Yeast Chromosome Analysis

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Running title: Yeast Chromosome Analysis
1. Introduction

The budding yeast, *Saccharomyces cerevisiae*, is the first eukaryotic organism whose genomic DNA sequence was determined, and efforts are in progress to characterize all of its ca. 5900 genes by disrupting the corresponding open reading frames. Many of the deletions have shown or are expected to show a cytological phenotype. Likewise, many known mutations show aberrant chromosome behaviour. Therefore, chromosome analysis is required as an important tool for the study of yeast mutants. Due to its powerful genetics and easy handling, yeast has also become the most thoroughly studied meiotic model system to date. Also for meiotic studies, cytological techniques were needed to complement genetical and biochemical analyses.

A major disadvantage of yeast is that it does not show condensed mitotic chromosomes, whose absence precluded the analysis of individual chromosome behaviour and limited yeast cytology to observations at the level of whole nuclei. Therefore, for many years, the primary and almost only tool in yeast cytology has been the staining of nuclei with DNA-binding fluorochromes such as DAPI, to monitor the passage of nuclei through mitotic and meiotic divisions which are accompanied by elongation and the equatorial constriction of nuclei. However, the recent years have witnessed a renaissance in yeast cytology. A wealth of techniques, including fluorescence in situ hybridization (FISH), GFP-tagging of chromosomal loci with green fluorescent protein (GFP) and immunostaining, are now being applied to yeast in order to localize uncondensed chromosomes or parts thereof. In addition, certain aspects of yeast cytology can be studied using compacted meiotic chromosomes or bivalents. At the pachytene stage of meiosis, the synaptonemal complex (SC), a proteinaceous ladder-like structure, is formed between pairing chromosomes. By staining the SC, which faithfully traces the course of bivalents, one can watch chromosomes indirectly at least. Staining of the SC complement has become possible due to the introduction of a nuclear spreading method (1).
Finally, also pulsed-field electrophoresis of chromosome-sized DNA molecules must be mentioned as a tool used in yeast chromosome analysis preferentially to detect chromosomal rearrangements.

2. Materials

2.1. Cell Growth and Preparation

1. Rich medium, YPD: 1% yeast extract, 2% peptone, 2% glucose in distilled water, autoclaved.

2. Presporulation medium, YPA: 1% yeast extract, 2% peptone, 1% potassium acetate in distilled water, autoclaved.


4. Zymolyase: Enzyme for dissolving the yeast cell wall. Prepare a 10 mg/ml stock solution of Zymolyase 100T (Seikagaku Co., Tokyo, Japan) in distilled water (see Note 1).

5. Digestion solution: 0.8 M sorbitol with 10 mM dithiothreitol (prepared from a frozen 1 M stock). Add 7 µl of Zymolyase stock solution per 500 µl.

6. Stop solution: 0.1 M 2-(N-morpholino)ethane sulfonic acid (MES), 1 mM EDTA, 0.5 mM MgCl₂, 1 M sorbitol in distilled water.

7. Fixative I: 4% formaldehyde solution made from a formaldehyde stock solution by diluting with distilled water.

8. Detergent: Prepare a 1% solution of “Lipsol Liquid Concentrate” (LIP Ltd. Shipley, England) in distilled water. The working solution can be stored for several months in the refrigerator. (see Note 2).

9. Fixative II: 4% paraformaldehyde supplemented with 3.6% sucrose (see Note 3).

10. 1× PBS: 130 mM NaCl, 7 mM Na₂HPO₄, 3 mM NaH₂PO₄, in distilled water (pH 7.5)

2.2. Immunostaining
1. Primary antibodies. Antibodies against various components of the yeast SC, kinetochores
and several chromosomal proteins have been raised and more are to follow. At present,
none of these is commercially available. For immunolabelling of microtubules, YOL1/34
monoclonal rat anti yeast tubulin antibody (refs 2, 3; purchased from Serotec, Kidlington,
England) produces good results.

2. Secondary antibodies: Purchased from various vendors.

3. DAPI (4′6-diamidino-2-phenylindole) as a DNA-specific counterstain. Prepare 1 mg/ml
and 20 µg/ml stock solutions in distilled water which can be stored at –20°C. DAPI is
applied at at final concentration of 0.2 – 0.5 µg/ml.

4. Antifade buffer to reduce bleaching: 245 mg diazabicyclo(2.2.2)octane + 200 µl 1 M
NaHCO₃ (ph8.0) + 800 µl distilled water + 9 ml glycerol. Alternatively, antifade buffers
can be purchased under the tradenames Vectashield (Vector Laboratories Inc., Burlingame,
CA) or Slow Fade (Molecular Probes Inc., Eugene, OR) (see Note 4).

5. Rubber cement for sealing slides, e.g., Fixogum (Marabuwerke GmbH, Tamm, Germany).

2.3. FISH

1. Cosmids and λ-phage clones. Clones from the desired chromosomal loci can be selected
from the Saccharomyces Genome Database (4) and purchased from the American Type
Culture Collection (Rockville, MD) (see Note 5).

2. Probes produced by PCR. Fragments of ca. 5 to 10 kb sizes are produced by long-range
PCR (suitable kits are available from various companies: e.g., Expand™ Long Template
PCR System (Roche Diagnostics, Basel, Switzerland); TaKaRa Ex Taq (TaKaRa Shuzo
Co., Ltd., Otsu, Japan)) with appropriate primers from the Saccharomyces Genome
Database (4) (see Note 5).

3. 10× labeling buffer: 500 mM Tris, pH 8.0, 50 mM MgCl₂, 500 µg/ml BSA (Sigma,
Chemical Co., St. Louis, MO).
4. 1 mM dATP/dCTP/dGTP mixture.

5. Labeled nucleotides: e.g. Cy3-dUTP, Cy5-dUTP (Amersham Pharmacia Biotech, Uppsala, Sweden), fluorescein-dUTP, tetramethylrhodamin-dUTP, digoxigenin-dUTP or biotin-dUTP (Roche Diagnostics, Basel, Switzerland).

6. 280 mM β-mercaptoethanol.

7. DNase I (Roche Diagnostics, Basel, Switzerland).

8. *E. coli* DNA polymerase I (10U/µl, BioLabs, Beverly, MA).


10. RNase, DNase free (Roche Diagnostics, Basel, Switzerland).

11. ST buffer: 4× SSC (0.6 M NaCl, 60 mM Trisodium citrate, pH 7.0), 0.1% Tween 20.

12. 20× SSC: 3.0 M NaCl, 0.3 M trisodium citrate, pH 7.0.


14. 3M LiCl.

15. Hybridization mixture: 50% formamide, 2× SSC, 10% dextran sulfate, 1 µg/µl salmon sperm carrier DNA.

16. Rubber cement, e.g., Fixogum (Marabuwerke GmbH, Tamm, Germany).

17. Heating block or a thermocycler capable of holding slides (HYBAID Ltd., Ashford England).

18. Blocking buffer: 3% BSA, 4× SSC.

19. Detection buffer: 1% BSA, 4× SSC, 0.1% Tween 20.

20. Detection reagents: E. g., Avidin FITC conjugate, Extravidin(R) FITC conjugate, Extravidin(R) Cy3 conjugate ; Biotin-conjugated anti-avidin monoclonal antibody (Sigma, Chemical Co., St. Louis, MO); anti-digoxigenin-fluorescein, anti-digoxigenin-Rhodamin, anti-digoxigenin-AMCA (Roche Diagnostics, Basel, Switzerland)

21. Vectashield anti-fading medium (as above).
22. DAPI (as above).

2.4. Silver Staining of Synaptonemal Complexes

1. AgNO$_3$ solution: 5 g AgNO$_3$ in 10 ml ultrapure water (see Note 6). Caution: Corrosive; wear eye protection and gloves!

2. Polyamide cloth: e.g., Nybolt PA-100/31 (Swiss Silk Bolting Cloth Mfg. Co. Ltd., Zurich, Switzerland).

2.5. Spread Preparations for Electron Microscopy

1. N-hexane for removal of immersion oil.

2. 1% Hydrofluoric acid. Prepared from 40% stock. Caution: Obey safety regulations of your institution. Wear eye protection and gloves. Work under a fume hood!

3. 1% solution of Formvar (or crumbled polystyrene labware) in chloroform.


3. Methods

3.1. Cell Growth

1. For the study of mitotic cells, inoculate 5 ml YPD with a small colony from a plate and grow to a concentration of $\sim 2\times10^7$ cells per ml in a shaker at 30°C.

2. To obtain meiotic cells, inoculate 10 ml YPA with a small colony of a diploid strain from a plate and grow to a concentration of $\sim 2\times10^7$ cells per ml in a shaker at 30°C (see Note 7). Centrifuge cell suspension for 4 min at 2000 rpm. Resuspend pellet in 5 ml SPM and incubate until 2-4% 4-nucleate cells appear in DAPI-stained preparations (3.2.1). At this
point the majority of cells will be at pachytene which is the most favourable stage for
cytological examination. Sporulation time varies considerably between strains. Strain SK1
(5) which is widely used for meiotic studies shows a maximum of pachytenes at ~4-5 h in
sporulation medium.

3.2. Cell Preparation

There are four preparation procedures which can be used to study yeast chromosomes
cytologically. Procedure 3.2.1 (Ethanol Fixation) is useful in combination with DAPI staining
and GFP labelling of chromosomes and other nuclear components as a quick orientation.
Procedure 3.2.2 (Formaldehyde Fixation) provides good preservation of the outer shapes of
cells and astral microtubules. It is to be used in combination with GFP labelling of
chromosomes and immunostaining (see ref. 3). Procedure 3.2.3 (Spreading) offers enhanced
cytological resolution but tends to disrupt cells. It is best suited for the preparation of SCs and
to be used in combination with GFP labelling (although GFP signals tend to become weak
after this procedure), Ag-staining and immunolabelling of SC components, and FISH.
Procedure 3.2.4 (Semi spreading) is a good compromise for obtaining a good spatial
resolution of nuclear contents and a reasonable maintenance of cell integrity.

3.2.1. Ethanol Fixation

1. Transfer 50 µl of a liquid cell culture to an Eppendorf tube and spin for 5 sec.
2. Remove supernatant and dissolve pellet in 96% ethanol. Spin again.
3. Dissolve pellet in 50 µl of water and add 1 µl of 20 µg/ml DAPI-stock. Alternatively,
dissolve pellet in glycerol-containing medium (antifade buffer supplemented with DAPI,
see Note 4). Due to higher viscosity, glycerol reduces Brownian motion of the cells, which
may be advantageous for microscopical inspection.
4. Spread 10 µl of cell suspension under a coverslip for examination in the fluorescence microscope.

3.2.2. Formaldehyde Fixation

1. Add formaldehyde stock solution to the cell suspension to give a final concentration of 4% formaldehyde.

2. Vortex and leave for 30 min at room temperature. For prolonged incubation with formaldehyde up to several hours, put suspension on ice.

3. Wash cells in 2% KAc.

4. Collect cells at a concentration of 1-4 × 10⁷ cells/ml in 2% KAc.

5. Add 10 µl of 1 M dithiothreitol and 7 µl of Zymolyase stock solution per 500 µl.

6. Spheroplast cells for 20 min at 37°C.

7. Wash cells in 2% KAc and resuspend at a concentration of 1-4 × 10⁷ cells/ml in 2% KAc.

8. Coat clean slides with polylysine (0.1% solution) to reduce the loss of cells.

9. Spread out 20 µl of cell suspension evenly on the surface of a slide with the help of a glass rod.

10. Leave preparation for 2-3 min in a moist chamber to allow cells to settle and stick to the polylysine-layer. Do not allow them to dry!

11. Transfer wet slides to a cuvette with ice-cold methanol and incubate them for 30 seconds.

12. Drain excess liquid and transfer slides to 1× PBS.

13. Mount preparation in anti-fading medium supplemented with DAPI (for observation of GFP staining) or proceed with immunostaining (procedure 3.4).

3.2.3. Spreading

The spreading protocol described here is a modification by Loidl et al. (6) of the method by Dresser and Giroux (1).
1. Take 5 ml of a cell suspension obtained according to procedure 3.1.

2. Spin and resuspend pellet in digestion solution (see 2.1.5).

3. Spheroplast cells for 20 min at 37°C.

4. Wash cells with stop solution (see 2.1.6) and collect them in 100 to 150 µl of the same medium (see Note 8).

5. Drop 20 µl of cell suspension onto a slide, add 40 µl fixative, 80 µl detergent and another 80µl fixative (see Notes 9, 10).

6. Spread out the mixture with a glass rod and put slides in a chemical hood for several hours (see Note 11).

7. Continue with one of the procedures 3.4, 3.5.2, or 3.8, or freeze slides at –20°C for later use.

**3.2.4. Semi-spreading**

1. Produce a suspension of formaldehyde-fixed cells as described in procedure 3.2.2, steps 1-4.

2. Spheroplast cells with Zymolyase by following procedure 3.2.3, steps 2-4.

3. Drop 20 µl of cell suspension onto a slide, add 80 µl detergent and 80µl fixative (see Note 9).

6. Spread out the mixture with a glass rod and put slides in a chemical hood for several hours (see Note 11).

7. Continue with one of the procedures 3.4, 3.5.2, or 3.8, or freeze slides at –20°C for later use.

**3.2.5. Three-dimensional Preparation**
Embedding of intact cells in a polyacrylamide matrix for maintaining the three-dimensional structure of cells can be used in combination with immunostaining and/or FISH and confocal microscopy. This is beyond the scope of this treatise. Protocols are given in Dernburg and Sedat (7) and Atkin (8).

3.3. Chromosome Labelling With GFP

At present, there exist two published constructs for chromosome-specific GFP-labelling. Both are based on the heterologous expression in yeast of GFP-tagged repressor molecules which bind to tandem repeats of operator sequences inserted in chromosomal loci. One is based on the bacterial Lac repressor/operator system (9), the other on the bacterial Tet repressor/operator system (10, 11). GFP fluorescence can be seen with the appropriate filter combination after preparation procedures 3.2.1. to 3.2.4. (Figs 1 and 2).

3.4. Immunostaining of Synaptonemal Complexes

Synaptonemal complexes (SCs) run along the axes of paired homologous chromosomes at meiotic pachytene and thus can be stained to trace meiotic chromosomes. Zip1 is an abundant protein of the SC (12) and is particularly useful as an antigen (Fig. 2) (A yeast strain expressing a Zip1-GFP fusion protein, which can be used for direct visualization of the SC according to procedures 3.2.3 and 3.3 was constructed in the lab of David B. Kaback, UMDNJ, New Jersey.) Pachytene bivalents are best visualized after preparation procedure 3.2.3. (spreading) by which they become well separated (Figs 2, 3 and 4). The technique for immunostaining SC proteins is the same as for other cellular structures (staining of the microtubules as shown in Fig. 1 was done according to the same procedure).

1. Wash slides that have been prepared according to one of the procedures 3.2.2. – 3.2.4. twice for 5 min in 1× PBS and drain excessive liquid.
2. Incubate slides with a drop of primary antibody (diluted in 1x PBS; the appropriate dilution, usually 1:50 to 1:200, has to be tested empirically) under a coverslip at 4°C overnight.

3. Rinse coverslip away with 1x PBS, wash slides twice for 5 min in 1x PBS and drain excessive liquid.

4. Incubate slides with a fluorochrome-conjugated secondary antibody (diluted in 1x PBS according to the instructions of the provider) under a coverslip for 90 min at room temperature.

5. Rinse coverslip away with 1x PBS, wash slides twice for 5 min in 1x PBS and drain excessive liquid.

6. Mount preparation in anti-fading medium supplemented with DAPI (Fig 2).

3.5. (Multicolor) FISH

3.5.1. Labeling of the Probes

The probes are labeled by the incorporation of Cy3-dUTP, Cy5-dUTP, fluorescein-dUTP, tetramethylrhodamin-dUTP (direct labeling) or digoxigenin-dUTP or biotin-dUTP (indirect labeling, see Note 12), using standard nick translation procedures (see Notes 13, 14).

In the following, a typical labeling reaction is described.

1. Mix 1-2 µg DNA, 10 µl 10x labeling buffer, 3.5 µl 280 mM β-mercaptoethanol, 2.5 µl 1 mM dATP/dCTP/dGTP mixture, 2.5 µl 1 mM labeled dUTP, 2.5 µl DNase I (~1:1000 from a 1 mg/ml stock; the optimal dilution must be empirically determined), 2 µl E. coli DNA polymerase I (10U/µl), and bring the reaction volume to 100 µl with dH2O (see Note 15).

2. Incubate the reaction mixture at 15°C for 90 - 120 min.

3. Stop the reaction by adding 1µl 0.5M EDTA and/or incubation for 10min at 65°C.
4. Purify the labeled DNA through a spin column (Sephadex G50) (optional) and store at -20°C until use.

3.5.2. Hybridization

1. Incubate slide produced by procedures 3.2.3 or 3.2.4 in distilled water until the sucrose-layer has dissolved.
2. Drain the slide and let it dry.
3. Apply 50 µl RNase (100 µg/ml in 2× SSC) to each slide, cover with a coverslip, and incubate for 30 min at 37°C in a moist chamber.
4. Incubate the slide in ST buffer at 37°C for 1 - 3 hours.
5. To denature chromosomal DNA, place the slide in 70% formamide solution (in 2× SSC, pH 7.0) at 60°C for 2 min, then immediately immerse in ice-cold 70%, 90%, and 96% ethanol for 5 min each, and air dry.
6. Ethanol-precipitate labeled probe DNA (3.5.1) with 1/10 volume 3M LiCl and 2.5 volumes of ethanol for at least 30 min at -80°C.
7. Spin and resuspend the DNA pellet at a concentration of 10 - 30 ng/µl in hybridization mixture.
8. Denature the probe DNA by heating to 95°C for 5 min and then chill on ice.
9. Apply 5 -10 µl of denatured probe mix onto each slide (use same amount of mix for each probe if 2 or 3 probes are applied at the same slide), place a coverslip (18 × 18 mm) over the sample and seal with rubber cement.
10. Put slides onto a thermocycler for slides and expose them for 5-10 min to 80°C (co-denaturation of chromosomal DNA with probe mix) and then to 37°C for 16 - 48 h (hybridization).
11. Peel off the rubber cement and gently rinse off the coverslip with 2× SSC.
12. Wash slides for 5 min in each of the following buffers: 50% formamide in 2× SSC (37°C), 2× SSC (37°C), and 1× SSC (room temperature).

13. After hybridization with directly labelled DNA, apply a drop of antifade medium with DAPI, and seal under a coverslip (see 2.2. agents 4 and 5).

14. After hybridization with digoxigenin- or biotin-labelled DNA, go to 3.5.3. (signal detection).

### 3.5.3. Signal Detection

1. After washing the slides (3.5.2., step 12), put a large drop of blocking buffer under a coverslip and incubate slide for 1 hr at 37°C in a moist chamber.

2. To detect biotinylated probes, rinse coverslip away with detection buffer and incubate slides with 50 µl FITC- or Cy3-conjugated (Extra-) avidin diluted in detection buffer under a coverslip for 1 hr at 37°C. Continue with steps 4 or 9.

3. To detect of digoxigenin-labeled probes, apply FITC-, rhodamin- or AMCA- conjugated anti-digoxigenin antibody (see Note 16) and proceed as with applying secondary antibody in procedure 3.4 (immunostaining), steps 3-6.

4. Weak signals of a biotin labeled probe can be amplified as follows:

5. Rinse coverslip away with ST buffer, wash slides twice for 5 min in ST buffer and drain excessive liquid.

6. Incubate preparation with a biotin-conjugated anti-avidin monoclonal antibody for 1 hour at 37°C as above.

7. Rinse coverslip away with ST buffer, wash slides twice for 5 min in ST buffer and drain excessive liquid.

8. Incubate preparation with FITC- or Cy3-conjugated (Extra-) avidin as in step 2.

9. Rinse coverslip away with ST buffer, wash slides twice for 5 min in ST buffer and drain excessive liquid.
10. Mount preparation in anti-fading medium supplemented with DAPI (Fig 3).

3.6. Simultaneous Immunostaining and FISH

1. Take pictures of immunostained cells (3.4) and record their coordinates.
2. For subsequent FISH, rinse off the coverslip with 1× PBS.
3. Fix cells for 5 – 10 min in fixative II.
4. Apply the standard FISH procedure (3.5.2, 3.5.3). In most cases, immunostaining is retained after FISH. If it has faded, however, merge FISH images electronically with the corresponding images previously taken from the same coordinates of immunostained nuclei (see ref. 13).

3.7. Microscopic Evaluation of Flurescent Signals

For GFP-, immunostaining and FISH an epifluorescence microscope equipped with a mercury lamp and appropriate filter sets for the excitation and emission of fluorescence spectra characteristic for the GFP-variants and fluorochromes used, is necessary to visualize signals. There are different mutant GFP proteins with modified spectroscopic properties available (14). The optimal combinations of excitation filter, beam splitter and emission filter must be tested. Filter selection is a compromise between narrow band width (weaker signals) and wide spectrum (other fluorochrome may "leak through"). See contribution by Lichter and Ried in this volume, Loidl et al. (15). Images are best recorded with a cooled CCD camera with high sensitivity to a wide spectrum of wave-lengths, including far-red (as emitted by Cy5).

3.8. Silver Staining of Synaptonemal Complexes (6)

1. Incubate slide (from procedure 3.2.3) in distilled water until the sucrose-layer has dissolved.
2. Drain the slide and let it dry (see Note 17).
3. Apply several drops of AgNO$_3$ solution to the slide and cover with a piece of polyamide cloth, trimmed to the size of a coverslip (see Note 18).

4. Incubate slides in a moist chamber at 60°C for 40 min.

5. Rinse away the cloth with distilled water and allow the preparation to dry.

6. Inspect the slide (see Note 19) without embedding under a coverslip, as most microscopical mounting media will bleach or dissolve the silver deposit. For prolonged storage, immersion oil can be removed from the slide by 1-3 min incubation in hexane.

3.9. Transfer of light-microscopical preparations to the electron microscope (EM)

Silver-impregnation produces highly contrasted structures which are suitable for inspection in the electron microscope (Figs 4, 5a). For this purpose, preparations on glass slides can be transferred to electron microscopical grids.

1. If required, remove immersion oil by incubating the slide for 1-3 min in hexane.

2. Dip slide in Formvar solution and retract slowly such that a thin plastic coating will deposit on the slide (see Note 20).

3. Identify at low magnification (no oil immersion!) regions of interest and mark them with a water-resistant marker pen.

4. Scratch the coating with a diamond glass-writer around the area with the markings. Put small drops of 1% hydrofluoric acid onto the slide; it will dissolve the glass surface and help to detach the plastic film together with the cells from the slide. Caution: Hydrofluoric acid and its vapours are corrosive and toxic. Work in a chemical hood and wear gloves and safety goggles!

5. Add water until the plastic film floats. Submerge the slide in a large bowl with water such that the coating will come off and float.

6. Place EM grids on the marked regions of interest.
7. Push the plastic film together with the grids under the surface with the edge of a piece of Benchkote paper (Schleicher & Schuell, Dassel, GER) such that the plastic film will attach to the smooth side of the paper and the EM grids will become sandwiched between the plastic film and the paper.

8. After the film has dried, pick off the EM grids together with the adhering plastic film with fine tweezers. For spread SCs in the EM see Figs 4 and 5a.

3.10. Electrophoretic Karyotyping

Pulsed-field gel electrophoresis (PFGE) of chromosome-sized DNA molecules can be regarded as a non-microscopical cytological technique. It allows the recognition of karyotypic features, which to identify by genetical approaches would be more complicated. For example, size changes of individual chromosomes due to duplications or deletions are easily seen. They occur quite frequently in chromosome XII due to unequal mitotic and meiotic recombination between the rDNA tandem repeats. Reciprocal translocations are diagnosed by the simultaneous gain and loss of pieces of the same size on the exchange partners which then appear as bands at new positions. Similarly, yeast artificial chromosomes (YACs) can be identified as additional bands. Aneuploidies (the loss or addition of chromosomes of the standard karyotype) are detectable. Also different laboratory strains or wild types of Saccharomyces cerevisiae feature considerable karyological differences, not to speak of differences between sibling species (yeast taxonomy).

In our laboratory we use a modification of the protocol by Gerring et al. (16) for the preparation of chromosome-sized DNA molecules and their separation by contour-clamped homogeneous electric field (CHEF) electrophoresis (15). Shortened versions of the protocol for DNA preparation with abbreviated or even without Zymolyase treatment were described by Ibeas and Jimenez (17) and Goldman and Lichten (18). Detailed protocols for PFGE are given in the contribution by Maule in this volume. Therefore we can here limit ourselves to
pointing out the use of PFGE as a valuable tool for yeast chromosome analysis. Fig. 5b shows a chromosomal translocation as detected by PFGE.

4. Notes

1. The Zymolyase stock can be stored at –20°C for several months and repeatedly frozen and thawed. The powder does not dissolve completely, therefore after thawing a pellet is present which has to be stirred up before use.

2. Lipsol is a laboratory cleaning agent, a mixture of nonionic and anionic detergents plus a chelating agent and builders (information by the manufacturer). Several standard laboratory detergents (Nonidet, Triton X-100, sodium dodecyl sulfate, N-lauroyl sarcosine) were tested as alternatives but gave unsatisfactory results in our hands.

3. 4 g paraformaldehyde is heated in 90 ml distilled water on a magnetic stirrer to 80°C (Caution: Formaldehyde vapours! Work under fume hood!). After 20-30 min the solution should become clear. If it stays opaque, add 1 M NaOH until it becomes clear. Add 3.4 g sucrose to the solution after cooling. If NaOH has been added, the solution has to be titrated back to pH 8.5. Fill up volume to 100 ml. If the fixative is not completely clear, it may be filtered. It can be stored for several months in the refrigerator.

4. It is convenient to prepare antifade medium containing 0.5 µg/ml DAPI so that embedding and DAPI staining can be done in a single step.

5. FISH probes should be carefully selected and their sequence be checked against the database to avoid the inclusion of repetitive genomic elements (e.g., transposons), which would result in unspecific speckled background staining.

6. Working solution can be stored for several months in the refrigerator. If a black or brown deposit is formed, the solution should be filtered. Old AgNO₃ or used AgNO₃ solution
should not be disposed to the environment. Check with a recycling lab; it can be collected together with photographic fixer for the recycling of silver.

7. Presporulation growth in YPA was introduced by Roth and Halvorson (19) to enhance synchronicity of sporulation.

8. This suspension can be stored on ice (for up to 1 day) until used for the preparation of slides.

9. The presence of sucrose in the fixative has the additional advantage that the mixture is hygroscopic and does not dry out completely. Therefore this kind of preparations can be used for immunostaining even after prolonged storage in the refrigerator.

10. Fixative is added to the slide before and after the detergent. A small amount of fixative present during detergent spreading prevents the disruption of spheroplasts but does not interfere too much with spreading. The relative amounts and order of application of nuclear suspension, detergent and fixative should be optimized by testing since the optimal spreading depends on the density of nuclei in the suspension, the degree of spheroplasting and the age of solutions. The process of spreading can be watched in the phase contrast microscope at small magnification without a coverslip. Spheroplasts should swell slowly, and turn continuously from white to black and then to grey. They should not "explode" instantly!

11. After spreading and semi-spreading (procedures 3.2.3 and 3.2.4), cells readily stick to the slides owing to their large surface. It is not necessary to coat slides with polylysine.

12. Since nucleotids tagged with fluorochromes (Cy3-dUTP, Cy5-dUTP, fluorescein-dUTP) have become commercially available, direct probe labeling is becoming more and more common and it is sufficient for most applications. However, if numerous different fluorochromes are needed for multicolor FISH or if fluorescence intensity of directly labelled probes is insufficient, it may be useful to work with indirectly labelled probes.
13. Direct labeling of the DNA probe with the fluorescent tags Cy3, Cy5 or FITC usually produces a sufficiently strong signal to be readily detected. For small probes, indirect labelling is recommended.

14. Due to the occasional unspecific staining of the nucleoli caused by the use of antibodies it is recommended to use directly labeled probes for FISH experiments on semispread (procedure 3.2.4.) nuclei.

15. Labeling reactions should be optimized to give labeled products of 100 - 500 bp length, by adjusting the DNase I concentration. The size of the product should be monitored on an agarose gel.

16. AMCA is a blue fluorescent dye. It cannot be used in combination with DAPI as a DNA counterstain. Use propidiumiodide (red) instead of DAPI.

17. Prior to Ag-staining, slides should be allowed to dry for several hours.

18. Polyamide cloth produces a homogeneous staining all over the coated area, whereas by using a coverslip instead, regions near the edges are stronger stained than the interior. A possible explanation for the enhancing effect, based on the chemical interaction of the polyamide with the staining reaction, is given by Herickhoff et al. (20).

19. If the silver staining turns out too pale, modify protocol by transferring slides to sodium tetraborate buffer (pH 9.2; Merck, Darmstadt, GER) for 30 seconds after step 1. Also, using different brands or batches of nylon cloth may have an influence on staining intensity. Increasing the time or temperature of the incubation with siver nitrate solution is not recommended as it tends to enhance unspecific precipitation of silver.

20. Work in a dry environment. Moist slides may cause holes in the plastic coating.

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References


Fig. 1. Double labelling of a mitotic yeast cell with anti-tubulin antibody (procedure 3.4) and GFP (procedure 3.3). Tet Repressor-GFP fusion protein binds to chromosomal sites containing repeats of the Tet Operator sequence (in this case close to the centromeres and to the telomeres of the long arm of chromosome V). Diploid cell at anaphase fixed according to procedure 3.2.2. a. DAPI staining of the nucleus. The contours of the mother cell and the bud are also visible. The nucleus has partially passed through the bud neck. Note also the DAPI-positive thread-like mitochondria. b. Chromosomal GFP signals plus DAPI counterstain. c. Anaphase spindle highlighted by anti-tubulin. Nucleus counterstained with DAPI. d. Interpretative drawing of the nucleus with segregating anaphase chromatids (black) moving with their centromeres at the leading edge of the spindle (grey) and carrying GFP-labelled Tet Operator repeats (white).
Fig. 2. Yeast pachytene bivalents prepared according to the SC spreading method (3.2.3). SC are labelled by anti-Zip1 immunostaining (procedure 3.4). In addition, the paired chromosomes V carry GFP-labelled Tet Operator repeats (bright spots – procedure 3.3, see also Fig. 1).

Fig. 3. Yeast pachytene karyotype. Yeast meiotic nuclei were spread according to procedure 3.2.3, centromeres (a) and telomeres (b) labelled with specific probes (21) by the FISH protocol (3.5), and counterstained with DAPI. Bivalents were excised and arranged according to decreasing size. The bulge on the second bivalent corresponds to the nucleolus (bivalent XII).

Fig. 4. Electron micrograph of spread yeast synaptonemal complexes stained with silver (procedures 3.2., 3.8 and 3.9). The electron-dense mass on top is the nucleolus which is associated with bivalent XII. Bar represents 2 µm.

Fig. 5. Cytological examination of a chromosomal translocation. (a) Heterozygous translocation quadrivalent t(VII/XVI) (see ref. 13) seen in the electron microscope after SC spreading (procedures 3.2.3, 3.8 and 3.9). (b) Translocation t(VII/XVI) as seen on a CHEF gel (see 3.10). Left lane: translocation strain, right lane: wild type (strain SK1). The two new translocation chromosomes (arrows) are larger and smaller, respectively, than the two wild-type chromosomes VII and XVI (open arrowheads). In addition, a polymorphism of chromosome IX is visible (see ref. 22). Fewer than 16 bands are visible because several chromosomes cannot be resolved as individual bands.
Figure 1