

Video Article

Surface Spreading and Immunostaining of Yeast Chromosomes

Jennifer Grubb¹, M. Scott Brown^{1,2}, Douglas K. Bishop^{1,2}

¹Department of Radiation and Cellular Oncology, University of Chicago

²Department of Molecular Genetics and Cell Biology, University of Chicago

Correspondence to: Douglas K. Bishop at dbishop@uchicago.edu

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Abstract

The small size of nuclei of the budding yeast *Saccharomyces cerevisiae* limits the utility of light microscopy for analysis of the subnuclear distribution of chromatin-bound proteins. Surface spreading of yeast nuclei results in expansion of chromatin without loss of bound proteins. A method for surface spreading balances fixation of DNA bound proteins with detergent treatment. The method demonstrated is slightly modified from that described by Josef Loidl and Franz Klein^{1,2}. The method has been used to characterize the localization of many chromatin-bound proteins at various stages of the mitotic cell cycle, but is especially useful for the study of meiotic chromosome structures such as meiotic recombinosomes and the synaptonemal complex. We also describe a modification that does not require use of Lipsol, a proprietary detergent, which was called for in the original procedure, but no longer commercially available. An immunostaining protocol that is compatible with the chromosome spreading method is also described.

Video Link

The video component of this article can be found at <https://www.jove.com/video/53081/>

Introduction

The budding yeast *Saccharomyces cerevisiae* offers many advantages for studies of molecular mechanisms of biological processes, including the study of proteins that control chromosome function. Although there are well-known advantages of budding yeast for genetic, molecular, and biochemical studies, cytological studies of the distribution of proteins in the cell's nucleus is complicated by its small size. The typical yeast nucleus has a diameter of less than one micron, which is only about 5 times the resolution limit of visible light. Thus, the amount of information about the distribution of nuclear proteins that can be obtained from conventional immunostaining or by using fluorescent protein tags, such as green fluorescent protein (GFP), is limited. A useful approach to characterizing the subnuclear distribution of proteins is chromosome surface spreading. This approach involves removing the cell wall, disrupting cell and nuclear membranes, and allowing the insoluble contents of the nucleus to settle onto the surface of a microscope slide. These insoluble components include the nuclear matrix and the chromosomes. In addition to allowing removal of soluble nuclear contents, which enhances the ability to detect chromatin bound proteins, the chromosome spreading method results in substantial decompression of chromosomes such that the spread nuclei have diameters of around 3 to 5 μ m (for diploid meiotic nuclei) and 2 to 3 μ m for diploid mitotic nuclei. This decompression allows detection of nuclear substructure that is relatively difficult or impossible to resolve in intact nuclei.

An obvious shortcoming to chromosome spreading is the possibility that the spreading procedure may partially or completely disrupt the structure of interest. Of particular concern is that a particular chromosome bound protein might be lost as a consequence of the spreading procedure. This potential complication should be kept in mind when interpreting data. One example of a protein that is sensitive to the spreading procedure is beta-tubulin. Under some conditions, the spindle, which is comprised mainly of tubulin, is preserved during spreading³. Visualization of the spindle is often useful to stage nuclei of interest. However, visualizing tubulin requires treatment with a high concentration fixative; spindles are lost under the standard conditions described below. This example illustrates that, when analyzing the distribution of a previously uncharacterized protein, it is important to vary the concentration of fixative to determine how sensitive the protein is to such variation. In spite of the concern regarding the impact of the spreading conditions on chromosome structure, the utility and power of the spreading method has been demonstrated in many contexts and has broad utility in characterization of mitotic and, especially meiotic cells⁴⁻⁷.

Two spreading methods have been used extensively. The first of these methods, developed by Dresser and Giroux⁸, avoids the use of detergent and can yield spread preparations that appear to have relatively well-preserved chromosome morphology when stained for the DNA-specific dye DAPI. However, this method is relatively difficult to perfect and the quality of the spread nuclei varies dramatically, when one region of a slide is compared to other regions. This problem can complicate quantitative approaches that involve imaging many unselected nuclei from one slide to avoid data acquisition bias. The second chromosome spreading method, developed by Loidl and Klein¹, involves balancing fixation by paraformaldehyde, with lysis and chromatin decompression promoted by a detergent solution. When properly performed, this method gives very reproducible results with less region-to-region variation compared to the Dresser and Giroux method. This presentation focuses on a modified version of the method of Loidl and Klein, because of its reliability and simplicity.

Chromosome spreading is not complicated or time-consuming; up to 100 slides can be prepared for immunostaining in a single day. Furthermore, the spread preparations may be stored in the freezer for years prior to immunostaining, and thus labs can develop a repository of frozen chromosome spreads that can be used when new biological questions arise or new staining reagents become available.

The chromosome spreading method is most commonly used in combination with immunostaining and widefield fluorescence microscopy, but it is also possible to prepare slides for super-resolution light microscopic methods such as stimulated emission depletion (STED) microscopy.

Protocol

NOTE: Some steps of the protocol below require working in a clean fume hood. In addition, the method requires a yeast tetrad dissection microscope equipped with a 10X long working distance objective to monitor spheroplasting. The microscope should be set up in the hood ahead of time. Remove the micromanipulator arm and plate holder from the scope and place these items in a safe place away from the work area.

1. Preparation of Spheroplasts

1. Grow yeast under desired conditions. Use about 2×10^8 cells for each sample, e.g. 4 ml of a culture at OD600 1.4 = 5×10^7 cells/ml. Although immediate processing of samples may be preferable in some cases, for most applications, cell aliquots can be stored on ice for up to 8 hours before being processed.
2. Transfer cell suspension to a 15 ml centrifuge tube and spin in a clinical centrifuge at setting 5 (2345 rpm/857.5 x g) for 3 min to pellet the cells.
3. Decant the medium taking care not to lose cells from the pellet. Gently resuspend the cells in 1 ml ZK buffer and then add 40 μ l 1 M dithiothreitol (DTT). Incubate 2 min with gentle mixing. Pellet cells as before (see step 1.2).
4. Resuspend pellets in 1 ml ZK buffer. Add 5 μ l of a freshly prepared solution of zymolyase 100T that has been thoroughly mixed. Incubate for 20-30 min at 30 °C to remove the cell wall and produce spheroplasts.
5. Assay a 10 μ l droplet of cell suspension on a microscope slide to determine if spheroplasting is complete. Cells viewed under the dissection microscope without a coverslip. Cells should appear bloated and round rather than slightly oblong and should lyse following addition of 20 μ l of water. If cells fail to lyse, return the sample to 30 °C and re-assay every 10 min until water induced lysis is readily observed. Pellet cells as before (see step 1.2).
6. Gently resuspend and wash cell pellet in 2.5 ml cold MES/sorbitol buffer. Pellet cells as before (see step 1.2). Gently resuspend cell pellet in 300-400 μ l cold MES/sorbitol buffer. Cells can be kept on ice at this stage for several hours.

2. Chromosome Spreading

NOTE: The glass surfaces upon which chromosomes will be spread—either slides or coverslips—should be prepared ahead of time. Each slide or coverslip should be submerged in water, then EtOH, then allowed to dry, and polished with lens paper. If chromosomes will be spread on coverslips, the coverslip should be affixed to a slide with Scotch tape or rubber cement. Unless otherwise mentioned, the rest of the protocol will describe spreading on either a slide or coverslip

1. Using a P20 pipettor, pipet 20 μ l of cell suspension onto the surface of a clean slide.
2. Using a P200 pipettor, add 40 μ l of the 3% PFA/sucrose solution and gently mix the solution by “swirling” the slide with the hand until Schlieren lines disappear. Place slide under microscope and confirm that cells are in focus. The PFA solution should be freshly-prepared in the fume hood on the day of use.
NOTE: If the protein of interest has not been examined by the method previously, it is advisable to prepare additional slides using solutions that contain 2 and 4% PFA to determine if retention of the protein is sensitive to fixation conditions. A 4% PFA/sucrose solution is used in cases where it is desirable to visualize associated tubulin microtubules. The disadvantage of the higher fixative concentration is that the chromosomes will be “underspread,” 2%PFA also tends to yield “underspread” chromosomes.
3. Using a P200 pipettor, add 80 μ l of 1% Lipsol, swirl as before to mix solutions as completely as possible, start a timer. If Lipsol is not available, 80 μ l 1% NP40 or 80 μ l dH₂O may be substituted (see Representative Results, Figure 2).
4. Watch the cells carefully and gently swirl the slide every 15 sec. When roughly 80% of cells have lysed (disappeared) stop the timer, immediately remove the slide from the microscope, add 80 μ l of PFA/sucrose solution, and swirl to mix. Lysis should occur from between 30 and 90 sec after starting the timer.
NOTE: If several slides show lysis at about the same time, one can eventually omit microscopic monitoring for the remainder of the slides and simply carefully time the period between addition of lipsol and addition of the final aliquot of fixative. However, this short cut is only reliable when preparing multiple slides from the same sample. It is best to monitor lysis of each sample microscopically when different slides are prepared from samples taken at different times or from different cultures.
5. Place the slide on a clean flat surface and spread the liquid across the entire unfrosted surface of the slide using the side of a clean, disposable Pasture pipet held below the meniscus of the “puddle” but above the surface of the slide itself, i.e. do not “rake” the surface of the slide with the pipet. Do not reuse pipet on different slides to avoid contamination of samples.
6. Leave the slides to dry overnight in the fume hood. Insoluble nuclear components will settle onto the slide surface and bind to it. For large experiments, planning the use of space for the drying step is important.
7. Once dried, optimal results are obtained by progressing to the immunostaining stage on the same day. However, the dried sucrose solution embeds the spread chromosomes in a “honey” that allows freezing of samples: slides can be transferred to a plastic slide box and stored at -20 °C for years.

3. Immunostaining

1. Dip slides in 0.2% Photo-Flo for 30 sec. to remove honey. Lean slides on an edge, with the edge resting on a paper towel, to remove residual Photo-Flo.
2. Dip slides in 1x TBS for 5 min to wash. Remove excess liquid by leaning slides on an edge, but do not let them dry out.
3. Lay slides frosted side up, pipet 300 μ l TBS/BSA onto the slide across the unfrosted portion of the slide.
4. Incubate in a moist chamber at RT for 15 min. (large plastic container lined with water saturated paper towels under a perforated metal plate or plastic slide box with saturated paper towels).
5. Drain slides by resting a short edge of the slide on a paper towel and leaning the slide against an appropriate support (such as a test tube rack). Do not allow surface to dry.
6. Immediately apply 80 μ l of TBS/BSA buffer containing the appropriate dilution of primary antibody. If antibody is limiting, use 40 μ l and a 22 x 22 mm coverslip. For crude rabbit serum, this is typically between a 1/50 and 1/500 dilution.
NOTE: Perform titrations for new antibody preparations. The most dilute preparation that yields high signal above background is chosen. To control for the level of background staining, nuclei should be prepared from the appropriate deletion mutant strain and/or duplicate slides should be stained with pre-immune serum.
7. Place a polished 22 x 50 mm coverslip on the slide avoiding bubbles. Do this by holding the coverslip between the thumb and index finger along the long edge at positions near one short edge. Holding the coverslip at a 30° angle relative to slide, the short edge farthest from the fingers is lowered until it rests on the slide just inside the edge of the "puddle" closest to the frosted region of the slide. Then slowly lower the coverslip in a smooth motion until the fingers holding the slide touch the bench, and then release. Do not attempt to adjust the position of the coverslip once it lands.
NOTE: If chromosome spreads were prepared on affixed coverslips (rather than directly on the surface of slides), this sample coverslip will remain affixed to the slide throughout the staining and washing steps. An additional coverslip will be placed on top of the sample coverslip to evenly distribute the antibody solution during staining and then discarded.
8. Place slides in a sealed moist chamber and incubate overnight at 4 °C.
9. Holding the frosted edge transfer the slides to a glass slide staining rack and submerge in a staining jar containing TBS.
10. Gently dip the slide up and down to remove the coverslip. Try not to use too much force to do this. Wash the slides 2x 10 min by submerging in TBS. Remove slides from rack and drain excess liquid by touching edge to paper towel. Do not let surface dry.
11. Working under subdued light, immediately add 80 μ l TBS/BSA containing a 1/1,000 fold dilution of fluorochrome conjugated secondary antibody (or 40 μ l with a 22 x 22 mm coverslip). Add coverslip as before and incubate at 4 °C for 2 hr in the moist chamber in the dark.
12. Remove coverslips as before, drain slides, and allow surface to air dry for 1 to 2 hr in the dark. Lean slides on paper towels as before.
NOTE: If spreading was performed on a mounted coverslip, detach the coverslip from the slide before drying. Coverslips are then dried as described for slides in the previous step.
13. Working under subdued light, add about 30 μ l of mounting medium containing DAPI (three 10 μ l droplets) and then carefully lower a coverslip. If the spreads are on coverslips, place the droplets of mounting medium on a clean slide and lower the coverslip, chromosome side down, onto the slide.
14. Still under subdued light, wait 2 min for coverslip to settle and seal edges of coverslip with clear nail polish. Place slides in a flat slide box.
NOTE: For STED microscopy, mount with ProLong Gold. Use 30 μ l of ProLong Gold without DAPI and allow to cure overnight. Sealing with nail polish is unnecessary.
15. View slides by widefield epifluorescence microscopy with a 40 or 100X objective using a filter set appropriate for DAPI to focus. Optionally store the slide in the dark at 4 °C for several weeks. Do not freeze.

Representative Results

The appearance of spread nuclei critically depends upon the balance between chromosome fixation and de-compaction. Even when the reagents are properly balanced, variation in the degree of chromosome de-compaction can occur in different regions of the same slide and/or between different slides. Thus, the quality of spreads in a given region of a slide should be assessed before images are interpreted.

The effects of "overspreading" and "underspreading" can be illustrated using antibodies against meiotic recombination proteins. In **Figure 1**, meiotic nuclei are double immunostained for the two eukaryotic strand exchange proteins Rad51 and Dmc1 and counter stained for DNA with DAPI. Two examples of optimally spread nuclei are shown in **Figure 1A**. **Figure 1B** shows an example of an underspread nucleus and **Figure 1C** examples of two overspread nuclei. Note that fewer foci of Rad51 and Dmc1 are observed in both over and underspread nuclei compared to properly spread nuclei. In the case of underspread nuclei, some foci are out of focus because the sample is not sufficiently flat. In addition, epitope accessibility may contribute to the failure to detect all foci. Furthermore, the smaller diameter of the spread can preclude resolution of closely spaced structures. In the case of overspread nuclei, protein may be lost because of insufficient fixation and/or excessive detergent treatment.

Variations of the standard method can avoid the use of Lipsol. The original version of the spreading method described by Loidl et al. makes use of Lipsol, a propriety laboratory glassware detergent¹. Although this detergent is currently used for chromosome spreading in many laboratories, the original formulation is no longer commercially available and the product currently sold under that name has been re-formulated. Furthermore, the original formula for Lipsol is also not available (Franz Klein, personal communication). In an effort to overcome this problem, we carried out experiments in which the commercially available and chemically defined detergent NP40 was used in place of Lipsol; this modification of the standard protocol was found to give satisfactory results for an experiment in which spread meiotic nuclei were stained for Rad51 and Zip1, a component of the central region of the synaptonemal complex (**Figure 2A**). Interestingly, replacing the Lipsol solution with the same volume of dH₂O also yielded satisfactory results (**Figure 2B**). Although these findings indicate that the spreading method described above may be employed successfully without Lipsol, it is possible that some previously described results depend on use of Lipsol and will not be reproducible if NP40 or H₂O is used in its place. An individual learning the spreading method might reasonably choose to begin by using NP40 and/or H₂O in place of Lipsol. In the event that difficulties are encountered, the investigator may request an aliquot of Lipsol from a lab that obtained a stockpile

of the reagent before it was discontinued; Lipsol was inexpensive and the minimum amount one could order was sufficient to generate millions of slides.

The spreading method is suitable for use with super-resolution light microscopy methods. The synaptonemal complex consists of two linear axial/lateral elements each of which organizes a pair of sister chromatids into a set of loop arrays, with the base of each loop bound to an element. Pachytene chromosomes have synapsed pairs of lateral elements held in parallel at a distance of 100 nm by proteins that form the central region of the synaptonemal complex. These paired lateral elements cannot be resolved by conventional widefield epifluorescence microscopy, but can be resolved by super-resolution methods. In **Figure 3**, a pachytene nucleus is shown that is stained with the same antibody for Zip1 protein, used for the experiment in **Figure 2**. The sample was also stained for Red1 protein, a component of axial/lateral elements. The result reveals that the Zip1 antibody recognizes the lateral element binding region of Zip1, rather than the elongated alpha helical coiled-coil domain that lies between lateral element binding regions. Thus, the staining pattern observed with this Zip1 antibody reveals the parallel paths of the paired lateral elements. The distribution of Red1 foci along lateral elements is relatively sparse compared to Zip1 foci, but the double staining method shows that Red1 foci generally lie near the linear paths defined by Zip1 foci. These findings are consistent with previous work in which the same spreading method was used to prepare samples for analysis by electron microscopy; the tripartite structure of the synaptonemal complex is preserved during the spreading procedure. The image shown in **Figure 3** was generated by STED microscopy⁹.

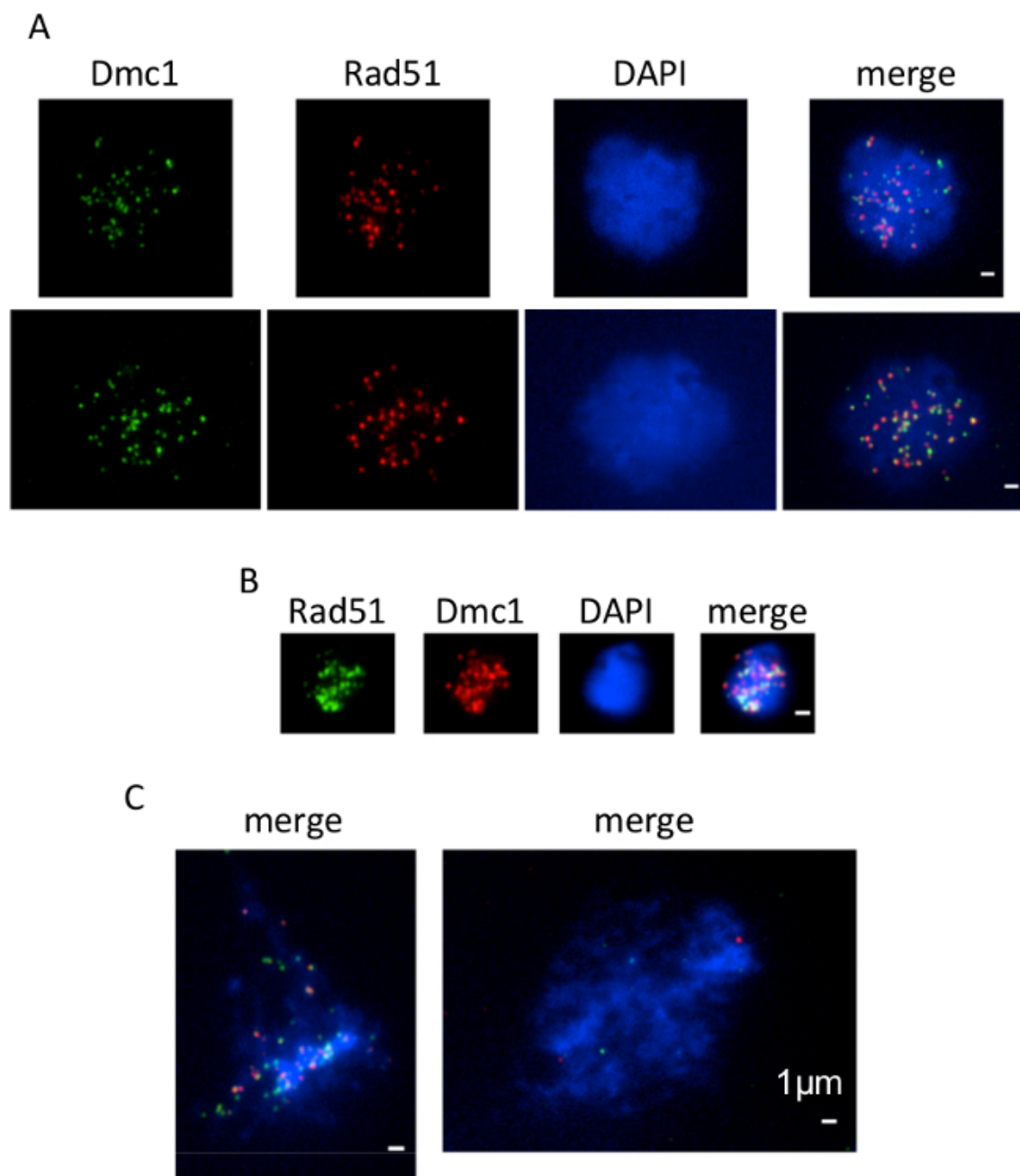
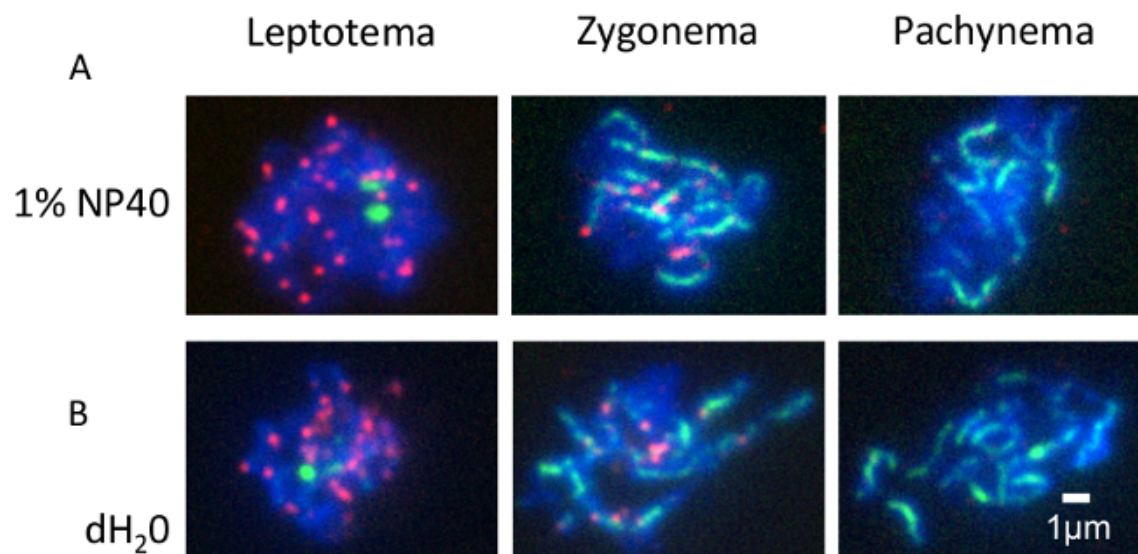
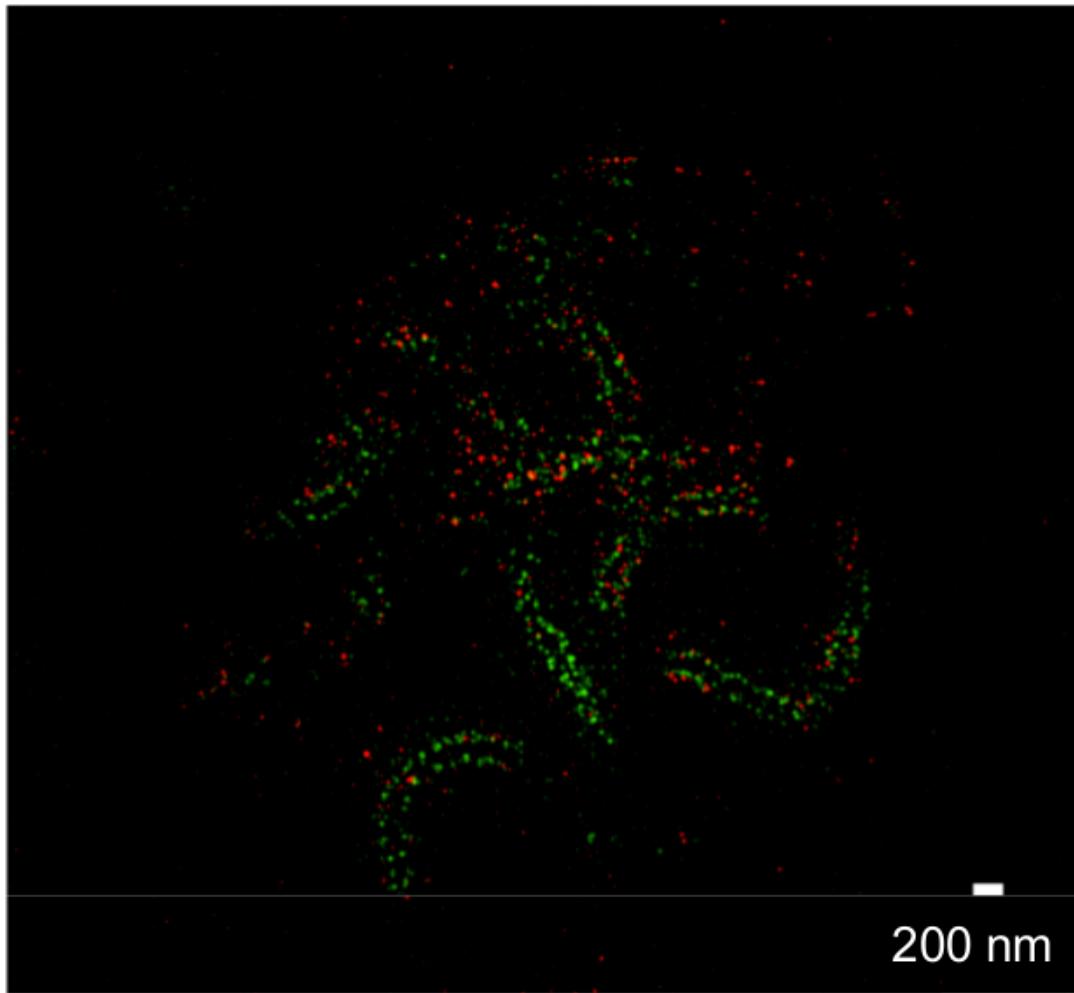


Figure 1: Examples of spread nuclei. Meiotic nuclei stained for Dmc1 (green), Rad51 (red), and DNA (DAPI, blue). Bar = 1 μ m. **(A)** 2 examples of optimally spread nuclei. **(B)** An example of an underspread nucleus. **(C)** Two examples of overspread nuclei. The example on the left is a nucleus in which only the upper portion is overspread.



Rad51=red, Zip1=green, DAPI=blue

Figure 2: Meiotic nuclei prepared without use of Lipsol. Spread meiotic nuclei from the stages indicated stained for Rad51 (red), Zip1 (green), and DNA (DAPI, blue). Note that the chromosome compaction that occurs as cells transition from leptotema to pachynema is largely preserved.



Red1=red, Zip1=green

Figure 3: Visualization of the synaptonemal complex via STED microscopy. A pachytene nucleus stained for Zip1 (green) and Red1 (red). The data were processed using the DeconvolutionLab ImageJ plugin¹⁰ to run 100 iterations using the Richardson-Lucy model with a measured STED PSF.

Discussion

The appearance of spread nuclei critically depends upon the balance between fixation and lysis/detergent treatment. As discussed above, the preservation of varying cellular structures requires the use of different PFA concentrations. For most proteins, 3% PFA is optimal. However, preservation of spindles requires use of 4% PFA. Even with a single set of reagents, the timing of lysis relative to fixation can also affect the quality of spread nuclei. For the most consistent results, the time of lysis should be normalized for each spheroplast sample by direct observation of lysis with a light microscope. To the trained eye, round spheroplasts darken as they swell before lysis; after lysis cellular remnants appear as floating “ghosts”. The combination of experimentally optimized reagent concentrations, and inter-sample standardization of timing, yields consistently well-spread nuclei.

The choice of fluorophore and microscope used to visualize spread chromosomes depends on the desired structure. In general, spread nuclei are very thin (about 200 nm or less) and soluble protein is removed by the procedure. Thus, the reduction of out-of-focus fluorescence provided by confocal or total internal reflection fluorescence (TIRF) microscopy yields little benefit. In fact, if manual focusing reveals staining in multiple focal planes, the nucleus is likely underspread. Although any fluorophore compatible with the microscope can be used for staining, shorter wavelength fluors will improve spatial resolution. For example, spatial resolution progressively decreases as the same structure is stained with a green (Alexa 488), red (Alexa 594), far-red (Alexa 647), or infrared (Alexa 750) fluor. This relationship should be considered in the planning and interpretation of experiments. If an even greater level of structural detail is desired, a super-resolution microscope is required. The details of the staining procedure required for each of these applications differ from those described above and the appropriate staining protocol should be followed⁹.

Finally, although direct spreading of nuclei on microscope slides is relatively easy, spreading on slide-mounted coverslips yields significantly improved resolution as it avoids viewing the chromosomes through a layer of mounting medium that contains a high concentration of glycerol.

Mounting coverslips on slides requires more dexterity and patience than spreading on slides, but this extra effort can be worthwhile depending on the type of structures being analyzed.

Disclosures

The authors declare no competing financial interests.

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