A protocol for measuring mitotic chromosome condensation quantitatively in fission yeast cells

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This is the unedited version of the manuscript published in final form in Methods in Molecular Biology, Volume 1515, 245-255, 29. October 2016.

Summary

Even though the formation of compact cylindrical chromosomes early during mitosis or meiosis is a prerequisite for successful segregation of eukaryotic genomes, little is known about the molecular basis of this chromosome condensation process. Here, we describe in detail the protocol for a quantitative chromosome condensation assay in fission yeast cells, which is based on precise time-resolved measurements of the distances between two fluorescently labeled chromosome positions. In combination with an automated computational analysis pipeline, this assay enables the study of various sets of candidate proteins for roles in regulating genome topology during cell divisions.

1. Introduction

The formation of compact rod-shaped chromosomes during mitotic and meiotic cell divisions is a key step during the segregation of eukaryotic genomes. The molecular mechanisms behind the chromosome condensation process have, however, remained largely elusive. One major reason for the still limited understanding of chromosome condensation is the shortage of dedicated assays that can capture the highly dynamic nature of this process with quantitative precision.

A number of different methods, based either on imaging or molecular biology approaches, have been developed to monitor the changes in chromosome architecture that take place during chromosome condensation in different eukaryotic model organisms (**Table 1**). Even though the expansion of novel high-throughput molecular biology technologies, including proximity ligation methods like 5C and Hi-C, have provided important new insights into the architecture of mitotic chromosomes ***(1)***, these methods are limited by the temporal resolution that can be achieved by synchronizing cell populations. Live cell imaging, in contrast, captures the dynamics of chromosome condensation in single cells in real time and is therefore the method of choice for studying the temporal and spatial features of condensation.

Here, we describe in detail a protocol for quantitatively measuring mitotic chromosome condensation dynamics in live fission yeast cells. Unlike the relatively short chromosomes of the budding yeast *Saccharomyces cerevisiae* ***(2)***, the three chromosomes of the fission yeast *Schizosaccharomyces pombe* undergo pronounced longitudinal compaction during cell divisions, irrespective of the genomic positions that are being monitored ***(3)***. *S. pombe* furthermore combines the ease of genetic engineering of yeast cells with genomic features that are more similar to those of metazoan cells, including complex centromeres ***(4)***, organization of chromosome arms into structured globular domains ***(5)***, and RNAi-dependent heterochromatin formation ***(6)***. In addition, mitotic chromosome condensation in fission yeast and mammalian cells share common characteristics. For example, chromosomes in fission yeast cells arrested at a pre-anaphase stage continue to condense, similar to

Method	Key variable(s)	Model organism(s)	References
Imaging-based methods			
Whole-chromatin labeling (Hoechst or histone-FP)	Intensity, distance	Human cultured cells	Reviewed in ***(19)***
Histone-YFP and -CFP	FRET, FLIM	Human cultured cells	***(21)***
FROS	Euclidean distances	Budding and fission yeast	***(2, 18)***
Molecular biology-based methods			
Histone site-specific cross-linking	Cross-linking efficiency by western blotting	Budding yeast	***(22)***
5C and Hi-C	DNA proximity ligation probability	Human cultured cells	***(1)***

Table 1. Overview of methods used to measure chromosome condensation.

those in human cells ***(3)***. This is in contrast to budding yeast chromosomes, which have been reported to lose their condensed architecture under these conditions ***(2)***. Fission yeast is hence an ideal system for the study of chromosome condensation in a model organism amenable to genetic screens.

However, the small size of mitotic fission yeast chromosomes – when compared to those of mammals – and their dense packing within the nucleus during 'closed' mitotic divisions make it difficult to resolve whole chromosomes by conventional light microscopy. The chromosome condensation assay we describe is therefore based on labeling two discrete loci located on the same chromosome using a fluorescent repressor–operator system (FROS) ***(7)***. FROS takes advantage of the specific binding of the *Escherichia coli* lac repressor protein (LacI) to the lac operator sequence (*lacO*) ***(8)*** and of the tet repressor protein (TetR) to the Tn10 Tet operator sequence (*tetO*) ***(9)***. Both systems have previously been established to label chromosome loci in yeast, fruit flies and cultured human cells ***(10-13)***. Recently, alternative systems that are based on the binding of the lambda phage repressor to its operator sequence ***(14)***, on the spreading of the prokaryotic ParB proteins along their *parS* target sites ***(15)***, or on CRISPR/inactive Cas9 ***(16)*** have been established for chromosome labeling in eukaryotes.

To employ FROS to quantitatively measure chromosome condensation in live fission yeast cells, we integrated tandem arrays of *lacO* and *tetO* sequences into different positions of the longest fission yeast chromosome arm (chromosome I) and labeled them by the simultaneous expression of enhanced green fluorescent protein fused to LacI (EGFP-LacI) and TetR fused to a tandem copy of the red fluorescent protein Tomato (TetR-tdTomato). The two loci are visible as diffraction-limited green and red foci by fluorescence microscopy. Axial shortening of this chromosome arm during condensation can then be quantified by measuring in three dimensions the Euclidean distance between the centers of the foci. By probing genomic distances at the mega-base scale, the assay assesses global chromosome folding rather than local changes in chromosome architecture, caused, for example, by short-range chromatin looping. The use of two spectrally distinct colors enables the accurate determination of the centroid of both fluorescent marker foci at sub-diffraction limited resolution, even when the foci are in close proximity.

Chromosome condensation is measured by live cell microscopy in cell populations that have been enriched for cells in late G2 phase of the cell cycle. This enrichment step is advantageous, since chromosome condensation takes place during an ~8 minutes time window of the ~3 hours fission yeast cell cycle and image acquisition time must be limited to prevent phototoxic effects of prolonged exposure to short wavelength light. Enrichment of fission yeast cells in G2 phase can be achieved by lactose gradient centrifugation with little perturbation of the cells ***(17)***. The splitting of the centromere-proximal sister marker arrays upon anaphase onset can then be used as a

reference point to align the time series measurements of individual cells in one experiment. Considering the high mobility of chromosome arms and random fluctuations in chromosome movements, it is necessary to combine measurements from at least 20 individual cells into an average distance time series to obtain statistically significant measurements.

2. Materials

2.1 Fission yeast strains

Fission yeast strains expressing EGFP-LacI and TetR-tdTomato and carrying *lac* and *tet* operator sequences at different base distances are available upon request (**Table 2** and **Note 1**).

2.2 Media and solutions

1. YE5S media: Dissolve 5 g yeast extract (Becton Dickinson), 30 g glucose, 225 mg adenine, 225 mg histidine, 225 mg leucine, 225 mg lysine, and 225 mg uracil in 900 ml of ddH_2O. Once dissolved, add ddH_2O to one liter. Sterile filter and store at room temperature.
2. BS1 solution: Dissolve 2 mg lectin from *Bandeiraea simplicifolia* (Sigma) in 1 ml ddH_2O. Store BS1 solution in aliquots at -20 °C and keep the aliquot currently in use at 4 °C.
3. 7.0% and 30.0% lactose stock solutions: Dissolve 7 g or 30 g lactose in ddH_2O and bring to 100 ml. Stock solutions can be stored at room temperature. Precipitates that form over time can be solubilized by short heating in a microwave.

2.3 Microscopy equipment

1. 35-mm glass bottom dishes (10 mm micro-well, number 1.5 cover-glass)
2. A wide-field epifluorescence microscope with an automated stage and excitation filter wheel, equipped with an excitation filter for each band to allow selection of individual channels (e.g. a dual-band dichroic mirror, FWHM 520/25 and 630/50), a 100× objective (see **Note 2**), and a temperature-controlled environment. Advantages and

Strain	Genotype
C2566	*h-, lys1::lacO::lys1$^+$, his7::EGFP-LacI::his7$^+$, ChrI 1.5Mb::tetO::hphMX, Z locus::TetR-tdTomato::natMX, leu1-32, ura4-D18, ade6-M210*
C2568	*h-, lys1::lacO::lys1$^+$, his7::EGFP-LacI::his7$^+$, ChrI 1.95Mb::tetO::hphMX, Z locus::TetR-tdTomato::natMX, leu1-32, ura4-D18, ade6-M210*
C2570	*h-, lys1::lacO::lys1$^+$, his7::EGFP-LacI::his7$^+$, ChrI 2.49Mb::tetO::hphMX, Z locus::TetR-tdTomato-natMX, leu1-32, ura4-D18, ade6-M210*
C2572	*h-, lys1::lacO::lys1$^+$, his7::EGFP-LacI::his7$^+$, ChrI 3.6Mb::tetO::hphMX, Z locus::TetR-tdTomato-natMX, leu1-32, ura4-D18, ade6-M210*
C2574	*h-, lys1::lacO::lys1$^+$, his7::EGFP-LacI::his7$^+$, ChrI 3.0Mb::tetO::hphMX, Z locus::TetR-tdTomato::natMX, leu1-32, ura4-D18, ade6-M210*
C2724	*h-, ChrI 3.0Mb::lacO::natMX, his7::EGFP -LacI::his7$^+$, ChrI 1.5Mb::tetO::hphMX, leu1::TetR-tdTomato::leu1$^+$, lys1-131, ura4-D18, ade6-M210*
C2774	*h-, ChrI 1.95Mb::lacO::natMX, his7::EGFP -LacI::his7$^+$, ChrI 1.5Mb::tetO::hphMX, leu1::TetR-tdTomato::leu1$^+$, lys1-131, ura4-D18, ade6-M210*
C2779	*h-, ChrI 2.49Mb::lacO::natMX, his7::EGFP -LacI::his7$^+$, ChrI 1.5Mb::tetO::hphMX, leu1::TetR-tdTomato::leu1$^+$, lys1-131, ura4-D18, ade6-M210*
C2926	*h-, lys1::lacO::lys1$^+$, his7::EGFP -LacI::his7$^+$, ChrI 2.49Mb::tetO-hphMX, Z locus::TetR-tdTomato::natMX, leu1-32, ura4-D18, ade6-M210*

Table 2. Available fission yeast strains containing *lacO* or *tetO* arrays and expressing EFGP-LacI and TetR-tdTomato (see ref. ***18***).

disadvantages of different microscopy setups are discussed in depth in ref. *7*.

2.4 Software

Fiji image analysis software is freely available for download from http://fiji.sc/Fiji and the required plugin is freely available from https://github.com/cmci/BoryProject.

3. Methods

We describe the experimental setup for a temperature-sensitive fission yeast strain that is cultured at a permissive temperature of 25°C and then imaged at a restrictive temperature of 34°C. Growth temperatures can be adjusted depending on the strain to be studied.

3.1 Preparation steps

1. Grow a 50 ml fission yeast cell culture in YE5S to 0.4–1.0×10^7 cells/ml at 25°C (permissive temperature).
2. Equilibrate three 2 ml tubes with 1 ml YE5S at 25°C (permissive temperature) and two 2 ml tubes with 2 ml YE5S at 34°C (restrictive temperature).
3. Pipet 200 µl BS1 solution onto the glass surface of a glass bottom dish such that the surface is completely covered. Immediately take off the solution and let any residual solution dry.
4. Prepare a 7.0–30.0% lactose step gradient:
   a. Pipet 1.5 ml 30.0% lactose solution to the bottom of a 15-ml conical tube, using a 1-ml pipet with a cut-off tip to reduce dynamic pressure during pipetting. Avoid entrapping air at the bottom of the tube.
   b. Carefully overlay with 1.5 ml 27.1% lactose solution using a cut-off pipet tip without mixing the two layers. The interface between the layers should be clearly visible.
   c. Repeat the previous step with 1.5 ml of each of the rest of the lactose solutions listed in **Table 3** with decreasing lactose concentration.

   The lactose gradient should be used within 1 hour after preparation. Alternatively, a linear lactose gradient can be prepared using a gradient maker or a freezing/thawing protocol ***(17)***.
5. Equilibrate the microscope temperature to 34°C (restrictive temperature).

3.2 Enrichment of G2 phase cells by lactose gradient centrifugation

1. Transfer the fission yeast cell culture to a 50 ml conical tube and pellet cells at 2,200×g for 2 min at room temperature.
2. Discard the supernatant and re-suspend the cell pellet in 50 ml ddH_2O at room temperature to remove residual sugar from the cells. Pellet cells again at 2,200×g for 2 min at room temperature.
3. Discard the supernatant and re-suspend the cell pellet in 500 µl ddH_2O.
4. Carefully pipet the cell suspension onto the top of the lactose gradient. Centrifuge at 220×g for 8 min at room temperature with slow acceleration and slow deceleration settings. Start a timer as soon as the centrifuge run is complete.
5. Take off 3×300 µl from the layer of the highest cell density (judged by the highest optical density in the gradient) by penetrating the upper layers with a

Final lactose conc. (%)	Volume 30 % lactose stock (ml)	Volume 7 % lactose stock (ml)
30.0	10.00	0
27.1	8.75	1.25
24.3	7.50	2.50
21.4	6.25	3.75
18.5	5.00	5.00
15.6	3.75	6.25
12.8	2.50	7.50
11.0	1.25	8.75
7.0	0	10.00

Table 3. Lactose step gradient.

pipet tip and aspirating the suspension (see **Note 3**). Add each 300 µl fraction to one of the tubes containing 1 ml YE5S at 25°C. To limit osmotic stress, this step should be performed immediately after centrifugation.

6. Pellet cells at 2,200×g, discard the supernatant and re-suspend each cell pellet in 200 µl YE5S. Inspect 3 µl of each sample in a transmission light microscope and select the sample that contains the highest number of elongated cells and the lowest number of cells with septa. Discard the other two samples.
7. Pipet 200 µl of the selected cell suspension onto the dried glass bottom dish glass surface. Steps 5-7 should not have taken more than 5 min.
8. Incubate the glass bottom dish at 34°C (restrictive temperature) and let the cells settle for 5-10 min.
9. Take off 180 µl YE5S and discard. Carefully rinse the cells with 400 µl fresh YE5S pre-warmed to 34°C by pipetting onto one spot at the edge of the glass surface using a 1 ml pipette. This will detach loose cells and result in a monolayer of cells (see **Note 4**). Take off the 400 µl YE5S and fill the dish with 2 ml fresh YE5S pre-warmed to 34°C.
10. Place the dish onto the microscope stage equilibrated to 34°C.

3.3 Image acquisition

1. Find one or more position(s) with a suitable cell density (approx. 50 cells per field of view) using transmission illumination.
2. Place the dual band dichroic filter into the light path. Set the microscope software to switch between EGFP and tdTomato channels by changing the excitation band pass filters. Emission filters are not required.
3. Set the software to acquire 10 z-slices spaced by 400 nm (to cover the thickness of the cell monolayer) for each channel and exposure times of 100 ms, using a 33% neutral density filter to avoid photo toxicity and bleaching (see **Note 5**). We use 2×2 pixel camera binning. Depending on the microscope, imaging the complete z stack for a channel before switching channels might speed up image acquisition.
4. Start imaging 1 h after the end of the lactose gradient centrifugation run.

   Image for 1 h at a frame rate of 1/45 s. Depending on the type of microscope, multiple positions can be defined and imaged successively. Manually or, if possible with the microscope setup, automatically correct z-drift.

3.4 Image processing and data extraction

1. In Fiji, open the region of interest (ROI) Manager window (Analyze > Tools > ROI Manager…) and activate the *Show All* checkbox to highlight selected regions.
2. Set preprocessing FFT parameters (Plugins > EMBLtools > Bory > PreProcess > Set FFT Parameters) to *Filter large structures down to* 10 pixels, *Filter small structures up to* 2 pixels and *Tolerance of direction:* 5%.
3. Set the fluorescent foci segmentation parameters (Plugins > EMBLtools > Bory > Set DotSeg Parameters) to the following values:

   Segmentation Method: AutoThreshold,
   Segmentation Min Spot Size: 20,
   Measurements Min Spot Size: 20,
   Segmentation Max Spot size: 200,
   Min Volume Sum for Segmentation: 5,
   Max Volume Sum for Segmentation: 80,
   Min Object Number for Segmentation: 1,
   Max Object Number for Segmentation: 4,
   Maximum Loop exit for threshold adjustment: 50.
4. Import the image data into Fiji using the Bio-Formats Importer plugin (Plugins > Bio-Formats > Bio-Formats-Importer).
5. In the video, find a dividing cell and encircle it using the freehand selection tool (see **Note 6**). Avoid

signals from neighboring cells and check that the fluorescent foci are visible throughout the video. Add the region of interest to the ROI Manager (Add [t]).

6. Duplicate the ROI (Image > Duplicate… Check *Duplicate hyperstack*).
7. Preprocessing:
   a. Separate the two channels of the duplicated image (Image > Color > Split Channels). Select the image containing the first channel data. Preprocess the data (Plugins > EMBLtools > Bory > PreProcess > PreProcess ChromosomeDots). This will open a window named DUP_ containing the preprocessed image. Repeat this procedure for the second channel.
   b. Merge the preprocessed images (Use Image > Color > Merge Channels…). Add the ROI by clicking first on the merged image and then on the ROI in the ROI Manager.
   c. Delete regions outside the ROI by Edit > Clear Outside. Save the resulting image under the same name as for the original image preceded by ppcd_ to mark it as preprocessed.
8. Fluorescent focus segmentation, position measurement and distance calculation:
   a. Split the channels of the preprocessed image (Image > Color > Split Channels and Plugins > EMBLtools > Bory > Do Segmentation & Measurement). Chose the respective images for channel 1 and channel 2 and start the 3D segmentation by clicking OK. When complete, the script will output a side-by-side z-projected image (Detected dots), a results table containing dot positions for each frame in channel 1 (Statistics_Ch0) and for channel 2 and a results table Statistics_Distance containing the distance between the foci (ch0-ch1_dist) for each focus pair.
   b. Wrong segmentations can be deleted from the Statistics_Distance results table using delete or backspace keys.
   c. Save the Statistics_Distance results table as comma separated value (.csv) file.

3.5 Data analysis

Data analysis can be performed in any data analysis software of choice. To make data analysis accessible to as many researches as possible, we describe a method for Microsoft Excel™.

1. Copy all results .csv files into one continuous Excel table, making sure that columns match. Use Excel's 'Pivot Table' function with Row Labels 'time' and values average of ch0-ch1_distance.
2. The resulting table can be plotted as an x-y scatter plot (**Figure 1**).

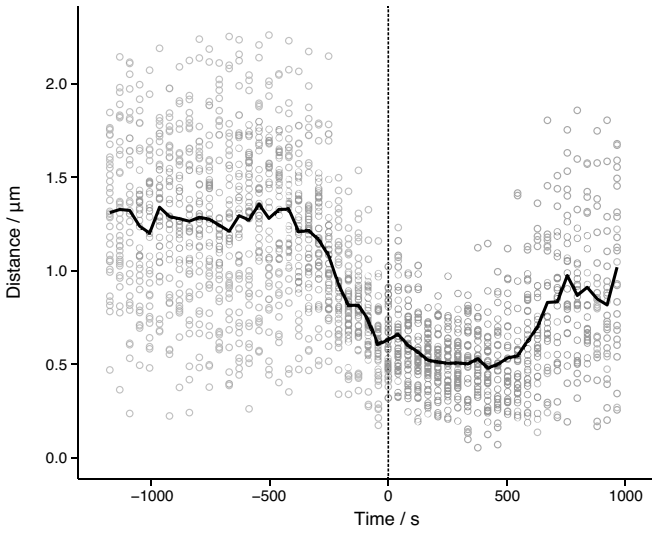

Figure 1. Example condensation graph generated from distance measurements of 45 individual cells (circles) of a fission yeast strain with *lacO* and *tetO* arrays separated by 1 Mb of DNA (C2779). Measurements of individual cells were aligned to anaphase onset (t = 0) to generate a mean distance curve (line).

4. Notes

1. Genetic crosses of fission yeast strains containing *lacO* and *tetO* arrays is in principle possible, but we frequently observed reduced LacI-GFP or tdTomato-TetR signals due to meiotic recombination events within the repetitive arrays.

We therefore recommend introducing the operator arrays and fluorescent repressor expression cassettes into the strain(s) of interest or introducing the mutation(s) of interest into the available reporter strains ***(18)***.

2. Alternatively, a 63× objective can be used; x–y pixel size should be around 130×130 nm. Since chromosome condensation, like all physiological reactions, is temperature dependent, it is essential to use a microscope with an efficient temperature-controlled environment irrespective of whether temperature-sensitive mutants are measured or not.
3. Instead of penetrating the upper layers of the gradient with a pipet tip, the layers can also be carefully aspirated.
4. It is important to obtain a monolayer of cells that adheres stably to the glass surface. The two factors influencing this adherence are the amount and quality of lectin used to coat the glass surface and the pressure applied when rinsing the dish (**Figure 2**). Do not use lectin solutions of less than 2 mg/ml.
5. *S. pombe* is, like most cells, sensitive to extended exposure of short wavelength light ***(19)***. A good indication for photo toxicity caused by overexposure is that cells cease to divide.
6. Unbound tdTomato-TetR produces a faint signal filling the nuclear volume, as reported ***(20)***. This background signal can be used to identify the positions and sizes of nuclei. It can also be used to score completion of nuclear division.

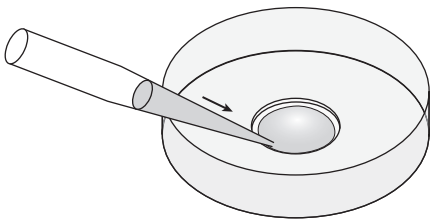

Figure 2. Rinse the cells adhered to the microscopy dish by pipetting media to the edge of the glass surface.

Acknowledgements

We are grateful to Kota Miura (CMCI, EMBL Heidelberg) for advice and developing the Fiji software plugin for data analysis and to the EMBL Advanced Light Microscopy Facility (ALMF) for assistance. Work in the authors' laboratory is funded by EMBL and grants HA5853/1-2 and HA5853/2-1 from the German Research Foundation.

References

1. N. Naumova, M. Imakaev, G. Fudenberg, et al. (2013) Organization of the mitotic chromosome, Science (New York, NY). 342, 948–953.
2. A.C.J. Vas, C.A. Andrews, K. Kirkland Matesky, et al. (2007) In vivo analysis of chromosome condensation in Saccharomyces cerevisiae, Molecular biology of the cell. 18, 557–568.
3. B. Petrova, S. Dehler, T. Kruitwagen, et al. (2013) Quantitative analysis of chromosome condensation in fission yeast, Molecular and cellular biology. 33, 984–998.
4. A.L. Pidoux and R.C. Allshire (2004) Kinetochore and heterochromatin domains of the fission yeast centromere, Chromosome research : an international journal on the molecular, supramolecular and evolutionary aspects of chromosome biology. 12, 521–534.
5. T. Mizuguchi, G. Fudenberg, S. Mehta, et al. (2014) Cohesin-dependent globules and heterochromatin shape 3D genome architecture in S. pombe, Nature. 516, 432–435.
6. M. Zofall and S.I.S. Grewal (2006) RNAi-mediated heterochromatin assembly in fission yeast, Cold Spring Harbor symposia on quantitative biology. 71, 487–496.
7. I. Lassadi and K. Bystricky (2011) Tracking of single and multiple genomic loci in living yeast cells, Methods in molecular biology (Clifton, N.J.). 745, 499–522.
8. M. Lewis, G. Chang, N.C. Horton, et al. (1996) Crystal structure of the lactose operon repressor and its complexes with DNA and inducer, Science (New York, NY). 271, 1247–1254.
9. P. Orth, D. Schnappinger, W. Hillen, et al. (2000) Structural basis of gene regulation by the tetracycline inducible Tet repressor-operator system, Nature structural biology. 7, 215–219.
10. C.C. Robinett, A. Straight, G. Li, et al. (1996) In vivo localization of DNA sequences and visualization of large-scale chromatin organization using lac operator/repressor recognition, The Journal of cell biology. 135, 1685–1700.
11. T. Sakuno, K. Tada, and Y. Watanabe (2009) Kinetochore geometry defined by cohesion within the centromere, Nature. 458, 852–858.
12. J. Vazquez, A.S. Belmont, and J.W. Sedat (2001) Multiple regimes of constrained chromosome motion are regulated in the interphase Drosophila nucleus, Current biology : CB. 11, 1227–1239.
13. S.L. Thompson and D.A. Compton (2010) Proliferation of aneuploid human cells is limited by a p53-dependent mechanism, The Journal of cell biology. 188, 369–381.
14. I. Lassadi, A. Kamgoué, I. Goiffon, et al. (2015) Differential chromosome conformations as hallmarks of cellular identity revealed by mathematical polymer modeling, PLoS computational biology. 11,

e1004306.

15. H. Saad, F. Gallardo, M. Dalvai, et al. (2014) DNA dynamics during early double-strand break processing revealed by non-intrusive imaging of living cells, PLoS genetics. 10, e1004187.
16. B. Chen, L.A. Gilbert, B.A. Cimini, et al. (2013) Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system, Cell. 155, 1479–1491.
17. D.D. Luche and S.L. Forsburg (2009) Cell-cycle synchrony for analysis of S. pombe DNA replication, Methods in molecular biology (Clifton, N.J.). 521, 437–448.
18. B. Petrova (2012) In vivo analysis of chromosome condensation in Schizosaccharomyces pombe, 147 S.
19. F. Mora-Bermúdez and J. Ellenberg (2007) Measuring structural dynamics of chromosomes in living cells by fluorescence microscopy, Methods (San Diego, Calif). 41, 158–167.
20. F. Hediger, A. Taddei, F.R. Neumann, et al. (2004) Methods for visualizing chromatin dynamics in living yeast, Methods in enzymology. 375, 345–365.
21. D. Llères, J. James, S. Swift, et al. (2009) Quantitative analysis of chromatin compaction in living cells using FLIM-FRET, The Journal of cell biology. 187, 481–496.
22. B.J. Wilkins, N.A. Rall, Y. Ostwal, et al. (2014) A cascade of histone modifications induces chromatin condensation in mitosis, Science (New York, NY). 343, 77–80.