Methods for Visualizing Chromatin Dynamics in Living Yeast

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Introduction

It is now well established that chromatin is not randomly organized within the nuclei of eukaryotic cells. Its arrangement varies broadly among species and cell types, yet conserved aspects of chromosomal organization are thought to facilitate common nuclear functions, such as transcription, replication, and repair. One obvious example of a subnuclear domain with a specialized activity is the nucleolus, a prominent nuclear substructure that serves as the unique site of ribosomal RNA transcription, processing, and assembly into ribosomal particles. Subnuclear positioning may also contribute to the establishment of differentiated patterns of gene expression or to the maintenance of heritable epigenetic controls over expression states.\(^1\,^2\)

In higher eukaryotic cells and particularly in primate species, chromosomes occupy discrete territories, and their placement tends to correlate with the density of genes and timing of replication: gene-rich, early-replicating chromosomes are generally more central in the nucleus than gene-poor, late-replicating chromosomes.\(^3\) Moreover, within chromosome territories, specialized subdomains like centromeres and telomeres, or Giemsa-dark bands and R-bands, often occupy distinct subdomains. In some cells, a radial organization can be detected within chromosome territories, with heterochromatin being frequently associated with the nuclear periphery.\(^4\) Despite evidence suggesting certain reproducible patterns of nuclear organization, the mechanisms that move chromosomes in interphase and govern their positioning remain largely a mystery.

Budding and fission yeast genomes are nearly 200-fold less complex than those of vertebrate and mammalian species, and both yeasts have been extremely useful for correlating function with the position of chromatin domains. For example, in both organisms centromeres and telomeres are clustered in distinct subnuclear sites, each reflecting a different

mechanism of subnuclear localization. Budding yeast telomeres cluster at the nuclear periphery in groups of four to six, forming discrete subcompartments in which histone-binding silencing factors concentrate.⁵ Centromeres, on the other hand, cluster around the spindle pole body, being held in place by microtubules.⁶⁻⁷ Genetic manipulations have revealed functional links between the formation and maintenance of silent chromatin and the positioning of telomeres, and artificial means to anchor DNA domains at the yeast nuclear envelope (NE) have helped implicate subnuclear position in the regulation of nuclear functions.⁹ It was shown, for instance, that late-replicating DNA is found preferentially at the nuclear periphery in G₁ phase, although this localization need not persist into S phase to maintain late replication status.⁹ Consistently, the anchoring of an origin at the periphery through a transmembrane ligand will not necessarily render it late-firing, although the nucleation of silent chromatin will.¹⁰ Nevertheless, tethering a potential silencer element at the nuclear periphery does enhance its ability to repress transcription.⁹

The notion that chromatin is generally immobile, and thus can provide anchorage sites for various nuclear processes, appears repeatedly in the literature on nuclear organization despite an absence of supporting evidence. In fact, a large body of data from live imaging of tagged chromosomal loci in interphase nuclei of fly, mammalian, and yeast cells shows the contrary; chromatin is highly dynamic with specific tagged loci moving in a rapid, random walk, which in some cases is coupled with a slow directional movement of the chromosomal domain.¹¹⁻¹³ Determining the exact nature of chromatin movement and its impact on nuclear functions is an area of intense pursuit. Similar efforts focus on identifying the proteins that anchor certain specialized domains of chromosomes, such as telomeres and centromeres in yeast,¹⁴ and other periodic anchorage sites along chromosomes in flies.¹⁵ Only with the identification of bona fide structural

proteins can the mechanisms of chromosome positioning and the functional implications of anchoring and dynamics be rigorously tested.

The protocols included here describe common techniques for the visualization of chromatin in living cells (primarily in budding yeast), while pointing out pitfalls and artifacts that can arise during live cell imaging. We also present analytical tools that have been developed for the quantitation of data generated by digital imaging. These tools allow us to define a new field of quantitative analysis: the dynamic behavior of DNA in real time. As these approaches are new, we expect there to be ongoing developments both in analytical tools and in high-resolution fluorescence microscopy. Half a century after the model was proposed for the structure of the DNA helix, and 10 years after the first eukaryotic chromosome was sequenced (i.e., Chr 3 of yeast), it is hoped that spatial and dynamic analyses may uncover novel mechanisms working to control the expression, replication, and repair of the genome.

Visualization of Specific Chromosomal Loci in Yeast

Tagging Genomic Loci

The precise identification of specific chromosomal loci in living cells was initially rendered possible by the development of a green fluorescent protein (GFP)-tagged lac repressor–operator system for site recognition.\textsuperscript{16} This system exploits the high affinity and highly specific interaction of the bacterial lac repressor (lac\textsuperscript{C}) for its recognition sequence called the lac operator (lac\textsuperscript{OP}).\textsuperscript{17} Directed insertion of an extended array of lac\textsuperscript{OP} (usually 256 copies or roughly 10 kb) and expression of a fusion construct between lac\textsuperscript{C} and a fluorescent protein like GFP, enables in vivo visualization of a defined DNA locus (Fig. 1A and B). Naturally, the specificity of targeting depends on the ability of the organism to carry out site-specific homologous recombination, an event that is extremely efficient in budding yeast.

To visualize the lac\textsuperscript{C} repressor in yeast, its gene is fused in frame to sequences encoding a nuclear localization signal, and the S65T derivative of natural GFP, which has a red-shifted excitation spectrum and higher emission intensity. Fusions to optimized forms of cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP) (or ECFP and EYFP; Fig. 2).


Fig. 1. Representative images of G1-phase budding yeast cells in which a lac\textsuperscript{50} array has been inserted near telomere 14L [(A and B); Hodiger \textit{et al.}\textsuperscript{15}] or near \textit{MATa}, the mating type locus at Chr 3 [(C and D); K. Dubrana, unpublished data, 2003]. The cells express the lac\textsuperscript{50}-GFP fusion and either a Nup49-GFP fusion (A and B) or a Tet\textsuperscript{R}-GFP fusion (C and D). Nup49-GFP gives a ringlike signal that allows precise determination of the nuclear volume, but interferes with detection of the tagged DNA locus when the latter is near the nuclear envelope (B). Tet\textsuperscript{R}-GFP fusion gives uniform diffuse nuclear staining, which is easily discernible from the spot signal [compare (B) and (D)]. The nuclear center can be accurately determined from the Tet\textsuperscript{R}-GFP signal, although a precise determination of the nuclear periphery is not possible. Scale bars: 2 \textmu m.
are also successfully used. The lac repressor itself is modified to prevent tetramerization, thus minimizing artifactual higher order interactions between lac\textsuperscript{op} sites. The minimal detectable cluster of lac\textsuperscript{op} sites is less than 24 operators,\textsuperscript{18} although longer time-lapse series are facilitated by the insertion of larger arrays. Long arrays of lac\textsuperscript{op} sites can be assembled into nucleosomes, yet these are largely disordered. Mild micrococcal nuclease analysis of these sequences reveals primarily monomeric and dimeric nucleosomal patterns (F. R. Neumann, unpublished data, 2002). This underscores the fact that lac\textsuperscript{op} inserts may not form normal nucleosomal arrays, yet in our hands 10-kb arrays did not grossly affect the timing of replication of either late- or early-replicating domains.\textsuperscript{8}

The repetitive 10-kb lac\textsuperscript{op} arrays are difficult to propagate both in bacteria and in yeast. Amplification of these constructs in Escherichia coli often results in plasmids that have lost many repeats. To avoid this, it is recommended that growth temperatures be reduced to 24 or 30\textdegree, storage of colonies be minimized, and recombination-deficient bacteria strains be used. Even after integration in the yeast genome, lac\textsuperscript{op} repeats are subject to recombination events that shorten or even eliminate the array. To have sufficiently strong fluorescent signals, we usually screen individual transformants for the brightest signal and freeze these isolates immediately. When

\textsuperscript{18} A. S. Belmont, Trends Cell Biol. 11, 250 (2001).
strains are recovered from frozen stocks, they are grown on selective media if possible, to avoid recombination events that would eliminate both the lac⁹⁰ repeats and the integration marker.

When comparing the position or mobility of two different genomic loci, one should avoid tagging both with the same repeats. It has been shown that two integrated identical arrays undergo an ill-defined pairing event that, at least in the case of the Tet system, depends on the expression of the repressor.¹⁹,²⁰ These may interfere with the positioning or dynamics of normal chromosomal loci. By using the Tet repressor (TetR)–operator combination for one site, and lac³–lac⁹⁰ sequences for the second, the risk of spurious pairing is eliminated.¹⁹ We have successfully used fusions between either the TetR or lac with EYFP or ECFP and found no interference for binding or fluorescent signals (K. Bystricky, personal communication, 2002). A monomeric red fluorescent protein has become available (mRFP1),²¹ which is functional when fused to lac³ and which can be easily distinguished from the other fluorescent proteins.

We note that in contrast to the lac³–GFP fusion, TetR–GFP gives a high and generally diffuse background of nuclear staining in yeast. This has two consequences: it can render the detection of a small array more difficult to detect, but at the same time, it can be useful for identifying the global nuclear volume and for calculating the center of the nuclear sphere (see below and Fig. 1C and 1D).

**Nuclear Detection**

To correct for any movement of the cell or the nucleus, or for mechanical vibration due to the instrumentation, it is necessary to determine the precise coordinates of the nuclear position in each acquired frame. Two alternative approaches have been used with success. In the first, a Nup49–GFP fusion is introduced into the yeast genome, generating a characteristic perinuclear ring due to the regular distribution of nuclear pores within the NE.²² The second makes use of a TetR–GFP fusion in the presence or absence of an integrated TetG array. TetR–GFP produces a uniform low-intensity fluorescence throughout the yeast nucleoplasm, including the nucleus. This background allows for a precise calculation of the nuclear center within each frame, and permits ready detection of a tagged locus near the nuclear periphery. Its disadvantage is that it does

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not provide a precise determination of the nuclear limit. The GFP–Nup49 fusion, on the other hand, defines the nuclear periphery clearly, but renders detection of perinuclear GFP-tagged loci difficult (Fig. 1). This can be overcome by using a CFP–Nup49 fusion, although this requires double imaging of the nucleus at each time point.

**Growth Conditions**

Strains are grown to an early exponential phase of growth (not more than $0.5 \times 10^7$ cells/ml) in synthetic or YPD medium. Cells are usually washed once before observation if grown in YPD, because this medium gives an autofluorescent signal.

We observe highly significant differences in the dynamics of internal DNA loci under different growth conditions. As little as one doubling of the culture (i.e., from a concentration of $1 \times 10^7$ to $2 \times 10^7$ cells/ml) was shown to produce a sharp drop in the dynamics of an internal tagged locus on Chr 14. Less quantitative analyses suggest that this is true for other sites, as long as they are not tethered due to their proximity to a telomere or centromere. This drop in dynamics occurs just before the so-called diauxic shift, which entails a major reprogramming of the transcriptional pattern of genes involved in glycolytic metabolism, resulting in the induction of genes required for oxidative respiration. It may reflect a general drop in global ATP levels or changes in ratios of other small molecules such as NAD/NADH. Treatment of yeast cells for as little as 15 min with the uncoupler carbonyl cyanide chlorophenyl hydrazone (this depletes both mitochondrial and plasma membrane potentials, inducing a hydrolysis of ATP) also provokes a significant drop in internal chromatin movement.

Growth in medium containing galactose, rather than glucose, as carbon source has also been observed to shift specific loci on Chr 3R to more perinuclear positions (K. Dubrana, M. Gartenberg, and K. Bystricky, personal communication, 2002). It is not known how general this phenomenon may be. These observations nonetheless stress the importance of using identical growth conditions and of including appropriate controls for growth density when pursuing comparative studies of nuclear dynamics.

**Cell Preparation**

Because we use cultures with a low cell concentration, a gentle centrifugation step (1 min at $\leq 5000g$) is recommended to ensure a convenient cell

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density on the slide. For short periods of observation (i.e., minutes), cells can be deposited on depression slides filled with 1.4% high-quality agarose dissolved in synthetic medium containing 4% glucose. The agarose is necessary to prevent deformation of the cells due to pressure between coverslip and slide (Fig. 3A). However, these anaerobic conditions and the production of CO₂ from cellular respiration can perturb extended observations. As an alternative, we recommend the use of a cell chamber system, such as the Ludin chamber (Life Imaging Services, Reinach, Switzerland). The chamber consists of a round 18-mm glass coverslip on which cells are deposited, mounted in a stainless steel chamber of 0.75 ml volume. For yeast, this slide must be coated with concanavalin A (1-mg/ml solution in H₂O; Sigma, St. Louis, MO) to allow cells to stick efficiently. The chamber can be closed by an upper glass coverslip and two perfusion lines permit medium changes or drug addition (Fig. 3B). With this system, the distance between the objective and the sample is separated only by the thickness of the coverslip, which improves resolution and prevents loss of focus.

The optimal temperature control can be achieved with an incubator box that encloses not only the specimen, but also the stage and much of the microscope. This allows a tight regulation of the specimen conditions and also avoids physical changes in microscope stand, stage, and objective due to thermal variations. We have not systematically analyzed the effects of temperature on chromatin dynamics, but imaging conditions should be standardized by using a well-thermostatted microscopy room (temperature, 22 ± 2°C). Work with thermosensitive yeast strains can be performed by locally heating the stage, the specimen, and the slide, if an incubator box is not available.

Fig. 3. (A) Glass slides containing a spherical depression are used for short time-lapse imaging. The depression is filled with synthetic medium containing 4% glucose and 1.4% agarose, on which cells are deposited. This avoids cell movement and deformation due to coverslip pressure. (B) The stainless steel Ludin chamber (Life Imaging Services, Reinach, Switzerland) permits deposition of the cells on an 18-mm coverslip precoated with concanavalin A. The chamber is then filled with 0.75 ml of medium. Two perfusion lines (arrows) allow medium or gas exchange.
Time-Lapse Acquisition

General Considerations. Because chromatin movement in yeast can be fast (we frequently detect movements >0.5 μm in less than 10 s), it is important to capture cell images very quickly. A compromise between resolution, the number of z frames, intervals between frames, exposure time, and bleaching must be found. It is also critical to control for laser- or light-induced damage to the organism. This is usually done by comparing the time required for an imaged cell to complete a division cycle with that of a nonimaged cell. We find that for rapid time-lapse imaging, the Zeiss (Thornwood, NY) LSM510 scanning confocal microscope is optimal for achieving fast acquisition, low bleaching, and minimal cell damage. To reduce the risk of damage by illumination, we keep the argon laser transmission as low as possible (0.1–1% for GFP), and image the cell as fast as possible by limiting the acquisition area to a minimal region of interest (ROI). High scanning speeds are used as described below.

The simplest approach is to capture “2D movies” (xy images over time) by manually maintaining the moving spot in focus during the acquisition. Image capture (average of four scans) requires approximately 500 ms for an ROI of 7 × 7 μm or roughly 250 ms for 3 × 3 μm, which is sufficient for a yeast nucleus. Image capture is repeated every 1.5 s. Using this method with minimal laser intensity, we were able to collect time-lapse series over 12 min without detectable effects on cell growth. For 3D time-lapse stacks, we typically take six to eight focal planes in z, each at a spacing of 300 to 450 nm, using an ROI of 3 × 3 μm. The stack is repeated every 1.5 s. Again, we could acquire up to 12-min movies (i.e., 480 stacks) without impairing cell division. The 3D time-lapse information has two advantages: (1) the spot is always present in one of the focal planes, meaning that after a maximal projection of the z axis, a complete 2D time-lapse sequence is obtained without ever losing the focal plane of the GFP spot; and (2) by image reconstruction, one can calculate distances and volumes in three dimensions. Three-dimensional measurements are nonetheless compromised by the reduced optical resolution in z (≥0.5 μm for 488-nm wavelength light).

When imaging a DNA locus as a 2D series over time (moving the focal plane when necessary), it should be noted that we generally exclude time-lapse series in which the GFP spot is within 0.5 μm of the top or bottom of the nucleus. In this zone, the spot is not easily distinguishable from GFP–Nup49, nor is the center of the nucleus readily determined. Particularly for measuring radial movements (see below), 2D time-lapse imaging is generally restricting to a 1-μm central zone of these nuclei (diameter, 2 μm).
Zeiss LSM510 Settings. The specific settings for a Zeiss LSM510 on an Axiovert 200M, equipped with Plan-Apochromat ×100/NA 1.4 oil immersion and Plan-Fluar ×100/NA 1.45 oil immersion lenses, an argon laser, and hyperfine motor HRZ 200, are as follows:

**Laser:** Argon/2: 458-, 488-, or 514-nm tube; current, 4.7 A; output, 25%

**Filters:** Channel 1, Lp 505 for GFP alone; channel 1, Lp 530; channel 3, Bp 470–500 for YFP/CFP single-track acquisition

**Channel setting:** Pinhole, 1–1.2 Airy units (corresponding to optical slice of 700 to 900 nm); detector gain, 950 to 999; amplifier gain, 1–1.5; amplifier offset, 0.2–0.1 V; laser transmission AOTF (acousto-optic tuned filter) = 0.1–1% for GFP alone, 5–15% for YFP, and 10–40% for CFP in single-track acquisition. The pinhole must be regularly aligned to use minimal laser transmission

**Scan setting:** Speed, 10 (0.88 µs/pixel); 8 bits one scan direction; 4 average/line; zoom, 1.8 (pixel size, 100 × 100 nm), using an ROI of 3 × 3 to 4 × 4 µm

**z settings:** Hyperfine HRZ 200; six to eight optical slices of 300 to 450 nm each

**Imaging intervals:** 1.5 s

If CFP and YFP signals are weak, one can acquire both signals sequentially on the LSM510 channel 1 (which is more sensitive than the others), using the multitrack mode to allow the use of broader filters. In this case, CFP signal is recovered through long-pass filter Lp 475 and YFP through Lp 530. Alternatively, and to avoid any cross-talk, YFP signal is recovered as before but CFP is recovered on channel 3 through bandpass filter Bp 470–500. Obviously, these latter parameters slow the imaging process.

**Wide-Field Microscopy Imaging with Deconvolution.** An efficient alternative to confocal imaging of chromatin dynamics is the use of a sensitive monochrome charge-coupled device (CCD) on a wide-field microscope equipped with a piezoelectric translator [PIFOC; Physik Instrumente (PI), Karlsruhe, Germany], xenon light source, monochromator, and rapid imaging software, such as Metamorph (Universal Imaging, Downingtown, PA). The advantage is that the most recent cooled CCD cameras have high sensitivity and speed, allowing for less than 50-ms exposures to acquire images up to 1392 × 1040 pixels. This makes this system convenient for imaging several cells at once. Here, the limiting step is the speed of signal transfer from the CCD chip to the computer, which depends on the image size. The monochromator allows the continuous regulation of incident light wavelength over a larger range of values than does the AOTF and the confocal laser system. Because out-of-focus haze makes wide-field images noisier than confocal images, deconvolution of the 3D stack is
recommended to reassign blurred intensities back to their original source. This can be readily performed by Metamorph software, although other deconvolution packages are also available.

The instrumentation that we generally use is as follows: an inverted Olympus IX70 microscope equipped with a piezoelectric translator (PIFOC; PI) placed at the base of a Planapo × 60/NA 1.4 objective, a polychromator (Polychrome II; TILL Photonics, Gräfelfing, Germany) and a CoolSNAP-HQ digital camera (Roper Scientific, Tucson, AZ). Suppression of stray light may require an additional shutter. The conditions of 3D time-lapse series capture are as follows: 5–11 optical z slices taken every 1 to 4 min; optical sections are 200 to 400 nm in depth and have 50-ms exposure. Using these settings, we are able to capture up to 300 stacks of 5 sections each (1500 frames) at 1-min intervals, which corresponds to 2.5 cell cycles, without affecting cell cycle progression. More rapid sampling with this system, on the other hand, leads to bleaching and potential cellular damage. Until this can be remedied by more rapid CCD cameras, we recommend confocal microscopy for rapid time-lapse imaging (intervals ≤ 2 s) on small regions of interest (typically, one yeast nucleus) and wide-field microscopy for less rapid time-lapse imaging (intervals ≥ 60 s) on larger fields.

Positional Information

Because time-lapse movies are necessarily limited to tens of cells for each condition tested, other more statistically rigorous methods are recommended to obtain an accurate determination of the subnuclear position occupied by a tagged locus with relation to a nuclear landmark or in relation to another site. This is performed by taking one stack of 17 to 19 images (exposure, 200 ms; step size, 200 nm) through a field of yeast cells that are either growing on agar or placed in a Ludin chamber, using the wide-field microscope equipped with a cooled, high-sensitivity CCD camera as described above. Analysis of position is generally performed on 200–300 cells, and cells are classified by their position in the cell cycle.

Position is routinely determined in relation to the nuclear envelope, which is usually tagged with the same fluorescent protein as the locus. In this way, both the DNA and the NE are imaged in each frame of the stack. If a tagged locus is to be localized with respect to a second spot or another specific nuclear landmark [e.g., spindle pole body (SPB) or nucleolus], it is preferable to tag the two with different fluorescent proteins to eliminate confusion during analysis. A bright-field or phase image of each cell is also essential to determine its cell cycle stage.

The position of the GFP-tagged locus is monitored relative to the middle of the Nup49-GFP ring. To do this, we measure from the center
of intensity of the GFP spot to the nearest pore signal along a nuclear diameter, as well as measuring the nuclear diameter itself. By dividing the first value by half of the second (i.e., the radius), we can classify each spot falling into one of three concentric zones of equal surface, as depicted in Fig. 4. The most peripheral zone (zone I) is a ring of width 0.184 × the nuclear radius (r). Zone II lies between 0.184r and 0.422r from the periphery and zone III is a central core of radius 0.578r. The three zones are of equal surface no matter where the nuclear cross-section is taken. We usually eliminate nuclei in which the tagged locus is at the very top or bottom of the nucleus, because the pore signal no longer forms a ring but a surface.

Once the distribution of a given locus is determined, it can be compared with either the predicted random array or another distribution by $\chi^2$ analysis or by proportional analysis, which compares percentages in one zone for different conditions. Statistical significance is determined using a 95% confidence interval.

Quantitative Approaches to Motion Analysis

Reproducible variations in chromatin mobility have been detected between G1- and S-phase nuclei, making it important to monitor the cell cycle stage of each imaged cell. In budding yeast, the cell cycle stage is easily determined by monitoring bud presence and size, together with nuclear position, in bright-field images taken before and after the fluorescence imaging series. Such analysis will also confirm that the imaged cell is progressing normally through the cell cycle. Figure 5 summarizes the characteristics used to classify budding yeast cells as G1, early S, mid-to-late S, G2, mitosis, and telophase. Unbudded cells are considered G1 phase; this category includes posttelophase stages in which two equal-sized cells remain attached,
Fig. 5. Nuclear shape, position, and bud size provide elaborate criteria for identifying the cell cycle stage of individual *Saccharomyces cerevisiae* cells. Round nonbudding cells are in G1 phase; early S phase cells (eS) have a very small bud. Other budded cells are classified into mid-to-late S phase (mS). We consider cells to be in G2 phase when they have a large bud (about two-thirds of the mother cell) and when the nucleus has moved to the bud neck. Most of the nuclei in G2 cells start to elongate. Mitotic cells (M) are characterized by intrusion of the nucleus into the bud. Once the two nuclei are clearly separated but not yet round, and still linked through residual nuclear envelope structures, cells are considered to be in telophase (T). As much of the dynamics and positional studies are based on the assumption that nuclei are round, we usually do not analyze G2, M, and T cells.

although their nuclei are clearly separated and round. Cells with an emerging bud and small-budded cells are classified as early S phase, and all other budded cells, in which the bud is big enough to form a ring at the neck and the nucleus is still round, are grouped as mid-to-late S phase. Once the nucleus moves to the bud neck and the bud is two-thirds the size of the mother cell, the cells are considered G2-phase cells. As soon as the nuclear envelope begins to extend into the daughter cell as a result of spindle extension, cells are considered to be mitotic (Fig. 5). Telophase can be defined as a state in which two distinct nuclei are visible, but remain connected via residual NE structures. In addition to cell cycle effects, we observed a reduced frequency of large movements (defined below) in G1 daughter nuclei compared with G1 mother nuclei, which can be attributed to the difference in nuclear size (nuclear diameters in G1 average 1.68 ± 0.2 μm, whereas it is 1.95 ± 0.3 μm in mother cells\(^2\)). For this reason, we include only mother cells in our G1-phase analyses.

*Tracking*

The detection of chromatin movement is of interest if it can be correlated with physiological changes; therefore, it is necessary to quantify its behavior under different controlled states. To this end, it is important
to accurately identify the position of the tagged locus relative to the nuclear center for each frame of a typical time-lapse movie. This laborious task was originally done by hand with simple measurement tools (AIM tool, LSM510 software; Zeiss). It is now greatly simplified by the development of a new tracking algorithm developed in collaboration with D. Sage and M. Unser (Swiss Federal Institute of Technology, Lausanne, Switzerland).25

The automated image analysis software consists of three components.

Alignment phase: The first step of the program is an alignment module that compensates for the movement of the nucleus or the cell. This is achieved by a threshold on the image and the extracted points are fit within an ellipse using the least-squares method of Fitzgibbon et al.26 Each image is then realigned with respect to the center of the ellipse.

Preprocessing phase: To facilitate detection of the tagged locus, the images are convolved with a Mexican-hat filter. This processing compensates for background variation and enhances small spot-like structures.

Tracking phase: The final component is the tracking algorithm, which uses dynamic programming to extract the optimal spatiotemporal trajectory of the particle. Detection of the spot is a difficult task mainly because of the low signal-to-noise ratio of the images and the similarity between the DNA spot and the nuclear pore signal. We take advantage of the strong dependency of the spot position in one image on its position in the next: all endogenous chromosomal loci observed so far show a constrained motion within the nucleus and a spot localized at a certain time point is generally not found more than 2 µm away at the next time point (1.5 s). The algorithm evaluates all possible trajectories over the sequence and the one that maximizes an appropriate set of criteria is kept. The criteria used are as follows:

1. Maximum intensity is favored (i.e., the tagged DNA is usually brighter than the pore signal).
2. Smooth trajectories are favored.
3. Positions that are closer to the center of the nucleus are favored. This is because the Nup49 staining can give signal similar to that of

the tagged locus when it is located at the nuclear periphery; a bright signal found inside the nucleus, on the other hand, is unlikely to be anything other than the tagged locus.

Importantly, these three parameters can be modulated individually in order to optimize the tracking for different situations (loci that are more mobile, more peripheral, of variable intensity, etc.). The program also has the option of further constraining the optimization by forcing the trajectory to pass through a manually defined pixel. In other words, the user can move or add nodes interactively when the spot is not well defined automatically and the new optimal trajectory is recomputed quasi-instantaneously. This tracking method proves to be extremely robust because of its global approach: the decision for spot definition is based not only on the present and the recent past but on the future as well, taking the data set as a whole into consideration.

This complete system has been implemented as a Java plug-in for the public domain ImageJ software. Application to time-lapse series of *S. cerevisiae* nuclei produces results that are equal to—if not better than—manual tracings, with the enormous advantage of being reproducible and analyzer independent. This new algorithm reduces the tracking time from 10 min for a typical experiment, when it is done manually, to a few seconds (Pentium IV, 1 GHz) if no user intervention is required. The spatiotemporal trajectory is exportable to a spreadsheet and will soon be implemented for 3D image stacks over time. Some software applications commercially available are able to track objects [Imaris (Bitplane, Zurich, Switzerland); Volocity (Improvision, Lexington, MA)], but in addition to being expensive, tracking efficiency is variable and usually requires uniformly high-quality images. Available algorithms are mostly based on threshold principles, and are rarely modifiable or interactive.

**Controls for Nuclear Rotation**

Before the analysis of the tagged locus dynamics, certain artifacts of spatial analysis must be eliminated by control studies. For instance, it is necessary to demonstrate that the yeast nucleus is not rotating on itself, as nuclear movement might be misinterpreted as tagged locus movement. To do this, we track the movement of a fixed point within the nuclear envelope, using an integral component of the spindle pole body (SPB) called Spe42,28 fused to CFP. Time-lapse analysis of SPB shows little movement over time, and individual steps are generally restricted to changes smaller

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than the optical resolution size (~0.2 μm). A second control for nuclear rotation uses fluorescence recovery after photobleaching (FRAP). A restricted zone of the NE visualized through the Nup49-GFP signal is bleached by repeated irradiation (50 iterations of 100% power pulses with the 488-nm laser). Subsequent imaging shows that the bleached zone persists in the same position for 2–3 min and is then “invaded” by one or two discrete pores, which diffuse from the edges of the bleached zone. This shows that there is no general rotation of the nucleus, for this movement would have shifted unbleached pores into the photobleached zone. Moreover, pore diffusion is much slower than chromatin movement. Such controls should be routinely performed before attributing movement to a tagged chromosomal locus, rather than to the nucleus in general.

**Characteristic Parameters of Movement**

Once the xy or xyz coordinates for each time point of a time-lapse series are exported, which will identify the center of the nucleus and the center of the tagged locus for each frame, the moving particle can be characterized by many different parameters. Those presented here were selected because they accurately discriminate between visually different dynamics. These analyses can be carried out with a variety of programs designed for advanced calculations, including Excel, Mathematica, or MATLAB. Macros for implementation can be obtained by e-mail from our laboratory.

**Trace.** For each time-lapse series, we sum the total distance traveled by the particle over 5 min of 1.5-s intervals, assuming a straight trajectory between sequential positions (Fig. 6A). This gives the total track length for a given time period, which should be averaged over 8–10 similar movies. Alternatively, the movies and tracks can be “added,” such that the sum represents the track length over 40 to 50 min of time-lapse imaging (1600 to 2000 frames). From this, one can calculate an “average velocity” for spot movement with some reliability. Analysis of individual movies (i.e., one cell within a 5-min time period) is too anecdotal for any reliable conclusions to be drawn. Examples of track length variation for internal chromosomal sites versus telomeric or centromeric sites, and an internal site in G1- versus S-phase cells, are available in Ref. 23.

**Step Size Distribution.** For small changes in dynamic behavior, the trace measurement is sometimes not sufficiently discriminating and a statistical approach is preferable. Position-to-position distances can be averaged over one or multiple movies and the standard deviation calculated. These parameters can be compared for different groups of time-lapse series with statistical tests (e.g., ANOVA) and even small but reproducible differences
Fig. 6. (A) A representative trace of a tagged telomere on Chr 14L in a G1-phase yeast cell is shown. Images were captured over 5 min with frames taken every 1.5 s. The position of the spot was tracked after alignment of the nuclei on the basis of their nuclear envelope fluorescence, using the tracking algorithm. The trajectory of the locus is projected in red on a single focal section of the nucleus. The mean length of the path in micrometers for a 5-min movie (200 frames) averaged over 8 movies is indicated. Scale bar: 1 μm. (B) Radial movement of the GFP-tagged telomere 14L relative to the nuclear envelope (NE) was monitored by measuring the distance from the middle of the spot to the middle of the pore signal in each frame. The red box indicates radial movement > 0.5 μm within 10.5 s or 7-frame intervals. (C) Mean square difference (MSD) of actual point-to-point distances is shown for the time-lapse series pictured in (A) and (B). For each time interval (1.5 s to 150 s), the mean of the absolute distance (Δd) covered by the spot is calculated and plotted against time intervals. (D) The MSD of radial distances is shown for the same time-lapse series. For each time interval (1.5 s to 150 s), the mean of the radial distance (Δdr) covered by the spot is calculated and plotted against time intervals. Telomere 14L moves significantly along the nuclear envelope and thus appears less
can be documented. Because of the large number of measurements taken into consideration, small differences can nonetheless be highly significant.

*Large (Radial) Movements.* Some loci do not appear to move with a significantly higher average speed, but the frequency of large rapid jumps (movements ≥ 0.5 μm within a few frames; see Fig. 6B) varies greatly among different loci and points in the cell cycle. This criterion has been found to be one of the most useful for distinguishing patterns of mobility. Any threshold over 0.2 μm can be chosen for the definition of a “large” movement, but in our experience a useful criterion for comparison has been the frequency of radial movements ≥ 0.5 μm within a 7-frame interval, that is, within 10.5 s. Once radial distances are plotted over time, the scoring of these can readily be done by hand and confirmed by computation, although this analysis is also readily automated. A similar quantification of the frequency of point-to-point movements over a certain size is also possible, although, in this case, we have no standard to recommend.

*Relative Surface Coefficient.* The fraction of the nuclear surface occupied by a particle tracked by 2D imaging can be calculated for a given time period. In the case of chromosomal loci, these show a restricted zone of movement, which is significantly different for “tethered” sites, such as telomeres, and internal chromosomal loci. The relative surface coefficient (RSC) defines the minimal ellipse that encloses 95% of the individual particle positions during a given movie. The surface of the ellipse is divided by the average surface of the nucleus. This number must be determined movie by movie, but the RSC of a group of time-lapse series can be averaged. The surface coefficient quantifies graphically the freedom of movement of a given tagged locus, and these values generally agree well with results from the mean square displacement analysis (see below).

*Mean Square Displacement Analyses.* The observation of a moving DNA locus over time gives information not only about the rate of movement but also about the area or volume that it occupies during this period of time. It has been amply shown that certain chromosomal domains or loci are able to move locally, but remain in a given subvolume of the nucleus.\(^{11-13}\) The fact that they move within a defined area can be considered to be a constraint on their apparently random local motion. This particular behavior can be analyzed by a calculation based on the random walk model that describes a linear relationship between time intervals and the square of the distance traveled by a particle during this period of time (mean square displacement = MSD or \(<\Delta d^2>\), where

constrained when the MSD is calculated from absolute distance as compared with radial distances. The fact that telomere 14L is restricted to a small volume close to the nuclear periphery is shown by the low plateau in the radial MSD graph. (See color insert.)
\[ \Delta d^2 = [d(t) - d(t + \Delta t)]^2 \] The slope reflects the diffusion coefficient of the particle. The linearity of the curve is lost, however, if the particle is restricted in its freedom of movement (obstructed random walk), or when a global directional movement is observed (random walk with flow). A particle moving in a closed volume (as a cell nucleus) will show a plateau in the MSD analysis, and the height of this plateau is related to the volume in which the particle is restricted (see red dashed line in Fig. 6).

The slope of the MSD relation is directly correlated with the diffusion coefficient \( (D) : < \Delta d^2 > = 2nD \Delta t / d_{w}^{2/3} \) (where \( n \) is the number of spatial dimensions and \( d_{w} \) is the anomalous diffusion exponent). \( d_{w} \) can be calculated by plotting \( \log(\Delta d^2 / \Delta t) \) versus \( \log(\Delta t) \) and is larger than 2 if the diffusion is obstructed. In enclosed systems, the diffusion coefficient decreases with increasing \( \Delta t \) because of space constraints exerted on the particle dynamics. Nevertheless, the maximal diffusion coefficient can be calculated for short time intervals and reflects intrinsic mobility of particles (see green dashed line in Fig. 6C and D). Diffusion coefficients of nuclear components have been calculated by MSD or FRAP and values can range from \( \sim 60 \, \mu m^2/s \) for EGFP in hamster cells,\(^{31} \) to \( \sim 1 \times 10^{-4} \, \mu m^2/s \) for freely diffusing Cajal bodies in HeLa cells.\(^{32} \) We observe diffusion coefficients in the range of \( 1 \times 10^{-2} \) to \( 1 \times 10^{-3} \, \mu m^2/s \) for chromosomal loci in yeast. Of course, in an obstructed random walk diffusion situation, the calculated \( D \) will depend on the time interval used to acquire images, as \( D \) decreases with increasing time intervals.

In practical terms, the distances traveled by the spot for each time interval (1.5 s, 3 s, 4.5 s, \ldots) are calculated and the square of their mean is plotted against increasing time intervals (Fig. 6C and D). The original slope of MSD is equal to \( 2nD \Delta t \) (\( D \) is the diffusion coefficient), such that \( n = 1 \) for radial measurements and \( n = 2 \) for absolute measurements. With respect to position-related questions, it is of interest to plot MSD for radial distances. In this case, the MSD plateau will reflect the tendency of the particle to be associated with the nuclear periphery, discriminating loci that are moving freely in the nucleoplasm from loci that move in a restricted zone near the nuclear envelope. The graphs in Fig. 6C and D are based on the same time-lapse data from telomere I4L, but show MSD calculations using

absolute distances and radial distances, respectively. The low plateau in Fig. 6D reflects the fact that telomere 14L is constrained close to the NE.

The MSD analysis has been used to characterize the dynamic behavior of chromosomal loci in yeast, Drosophila, and mammalian cells, and is a useful means to compare the levels of constraint experienced by chromatin in these different organisms. In other organisms, distances between two separate moving loci have been used, in which case $<\Delta d^2>$ reflects two times the MSD of an individual spot or locus moving relative to a fixed point. For a more theoretical treatment of this parameter, the reader is referred to Ref. 33.

Discussion

We have described techniques that allow a quantitative evaluation of the position and mobility of specific DNA sites in the yeast nucleus and give the details of how we and others analyze such results. These analytical methods are applicable to data from any organism. The most striking conclusion that arises from this type of analysis is that chromatin mobility is nearly identical in the three organisms studied in detail to date. Notably, tagged sites along yeast chromosomes (but not telomeres or centromeres), sites on the X chromosome in Drosophila spermatocytes and various insertions at random positions on human chromosomes show similar dynamics. In general, one detects both continuous small oscillations of 0.2 to 0.4 μm/s in random directions and, less frequently, larger movements of >0.5 μm, which are not strictly random: these are often followed by movements in the opposite direction. In contrast to these chromosomal loci, yeast telomeres and centromeres and inserts near human nucleoli or satellite repeats have significantly fewer large movements and are more spatially constrained.

One might question the significance of motion within this range, as the smaller movements approach the focal resolution of a confocal microscope (0.2 to 0.3 μm). However, these distances are large compared with chromatin structure. A movement of 0.5 μm spans half the radius of a yeast nucleus and is equivalent to about 100 kb of interphase chromatin (based on a linear compaction ratio of ~70-fold). We stress that these hyperfine movements are, to a large degree, sensitive to ATP levels in yeast, and that the conditions that cells are exposed to during imaging may influence

35 K. Bystricky et al., submitted (2003).
the mobility of a given locus. DNA damage from short-wavelength light may provoke changes in chromatin dynamics as a result of the cellular response to DNA damage. For the analysis of chromatin position within a nucleus, it is strongly advised that time-lapse analyses (which give only a temporally restricted sampling of a single cell, usually averaged over a limited number of movies) be complemented with more accurate analyses of specific cell populations (>200 cells), monitored at a single time point. Both sets of data are necessary to understand chromatin position and behavior in living cells.

Present efforts now focus on linking chromatin mobility with either active transcriptional states or the potential for transcription. The source of movement does not appear to be RNA polymerase II elongation, but possibly the events of chromatin remodeling that render promoters accessible to the machinery of transcription. The ATP dependence of chromatin-remodeling machines (SWI/SNF, etc.) correlates well with the sensitivity of large chromatin movements to ATP depletion and changes correlated with the diauxic shift. Thus, the near future promises at least some enlightenment on the physiological implications of chromatin dynamics in living cells.

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Direct Visualization of Transcription Factor-Induced Chromatin Remodeling and Cofactor Recruitment *In Vivo*

*By Anne E. Carpenter and Andrew S. Belmont*

Introduction.

The application of chromatin immunoprecipitation procedures, as described elsewhere in this volume, has greatly facilitated *in vivo* measurement of recruitment of various transcriptional activators and cofactors to specific *cis* sequences in a wide range of biological systems. The impact on the field of chromatin structure and gene regulation has been tremendous. However, this method does have two specific limitations. Not all proteins will cross-link efficiently to nearby DNA sequences. More importantly, perhaps, chromatin immunoprecipitation is a biochemical procedure involving averaging among cells in a population, precluding single-cell observations. This is a particular limitation when cell heterogeneity is present and/or when fine temporal ordering of events is important. Similarly, biochemical and molecular probes for chromatin remodeling have been limited to methods that measure averages over cell populations.

Our laboratory and others have begun to develop assays for chromatin remodeling and transcription factor and coactivator recruitment based on direct, microscopic observations within individual live cells. The experimental basis for this approach involves the visualization of chromosome regions containing multiple transgene copies. To date, two approaches have been used. The first uses direct repeats of bacterial operators to bind repressor fusion proteins as a means of tethering specific transcription factors to specific chromosomal sites. The second uses repeats of transgenes containing viral promoters with binding sites for known transcription factors. We anticipate a natural experimental progression will be to combine both of these approaches, using the bacterial operator repeats for tagging the chromosome regions, while using transgene repeats containing specific promoters with known transcription factor-binding sites and reporter constructs for monitoring gene expression.

These methods promise to allow real-time visualization of transcription factor dynamics and their relationship to changes in chromatin structure and gene expression. Here, we review previous work using these methods. We then discuss key methodologies used in these experiments together with ongoing technological developments in our laboratory. We conclude...
HEDGER ET AL., CHAPTER 22, FIG. 2. Excitation and emission spectra of enhanced cyan, green, yellow, and red fluorescent proteins. Excitation spectra are represented as dashed lines, emission spectra as unbroken lines. ECFP, EGFP, and EYFP are all variants of the green fluorescent protein from *Aequorea victoria*. DsRed is derived from a coral of the *Diceromona* genus (for more information, see www.clontech.com).

HEDGER ET AL., CHAPTER 22, FIG. 4. Schematic representation of the Nup49-GFP and DNA tagged locus fluorescent signals. For hundreds of different cells, the distance from the middle of the spot to the middle of the envelope signal ($x$), and the nuclear diameter ($y$), are measured. By dividing $x$ by $y/2$ ($p = 2x/y$), we can classify the spot position into three concentric zones of equal surface. The outer-most zone (I) contains peripheral spots ($p < 0.184$). Zone II regroups intermediate positioned spots ($0.184 < p < 0.422$). Zone III contains internal spots ($p > 0.422$).
A representative trace of a tagged telomere on Chr 14L in a G_1-phase yeast cell is shown. Images were captured over 5 min with frames taken every 1.5 s. The position of the spot was tracked after alignment of the nuclei on the basis of their nuclear envelope fluorescence, using the tracking algorithm. The trajectory of the locus is projected in red on a single focal section of the nucleus. The mean length of the path in micrometers for a 5-min movie (200 frames) averaged over 8 movies is indicated. Scale bar: 1 μm. (B) Radial movement of the GFP-tagged telomere 14L relative to the nuclear envelope (NE) was monitored by measuring the distance from the middle of the spot to the middle of the pore signal in each frame. The red box indicates radial movement > 0.5 μm within 10.5 s or 7-frame intervals. (C) Mean square difference (MSD) of actual point-to-point distances is shown for the time-lapse series pictured in (A) and (B). For each time interval (1.5 s to 150 s), the mean of the absolute distance (Δd) covered by the spot is calculated and plotted against time intervals. (D) The MSD of radial distances is shown for the same time-lapse series. For each time interval (1.5 s to 150 s), the mean of the radial distance (Δr) covered by the spot is calculated and plotted against time intervals. Telomere 14L moves significantly along the nuclear envelope and thus appears less constrained when the MSD is calculated from absolute distance as compared with radial distances. The fact that telomere 14L is restricted to a small volume close to the nuclear periphery is shown by the low plateau in the radial MSD graph.