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Preparation and fluorescent analysis of plant metaphase chromosomes

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Abstract

Good preparations are essential for informative analysis of both somatic and meiotic chromosomes, cytogenetics, and cell divisions. Fluorescent chromosome staining allows even small chromosomes to be visualized and counted, showing their morphology. Aneuploidies and polyploidies can be established for species, populations, or individuals while changes occurring in breeding lines during hybridization or tissue culture and transformation protocols can be assessed. The process of division can be followed during mitosis and meiosis including pairing and chiasma distribution, as well as DNA organization and structure during the evolution of chromosomes can be studied. This chapter presents protocols for pretreatment and fixation of material, including tips of how to grow plants to get good and healthy meristem with many divisions. The chromosome preparation technique is described using proteolytic enzymes, but acids can be used instead. Chromosome slide preparations are suitable for fluorochrome staining for fast screening (described in the chapter) or fluorescent in situ hybridization (*see* Schwarzacher and Heslop-Harrison, *In situ hybridization*. BIOS Scientific Publishers, Oxford, 2000).

Key words Chromosome, Heterochromatin, Metaphase, Meiosis, Proteolytic enzyme, Fluorochromes, DAPI

1 Introduction

Chromosome number and their shapes and sizes are important characters of species and have been investigated since the early days of microscopy [1]. Initially, transmitted light microscopy was used and chromosomes were stained with carmine, orcein, or Feulgen [2] resulting in simple uniformly stained chromosomes showing the typical constrictions at the centromeres and secondary constrictions at the nucleolus-organizing regions (NORs) [3]. If specific pretreatments are used, characteristic bands, e.g. Giemsa C-banding for heterochromatin, are produced [1, 4]. Later, fluorescence microscopy and fluorochromes (*see* Table 1; Figs. 1 and 2) have been introduced that bind directly to DNA, either to AT-rich or GC-rich DNA sequences or uniformly ([5]; *see* Table 1). Classic

Table 1**Characteristics of fluorochromes and fluorescent filter set specifications**

| Fluorochrome | Characteristics | Color | Filter set specifications | | |
|--|----------------------|--------------|------------------------------|-----------------|----------------------------|
| | | | Approximate excitation range | Dichroic mirror | Approximate emission range |
| DAPI | AT-selective binding | Blue | 350–375 nm (UV) | 400 nm | 435–485 nm or above 400 nm |
| Chromomycin A3 (CA3 or CMA) ^a | GC-selective binding | Yellow-green | 430–480 nm (blue) | 510 nm | 540–590 nm or above 520 nm |
| YOYO-1 | Total DNA | Green | 480–500 nm (green) | 505 nm | 505–525 nm |
| TOTO-1 | Total DNA | Red | 490–520 nm | 530 nm | 530–550 nm |
| Propidium iodide (PI) ^b | Total DNA | Red | 510–550 nm (green) | 565 nm | 590–630 nm |

Different manufacturers use slightly different filters in predesigned cubes that they specify for the common fluorochromes in use, but they should fall within the ranges given

^aCA3 will appear green in filter sets that cut off emission above 560 nm, but yellow if a wider band or long-pass filter is used. Normally the filter set specified for Fluorescein (FITC) can be used

^bPI can also be excited with other filter sets of lower wavelength range allowing visualization of two fluorochromes simultaneously, but it will be less strong

stains include DAPI, Chromomycin A3, and propidium iodide, but many new, strong, and stable fluorochromes are regularly introduced. For example, the dimeric cyanine nucleic acid stains TOTO and YOYO from Molecular Probes are some of the high-affinity nucleic acid stains available and single molecules and DNA fibres can be stained (*see e.g.* [6]), but they also produce characteristic bands on chromosomes (*see Fig. 2d*). In many species, repetitive sequences have the same GC/AT ratio as euchromatin and are not distinguished (*see examples in Fig. 1*). NORs often show Chromomycin A3-positive and DAPI-negative staining due to the high content of the repetitive 45S rDNA genes that are GC-rich as genes in general (*see Fig. 2a, b*). In many species, bulk differences in DNA composition (AT:GC ratio) mean stains can characterize heterochromatin in more detail and can produce bands (*see Fig. 2c, d*), potentially defining several classes of heterochromatin (e.g. [7]).

Both banded or uniform, colorimetric or fluorochrome-stained chromosomes have been used extensively to establish karyotypes of many plant species and to describe evolutionary relationships. In particular, chromosome base numbers in polyploidy series, interspecies diversity, and chromosome rearrangements have been determined [7–11]. The simple staining techniques are still of value for initial cytogenetic screening when studying a new species complex, and when ploidy level or aneuploidies need to be established for tissue culture, mutant, or hybrid and backcross lines. In

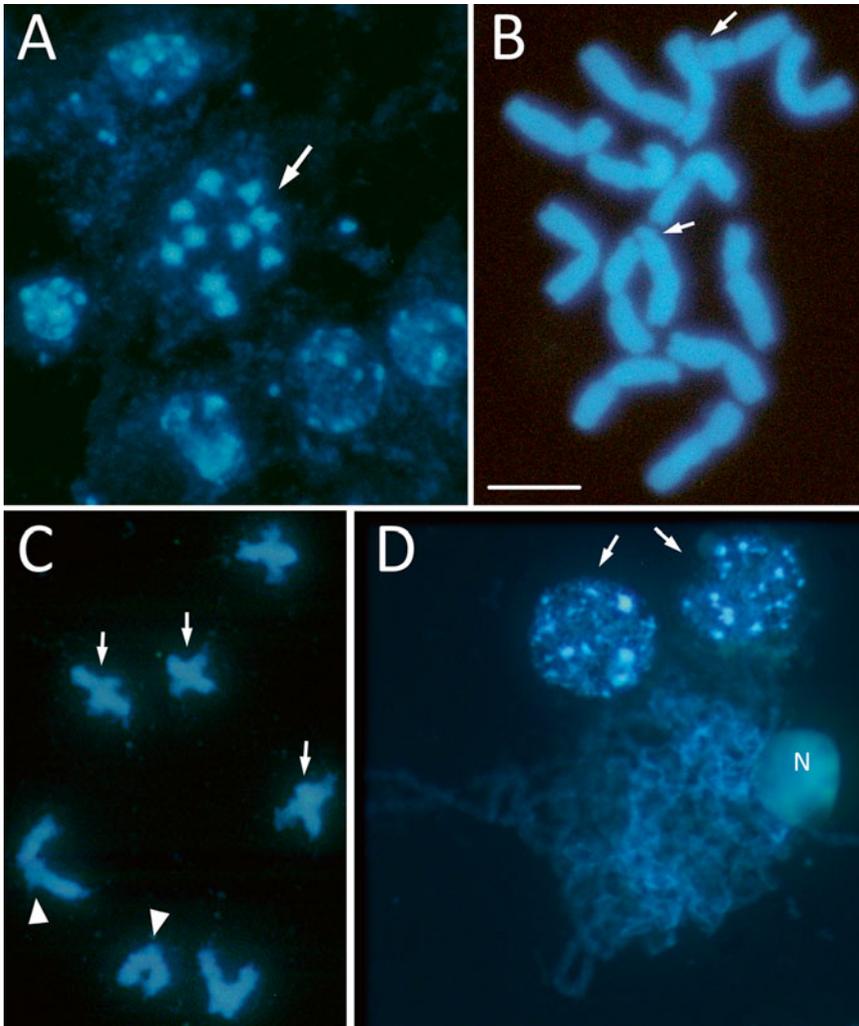


Fig. 1 Chromosomes stained with DAPI. **(A)** *Arabidopsis thaliana* ($2n=10$) flower bud meristem metaphase chromosomes (*arrow*) and four interphase nuclei with several strong chromocentres stained brightly. **(B)** *Secale cereale* ($2n=14$) root tip metaphase with much larger chromosomes showing clear gaps at the primary constriction, the centromere and the NORs of chromosome 1R (*arrows*). **(C)** Meiotic pollen mother cell of *Rosa canina* ($2n=10$) at metaphase I showing seven bivalents with each one proximal (*arrow*) or distal (*arrowhead*) chiasma. **(D)** Male meiotic prophase of diploid *Musa acuminata* ($2n=22$) showing thicker paired and thinner unpaired chromosomes in the middle; the opaque fluorescing round body is the nucleolus (N) while two somatic interphases with strong positively fluorescing chromocentres are near the top (*arrows*). Bar represents 2 μm in **(A)** and **(C)** and 5 μm in **(B)** and **(D)**

many karyotype studies, the more advanced molecular cytogenetic technique of in situ hybridization is used to analyse chromosome complements, locate the 5S and 45S rDNA genes, and localize satellite and dispersed repeats (*see e.g.* [3, 12] and references therein). Fluorochrome staining following chromosome preparation allows many more preparations to be examined compared to in situ hybridization and hence is advantageous for screening aneuploid lines or cytotaxonomy.

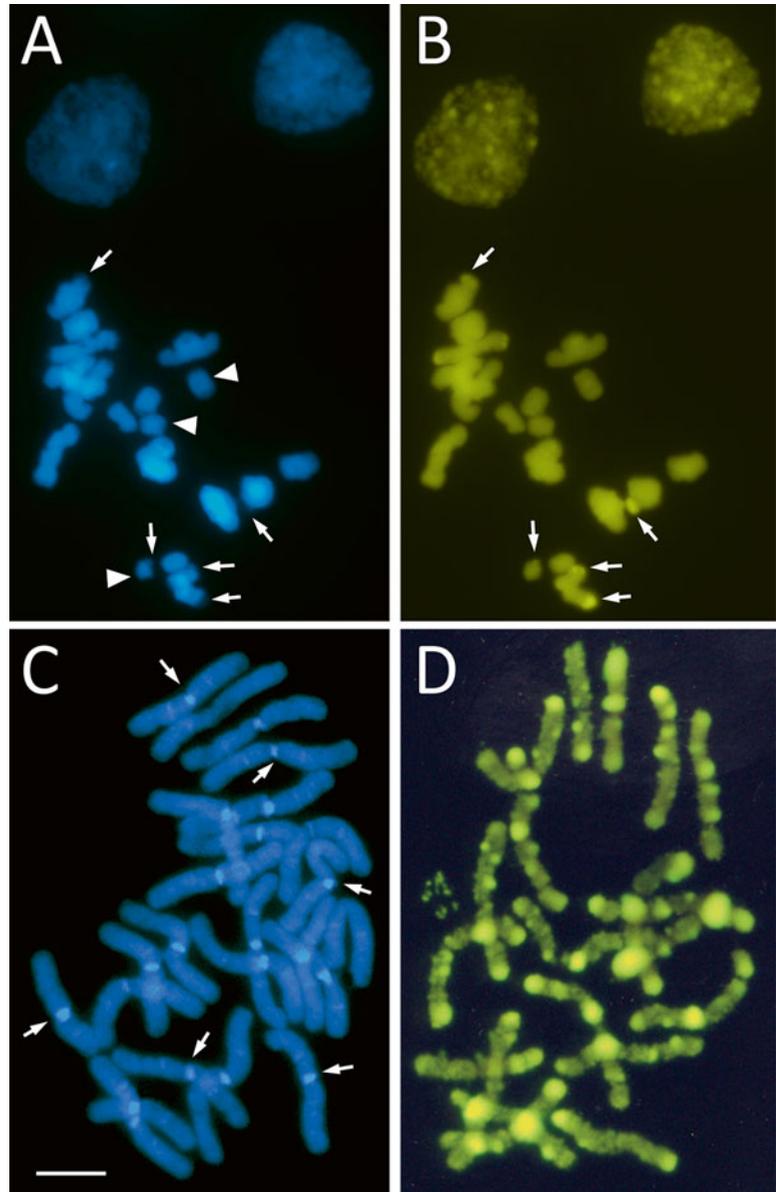


Fig. 2 Fluorescent chromosome banding. (A, B) The same metaphase I of meiosis of *Camellia japonica* × *C. saluenensis* ($2n=30$) showing bivalents and some univalents (arrowheads) after staining with DAPI (A) and Chromomycin A3 (B). Several terminal bands are positive with Chromomycin, but negative with DAPI (arrows) and most likely correspond to 45S rDNA sites (Image courtesy of Kazumi Furukawa). (C, D) Root tip metaphases of *Pinus elliotii* ($2n=24$) stained with DAPI (c) showing strong staining at the centromeres (some marked by arrows) and with YOYO-1 (D) showing several strong intercalary and terminal bands (Images courtesy of Pat Heslop-Harrison). Bar represents 5 μm in (A) and (B) and 10 μm in (C) and (D)

In this chapter, I describe the basic methods of chromosome preparations including the treatment of material before fixation. As most chromosome analyses rely on metaphases, dividing tissue is essential and is best obtained from healthy, disease-free, and rapidly growing plants. Root tips from young seedlings, freshly appearing roots at the edge of pots of older plants, or hydroponic cultures are all suitable. Great care needs to be taken when germinating seeds and watering young seedlings. The quality of water is important and it should not contain chlorine, heavy metal ions, or other water purification media or toxins, but it should have some salts and buffering capacity. Meristematic cells from young shoots, leaves or emerging buds or liquid tissue culture cells can also be used, but finding dividing cells within callus tissue is difficult.

In order to achieve good chromosome preparations with many metaphases, the material is pretreated with metaphase arresting agents before fixation. This step not only accumulates metaphases and condense the chromosomes, but importantly destroys the spindle microtubules allowing better spreading of the chromosomes. The spindle microtubule inhibitor colchicine gives heavily condensed chromosomes and is preferred for chromosome counting, while others such as ice water or 8-hydroxyquinoline give more extended chromosomes [12]. Alternative reagents, including spindle poisons used as herbicides, are also effective [13]. For meiotic studies, spindle arresting agents are not used so they do not interfere with pairing and disjunction (*see* Fig. 1c, d).

Most protocols use ethanol/acetic acid fixation that preserves chromosomes well while removing some of the chromatin proteins that would otherwise hinder the access to the DNA packaged within the chromosome. After fixation, material is either hydrolysed with acid [2] or digested with proteolytic enzymes [14] to soften the tissue, remove cell walls and allow spreading of chromosomes. Staining material with orcein or acetocarmine before spreading gives instant chromosome preparations and is often sufficient for chromosome counts. For small chromosomes or when much cytoplasm is present, fluorochromes are recommended as the unbound dye is essentially non-fluorescent and its fluorescence is a magnitude greater on binding to DNA (e.g. *Arabidopsis thaliana*, *see* Fig. 1a). Chromosome preparations as described here can also be used subsequently for fluorescent in situ hybridization (*see* [12]).

2 Materials

2.1 Preparation of Plant Material

1. Seeds or growing plants in pots.
2. Depending on material.
 - (a) Petri dishes and filter paper.

- (b) Plant pots of suitable size for material.
 - (c) Sterilization agent: 1 % (w/v) bleach in water.
 - (d) Soil or vermiculate.
 - (e) Agar minimal medium: e.g. Murashige and Skoog without sugar.
 - (f) Plant nutrient: commercial “complete” plant fertilizers (e.g. Phostrogen) made up in water to 1/10 the strength recommended.
3. Drinking water or bottled water (not distilled nor deionized) for germination and watering small plants.

2.2 Fixation of Material

1. Small tubes: 5–10 ml with tight caps (e.g. bijou tubes, freezer vials, or microcentrifuge “Eppendorf” tubes; *see Note 1*).
2. Forceps and scissors; forceps for handling unfixed material must be very clean and particularly not have residue of fixative (see Note 1).
3. Metaphase arresting agent: choose one of the following (*see Note 2*):
 - (a) Ice water at 0 °C (typically for seedlings of temperate plants, e.g. grasses): fill a small tube with bottled water or distilled water, shake vigorously to aerate or bubble air through, keep at –20 °C until freezing, shake again and store in an bucket of ice (*see Note 3*).
 - (b) 2 mM 8-hydroxyquinoline (for many species particular with small chromosomes): Make 500 ml or 1 l. Powder might take several hours to dissolve; stir, but do not heat. Store in dark at 4 °C (e.g. wrapped in foil in a refrigerator) for up to 1 year.
 - (c) 0.05–0.1 % (v/w) colchicine (for most plants): prepare a 0.05–1 % (v/w) solution in water; store in dark at 4 °C for up to 1 year.
 - (d) α -Bromo-naphthalene (for most plants): Overlay 10–20 ml with 3× volume of distilled water and shake vigorously to make saturated water; allow to settle for a few minutes and take upper water phase for use.
4. Fixative: three parts 96 % or 100 % ethanol, one part glacial acetic acid; prepared fresh, less than 1 h before use.
5. Optional 70 % ethanol.

2.3 Chromosome Preparation

1. 10× Enzyme buffer (pH 4.6): 40 ml 100 mM citric acid and 60 ml 100 mM tri-sodium citrate. Can be stored at 4 °C for a few days. Dilute to 1× with distilled water for use.
2. Enzyme solution: 2–4 % (w/v) cellulase (e.g. Sigma C1184; final concentration 10–20 U/ml), 0.2 % (w/v) “Onozuka” RS

cellulase (final concentration 10 U/ml), 3 % (v/v) pectinase (e.g. Sigma P4716 from *Aspergillus niger*; solution in 40 % glycerol, final concentration 15–20 U/ml). Make up in 1× enzyme buffer. Store in 2–5 ml aliquots at –20 °C (*see Note 4*).

3. 5–10 ml 45 % (v/v) and 60 % (v/v) acetic acid in distilled water.
4. Optional: acetic orcein or aceto-carmin (*see Note 5*).
5. Petri dishes.
6. Fine forceps and dissecting needle.
7. Microscope slides: normally 25 mm × 75 mm, either pre-cleaned (e.g. Super frost) or cleaned with strong acid (*see Note 6*).
8. Coverslips: 18 × 18 mm No. 1 (*see Note 7*).
9. Dissecting microscope.
10. Phase-contrast microscope.
11. Dry ice or liquid nitrogen (*see Note 8*).

2.4 Staining and Mounting Slides

1. McIlvaine's buffer (pH 7.0): 41 ml of 200 mM Na₂HPO₄ and 9 ml 100 mM citric acid.
2. Optional: Phosphate buffered saline (PBS): buy ready mixed concentrate or tablet, or prepare a 10× stock solution of 1.3 M NaCl, 70 mM Na₂HPO₄ and 30 mM NaH₂PO₄, adjust pH to 7.4 with 1 M NaOH or HCl and autoclave. Dilute to 1× before use (*see Note 9*).
3. Fluorochrome solution: choose as required (*see Notes 10 and 11*).
 - (a) DAPI (4',6-diamidino-2-phenylindole). Prepare 100 µg/ml stock solution in water. Aliquot and store at –20 °C (it is stable for years). Prepare a working solution of 1–2 µg/ml by dilution in McIlvaine's buffer, aliquot 0.5–1 ml and store at –20 °C.
 - (b) CMA (Chromomycin A₃): 0.5 mg/ml in McIlvaine's buffer diluted 1:1 with 5 mM MgCl₂. Add the buffer slowly to the powder without stirring and leave at 4 °C overnight. Store at 4 °C (*see Note 12*).
 - (c) PI (propidium iodide). Prepare a 100 µg/ml stock solution in water. Aliquot 50 µl or 100 µl in 1.5 ml microcentrifuge tubes and store at –20 °C. Dilute before use with 1× PBS to 0.1–5 µg/ml (*see Note 13*).
 - (d) TOTO-1, YOYO-1 or derivatives (Molecular Probes); normally supplied as 1 mM stock solution in dimethylsulfoxide (DMSO) that needs diluting in PBS 10,000- to 100,000-fold (*see Note 14*).

4. Antifade mounting medium for fluorescence microscopy: e.g. Vectashield (Vector Laboratories), Slow Fade and ProLong (Life technologies), Fluorguard, Fluorshield and Fluoromount (Sigma) or Citifluor AF1 (Agar Scientific or Citifluor, Leicester).
5. Plastic coverslips cut to size from autoclavable bags.
6. Glass coverslips: No. 0 24 × 30 or 24 × 40mm (*see* **Note 15**).

2.5 Microscopy

1. Epifluorescent microscope with camera; in most cases this will now be an electronic (CMOS or CCD) camera (*see* **Note 16**).
2. Image analyzing program, e.g. Adobe Photoshop, NIH imaging or microscope software.

3 Methods

3.1 Plant Material Preparation

1. Depending on species and availability of material choose one of the options below to generate new growth and meristem formation for collecting somatic divisions. For meiosis, anthers from developing flower buds are best.

(a) Root tips can be obtained with one of the following methods (*see* **Note 17**).

- Germinating seed or young seedlings (*see* **Note 18**).
- Put seeds onto filter paper saturated with drinking or bottled water, typically at 20–25 °C in the dark and leave until roots are about 10–20 mm long. Small seeds can be germinated under sterile conditions on agar minimal medium (*see* Subheading 2.1) in Petri dishes. Ensure the germinating seeds do not have any exposure to fixative fumes.
- Newly grown roots at the edge of plant pots.
- Plant seedlings in small pots with soil or vermiculate and wait until roots appear at the edge. Or repot established plants to generate new root growth. In some cases, applying compost on the surface around the stem is effective.

Hydroponic culture.

- Suspend plantlets or bulbs cleaned from soil above aerated plant nutrient or place on a gravel bed.

(b) Alternatively, flower buds, anthers, carpels, or leaf or apical meristems can be used. Cut plants back if necessary to generate new growth.

(c) For meiotic tissue, collect anthers at different developmental stages.

2. Make sure that seeds and plants are moist and at the correct temperature and light regime. Water regularly, but do not over water (*see* **Note 19**).

3.2 Fixation of Material

Use small glass or plastic containers, fill typically two-thirds with solution (metaphase arresting agent and fixative as appropriate) and allow a generous amount of solutions for the specimen to be fixed, typically two times as much. Use clean forceps or pipettes to transfer material (*see* **Notes 1** and **17**).

1. To accumulate metaphases, treat excised root tips (5–20 mm long) or other material with one of the metaphase arresting agents as follows (*see* **Notes 2** and **20**):
 - (a) Aerated ice water for 24 h (*see* **Note 3**):
 - (b) 8-Hydroxyquinoline for 1–2 h at room temperature, then 1–2 h at 4 °C.
 - (c) Colchicine for 3–6 h at room temperature or 10–24 h at 4 °C.
 - (d) α -Bromonaphthalene saturated water for 2–6 h at room temperature.
2. For meiotic analysis, anthers are collected without pretreatment (*see* **Note 21**).
3. Blot material and transfer to fixative (*see* **Note 22**).
4. Fix for at least 16 h at room temperature. If fixed material is to be kept (up to several months), leave for 2 h at room temperature and then transfer to new fixative or 70 % ethanol and store at –20 °C.

3.3 Chromosome Preparation

Material is carefully transferred by forceps or, if small roots or buds are used, with a pipette. Alternatively, fluids can be removed with a pipette and replaced with the next required solution.

1. Wash 2–10 root tips or buds two or three times for 10 min in 2–5 ml enzyme buffer (*see* Subheading **2.3**) in a small Petri dish to remove the fixative (until they sink). Make sure roots have intact tips and cut away any access material (e.g. outer layer of buds and only 1 cm of root is needed). Remove any dirt or soil remains.
2. Transfer material into 1–2 ml enzyme solution and digest at 37 °C until the material is soft, usually 45–90 min (*see* **Notes 4** and **23**).
3. Wash material in enzyme buffer for at least 15 min. Material can be stored at 4° for up to 24 h.
4. Transfer enough material for one preparation (typically one root, small bud, or anther) into 45 % acetic acid in an embryo dish, separate slide, or small Petri dish for 1–5 min (*see* **Note 24**).
5. Make chromosome preparations on clean microscope slides. Under the stereo microscope, in 1 drop (10–30 μ l) of 60 % acetic acid, dissect the meristematic tissue or pollen mother cells by removing as much of the other tissue as possible, e.g.

remove the root cap or cut the anther and tease out the cells in the remaining material (*see Note 25*).

6. Apply a small coverslip to the material without trapping air bubbles. Blot away excess liquid. Carefully disperse the material between glass slide and coverslip by tapping the coverslip gently with a needle or flat back of a pencil. Fold a filter paper in half around the slide, put it on a flat surface and then squash the cells, using the thumb to press as hard as possible onto the filter paper above the area of the coverslip (*see Note 26*).
7. Check the slide under a phase-contrast microscope. If not sufficiently flat squash again (*see Note 27*).
8. Place the spread slide onto dry ice for 5–10 min, then flick off the coverslip with a razor blade immediately before it warms up.
9. Allow the slide to air-dry. Screen slides to choose suitable preparations and store desiccated for up to 3 months at 4 °C or –20 °C (*see Note 28*).

3.4 Staining and Mounting Slides

1. Put 100–200 μl of fluorochrome solution (*see Subheading 2.4*) on the marked area of the preparation on each slide. Cover with a coverslip (either plastic cut from autoclavable bags or a large glass coverslip). Incubate at room temperature for 30 min in the dark avoiding bright light (*see Notes 29 and 30*).
2. Remove coverslips and wash slides briefly in fluorochrome buffer and shake excess solution off without letting the slide dry out.
3. Add one or two drops of antifade solution to each preparation and cover with a large, thin coverslip avoiding bubbles (*see Note 15*).
4. Allow to settle for a few minutes (or leave for hours as convenient) and then gently, but firmly, squeeze excess antifade from the slide between several sheets of filter paper.
5. Store slides flat at 4 °C until examination by fluorescent microscopy (*see Note 31*).

3.5 Microscopy

This section is only a brief guide and does not replace microscope manuals or books. Most advanced fluorescent microscopes and digital camera systems are increasingly operated through a computer interface that allows you to change objectives and filter blocks automatically as well as capturing and processing images. Detailed operation manuals are normally supplied.

1. Warm the slides to room temperature avoiding condensation on the coverslip.
2. Make sure that antifade is squeezed from underneath the coverslip and that the coverslip is clean.

3. Switch on the fluorescence lamp and wait until it is stabilized.
4. Add a drop of fluorescence immersion oil to the middle of the coverslip. We recommend using oil objectives for both low (20×) and high power (*see Note 32*).
5. Carefully load the slide onto the stage. Best is to leave the stage at the current height, but swing the objectives to the side. Carefully, while watching, swing in the 20× or 25× objective into place. The slide can be easily broken if it is not loaded properly, or the stage is too high.
6. Turn the filter wheel to the correct filter position (*see Note 33* and Table 1).
7. Open instrument and microscope shutters and make sure that the optical path switch levers (camera versus eyepiece) are in the correct position.
8. Focus images by using the manual or automatic focus button.
9. Scan slides using the stage controls.
10. When you find a good metaphase change to a higher power objective (63× or 100×). Note that particular UV excitation fades all fluorochromes and should not be used for more than a few seconds before photography, other than with low power objectives.
11. Take images following your microscope instructions and operation software guidance. Check histograms of pixel brightnesses to ensure you use the full range of levels available without clipping of bright areas. Make sure you save raw images before image manipulation (*see Note 34*).
12. Use image analysis to enhance weak signal and overlay images from different fluorochrome if necessary. Only use those function that treat all pixels in the image equally. Save images frequently in programme format and TIF, avoid formats that lose information (e.g. jpg) (*see Note 34*).
13. Slides can be de- or re-stained after photography (*see Note 35*).

4 Notes

1. It is important to have clean tools and containers during germination of seeds and pretreatment, to work away from fixatives and other toxins and to use airtight lids to exclude fumes, to avoid low metaphase index.
2. Different species and material, e.g. thickness of roots or size of buds, respond differently to metaphase arresting agents. Type

and concentrations need be established by trial and error. We prefer aerated ice water for small grained cereals and hydroxyquinoline for all other species and approximate conditions are given, but colchicine or α -bromonaphthalene can also be tried. Alternative reagents, including spindle poisons used as herbicides, are also effective ([13], and *see* **Notes 20** and **21**).

3. In order to make sure the ice water is at 0 °C, both aerated water and ice need to be present. Make sure that the bucket with ice is also partly liquid to allow for a tight seal around the tubes and not trapped air that might be warmer than 0 °C. For long storage and treatment keep the ice bucket in the cold room.
4. Enzyme concentrations are often given in percentage, but activity units are more important and so we give a range here. Batches of the same enzyme product can differ in strength and composition, while distributors may change isolation protocols or suppliers. Onozuka cellulase is a very pure enzyme and has a constant activity per weight. But purified enzymes (typically protoplast grades) in general give less good results than crude enzyme grades that contain a mixture of proteolytic enzymes. If enzymes are not available, hydrolysis in 1 M HCl for 10 min at 60 °C can be used. Enzyme mixtures can be reused several times: after use, centrifuge in a microcentrifuge, transfer the supernatant to a new tube, mark for reuse and freeze (not recommended for screening lines of similar material since cells may remain in the solution). Digestion time might need to be slightly increased after each round of use.
5. Dissolve 1 % orcein or carmine in 45 % acetic acid. For root tips, the addition of a part 1 N HCl to 10 part standard solution is recommended and gentle heating of the tissues in a drop of the mixture to assist the softening and separation of cells [2].
6. Microscope slides: good quality slides are needed, where no marks are left from the manufacturing process. Some come pre-cleaned, some charged or coated. To clean them yourself, put into 6 N HCl overnight and rinse in several changes of water, dip into distilled water and air dry.
7. 18 × 18 mm glass coverslips of medium thickness are used. Larger coverslips are not recommended, as well spread metaphases are often near the periphery of the coverslip; slightly larger coverslips need then to be used for staining and visualization (e.g. 24 × 30 mm or 24 × 40 mm) so that all cells can be probed and examined. The coverslips should be free of dirt, but should not be cleaned with alcohol or acid; otherwise material will stick to the coverslip and will be lost when the coverslip is removed (Subheading 3.3, step 8).

8. If dry ice or liquid nitrogen are not available, slides can be frozen on a metal plate in a -80°C freezer.
9. Several formulations are available for PBS with the NaCl concentration either 138 mM or 145 mM and some include 2.7 mM KCl, but this is not necessary for this application.
10. Choice of fluorochrome depends on the type of question you want to answer, size of chromosomes and microscope availability. *See* Table 1 and Figs. 1 and 2 for examples.
11. Most fluorochromes are potential carcinogens. To avoid weighing out the powder, order small quantities and use the whole vial to make the stock solution.
12. Chromomycin A3 fluorescence is more stable and stronger with older solutions. Adding 4 % paraformaldehyde to avoid bacterial contamination is recommended by some authors (e.g. [15]).
13. PI does not keep in diluted form and even after a few hours fluorescence reduces quickly. Sometimes restaining is required after slides have been stored; but it can be used as advantage when overstaining has occurred as just a short rinse with water reduces PI strength.
14. It is best to first prepare a working stock solution of $1\ \mu\text{M}$ and then dilute further to 2–10 nM. Test a quite wide range of dilutions before staining the best slides (*see* also **Note 29**).
15. Thin coverslips No. 0 (0.1 mm) are thinner than the most widely used specification (No. 1, 0.13–0.16 mm) and are essential for high-power fluorescent microscopy using oil objectives. Choose a size that is larger than the specimen area and used for making the preparations (*see* **Note 7**).
16. Main microscope manufacturers are Zeiss, Leica, Nikon, and Olympus. I have worked with all of them and cannot recommend one over the other. It is best to get several demonstrated and choose according to offers made and importantly after sale services. Also consider what you want to use the microscope for, routine fluorochrome staining for chromosome counts and ploidy checks or also high resolution in situ hybridization using several fluorochromes at once. The former can be done with a bottom-end microscope, while for the later the top of the range including camera and software are recommended. An important factor will be the location of the microscope on a stable bench in a room that can be darkened to see faint signal.
17. It is very important not to expose seedlings, roots, and plants during germination and growth induction and later during metaphase-arrest to chemicals and fumes, particularly fixatives (e.g. in a cold room also used for chemical storage) and to use

clean lab ware with tight lids (disposable plastic is ideal), clean forceps, and distilled water or bottled water (*see* also **Note 1**).

18. Some seeds are difficult to germinate and might need priming by moving between 4 and 25 °C every 3–14 days. Seed suppliers can also give advice about germination of difficult species. If seeds are prone to infection sterilize in diluted bleach by submerging for 5–10 min and then rinsing well until the smell has disappeared.
19. It is important to check seeds germinating in Petri dishes, so they have the correct amount of water; plants in pots can be put on a gravel bed and should be watered the day before collecting roots and not just prior to collection. For all material, make sure they are not stressed by lack of water or roots by lack of oxygen due to stagnated water created by overwatering. Root tips from germinating seeds, and plants grown in controlled conditions, often show waves of cell division that may follow internal or environmental rhythms (e.g. light) or correlate with root length. At certain times, there may be no divisions at all, so it may be helpful to make several collections.
20. Representative times are given. For best results fix material after different times of treatment, experiment with different reagents and check the mitotic index by making chromosome preparations. Treating material for too long in arresting agents, particularly colchicine, results in over-condensation of the metaphase chromosomes which might be desirable for counting chromosomes, but not where spatial resolution along chromosomes is wanted.
21. Meiosis is best analyzed in male pollen mother cells as more divisions are available. It is critical to check a few anthers before making large fixations to assess the correct stage. Meicytes undergo meiotic division in synchronously and therefore different sizes of anthers need collecting, at different times of day, for analysis of pachytene or metaphase I. As a norm anthers are normally whitish or light green and at meiosis before sepals or petals develop. In some species meiosis can take place a long time before flowering and anthesis, e.g. in *Crocus* where it happens in autumn for both autumn and spring flowering types. Some species will need a cold period for vernalization before flowering is induced.
22. Fixative should not be contaminated with water, so careful blotting or an extra rinse in fixative is advised.
23. Sometimes the ratio of cellulase to pectinase needs to be adjusted or different enzymes such as macerozymes, visco-

zyme, or pectolyase can be included. For meiotic tissue we recommend to add cytohelicase (2–5 %) to the enzyme mixture [16] which helps to remove the cell wall and cytoplasm. The enzyme digestion step needs to be adjusted to the material and species used, by changing the time of digestion; aim at a digestion time of 45–90 min; otherwise change the strength of enzyme. Ideally, cell walls should be weakened, so that the cells can be separated easily, and chromosome spreads are clean of cytoplasm. In most cases, the meristematic cells will be digested faster than the non-dividing tissue. The material should remain intact to handle, otherwise the dividing cells are lost into the medium. Once transferred to buffer, digestion will continue slowly; if roots are quite hard, they can be left overnight in buffer and will soften further. If material has been fixed for several weeks, the material becomes harder and needs longer digestion.

24. Acid disperses the material and so when the material is very soft it is better treated with 45 and then 60 % directly on the final slide. If material is hard it can be treated in acid for longer. Larger roots can be divided to make several slides.
25. The teasing out of good cells can be compared to extruding a sausage from its skin.
26. It is absolutely essential that the coverslip does not move sideways creating a “noodling” effect. So tap and press vertically only. The folded filterpaper will help.
27. If there is much cytoplasm and cells are not squashed properly, a drop of acetic acid can be added to the side of the coverslip and the slide warmed over an alcohol burner before squashing again. Be careful that the solution does not get to hot or boil; just touchable to the finger is fine.
28. Slides need to fully dry out before storing and if used immediately they benefit for 1–2 h at 60–80 °C.
29. Fluorochrome concentrations might need adjustment, but note that DAPI has two modes of binding to DNA: in the minor groove, which occurs at high stain concentrations; and, at lower DAPI concentrations, the alternative of intercalation between AT-nucleotide tracts in the DNA [17]. The latter binding gives the greater increase in fluorescence compared to the unbound molecule. Hence, increasing stain concentration has sometimes the unusual effect of reducing signal, and weak signal may mean too high stain concentration. PI is not stable in diluted form (*see Note 13*).
30. For quick examination with DAPI, slides can be viewed directly without rinsing and mounting.

31. Fluorochromes stabilize with storage, e.g. ChromomycinA3; so we normally wait 2–3 days before examination and photography.
32. 20× or 25× oil objectives are expensive, but very bright and convenient for scanning. Using non-oil low power objectives is tricky when high power lenses demand oil are used at the same time. Make sure you use immersion oil for fluorescence. High temperatures or moisture can make oils opaque. Also do not mix different types of oils.
33. Filter set specifications are summarized in Table 1, but manufacturers will also have tables and several choices of differently priced filter sets. Often fluorochromes can be excited in a wide range of filters and testing several before buying or simple try and error with what is available is recommended.
34. Store images in lossless forms and compatible with different programmes (e.g. TIF format as well as the programme format); use JPG format only for incorporation into presentations or for sending via e-mail. Make sure you use short file names that do not lose vital information in case of computer and disk failure. Make frequent back-ups.
35. Remove the coverslip as follows:
 - (a) With immersion oil on the coverslip, warm slide to 37 °C, gently but firmly pull off the coverslip being careful not to get oil on the slide surface and rinse the slide in 70 % ethanol.
 - (b) Without oil, help the coverslip to fall off in 70 % ethanol. Transfer slides through a 70, 90, and 96 % ethanol series (2 min each) and air-dry.

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