grade, Nakalai Tesque). Add DAPI stock solution (100 μg/mL) to a final concentration 1 μg/mL.

2.3. Preparation of Nuclei

1. 1× nuclei solution: 10 mM KCl, 10 mM PIPES buffer (pH 7.4), 1.5 mM $MgCl_2$, 1 mM DTT, 10 μM cytochalasin B, 1× CLAP, 100 μM PMSF.
2. 1000× CLAP: 5 mg each of chymotrypsin, leupeptin, aprotinin, and pepstatin A were dissolved in 5 mL of DMSO.
3. Syringe attached by 21G needle (needle tips were bent by 10–20°).
4. 30 % sucrose solution: 3 g sucrose dissolved with 10 mL 1× nuclei solution.

5. Buffer A: 250 mM sucrose, 80 mM KCl, 20 mM NaCl, 5 mM EGTA, 15 mM PIPES (pH 7.4), 1 mM DTT, 0.5 mM spermidin, 0.2 mM spermin, 100 μM PMSF, 1× CLAP, 50% Glycerol.
6. Incomplete KPM: 50 mM KCl, 50 mM PIPES (pH 7.0), 10 mM EGTA, 1.92 mM $MgCl_2$.
7. Complete KPM: 1 mM DTT, 20 μM cytochalasin B, 1× CLAP, 100 μM PMSF were added to incomplete KPM.
8. Aphidicolin (Sigma).
9. Nocodazole (Sigma).
10. 10× MDB buffer: 0.5 *M* NaCl, 20 μM $MgCl_2$, 50 mM EGTA, 100 mM PIPES, 100 mM DTT.
11. SYTO 59 (Molecular Probes, Invitrogen Corp., Carlsbad, CA).
12. Human stable cell line MDA-AF8 expressing EGFP-CENP-A (11).
13. ATP plus a regeneration system: mix equal amount of ATP/creatine just before use.
14. Phosphate solution (40 mM ATP, 200 mM phosphocreatine, 10 mM PIPES, pH 7.0) and creatine kinase solution (1 mg/mL creatine kinase, 10 mM PIPES, pH 7.0, 1 mM DTT, 50 mM NaCl).
15. 35-mm glass base dish (Iwaki Glass Co. Ltd.).

2.4. Time-Lapse Imaging System

1. Inverted fluorescent microscope: Eclipse TE300 or TE2000-U (Nikon, Tokyo, Japan).
2. Objective lens: PlanApo VC 60×, NA1.40 (Nikon) (*see* **Note 2**).
3. Stage-top incubator: INU-NI-F1 (Tokai Hit, Fujinomiya, Sizuoka, Japan).
4. CCD camera: ORCA-ER (Hamamatsu Photonics, Hamamatsu, Sizuoka, Japan).
5. Filter wheels and *z*-axis motor: BioPoint MAC5000 (Ludl Electronic Products, Hawthorne, NY).
6. Excitation and emission filters: 436/20 and 472/30 nm for ECFP, 484/15 and 520/35 nm for EGFP, 532/10 and 556/20 nm for mKO, and 580/13 and 630/60 nm for DsRed (Chroma Technology, Rockingham, VT; Semrock, Rochester, NY) (*see* **Note 3**).
7. Dichroic: 86006bs for CFP/YFP/DsRed (Chroma Technology) (*see* **Note 4**).
8. Image capturing and analyzing software: LuminaVision for MacOSX (Mitani Corporation, Fukui, Japan).

3. Methods

3.1. Cell Culture and Electroporation

1. Culture human MDA435 cells in DMEM with 10% fetal bovine serum (FBS, Biowest, Nuaille, France) in 90-mm dishes (usually four dishes for each preparation).
2. Rinse the cells with 4 mL PBS(–).
3. Trypsinize the cells with 1 mL 0.25% trypsin.
4. Suspend the cells with 4 mL of DMEM with 10% FBS.
5. Transfer into 15 mL tube.
6. Count the cell numbers with a hematometer.
7. Spin the cells 1000 rpm 5 min at room temperature (RT) with table-top centrifuge.
8. Resuspend the pellet with 12 mL PBS(–).
9. Recentrifuge 1000 rpm 5 min.
10. Repeat the resuspension and centrifugation step.
11. Resuspend the cells with ice-cold K-PBS to 1.2×10^7 cells/mL.
12. Transfer 0.45 mL aliquots of cell suspension into each eppendorf tube.
13. Add 16 μg of plasmid DNA and keep on ice for 10 min. For selection with drugs other than G418, add 7 μg of the appropriate selection plasmid as well.
14. Transfer into an ice-cold 0.4 cm electroporation cuvette.
15. Pulse with 960 μF at 0.22 kV with Gene Pulser and capacitance extender.
16. Keep the cuvette on ice for 10 min.
17. Add 0.5 mL of DMEM
18. Put a lid on the cuvette and turn upside down for mixing.
19. Keep at RT for 10 min.
20. Dilute the cell suspension into 4 mL of DMEM with 10%FBS in 50-mm dish.
21. Add 1–2 mL aliquots of the dilution into 8 mL of DMEM with 10%FBS in 90-mm dish.
22. Drop 0.5 mL aliquot of the cell suspension onto 18× 18 mm coverslip in 35-mm dish.
23. Incubate 35- and 90-mm dishes in CO_2 incubator for 1–2 days.
24. Add G418 stock solution to 0.8–1.2 mg/mL and culture for 2–3 weeks to obtain single colonies. For the

construction of multi-color cell lines, stable transformants are sequentially selected by hygromycin B (200 μg/mL) and puromycin (20–50 μg/mL).

3.2. Screening of Fluorescent Transfomants

1. Rinse the cells with 4 mL PBS(−).
2. Put cloning rings (7 mm in diameter) on each single colony.
3. Trypsinize the cells with a few drops of 0.25% trypsin.
4. Suspend the cells with a portion of 2 mL of DMEM with 10% FBS in each well of 12-well plates.
5. Grow the cells on coverslip (12 × 12 mm) for several days.
6. Transfer each coverslip into another 12-well plate.
7. Fix the cells with ice-cold 4% paraformaldehyde for 20 min.
8. Rinse with PBS(−) for 5 min twice.
9. Pick up and put each coverslip upside down on 5 μL drop of DAPI-containing glycerol on slide glasses.
10. Observe the cells under a fluorescent microscope.

3.3. Preparation of Isolated Nuclei

1. Culture human cells in DMEM with 10% FBS in three T75 cultures for each preparation.
2. Spin the cells at 1000 rpm for 5 min at RT with table-top centrifuge.
3. Resuspend the pellet in 30 mL PBS(−) into a 50-mL tube.
4. Recentrifuge at 1000 rpm for 5 min at 4°C.
5. Resuspend in 2 mL 1× nuclei solution.
6. Transfer cell suspension (1 mL) into two eppendorf tubes
7. Recentrifuge at 3000 rpm for 5 min at 4°C.
8. Resuspend in 1.5 mL each with 1× nuclei solution.
9. On ice for 20 min (with occasional shaking).
10. Up and down 30 times with shringe attached by 21G needle (*see* **Notes 1**).
11. Layer 750 μL of suspension over 500 μL 30% sucrose solution (3 g with 10 mL 1× nuclei solution).
12. Take care not to disturb sucrose solution.
13. Centrifuge with swing rotor at 3000 rpm 10 min at 4°C.
14. Discard supernatant.
15. Resuspend the pellet in 1 mL 1× nuclei solution.
16. 5000 rpm 5 min at 4°C.
17. Discard supernatant.

18. Resuspend the pellet in 120 μL buffer A.
19. Aliquot 20 μL × 5 tubes; store at –20°C.

3.4. Preparation of S/M Extracts

1. Culture chicken DU249 cells (12×T150 flasks) and presynchronize in S phase with aphidicolin at 2 μg/mL for 12 h.
2. Release from the block for 6 h and synchronize in mitosis with nocodazole at 100 ng/mL for 3 h.
3. Harvest mitotic cells with 2000 rpm for 5 min at 4°C to make S/M extracts (extracts for apoptosis induction) from floating cells obtained by selective detachment after the nocodazole treatment.
4. Wash cells with 40 mL incomplete KPM.
5. Centrifuge and resuspend with 40 mL of complete KPM.
6. Centrifuge and resuspend with 1 mL of complete KPM.
7. Transfer to a grinder (KONTES 20 or 21).
8. Centrifuge and aspirate supernatant.
9. Freeze in liquid nitrogen.
10. Store at –80°C.
11. Thaw in cold water and grind with pestle on ice just during thawing (*see* **Note 6**).
12. Freeze again in liquid nitrogen, thaw in cold water, and grind again.
13. Transfer to ultracentrifuge tubes (7×20 mm polycarbonate tube).
14. Ultracentrifuge at 55,000 rpm for 1 h at 4°C using Beckman TL-100, TLA100 rotor.
15. Recover clear extract.
16. Take 1 μL to measure protein concentration.
17. Make 20 μL aliquots and freeze in liquid nitrogen.
18. Store at –80°C.

3.5. How to Stain the Nucleus by Adding a Fluorescent Dye

1. Suspend MDA-AF8 nuclei with 200 μL 1× MDB buffer.
2. Centrifuge at 5000 rpm 5 min at 4°C.
3. Discard supernatant.
4. Stain nuclei with SYTO 59 for 20 min at 0.5 μM at RT.
5. Centrifuge at 5000 rpm 5 min at 4°C.
6. Wash twice with 1× MDB buffer.
7. Centrifuge at 5000 rpm 5 min at 4°C.
8. Discard supernatant.

3.6. Induction of Apoptsis

1. Suspend MDA-AF8 nuclei stained with SYTO 59 into a 10 μL reaction (S/M extracts) supplemented with 1 μL ATP plus a regeneration system.

3.7. Time-lapse Imaging

There are two different systems to obtain the time-lapse images, wide-field and confocal microscopies. Here, we mainly describe the basic components for the former system, such as a high sensitive cooled CCD camera, excitation and emission filter wheels, *z*-axis motor, and a stage-top incubator (**Fig. 8.2**). For the

Fig. 8.2. A typical conventional wide-field microscope system. (**a**) The basic components contain a stage-top incubator INU-NI-F1 (Tokai Hit), a high sensitive cooled CCD camera ORCA-ER (Hamamatsu Photonics), a BioPoint MAC5000 excitation and emission filter wheels, and *z*-axis motor (Ludl Electric Products). (**b**) A 100 W halogen lamp is used a light source.

illumination, we have used a halogen lamp (1 and 13, *see* **Fig. 8.2b**), instead of Hg or Xe lamp. To obtain the confocal images, this system can be easily system-upped by applying combined laser beams (440, 488, and 561 nm) and setting a confocal scan unit (CSU10, Yokogawa Electric Corp., Tokyo, Japan) between the emission filter wheel and the CCD camera (**Fig. 8.3**). To operate this type of confocal microscopy system, we can use the same image capturing software (LuminaVision for MacOSX) as used for a wide-field microscopy (*see* **Note 7**).

1. Place the mixture on the bottom of a 35-mm glass base dish on the stage of a fluorescent microscope.

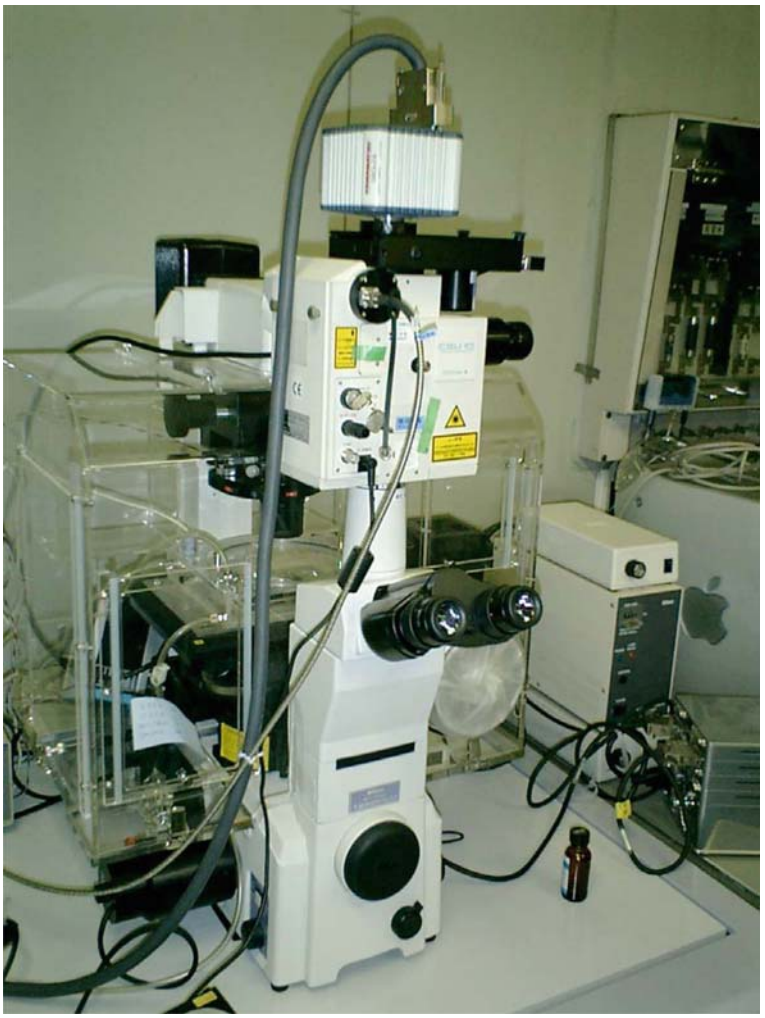

Fig. 8.3. System up to a laser confocal system. A laser scan unit CSU10 (Yokogawa Electric Corp.) is set between the microscope and the emission filter wheel. The combined laser beams of 440, 488, and 561 nm were used as a light source to illuminate ECFP, EGFP, and DsRed (*see* **Fig. 8.1d**).

2. Capture the images with a software (LuminaVision for MacOSX) controlling a cooled CCD digital camera (200–500 ms exposure for each filter) and rotating the excitation and emission filter wheels and *z*-axis motor with MAC5000, for collecting z-series optical sections (0.2-μm intervals).

4. Notes

1. Recently, a new 24- to 96-well type version, MXCell, is released. It is much convenient for determining the optimum condition to introduce plasmid DNA with this system.
2. The "VC" type of PlanApo 60× is recommended, rather than normal one. It is worth trying it if you want to obtain clearer images.
3. The filters with higher transmission (>95%) such as ion beam sputtering (IBS) thin-film coating filters (BrighLine, Semrock) or modified magnetron sputter-coated ones (ET Series, Chroma) are recommended. Sometimes, it is the easiest and most effective way to improve the signal of images, rather than obtaining an highly expensive EM-CCD camera.
4. The Dichroic 86006bs can be used for CFP/GFP/DsRed as well (14). Recently, higher transmitting multiband filter sets are available for CFP/YFP/HcRed (Semrock) and CFP/YPF/mCherry (89006, Chroma), but not yet for four living colors such as CFP/GFP/mKO/DsRed.
5. Check lysis with a phase-contrast microscope. If lysis is not complete, add 10 or 20 more strokes.
6. We prefer manual grinding to machine-driven grinding. Excess grinding will destroy the apoptotic activity.
7. It may be better to use the other excellent software (15) such as MetaMorph (Molecular Device, Sunnyvale, CA) or Volocity (Improvision a PerkinElmer Company, Coventry, UK) to operate the recent devices such as a precisExcite LED excitation (CoolLED Ltd., Andover, UK) or AURA or Spectra Light Engine (Lumencor, Inc., Beaverton, OR) and a Laser Merge Module, LMM5 (Spectral Applied Research, Ontario, Canada) or extremely sensitive EM-CCD cameras such as ImagEM (Hamamatsu Photonics) or Cascade II (Photometrics a division of Roper Scientific, Tucson, AZ). Recently, ORCA-R2 (Hamamatsu Photonics) is available for the new version of ORCA series, instead of ORCA-ER or AG.

Acknowledgments

The author would like to thank Mrs. Kouichi Amami (Tsukasa Giken, Osaka, Japan), Hideo Hirukawa (Yokogawa Electric Corp., Tokyo, Japan) and Ken Salisbury (formerly, Improvision, Coventry, UK) for the technical assistance for these microscopic analyses. This work is dedicated to the deceased Katsuji Rikukawa (Nikon Instech Co. Ltd., Japan).

References

1. Sugimoto, K., Senda-Murata, K., and Oka, S. (2008) Construction of three quadruple-fluorescent MDA435 cell lines that enable monitoring of the whole chromosome segregation process in the living state. *Mut Res* **657**, 56–62.
2. Brenner S., Pepper, D., Berns, M.W., Tan, E., and Brinkley, B. (1981) Kineochore structure, duplication, and distribution in mammalian cells: analysis by human autoantibodies from scleroderma patients. *J Cell Biol.* **91**, 95–102.
3. Rieder, C.L. (1982) The formation, structure, and composition of the mammalian kinetochore and kinetochore fiber. *Int Rev Cytol* **79**, 1–58.
4. Shaner N.C., Steinbach, P.A. and Tsien R.Y. (2004) A guide to choosing fluorescent proteins. *Nat Methods* **2**, 905–909.
5. Wyllie, A.H., Morris, R.G., Smith, A.L., and Dunlop, D. (1984) Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular systheswis. *J Pathol* **142**, 67–77.
6. Lazebnik, Y.A., Cole, S., Cooke, C.A., Nelson, W.G., and Earnshaw, W.C. (1993) Nuclear events of apoptosis in vitro in cell-free mitotic extracts: a model system for analysis of the active phase of apoptosis, *J Cell Biol* **123**, 7–22.
7. Samejima, K., Tone, S., Kottke, T.J., Enari, M., Sakahira, H., Cooke, C.A., Durrieu, F., Martins, L.M., Nagata, S., Kaufmann, S.H. and Earnshaw, W.C. (1998) Transition from caspase-dependent to caspase-independent mechanisms at the onset of apoptotic execution, *J Cell Biol* **143**, 225–239.
8. Liu, X., Kim, C.N., Yang, J., Jemmerson, R., and Wang, X. (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c, *Cell* **86**, 147–157.
9. Widlak, P., Palyvoda, O., Kumala, S., and Garrard, W.T. (2002) Modeling apoptotic chromatin condensation in normal cell nuclei. Requirement for intranuclear mobility and actin involvement, *J Biol Chem* **277**, 21683–21690.
10. Tone, S., Sugimoto, K., Tanda, K., Suda, T., Uehira, K., Kanouchi, H., Samejima, K., Minatogawa, Y. and Earnshaw, W.C. (2007) Three distinct stages of apoptotic nuclear condensation revealed by time-lapse imaging, biochemical and electron microscopy analysis of cell-free apoptosis, *Exp Cell Res* **313**, 3635–3644.
11. Sugimoto, K., Fukuda, R. and Himeno, M. (2000) Centromere/kinetochore localization of human centromere protein A (CENP-A) exogeneously expressed as a fusion to green fluorescent protein. *Cell Struct Funct* **25**, 253–261.
12. Sugimoto, K., Urano, T., Zushi, H., Inoue, K., Tasaka, H., Tachibana, M. and Dotsu, M. (2002) Molecular dynamics of Aurora-A kinase in living mitotic cells simultaneously visualized with histone H3 and nuclear membrane protein importin-alpha. *Cell Struct Funct* **27**, 457–467.
13. Sugimoto, K. (2006) Setting up a fluorescent microscope for live cell imaging in three colors. *Jikken Igaku* (*Expeimental Medicine*, in Japanese), **24**, 87–92.
14. Fukada, T., Inoue, K., Urano, T., and Sugimoto, K. (2004) Visualization of chromosomes and nuclear envelope in living cells for molecular dynamics studies. *BioTechniques* **37**, 552–556.
15. Sugimoto, K. (2006) Live cell imaging analysis of three-color cell-lines. *Jikken Igaku* (*Experimental Medicine*, in Japanese), **24**, 397–401.