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In Situ* Chromatin Binding Assay Using Epifluorescent Microscopy in *S. pombe

Jinpu Yang and Fei Li

Department of Biology, New York University, New York, NY 10003-6688, USA

Abstract

Chromatin-associated proteins play critical roles in many cellular processes, including gene expression, epigenetic regulation, DNA repair, recombination and replication. Especially, epigenetic landscape, shaped by a variety of chromatin-binding proteins, is dynamic and regulated in a context-dependent manner. *In situ* chromatin binding assay is a powerful but simple tool to investigate how proteins, such as epigenetic components, associate with chromatin. This approach relies on the fact that chromatin bound proteins are more resistant to detergent extraction. Here we describe a protocol for the *in situ* chromatin binding assay used in *Schizosaccharomyces pombe*.

Keywords

Epigenetics; *in situ* chromatin binding; H3; CENP-A; GFP; Fission yeast; *Schizosaccharomyces pombe*

1. Introduction

Eukaryotic DNA is packaged into chromatin by wrapping around histone proteins. The organization of chromatin is tightly regulated and can be divided into two major domains: heterochromatin and euchromatin. Whereas heterochromatin is a tightly packed form of chromatin containing few genes, euchromatin is a more open chromatin region where genes are actively transcribed. In addition to histones, a variety of proteins are also associated with chromatin and play important roles in a wide range of cellular activities, such as DNA replication, transcriptional regulation, chromatin remodeling, cell cycle progression, aging, tumorigenesis, development and differentiation. One major group of proteins associated with chromatin is epigenetic regulators. These proteins mediate epigenetic modifications on chromatin, such as histone modification, DNA methylation, and histone variants, which exert effects on cellular processes without changing genetic sequences. Epigenetic regulators are usually associated with chromatin in a dynamic manner. The epigenetic hallmark for

CORRESPONDING AUTHOR: Fei Li, Department of Biology, New York University, New York, NY 10003, USA, Phone: (212) 998-3746, Fax: (212) 995-4015, fl43@nyu.edu.

4. Notes

2. The optimal induction time for *nmt1* promoter should be determined empirically for specific GFP-tagged proteins. Before processing cells for *in situ* chromatin binding assay, take an aliquot and examine under epifluorescent microscope to make sure that the GFP signals are visible and proteins are properly expressed.

3. Proteins analyzed should have a GFP (or other fluorescent) tag; alternatively, proteins can be detected via indirect immunofluorescence as described.

heterochromatin is histone 3 lysine 9 methylation (H3K9me), conserved from fission yeast to human. H3K9me is essential for heterochromatin structure and function. In fission yeast, the modification is catalyzed by histone methyltransferase Clr4 (a human Suv39 homolog) and recognized by the conserved HP1 homolog, Swi6 [1]. The CLRC complex, which is composed of Clr4, Rik1, Cul4, Dos1/Raf1, Dos2/Raf2, and Lid2, is recruited to heterochromatin during S phase, and promotes heterochromatin assembly [2–6].

A distinct chromatin structure in all eukaryotes is the centromere that provides foundation for kinetochore assembly and is crucial for proper chromosome segregation during mitosis and meiosis. In most eukaryotes including fission yeast and humans, centromere is epigenetically defined by CENP-A, a centromere-specific H3 variant [4,7–9]. CENP-A loading to centromeres is cell cycle-dependent, and is mediated by multiple CENP-A loading factors. Analyzing the chromatin association of specific proteins is thus critical for elucidating the epigenetic mechanisms used to govern chromatin structures, such as heterochromatin and centromeres.

Chromatin immunoprecipitation (ChIP) is a widely used method to study the binding of proteins to chromatin, and has paved the way for better understanding of chromatin and epigenetic regulation. ChIP uses formaldehyde to create chemical crosslinks between proteins and DNA. The chromatin is mechanically sheared and subsequently precipitated by an antibody specific to the protein of interest. DNA fragments co-precipitated with the protein are analyzed by Southern hybridization or PCR [10–13]. However, the outcome of ChIP depends on the efficiency of crosslink, variation of immunoprecipitation and quality of antibodies. In addition, ChIP can only determine the protein binding ability to chromatin on average from a pool of cells. *In situ* chromatin binding assay has been developed to study the protein-chromatin interaction at the single-cell level in fission yeast. It originally was used to analyze the binding of replication and transcription factors to chromatin [14]. We have adapted it for use to study histone variants and histone modification complexes, such as centromere and heterochromatin regulators [6,15]. The method starts with partial digestion of fission yeast cell wall using zymolyase, followed by detergent extraction (washing with Triton X-100). As a result, soluble nucleoplasmic proteins and non-chromatin bound proteins are washed away, while proteins associated with chromatin remain, which can be detected using either a GFP (or GFP variants) tag, or indirect immunofluorescence (Fig. 1). Importantly, partial digestion of cell wall by zymolyase allows cells to maintain their structure. As an example, this technique has been used to examine the chromatin association of the human HP1 homolog, Swi6, in a *clr4* mutant background. Swi6-GFP can be washed away upon detergent extraction in *clr4* cells, demonstrating that Swi6 has little association with chromatin in the absence of H3K9me. On the other hand, the association of histone H3-GFP with chromatin is independent of Clr4 activity and therefore in *clr4* cells the nuclear localization of H3 is retained after the same detergent treatment (Fig. 2).

This *in situ* approach enables examination of protein distribution at the single-cell level while cell structure is largely maintained. In addition, since this method allows analysis of large cell population at single-cell level, cell cycle synchronization is not necessary, and comparative analyses between different mutants, time courses, or temperature conditions in fission yeast are simple and easy. When the protein of interest is directly tagged with a

fluorescent tag, this method is most straightforward. But indirect immunofluorescence can also be used to detect proteins of interest. The described protocol and buffer conditions are optimized for histone variants and histone modifying complexes. However, this approach can be adapted to study other chromatin binding proteins. Note that the detergent extraction conditions have to be determined empirically for specific proteins, and it also is important to have proper control samples in order to demonstrate the washing is effective but not excessive.

2. Materials

1. ZM buffer: 50 mM sodium citrate pH 5.6, 1.2 M sorbitol, 0.5mM MgAc.
2. STOP buffer: 0.1 M MES pH 6.4, 1.2 M sorbitol, 1 mM EDTA, 0.5 mM MgAc.
3. Extraction buffer (EB): 20 mM PIPES-KOH, pH 6.8, 0.4 M sorbitol, 150 mM KAc, 2 mM MgAc. Store at -20°C .
4. EBT buffer: 20 mM PIPES-KOH, pH 6.8, 0.4 M sorbitol, 150 mM KAc, 2 mM MgAc, 10% Triton X-100.
5. 20,000 U/g Zymolyase 20-T (Amsbio, cat. no. 120491-1), store at 4°C .
6. 10% Triton X-100 (in EBT buffer).
7. 2% Sodium dodecyl sulfate (SDS) (optional).
8. 1 M Dithiothreitol (DTT), store at -20°C .
9. 100% Methanol.
10. 100% Acetone.
11. Protease inhibitor cocktail 100X (Sigma, cat.no. P8215), store at -20°C .
12. Fixation solution: 3.7% formaldehyde, 10% methanol.
13. Mounting medium: Vectashield anti-fade mounting media with DAPI, (Vector, cat. no. H-1200), store at 4°C .
14. Microscope slides, precleaned Plain Microscope Slides, (Fisherbrand, cat. no. 12-550-A3).
15. Poly-L-lysine-coated cover slips. Soak 13-mm cover slips (ThermoFisher Scientific cat. No. 174950) in 0.1% poly-L-lysine (Sigma, cat. no. P 8920) for 10 min. Drain, dry, rinse in deionized water, and dry.
16. VWR micro cover glass, cat. no. 48366-227.
17. PEM buffer: 100 mM PIPES, 1mM EGTA, 1mM MgSO₄, adjust the pH to 6.9.
18. PEMSorb buffer: PEM, 1.2M Sorbitol.
19. PBSBAL buffer: PBS, 1% BSA, 100mM Lysine hydrochloride, and 0.01% NaN₃, pH 6.9.
20. Media:

YES media:

	For 1 Liter	Final concentration
Yeast extract	5g	0.5% w/v
Glucose	30g	3% w/v
adenine	225 mg	
histidine	225 mg	
leucine	225 mg	
uracil	225 mg	
lysine	225 mg	

PMG media:

KH Phthallate	3g	14.7 mM
Na ₂ HPO ₄	2.2g	15.5 mM
L-glutamic acid, monosodium salt	3.75g	
Glucose	20g	2% w/v
Salt stock (50x)	20mL	
Vitamin stock (1000x)	1mL	
Mineral stock (10,000x)	0.1mL	
adenine	225 mg	
histidine	225 mg	
leucine	225 mg	
uracil	225 mg	
lysine	225 mg	

50× Salt stock

	For 1 Liter	Final concentration
MgCl ₂ .6H ₂ O	53.3g	0.26 M
CaCl ₂ .2H ₂ O	0.735g	4.99 mM
KCl	50g	0.67 M
Na ₂ SO ₄	2g	14.1 mM

1000× Vitamin stock

	For 1 Liter	Final concentration
pantothenic acid	1g	4.2 mM
nicotinic acid	10g	81.2 mM
inositol	10g	55.5 mM

	biotin	10mg	40.8 μ M
10,000 \times Mineral stock			
	For 1 Liter	Final concentration	
boric acid	5g	80.9 mM	
MnSO ₄	4g	23.7 mM	
ZnSO ₄ ·7H ₂ O	4g	13.9 mM	
FeCl ₂ ·6H ₂ O	2g	7.4 mM	
molybdic acid	0.4g	2.47 mM	
KI	1g	6.02 mM	
CuSO ₄ ·5H ₂ O	0.4g	1.6 mM	
citric acid	10g	47.6 mM	

3. Methods

3.1 *in situ* Chromatin Binding Assay using GFP-tagged proteins.

1. Inoculate the fission yeast strain into 10 mL YES or PMG media, and grow overnight at permissive temperature. Culture should be in log phase when cells are collected for assay. In general, 10 mL of culture at OD_{595nm} = 0.2 (~10⁸ cells in total) is enough for one to three assays (See Notes 1~4).
2. Spin down an aliquot of cells by at 3000 \times g for 5 minutes. Fix cells by resuspending the pellet in fixation solution (See Note 5). These cells will be used as untreated control, and be examined in step 9.
3. Collect the remaining cells by centrifugation at 3000 \times g for 5 min. Resuspend the cells in 1 mL ice-cold ZM buffer containing 10 mM DTT (See Note 6). Transfer to a 1.5-mL Eppendorf tube.
4. Spin cells down at 3000 \times g for 5 min, and then resuspend in 0.5 mL ZM buffer containing 10 mM DTT and 1mg zymolyase 20-T (See Note 7). Incubate cells on a rocker platform at 32°C for 30 min. To test for adequate digestion, a few microliters of cells can be mixed with an equal volume of 2% SDS on a slide and

¹-Cells grow relatively healthier in rich (YES) medium than in minimum medium (PMG). However, for GFP-tagged proteins expressed on a non-integrated plasmid (such as pREP1), minimum medium lacking specific nutrient (for example, no leucine in the case of pREP1) should be used to make sure that the plasmid is properly maintained. We found that it is a good practice to culture cells in two-steps for cells carrying plasmid such as pREP1: *Step 1*) inoculate a single colony into 2 mL YES medium, and grow overnight. Collect cells by centrifugation at 3000 \times g for 3 minutes, followed by washing once with sterile water. pREP1 plasmid harbors *nmt1* promoter, which is repressed in the presence of thiamine. It is important to remove YES medium as much as possible because YES contains low level of thiamine. *Step 2*) inoculate cells from step 1 into 20 mL PMG without leucine, and culture at permissive temperature for 18~28 hours.

⁴-The Mg²⁺-containing EB is suitable for analysis of Swi6, CENP-A^{Cnp1} and H3 proteins in log phase cells. For other proteins, modifications of the buffer condition may be necessary by the end user.

⁵-Alternatively, cells can be fixed by resuspending in 2 mL ice-cold 100 % methanol for 10 mins. Spin cells down and then resuspend cells in 1 mL ice-cold 100% acetone. The best fixation procedure has to be optimized for specific protein examined.

⁶-DTT should add freshly to the ZM buffer prior each use. To avoid repeated thaw and freeze, store small aliquots (no more than 1mL) of 1M DTT in -20°C.

⁷-Zymolyase in ZM buffer recommended here reaches final concentration of 2mg/ml, or 20U per 10⁸ cells. Cells that were not adequately digested during the zymolyase digestion step appear bright under phase contrast microscope when examined by 2% SDS.

examined under phase contrast microscopy. Cells should go phase dark. (Aim for >90% cells going phase dark.) If digestion is not adequate with the previous described procedures, add more zymolyase (at 0.2mg interval) or extend incubation time (check every 5 minutes).

5. When adequate digestion has been achieved, wash cells 3 times with ice-cold STOP buffer. (All subsequent centrifugations from here to step 8 are at $1000 \times g$ for 1 min using an Eppendorf centrifuge at 4°C).
6. Resuspend cells in 1 mL ice-cold EB, and spin down.
7. Resuspend cells in 0.9 mL extraction buffer containing protease inhibitors. Transfer 0.45 mL of the cell suspension to a new 1.5-mL Eppendorf tube containing 50 μL EBT buffer. Mix and transfer to 20°C water bath for 7 min (See Note 8). Remaining cells will be used as unwashed control.
8. Spin both washed and unwashed cells down, and resuspend them in 20~50 μL ice-cold fixation solution. For proteins that are not directly tagged with GFP (or other fluorescent proteins), proceed to step 1 in Subheading 3.2.
9. To examine the cells by epifluorescence microscopy, spread a thin film of cells on microscope slide. Apply 1~2 μL Vectashield mounting solution and a cover slip to the sample. The cover slip can be sealed with nail varnish, but this is not necessary if the slides are to be viewed immediately (See Note 9). When obtaining images of cells, first check the cells under phase contrast to make sure cells within the field have been properly digested, then take a green fluorescent protein (GFP) image, and finally a DAPI image (See Note 10).

3.2 *in situ* Chromatin Binding Assay Using Indirect Immunofluorescence Staining.

1. Spin down cells prepared at step 8 in Subheading 3.1 at $1000 \times g$ for 3 min and remove the fixation solution. Resuspend cells in 0.5mL PEMSorb buffer, and wash three times with PEM.
2. Resuspend cells with 50 μL PEM by vortexing, and make sure that there aren't any cell clumps. Spread about 10 μL of the cell suspension onto a poly-L-lysine-coated 13-mm cover slip. Drain excessive liquid with kimwipes.
3. Transfer coverslips to a petri dish, wash in PBS for 5 mins.
4. Replace PBS with PBSBAL buffer, and incubate for 1 hour at room temperature (See Note 11).

⁸The exact time for washing with the detergent (1% Triton X-100) should be decided for individual proteins analyzed. We found that 7 minutes at 20°C is sufficient for washing away of Swi6 and CENP-A^{Cnp1}. It is highly recommended to use proper control groups to determine the optimal washing time, and to run control groups in parallel with each experiment. If it is suspected that the protein of interest may be hold in the nucleus by structures other than chromatin, such as nuclear matrix, it is recommended to include micrococcal nuclease digestion as an additional control to determine whether the retention of protein after detergent washing is chromatin-dependent. Method for micrococcal nuclease digestion control has been previously described [14].

⁹When cover slips are sealed with nail varnish, slides can be stored at 4°C for up to 7 days. When ready to analyze, warm the slides in room temperature until the condensation is gone.

¹⁰Other than epifluorescent microscope, flow cytometry analysis can be employed to further analyze the fluorescent signal within the cell population. Detailed methods have been previously described [14].

5. Drain off PBSBAL buffer, and add 10 μ L primary antibody (diluted in PBSBAL) to coverslip. Incubate for 1 hour at room temperature in a humid chamber.
6. Wash three times in PBSBAL (5 min each).
7. Apply 20 μ L secondary antibody to the cover slip (conjugated with fluorescent dye). Incubate in dark humid container for at least 1 h at room temperature (See Note 12).
8. Wash three times in PBS (5 min each).
9. Drain the cover slip well. Mount in Vectashield anti-fade mounting media with DAPI. Seal the cover slip with nail varnish (See Note 13). Obtain images of cells taking first a fluorescent image, and then a DAPI image for each field of cells.

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¹¹ This step is used to block nonspecific binding of the epitope. The appropriate time can be determined based on different antibodies. Coverslip can be rocked gently to speed up the process.

¹² Spin the secondary antibody before use to get rid of any fluorescent precipitate.

¹³ After nail varnish is completely dried, slides can be stored at –20°C.

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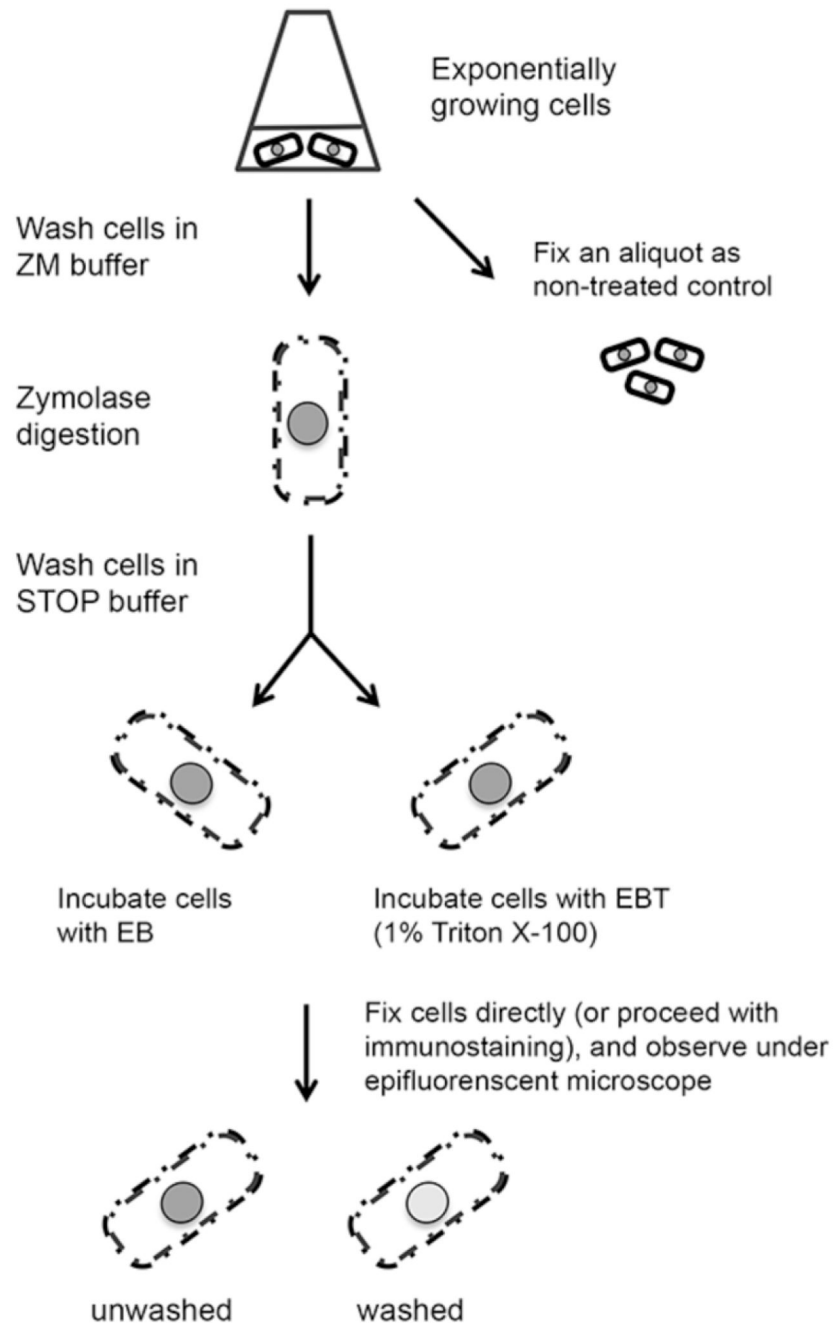


Figure 1. Schematic flow diagram for *in situ* chromatin binding assay.

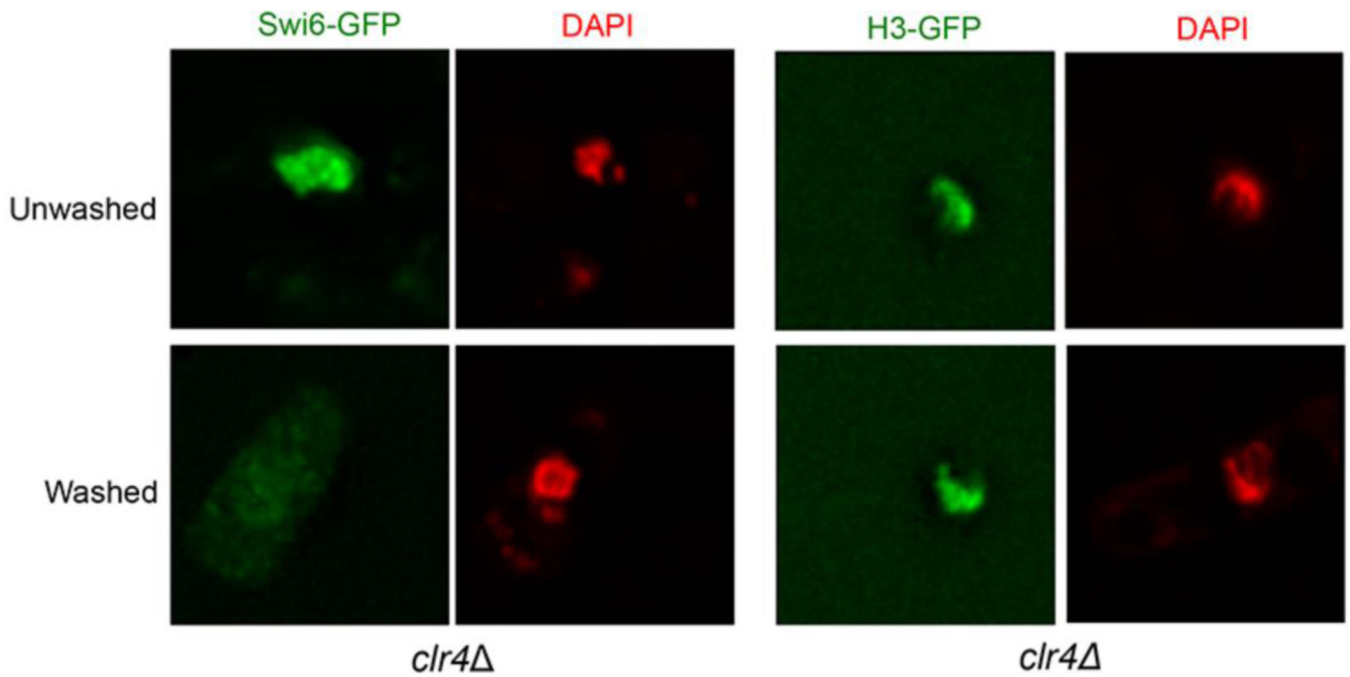


Figure 2. *In situ* chromatin-binding assay for *clr4* cells expressing either Swi6-GFP or H3-GFP. Swi6-GFP is diffused in the nucleus in *clr4* cells before washing with Triton X-100 (top panel). The signal can be readily removed upon washing with the detergent (bottom panel). In contrast, the H3-GFP signal in *clr4* cells is retained in the nucleus before (top panel) or after (bottom panel) Triton X-100 extraction, indicating that H3-GFP is stably bound with the chromatin. Cells are counterstained with DAPI (red) to visualize the nucleus.