CHAPTER TWENTY-TWO

Quantitative Localization of Chromosomal Loci by Immunofluorescence

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Contents

Abstract

DNA within the yeast nucleus is spatially organized. Yeast telomeres cluster together at the nuclear periphery, centromeres cluster together near the spindle pole body, and both the rDNA repeats and tRNA genes cluster within the nucleolus. Furthermore, the localization of individual genes to subnuclear compartments can change with changes in transcriptional status. As such, yeast researchers interested in understanding nuclear events may need to determine the subnuclear localization of parts of the genome. This chapter describes a straightforward quantitative approach using immunofluorescence and confocal microscopy to localize chromosomal loci with respect to well characterized nuclear landmarks.

Chromosomes within the yeast nucleus are spatially organized. Parts of chromosomes are associated with different subnuclear compartments such as the nucleolus, the nuclear envelope or the spindle pole body (Berger *et al.*,

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2008; Dorn *et al.*, 2007; Hueun *et al.*, 2001; Jin *et al.*, 2000). Localization to these subnuclear organelles is not static. Chromosomal DNA is in constant motion and exhibits varying degrees of constrained diffusion (Cabal *et al.*, 2006; Casolari *et al.*, 2004; Chuang *et al.*, 2006; Schmid *et al.*, 2006; Shav-Tal *et al.*, 2004). Sophisticated techniques have been developed to understand the dynamics of chromosomal elements in living cells (Chapter 21). However, to determine where a gene localizes within the nucleus and how its localization is affected by inputs of interest (such as transcriptional activation), simpler methods can be used. Here, we describe a quantitative method for localizing chromosomal loci with respect to subnuclear landmarks using established immunofluorescence methods.

1. Yeast Strain Construction

This protocol is based on binding of the lac repressor from *Escherichia coli* to an array of lac repressor-binding sites integrated near the chromosomal locus of interest (Fig. 22.1A; Robinett *et al.*, 1996; Straight *et al.*, 1996). Similar experiments can be carried out using the Tet repressor array (Abruzzi *et al.*, 2006; Cabal *et al.*, 2006; Chekanova *et al.*, 2008; Dundr *et al.*, 2007; Fischer *et al.*, 2004; Köhler *et al.*, 2008; Kumaran ad Spector, 2008). Depending on the sensitivity of the microscope being used, this method requires $\geq$100 binding sites. In our experiments, we readily visualize an array of $\sim$128 lac repressor-binding sites (lac operators/LacO array). To introduce this array at a locus of interest, the LacO array is first cloned into a plasmid with an appropriate selective marker (Fig. 22.1A). The LacO array was originally cloned in plasmid pAFS52, an integrating *TRP1*-marked plasmid (Brickner and Walter, 2004; Straight *et al.*, 1996). We have also moved the LacO array (as a *Hin*DIII–*Xho*I fragment) into pRS306 (*URA3*-marked integrating plasmid; Sikorski and Heiter, 1989) to create p6LacO128 (Brickner and Walter, 2004). Whenever cloning the LacO array, it is important to confirm that the array is the expected size (>5 kb) by restriction digestion, as the array is sometimes lost or reduced in size after propagation in *E. coli*.

The next step is to introduce a targeting sequence into this plasmid so that the LacO array can be integrated at a locus of interest by homologous recombination. Because the sequences in the plasmid will be duplicated upon recombination (Fig. 22.1A), it is important to consider carefully which targeting sequences to clone. When localizing genes, we usually use sequences at the 3′ end of the gene to introduce the LacO array downstream of the 3′UTR and to avoid duplicating the promoter. Also, to allow for homologous recombination, the targeting sequence must include a

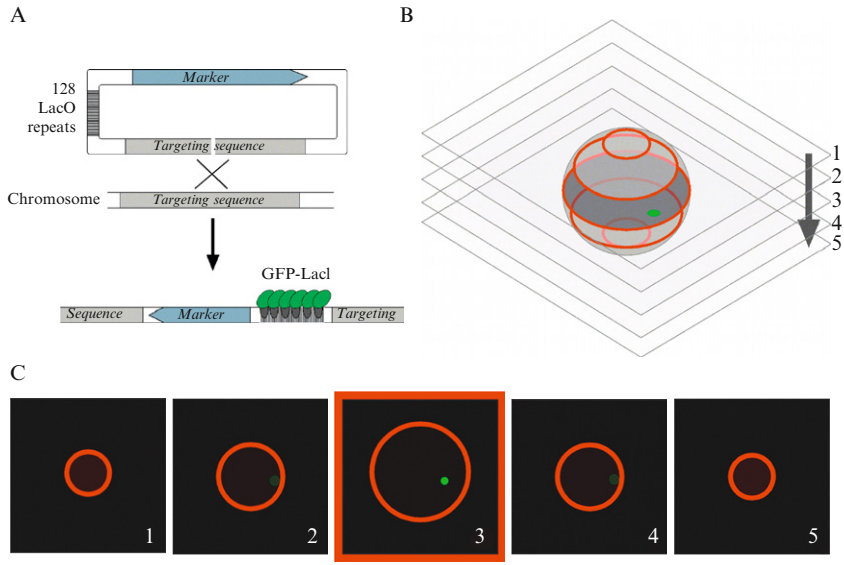

Figure 22.1 Schematic of the chromatin localization assay. (A) Strategy for marking a locus of interest with GFP. A plasmid containing the lac repressor array (LacO) and both a marker and a targeting sequence is digested at a unique restriction site within the targeting sequence. Transformation and homologous recombination introduce the marker and the lac repressor array into the yeast genome, flanked by the targeting sequence. The strain into which the plasmid is transformed also expresses the Lac repressor fused to GFP (GFP-LacI). (B) Confocal slices through a nucleus. The nuclear envelope is stained and a series of slices (numbered 1–5) are collected along the *z*-axis. Not shown in this representation is the staining of the cortical endoplasmic reticulum, which is visible when the Sec63-13myc marker is used. (C) Selection of the optimal slice. Slices 1–5 from panel (B) are shown. Slice #3 has the brightest, most focused GFP-LacI spot and is selected for scoring. The GFP-LacI spot in this cell is scored as nucleoplasmic.

restriction site that will be unique in the context of the LacO array plasmid. Table 22.1 shows a list of sites that are absent from the LacO array.

Once a targeting sequence has been introduced into the LacO array plasmid, it is digested at a unique restriction site within the targeting sequence and transformed into yeast. We typically start with a yeast strain that has previously been transformed with the GFP-Lac repressor (GFP-LacI) and, where necessary, additional tagged proteins localizing to other subnuclear domains (e.g., Sec63-13myc for the nuclear envelope/endoplasmic reticulum). The LacO array should be introduced last because not all transformants will possess a full-length array. To identify transformants that possess the full-length array, we screen through four or five clones to identify those that exhibit a clear green dot of GFP fluorescence. Once we have confirmed that the array is intact, we create a frozen stock of this strain.

Table 22.1 Sites absent from the LacO array

*Aar*I	*Bce*fI	*Bsm*AI	*Cla*I	*Hpy*188I	*Psh*AI	*Ssp*D5I
*Aat*II	*Bcg*I	*Bsm*BI	*Csp*6I	*Hpy*CH4III	*Psi*I	*Sth*132I
*Acc*65I	*Bci*VI	*Bsm*FI	*Cst*MI	*Hpy*CH4IV	*Psp*03I	*Sts*I
*Ace*III	*Bcl*I	*Bsp*24I	*Dde*I	*Kpn*I	*Psp*GI	*Stu*I
*Acl*I	*Bfr*BI	*Bsp*1286I	*Dpn*I	*Mae*III	*Psp*OMI	*Sty*I
*Acu*I	*Bgl*I	*Bsp*CNI	*Dra*I	*Mbo*I	*Psr*I	*Sty*D4I
*Afe*I	*Bgl*II	*Bsp*EI	*Dra*III	*Mbo*II	*Pss*I	*Swa*I
*Afl*II	*Blp*I	*Bsp*HI	*Drd*I	*Mfe*I	*Pvu*I	*Tai*I
*Age*I	*Bme*1580I	*Bsr*I	*Eag*I	*Mlu*I	*Pvu*II	*Taq*II
*Ahd*I	*Bmg*BI	*Bsr*DI	*Ear*I	*Mme*I	*Rle*AI	*Tat*I
*Ale*I	*Bmr*I	*Bsr*FI	*Eci*I	*Mse*I	*Rsa*I	*Tau*I
*Alf*I	*Bmt*I	*Bsr*GI	*Eco*HI	*Msl*I	*Rsr*II	*Tfi*I
*Alo*I	*Bpl*I	*Bss*HII	*Eco*ICRI	*Msp*A1I	*Sac*I	*Tse*I
*Alw*I	*Bpm*I	*Bss*SI	*Eco*57MI	*Mwo*I	*Sac*II	*Tsp*45I
*Alw*NI	*Bpu*10I	*Bst*API	*Eco*NI	*Nae*I	*San*DI	*Tsp*DTI
*Apa*I	*Bpu*EI	*Bst*BI	*Eco*O109I	*Nci*I	*Sap*I	*Tsp*GWI
*Apa*BI	*Bsa*I	*Bst*EII	*Fal*I	*Nco*I	*Sau*96I	*Tsp*RI
*Apa*LI	*Bsa*AI	*Bst*F5I	*Fau*I	*Nde*I	*Sca*I	*Tth*111I
*Asc*I	*Bsa*BI	*Bst*KTI	*Fmu*I	*Ngo*MIV	*Scr*FI	*Tth*111II
*Ase*I	*Bsa*JI	*Bst*NI	*Fnu*4HI	*Nhe*I	*Sel*I	*Unb*I
*Asi*SI	*Bsa*WI	*Bst*UI	*Fok*I	*Not*I	*Sex*AI	*Uth*SI
*Ava*II	*Bsa*XI	*Bst*XI	*Fse*I	*Nru*I	*Sfa*NI	*Vpa*K11AI
*Avr*II	*Bsc*AI	*Bst*YI	*Fsp*I	*Nsi*I	*Sfi*I	*Xcm*I
*Bae*I	*Bse*MII	*Bst*Z17I	*Fsp*AI	*Pac*I	*Sgr*AI	*Xma*I
*Bam*HI	*Bse*RI	*Bsu*36I	*Gdi*II	*Pas*I	*Sim*I	*Zra*I
*Ban*II	*Bse*YI	*Btg*I	*Hae*IV	*Pfl*MI	*Sma*I	
*Bbr*7I	*Bsg*I	*Btg*ZI	*Hga*I	*Pfo*I	*Sna*BI	
*Bbs*I	*Bsi*EI	*Bth*CI	*Hin*4I	*Pme*I	*Spe*I	
*Bbv*I	*Bsi*HKAI	*Bts*I	*Hpa*I	*Pml*I	*Srf*I	
*Bbv*CI	*Bsi*WI	*Cdi*I	*Hpa*II	*Ppi*I	*Sse*232I	
*Bcc*I	*Bsl*I	*Cha*I	*Hph*I	*Ppu*10I	*Sse*8647I	
*Bce*AI	*Bsm*I	*Cje*PI	*Hpy*99I	*Ppu*MI	*Ssp*I	

2. Immunofluorescence

Having tagged a locus of interest, it can be colocalized with respect to subnuclear structures either in live cells or in fixed cells. Chapter 21 describes methods for imaging chromosomal loci in live cells and defining their dynamic behavior. Here, we describe how to use immunofluorescence methods with populations of fixed cells to determine the localization of genes. The resulting localization represents a dynamic distribution and is

expressed as the fraction of the population in which the chromosomal locus and the protein marker. We focus on the localization of chromosomal loci with respect to the nuclear periphery or the nucleolus. We have successfully used several markers for the nuclear periphery. We have used the 9E10 anti-myc monoclonal antibody (Santa Cruz Biotechnology) to localize a 13myc-tagged Sec63 as a marker for the nuclear envelope (Brickner and Walter, 2004). This protein localizes throughout the endoplasmic reticulum (Gilmore, 1991). We have also used the 32D6 anti-Nsp1 monoclonal antibody from EnCor Biotechnology (Gainsville, FL) to stain nuclear pore complexes. This has the advantage that it does not require expression of a tagged protein. To stain the nucleolus, we have used the 37C12 monoclonal anti-Nop5/6 antibody from EnCor Biotechnology and we have used epitope-tagged versions of Spc42 as a marker for the spindle pole body.

3. Fixing Cells

Most immunofluorescence protocols use formaldehyde fixation. This works well for certain proteins and certain organelles. However, we have found that the shape of the nucleus can be poorly preserved by formaldehyde fixation (Fig. 22.2). Therefore, we use methanol fixation. This fixation

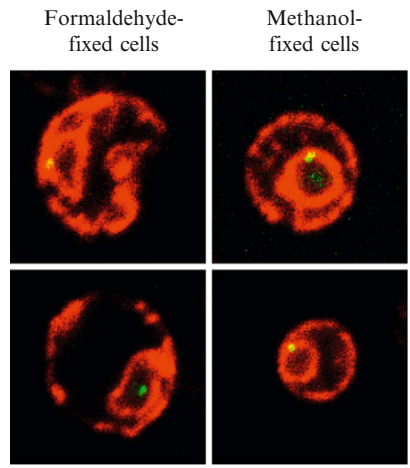

Figure 22.2 Methanol fixation versus formaldehyde fixation. Shown are representative examples of cells fixed using either methanol (as described in the protocol) or 4% formaldehyde (twice for 30 min). The ER/nuclear envelope was stained with Sec63-myc (red) and the chromosomal locus (in this case, the *INO1* gene) was stained with the GFP-Lac repressor (green). Note the nonspherical shape of the nucleus in the formaldehyde-fixed cells.

method causes cells to shrink somewhat but it preserves the spherical shape of the nucleus better than in formaldehyde-fixed cells.

1. Grow cells to $OD_{600} \sim 0.3$–0.7.

Note: This protocol has been used to examine cells from many different media, including both rich and synthetic media. Overgrown cells are more difficult to spheroplast and stain.

2. Harvest 10^7–10^8 cells by centrifugation and discard the supernatant. We typically harvest a culture of 10–15 ml at an $OD_{600} \sim 0.1$–0.7.
3. Suspend the cells in 1 ml of chilled methanol (store at -20 °C).
4. Incubate at -20 °C for ≥ 20 min.
5. Harvest cells by centrifugation and resuspend in 1 ml SHA (1 *M* sorbitol, 50 m*M* HEPES, pH 6.8, 1 m*M* NaN_3). The fixed cells can be stored at 4 °C (good for 4 or 5 days).

4. Spheroplasting

Before permeablizing cells, the cell wall must be removed. Once the cells are converted to fixed spheroplasts they should be processed for immunofluorescence immediately.

1. Harvest 5×10^7 cells by centrifugation, 30 s.
2. Resuspend in 1 ml SHA + 0.2% β-mercaptoethanol.
3. Add 2.5 μl (50 units) lyticase (Sigma catalog #L4025).
4. Incubate at 30 °C for 30 min. Invert tubes occasionally.
5. Harvest by centrifugation, 30 s.
6. Resuspend in 0.5 ml SHA + 0.1% Triton X-100. Incubate 10 min at room temperature.
7. Harvest by centrifugation, 30 s.
8. Resuspend in 250 μl SHA.

5. Preparing Slides

Ten well slides from Carlson Scientific (Peotone, IL; catalog # 101007) are used. Depending on the configuration of the microscope, we usually do not use the column of wells closest to the edge of the slides because they are more difficult to image. Between strains or treatments, we leave a column of wells empty to avoid cross-contamination. For washes, we add buffers with a Pasteur pipette and carefully remove the buffers by aspiration (using low vacuum and holding the tip at an angle beside the well).

1. Coat each well with 20 μl 0.1% polylysine (Sigma-Aldrich, catalog #P 8920).
2. Incubate ≥15 min at room temperature.
3. Remove by aspiration and let dry completely.
4. Add 20 μl fixed spheroplasts to each well and let settle for 15 min. Aspirate to remove excess liquid.
5. Wash cells twice with Buffer WT (1% nonfat dry milk 0.5 mg/ml BSA 200 m*M* NaCl 50 m*M* HEPES–KOH (pH 7.5) 1 m*M* NaN_3 0.1% Tween-20)

6. Antibody Incubations

1. Dilute the primary antibodies into Buffer WT.

Dilutions for antibodies we have used: anti-myc (1:200), anti-Nsp1 (1:200), anti-GFP (1:1000), anti-Nop5/6 (1:200), anti-HA (1:200).

2. Add 15 μl/well of diluted 1° Ab in Buffer WT.
3. Incubate 60–90 min at RT.

This incubation can be carried out overnight at 4 °C.

4. Wash five times with 20 μl Buffer WT.
5. Dilute secondary antibodies in Buffer WT.

We use Alexa Fluor® 594 goat antimouse IgG (Invitrogen catalog #A-11032) and Alexa Fluor® 488 goat antirabbit IgG (Invitrogen catalog #A-11008), diluted 1:200.

6. Add 15 μl of diluted secondary antibody in Buffer WT.
7. Incubate 60–90 min at room temperature in the dark.

This incubation can be carried out overnight at 4 °C.

8. Wash five times with 20 μl Buffer WT.

To stain for DNA, include 0.3 μg/ml DAPI in the third wash, incubate for 1 h at 4 °C (in the dark), and wash twice more.

7. Mounting and Storage of Slides

1. After aspirating most of the liquid from each well on a slide (without letting them dry completely), quickly add 1–2 μl of Vectashield mounting solution (Vector Laboratories, catalog #H-1000).

Aspirate and add mounting medium to each well before moving on to the next well.

2. Carefully cover wells with a clean 50 mm coverslip.

The mounting medium should fill the wells without overflowing into neighboring wells.

3. Seal the slide by painting the seams with clear fingernail polish. Let dry in the dark.
4. The slides may be stored in the refrigerator, but they look best when they are fresh.

8. Microscopy and Analysis

We use a Zeiss LSM510 confocal microscope with a 100× 1.4NA objective to image the cells. We have found that confocal imaging is the best system for accurate localization of genes within the nucleus. We routinely visualize fixed and stained cells using both a 30-mW 458/488/514 nm Argon laser and a 1-mW 543 nm Helium Neon laser. The pinhole is set to 146 nm, with the detector gain set to 750–900 and the amplifier offset is ~ -0.3. It is not necessary to collect three-dimensional stacks or to reconstruct whole cells. We use the motorized stage control to step through the nuclear volume to find a confocal slice (~ 0.7 μm in z dimension) that displays the most intense and most focused spot for the lac repressor-GFP (schematized in Fig. 22.1C; slice #3). Then we collect data from this slice in both channels. For experiments in which we are assessing peripheral localization, we only include cells in which the selected slice displays a clear ring staining for the nucleus (i.e., not at the top or the bottom of the nucleus, nor cells in which the nuclear envelope is incompletely stained).

For each cell, we compare the localization of the GFP-Lac repressor (GFP-LacI) spot and the marker of interest (e.g., nuclear envelope or nucleolus). In the case of the nuclear envelope, we bin cells into one of two classes (Brickner and Walter, 2004). If the center of the GFP spot is overlapping with the nuclear envelope, we classify the spot's localization as peripheral. If the center of the GFP spot is not overlapping with the nuclear envelope, we classify the spot's localization as nucleoplasmic. For each biological replicate, we collect data from 30 to 50 cells. For a given population, we determine the percentage of cells in which the spot localizes to the nuclear periphery.

The light microscope has a resolution of 100–200 nm in the X–Y dimensions (Born and Wolf, 1998; Pawley, 2006; Schermelleh *et al.*, 2008). The radius of the haploid yeast nucleus is approximately 1 μm. Therefore, GFP spots within the shell corresponding to the outermost $\sim 10\%$ of the radius will be scored as peripheral in our assay. The fraction of cells in which the GFP spot

would be expected to localize at the periphery by chance can be calculated from the fraction of the total volume represented by this outermost 10% of the radius. The volume of sphere is biased to the outside; comparing spheres having radii of 0.9 μm versus 1 μm, the small sphere has a volume (3.1 μm^3), that is 74% of the volume of the larger sphere (4.2 μm^3). Therefore, this outermost shell corresponds to 26% of the volume of the nucleus and an unbiased distribution within the yeast nucleus should result in ~26% colocalization with the nuclear membrane. This level of peripheral localization is the baseline for this assay. Peripherally localized chromosomal loci score as peripheral in 60–85% of cells using the scoring criterion above. For example, genes such as *INO1* and *GAL1* that are recruited to the nuclear periphery upon activation, localize at the periphery in ~30% of cells when they are repressed and in ~65% of cells when they are activated (Brickner and Walter, 2004; Brickner *et al.*, 2007; Casolari *et al.*, 2004, 2005; Schmid *et al.*, 2006). More stably associated peripheral loci such as telomeres localize at the nuclear periphery in ~85% of cells using this assay (our unpublished data).

To determine the variance in the peripheral localization for a particular locus, we use three or more biological replicates (i.e., cells harvested from independent cultures). From these measurements we determine the mean value and the standard error of the mean. As a negative control for peripheral localization, we use the *URA3* gene, a nucleoplasmic locus that localizes at the nuclear periphery in ~30% of cells (Brickner and Walter, 2004). To determine if peripheral localization is statistically significant, we use an unpaired *t*-test to compare the percentage of cells with peripheral localization of the locus of interest with the percentage of cells with peripheral localization of the *URA3* gene. In a typical experiment, the *URA3* gene localizes at the nuclear periphery in 30% ± 5% of cells and a peripheral gene like *INO1* localizes at the nuclear periphery in 65% ± 5% of cells ($P = 0.0078$).

We have used a similar strategy to localize chromosomal loci with respect to the nucleolus. The nucleolus in yeast is a single, crescent-shaped organelle that usually aligns opposite the spindle pole body (Stone *et al.*, 2000; Yang *et al.*, 1989). The rDNA repeats and tRNA genes localize within the nucleolus (Thompson *et al.*, 2003). Recent work using high-resolution probabilistic analysis has shown that, although most RNA polymerase II-transcribed genes are excluded from the nucleolus, some localize within the nucleolus (Berger *et al.*, 2008). In particular, the *GAL2* gene localizes within the nucleolus and becomes localized at the periphery of the nucleolus upon activation. We have also observed the nucleolar localization of *GAL2* using immunofluorescence (Fig. 22.3). Immunofluorescence using commercially available antibodies against the Nop5 and Nop6 proteins defines the nucleolus, which typically occupies approximately one-third of the nuclear volume and appears as a crescent or spherical shape. Using the same confocal microscopy analysis, we have quantified the colocalization of *GAL2* or *INO1* with the nucleolus. The *GAL2* gene colocalizes

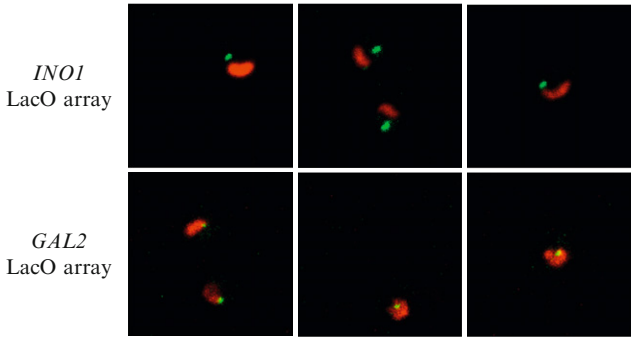

Figure 22.3 **Nucleolar colocalization. Cells were fixed and probed with anti-GFP and anti-Nop5/6 as described in the protocol. In the cells shown in the top panels, the LacO array was integrated beside the *INO1* gene. In the cells shown in the bottom panels, the LacO array was integrated beside the *GAL2* gene. Note the clear separation of the *INO1* gene from the nucleolus and the localization of *GAL2* within the nucleolus.**

with the nucleolus in most (~90%) of the cells in the population (Fig. 22.3; Gard *et al.*, 2009). In contrast, the *INO1* gene localizes within the nucleolus in ~10% of cells (Fig. 22.3; our unpublished data). Therefore, although the nucleolus occupies a significant fraction of the nuclear volume, the baseline colocalization of chromosomal loci with this structure is lower than expected. This suggests that cells in which a nonnucleolar chromosomal locus appears nucleolar because it is above or below the nucleolus is not a major source of background in these measurements. Therefore, this method readily distinguishes between two classes of RNA polymerase II-transcribed genes that differ in their localization with respect to the nucleolus.

The methods described in this chapter allow colocalization of chromosomal loci with respect to two major subnuclear compartments. It remains to be seen what fraction of the yeast genome localizes to particular subnuclear domains. It is conceivable that there are few parts of the genome that are randomly localized. As the spatial organization of the nucleus becomes better understood, we anticipate that these methods can be adapted and improved to allow the localization of loci with respect to additional subnuclear compartments.

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