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Analysis of chromatin binding dynamics using the crosslinking kinetics (CLK) method

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Abstract

Transcription factor binding sites in chromatin are routinely inventoried by the chromatin immunoprecipitation assay, and these binding patterns can provide precise and detailed information about cell state. However, some fundamental molecular questions regarding transcription factor function require an understanding of in vivo binding dynamics as well as location information. Here we describe the crosslinking kinetics (CLK) assay, in which the time-dependence of formaldehyde crosslinking is used to extract on- and off-rates for chromatin binding in vivo.

Keywords

formaldehyde; chromatin immunoprecipitation; dynamics; kinetic; method

1. Introduction

A major effort in the chromatin field for more than two decades has been to define where regulatory factors associate with the native DNA template and under what conditions of growth or differentiation they are found there. In general, the most robust methods for detection of binding to specific, single-copy loci involve isolation of chromatin fragments from cells using an antibody that detects the chromatin binding factor of interest [1, 2]. Native complexes can be isolated if the interactions are stable, but for many transcription factors, formaldehyde has been used to crosslink the constituents together so that the associated DNA can be obtained and assayed using locus-specific PCR, microarrays, or high throughput sequencing [3, 4]. This general approach, known as chromatin immunoprecipitation (ChIP¹), has revolutionized our understanding of chromatin structure

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¹Abbreviations: ChIP, chromatin immunoprecipitation; CLK, crosslinking kinetics; TF, transcription factor; QF, quench-flow instrument; BMN, benzyldene malanonitrile

and function by providing increasingly high resolution pictures of the chromatin interaction landscape in a wide variety of cell types and conditions [5, 6, 7].

The ChIP assay is a powerful approach for determining chromatin binding locations, and the magnitudes of ChIP signals obtained following a standard fixation time correlate with transcription factor (TF) affinities measured in vitro [8]. These results, which are discussed more fully below, suggest that conventional ChIP assays provide quantitative information about relative occupancy levels as well as position along the DNA fiber. However, the ChIP assay does not provide unambiguous information about how stable such interactions are. An increase in the ChIP signal obtained in one condition compared to another could be due in principle to more efficient capture because the complex is more stable, it could result from higher fractional occupancy in a cell population, or could even result from more efficient capture of a transient complex with a rapid on-rate. One approach to extend the ChIP assay so as to provide information on binding dynamics is to induce a differentially tagged isoform of the factor of interest and monitor the turnover of the constitutively expressed form compared to the induced form [9, 10, 11, 12]. This approach, known as competition ChIP, has provided information about relative complex stabilities, and more recently turnover dynamics have been used to extract actual rather than relative residence times [13]. An alternative approach utilizes metabolic labeling and has been used to measure nucleosome turnover in vivo [14]. However, competition ChIP is inherently limited by the time it takes to induce expression or activity of the competitor, which is on the order of 20–30 minutes. Thus, the method is well suited to measure dynamics occurring on the timescale of ~20 minutes or longer, but dynamics occurring more rapidly than that can't be measured by this method. Live cell imaging approaches have revealed that many chromatin complexes are short-lived, although it is not possible in most cases to monitor binding dynamics of a factor at a particular single-copy site by imaging. The rapid dynamics observed by live cell imaging approaches suggest that some, perhaps even most of the chromatin complexes identified by ChIP represent snapshots of short-lived interactions [15–19]. Although most live cell imaging studies have not defined binding kinetics at single copy loci, the conclusions of these studies are strengthened by the recent convergence of kinetic measurements obtained by multiple independent live cell imaging approaches [20]. The general conclusion from these studies is that the time-scales over which regulatory complexes form and persist in vivo are fundamentally important to understand in order to develop accurate molecular models for how gene expression occurs and is controlled in vivo.

2. Materials

2.1 Strain construction

Yeast strains (see below)

Yeast growth medium (YEP, SC supplemented with appropriate carbon source)

Plasmid for transcription factor overexpression (see below)

In-Fusion HD cloning kit (Clontech)

2.2 Whole cell extract

37% formaldehyde (Fisher Scientific)

2.5 M glycine (Bio-Rad) or 3 M Tris base pH 8.0 (Sigma)

Benoit Extraction Buffer (200 mM Tris-HCl (pH 8.0), 400 mM $(\text{NH}_4)_2\text{SO}_4$, 10 mM MgCl_2 , 1 mM EDTA, 10% glycerol, 7 mM β -mercaptoethanol, and protease inhibitors as described below)

Protease inhibitors: Roche Complete Protease Inhibitor Cocktail Tablet OR 1.0 mM phenylmethylsulfonyl fluoride, 2.0 mM benzamidine, 2.0 μM pepstatin, 0.6 μM leupeptin, and 2.0 μg of chymostatin per ml of buffer

SDS polyacrylamide gel electrophoresis and transfer system (mini-PROTEAN tetra cell, Bio-Rad)

Transcription factor-specific primary antibody

ECL anti-rabbit or –mouse horseradish peroxidase linked whole antibody (GE Healthcare)

Amersham ECL Prime western blotting detecting reagent (GE Healthcare)

2.3 ChIP procedure

2.3.1 Cross-linking

KinTek quench-flow apparatus (model RQF-3, KinTek Corporation) (requirement depends on transcription factor dynamics; see Section 4), exit tubing of various lengths (see Table 1 for examples)

37% formaldehyde (Fisher Scientific)

2.5 M glycine (Bio-Rad) OR 3 M Tris base pH 8.0 (see Section 3.3, Step 2)

TBS (40 mM Tris-HCl pH 7.5, 300 mM NaCl)

TBS + 125mM glycine

140 mM ChIP lysis buffer (50 mM Hepes pH 7.5, 140 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate) with protease inhibitors (see above)

Acid-washed 425–600 μm glass beads (Sigma)

Mini-Beadbeater (BioSpec)

18 gauge needles (BD PrecisionGlide)

Sonifier 250 with microtip (Branson)

Protein Assay Dye Reagent Concentrate (Bio-Rad)

2.3.2 Immunoprecipitation

1. Primary antibody and nProtein A Sepharose 4 Fast Flow beads (GE Healthcare)
OR

2. IgG Sepharose 6 Fast Flow and Sepharose 6 Fast Flow beads (GE Healthcare) 140 mM ChIP lysis buffer (50 mM Hepes pH 7.5, 140 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate) with and without protease inhibitors (see above)

ChIP lysis buffer (as for 140 mM ChIP lysis buffer but containing 500 mM NaCl)

LiCl wash buffer (10 mM Tris pH 8.0, 250 mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA)

1X TE (10 mM Tris-Cl pH 8.0, 1 mM EDTA)

Elution buffer (50 mM Tris-HCl pH 8.0, 1% SDS, 10 mM EDTA)

2.3.3 Real-time qPCR

QIAQuick PCR purification kit (Qiagen)

MyiQ real-time instrument (Bio-Rad)

iQ SYBR Green Supermix (Bio-Rad)

Locus-specific primers

3. Method

3.1 Overview of the method

The crosslinking kinetics (CLK) assay exploits the location precision afforded by the ChIP assay, but extends it so as to provide quantitative information about binding dynamics [21]. The CLK mathematical model describes the relationship between the ChIP signal at crosslinking time t as a function of the on- and off-rates for chromatin binding, the crosslinking rate, and the concentrations of formaldehyde and of the TF in the nucleus. The derivation of the model begins with a simple reaction describing the process of TF binding to DNA, followed by crosslinking:

In this reaction, the reversible binding of a TF to DNA is governed by the rate constants k_a and k_d , and formaldehyde crosslinking occurs at a rate defined by k_{xl} and is presumed to be irreversible under our experimental conditions. Formaldehyde crosslinking has been known for decades to be very stable [22] and irreversibility of the crosslinking reaction under the conditions described here is supported by direct measurements of the formaldehyde-mediated protein-DNA crosslink reversal rate [23]; discussed more below. The derived CLK model is very general, with the only assumptions being that (1) the ChIP signal is crosslinking dependent, (2) DNA binding by the TF precedes crosslinking, (3) the TF is in excess over chromatin sites and (4) that the unbound TF pool is not substantially depleted by nonspecific inactivation by formaldehyde. While the time-dependent increase in the ChIP signal is readily observed for a number of TF-chromatin complexes, the power of the CLK approach is its ability to distinguish binding dynamics from crosslinking kinetics in the time-dependent ChIP data. This is accomplished by comparing the time-dependent ChIP signals

obtained from otherwise identical cells harboring two different levels of the TF of interest. The contribution of binding dynamics to the time-dependent increase in the ChIP signal is discernable because crosslinking occurs much more rapidly (on the order of ~1 second) than binding dynamics, which occur in general on the tens of seconds to minutes (or longer) time scale [21]. Rapid crosslinking was reported in the first studies using formaldehyde to identify protein-DNA interactions in vivo [2, 22]. As a result, binding dynamics are qualitatively apparent in the data, as a change in the TF concentration will impact both the fractional occupancy and the on-rate by mass action [21]. The CLK method was developed using cells from the yeast *Saccharomyces cerevisiae*. In principle, the method is applicable to other types of cells, but this has not been explored thus far.

3.2 Construction and characterization of strains

The main steps involved in implementation of the CLK method are diagrammed in Figure 1. The first requirement is a strain expressing WT levels of the TF of interest, either in its native form if a ChIP-grade antibody is available, or epitope-tagged and suitably characterized for WT function. An assumption of the CLK model is that the TF is present in cells in excess over chromatin binding sites. It is often possible to provisionally assess the validity of this assumption using estimates of protein concentration from the literature (e.g. [24]) combined with estimates of the total number of binding sites from prior ChIP-chip or ChIP-seq experiments. Even TFs of relatively low abundance, such as Gal4, are suitable for analysis by the method as long as the number of binding sites is also low [21]. A related assumption is that the unbound TF pool is not depleted by formaldehyde treatment. To test this, prepare yeast whole cell extracts from cells treated with formaldehyde for 5, 10 or 15 minutes and quantify any changes in TF levels due to formaldehyde by western blotting. Prepare yeast whole extracts in the same way as the preparation of chromatin extracts for ChIP (except without the need for sonication; see below), but use of a higher ionic strength Benoit Extraction Buffer helps ensure good recovery of the TF. An advantage of Western blotting is that one can also assess whether formaldehyde treatment appreciably affects the mobility of the protein, which could suggest chemical alterations in activity. Formaldehyde-mediated protein-DNA crosslinks are reversed by heating [25], but most of the TFs we have analyzed by Western blotting require sample heating in order to visualize discrete bands on blots. We estimate from the reported crosslink reversal rate data [23] that heating samples for 5 minutes at 95° C results in a reduction of only about 7% of the crosslinks present in the sample prior to heating. For this reason, such brief heating can be done to ensure protein denaturation without substantial loss of crosslinks that may have been present in the sample. To date we have not observed by Western blotting any effects of formaldehyde on protein mobility, nor have we seen more than a two-fold effect of formaldehyde on soluble protein levels, although it is certainly possible that such effects may be observed for other TFs [21]. The absence of detectable effects of formaldehyde on protein mobility is consistent with prior work showing that neither glycine-peptide nor Tris-peptide conjugates were detectable in such reactions by mass spectrometry [26]. It is not known how sensitive Western blotting may be to formaldehyde monoadduct modification, but the monoadduct reaction is highly reversible under physiologic conditions [27], so such protein modifications would not be expected to persist.

Step 1: Strain construction—As mentioned above, the key to interpreting the crosslinking time-dependent increase in ChIP signal is to compare ChIP signals in a cell with WT levels of the TF to one in which the TF level has been increased. As the cells are otherwise identical, the resulting changes in ChIP signal can be modeled as effects on fractional occupancy and overall on-rate, both of which depend on the TF concentration. In general, we have found it easiest to increase the concentration of a given TF by introducing into the cells an additional plasmidborne copy of the gene that encodes it, giving rise to an overexpression (OE) strain. Usually the gene is placed under control of its native promoter, and if ChIP is performed using an epitope tag, the additional copy of the gene is tagged in exactly the same way. In principle, it need not matter how different the expression level of the TF is in the OE strain, but in practice we find that introduction of an additional copy of the TF gene on a CEN ARS plasmid works well, yielding a TF level increase of 2–3 fold over WT and giving rise to readily apparent increases in the ChIP signal. For reasons that are not understood, large overexpression changes such as those resulting from introduction of TF genes on multi-copy (2 micron) plasmids sometimes result in uninterpretable dosage-dependent changes in ChIP signal; such changes in the TF concentration may poison normal dynamic behavior by saturating interactions of the TF with other regulatory factors. An alternative approach that we have used successfully is to integrate an additional copy of the gene expressing the TF of interest at another location in the genome, under control of its own or a heterologous promoter and again tagged in the same way as the gene in the WT cell.

Step 2: Verification of overexpression by western blotting—Once the OE strain has been constructed, it is critical to measure the degree of TF protein overexpression compared to the levels in the WT strain. This is usually done by Western blotting using yeast whole cell extracts serially diluted so as to obtain bands of comparable intensity and within the linear range of sensitivity on the same blot from each of the two types of extracts. An example blot for TATA-binding protein (TBP) is shown in Figure 2, demonstrating that formaldehyde treatment did not deplete the soluble pool of TBP and allowing calculation of the TBP level present in extracts from the TBP overexpression strain. If the TF is GFP tagged, an alternative approach is to estimate its nuclear concentration by live cell imaging [21]. Quantitation of two- to three-fold differences in protein level by Western blotting can be challenging and requires quantitation of results from at least three independently prepared sets of extracts and normalization of the band intensities to total protein and/or the band intensities generated by use of an antibody to an unrelated protein as a loading control (for example, we have used as a loading control glucose-6-phosphatase, whose levels are unaffected by formaldehyde treatment and which can be detected by a very sensitive commercially available antibody [21]). Once it has been established that TF levels are not markedly depleted by formaldehyde treatment and the fold increase of the TF in the OE strain compared to WT has been estimated, crosslinking time-dependent ChIP experiments can be conducted.

3.3 Growth of cells and formaldehyde crosslinking by hand mixing

The ChIP procedure used for CLK analysis begins with growth and crosslinking of cells, and is very similar to standard ChIP procedures used in the field (Figure 3). The use of CEN

ARS plasmids for TF overexpression is convenient but requires selective media for plasmid maintenance. In some cases, we have crosslinked cells directly, whereas in others we have induced TF activity by shifting growth conditions (e.g. addition of copper for 90 min to induce Ace1 binding or galactose for 60 min to induce Gal4 binding). We have also found it useful in many cases to shift cells to non-selective media (YPD or YEP-galactose) for 60 min as required for measurements under particular conditions and/or to provide a consistent framework for comparison of the dynamic behaviors of different TFs under the same growth conditions [21]. In order to control for any media effects, WT cells carry an empty plasmid vector with the same marker and are grown in parallel under the same conditions as the OE strain. In principle, any number of systems for modestly over- or under-expressing a particular TF compared to its WT level could be feasible for applying this method to analysis of TF dynamics in other cell types.

Step 1: Growth of yeast cultures—For sample preparation, *S. cerevisiae* strains are typically grown overnight in 50 ml of the appropriate selective media at 30°C until an OD₆₀₀ of 0.8 is achieved. For each time point, two biological replicate cultures are prepared. Cells are then pelleted and resuspended in 100 ml of the desired growth medium and incubated for 1–1.5 hr until the OD₆₀₀ is ~1.0.

Step 2: Formaldehyde crosslinking

1. For hand mixing time points, formaldehyde is added to cultures (1% final concentration) with a rapidly mixing stir bar and quenched by addition of glycine (addition of one-tenth volume of 2.5 M glycine to achieve a final concentration of 250 mM). This can be done for time points as short as 5 seconds up to the longest incubation times of 30 minutes or more. For collection of time points between 5 and ~30 sec, it helps to use two automatic pipettors (one for dispensing formaldehyde and one for dispensing glycine solution), with the glycine quench solution pre-loaded prior to addition of formaldehyde to the culture. Crosslinking times shorter than 5 seconds are obtained using the KinTek quench-flow instrument (described in Section 3.4). Quenched cultures are incubated with glycine for ~5 minutes.

A technical note about the glycine quench: Published work including order-of-addition experiments and short crosslinking time data show that glycine can be used to effectively quench the formaldehyde crosslinking reaction when using yeast cells grown as previously described [21]. However, Tris is a better quencher than glycine, as glycine will not completely quench crosslinking reactions performed in vitro or using metazoan cells in tissue culture [26, 28]. The improved efficacy of Tris compared to glycine has been explained chemically [28]. For these reasons, we recommend going forward that investigators use Tris added to 750 mM final concentration rather than glycine, and control order-of-addition experiments should be performed to ensure that the quench is effective.

2. The cells are then pelleted in a clinical centrifuge at 4° C, washed with 50 ml cold (4° C) TBS with 125 mM glycine, followed by a wash with cold TBS. The pellet

from each culture is transferred to one screw-cap fast-prep tube, and it can now be stored at -80°C for later work-up.

Step 3: Preparation of the chromatin extract

1. Each cell pellet is resuspended in 600 μL ChIP lysis buffer (140 mM NaCl) with protease inhibitors added. For many factors, the Roche Complete Protease Inhibitor Cocktail Tablet is sufficient to prevent degradation of the TF of interest, but we find that more stable extracts are obtained by adding stocks of individual protease inhibitors (1.0 mM phenylmethylsulfonyl fluoride, 2.0 mM benzamidine, 2.0 μM pepstatin, 0.6 μM leupeptin, and 2.0 μg of chymostatin per ml of buffer).
2. Acid-washed 425–600 μm glass beads (Sigma) are added to just below the level of the liquid and the resuspended cells are lysed using a Biospec Mini-Beadbeater at 4°C set to “homogenize” for 45 seconds on, 1 minute off for 7 cycles.
3. Tubes are punctured with heated 18 gauge needles (BD PrecisionGlide), then placed in 13×100 mm glass tubes and the liquid is transferred from the fastprep tubes by centrifugation in a clinical centrifuge. The cell suspensions are then transferred to fresh eppendorf tubes on ice.
4. Samples are sonicated using a Branson Sonifier 250 with microtip probe at 30% output and 90% duty cycle for 7 cycles of 5 pulses each (with alternating cooling on dry ice and wet ice in between), followed by two rounds of centrifugation of 5 and 20 minutes at 4°C in an Eppendorf microfuge at $16,000 \times g$ to pellet debris and unlysed cells.
5. The supernatant is the chromatin solution and is quantified by Bradford Assay (Bio-Rad Protein Assay Dye Reagent Concentrate) using bovine serum albumin as the standard, followed by immunoprecipitation as described in Section 3.5.

3.4 Acquiring short time-scale data using quench-flow instrumentation

The requirement for quench-flow data is factor-dependent as outlined in Figure 1. In this section, the quench-flow procedure is described, but deciding whether quench-flow data are important for measuring dynamics of a particular TF-chromatin interaction is discussed below in Section 4.

3.4.1 Use of KinTek instrument—As described above, it is possible to incubate cells with formaldehyde for as little as five seconds by hand mixing. For shorter formaldehyde incubation times on the second or millisecond time scale, we adapted a KinTek quench-flow apparatus (model RQF-3, KinTek Corporation) for rapid mixing of a cell suspension and formaldehyde, followed by rapid quenching in glycine.

Step 1: Preparation of the KinTek machine: The temperature of the quench-flow instrument (QF) is controlled by a circulating water bath that maintains the temperature of the cells, reagents, and reactions at the same temperature as used for hand mixing experiments. Temperature control also provides the capability of crosslinking temperature-sensitive strains under particular desirable conditions. The temperature of the water bath

should be adjusted 15 minutes before crosslinking to allow the bath time to equilibrate. At this point the computer controlling the QF should also be turned on.

Step 2: Loading, ejecting, and flushing the drive syringes: The QF has 2 drive syringes that each hold 5 ml (Figure 4). The essentially instantaneous start and stop of the drive syringes ensures collection of precise time points. The KinTek RQF-3 also has a quench syringe, which is not used in this method. Instead, the quench solution (500 mM glycine) is placed in an Erlenmeyer flask and the exit tube is inserted into the quench solution just below the meniscus. As discussed above, although we have established glycine as an effective quench in our yeast system, Tris has been shown to be a better quencher and we recommend using Tris rather than glycine, particularly when using cells other than yeast. Different time points can be obtained by adjusting the length of the exit tubing (Figure 4). Although the instrument can be used as-is to obtain time points in the 3–150 ms time range, time points longer than 150 ms can be obtained by attaching calibrated tubing of specific lengths (see calibration below). The mixing chamber and the exit tubing need to be flushed between every run using the flushing mechanism built into the instrument.

There are two positions of the drive syringe valves: 'Load' and 'Fire'. When the drive syringes need to be filled, the valve should be kept in the 'Load' position and when they are ready for mixing, the valves are switched to the 'Fire' position. Although the reaction initiation is motor-driven and computer controlled, the valves need to be adjusted manually before every run as described below. To flush the drive syringes, the valves of both the syringes are moved to the 'Load' position and water is pushed into the syringe and back out manually. The mixing chamber also contains valves that can be placed in the 'Flush' or 'Fire' position. To flush the mixing chamber and exit tubing, tubes (located on either side of the exit tubing port) are placed in a beaker of water and the exit tube is attached to a vacuum pump. The vacuum pump (Dyna-Pump Model 3) is a separate piece of equipment attached to the QF. The vacuum pump is run until water is pulled from a beaker into the mixing chamber and pumped out through the exit tube into a waste container. Then the beaker of water is removed and the vacuum pump is run for few seconds to empty the mixing chamber and the exit tube of water. Once the drive syringes and the mixing chamber are flushed with water, one of the drive syringes is filled with 5 ml of the cell culture to be crosslinked while the other syringe is filled with 5 ml of 2% formaldehyde in water. A computer controlled stepping motor regulates the delivery of the syringe contents to the mixing chamber. The stepping motor speed should be set to 200, the steps per cycle to 60,000 and the number of cycles to 1 by selecting 'option 3' from the main menu. Before initiating the run, all the valves must to be set in the 'Fire' position to ensure that the sample is flowing through the mixing chamber and into the quenching solution via the variable length exit tube. Initiate the mixing reaction by pressing the 'Start' button. A 'dummy' run should be performed before each sample to make sure that the instrument is working properly. The settings are the same in the dummy run as described above except that the mixed contents are discarded. The mixing chamber is flushed and the syringes are re-loaded between every run. To load the syringes, reset them to their original positions. To do this, the platform for the stepping motor is moved in the reverse direction by selecting 'option 2' from the main menu. The syringes are then ready to be loaded. Five milliliters of culture can be processed with each

run of the Kintek device, so 12 runs are typically used to obtain 60 ml of crosslinked material for ChIP. Between runs, crosslinked cells are placed on ice, and when a particular sample has been fully processed by the QF, the cells are pelleted, washed with TBS as described earlier in section 3.3 Step 2.2, and stored at -80°C until processed as described in Section 3.5.

3.4.2 Calibration of exit tube lengths—To collect crosslinking time points longer than the inherent range of the KinTek instrument ($\sim 150\text{ms}$), variable length tubes are added to the exit port. In this case, the valve for adjusting the internal exit tube length is kept at its maximum setting (position 7). As it is necessary to maintain a rapid flow rate for efficient mixing, longer reaction times are achieved by increasing the length of the exit tube rather than decreasing the flow rate. Once a set of several exit tubes have been obtained corresponding to reaction times roughly spanning the range of 150 msec to 1.5 sec, these can be used repeatedly to obtain short crosslinking time data for many samples.

3.4.2.1 Calibration test reactions: Test reactions of the base-catalyzed hydrolysis of benzylidene malanonitrile (BMN) are performed to determine the reaction mixing time that corresponds to an exit tube of a given length. The procedure for doing this is adapted from the KinTek Corporation's instructions for performing a quench-flow test reaction. In the test reaction, BMN is reacted with NaOH at 20°C and quenched with HCl. The kinetics of hydrolysis are monitored by measuring the absorbance at 310 nm after diluting the quenched sample with water. The second order rate constant for BMN reacting with NaOH is $140\text{ M}^{-1}\text{ s}^{-1}$. The concentrations of BMN (in the 0.25–0.5mM range) and NaOH (20–40 mM) are chosen appropriately so that the reaction between them is in the observable OD range.

Step 1: Setting up BMN reactions: 1mM BMN is loaded into one drive syringe and 2X NaOH is loaded into the other. The loading of the reactants, flushing of the tubes and computer controls are done as described above. For each set of BMN and NaOH concentrations, OD_{310} at t_0 and t_{∞} are measured. The reaction of BMN with NaOH in the presence of HCl corresponds to t_0 and the reaction of BMN with NaOH for 1 minute and quenched with HCl is taken to be t_{∞} .

Step 2: Computing reaction times corresponding to exit tube length: Using the equation $[\text{BMN}]_t = [\text{BMN}]_0 * e^{-kt}$, where $[\text{BMN}]_t$ represents the concentration of BMN at time t and $[\text{BMN}]_0$ the concentration at $t = 0$ and using $k = 140 * [\text{NaOH}]$, time t for each variable length tube is calculated. For each tube, the reaction is repeated in triplicate and the time points obtained averaged and standard deviations calculated. These replicates can be done with single or different concentrations of NaOH, although the latter is recommended.

3.4.2.2 Reference exit tube lengths and practical limitations: The end-to-end lengths of the exit tubes and their corresponding time points in use in our lab are shown in Table 1. These provide rough guidelines for construction of appropriate length exit tubes of your own. Each tube is attached to the exit port by inserting it into the QF exit port tube with an overlap of 0.5 cm. Although the QF is well adapted for time points shorter than 100 msec, there is a practical limit for time points longer than $\sim 1.5\text{ sec}$ as the exit tube in such cases

would be very long, with the result that most of the crosslinked cell volume would be lost in the dead volume of the exit tubing.

3.5 Chromatin immunoprecipitation and real time PCR

To measure locus-specific TF binding, ChIP is used to isolate the proteins bound to DNA [29] followed by real time qPCR (RT-qPCR) using locus-specific primers. We recommend the following procedure:

Step 1: Immunoprecipitation of desired chromatin protein—Immunoprecipitation is performed overnight at 4°C using 1 mg chromatin protein for each sample, quantified by Bradford assay, and an appropriate amount of antibody. A negative control (lacking antibody) should be included for each sample. For TAP-tagged strains, we use IgG-conjugated beads and extracts from cells with and without the tagged TF, or the extracts from cells with the tagged TF are combined with sepharose beads with and without IgG. Each immunoprecipitation reaction should be adjusted to 500 µL final volume with ChIP lysis buffer (140 mM NaCl) with protease inhibitors added. At the same time, 100 µg of each chromatin sample should be aliquoted and saved in a separate tube at –80°C for later use to quantify the total target DNA amount added to the immunoprecipitation. After overnight incubation, 40 µL protein A sepharose beads (nProtein A Sepharose 4 Fast Flow, GE Healthcare) is added to new tubes (one for each sample) and washed three times with 1 ml ChIP lysis buffer (140 mM NaCl) with ~5 sec vortexing and ~5 sec centrifugation in between each wash, being careful to remove as much buffer as possible after the last wash. The reactions tubes are spun quickly (~10 seconds at max speed) to pellet any precipitate that may have formed. The supernatant is then transferred to the protein A sepharose bead-containing tubes and mixed end-over-end for 2 hours at 4°C.

Step 2: Removal of unbound proteins and isolation of chromatin—Next, samples are washed twice each with 1 ml of the following buffers: ChIP lysis buffer (140 mM NaCl), ChIP lysis buffer (500 mM NaCl), LiCl wash buffer, and 1X TE. After addition of each buffer, the tubes are vortexed for 5 sec and the beads pelleted by centrifugation for 5 sec in an Eppendorf centrifuge on max speed. The protein-DNA complexes are then eluted from the beads with 75 µL elution buffer and incubated at 65°C for 10 minutes; this elution is repeated and the eluates combined and incubated at 65°C overnight to reverse formaldehyde crosslinks. The samples of total input chromatin have 150 µL elution buffer added at this time and are also incubated at 65°C overnight.

Step 3: Quantification of ChIP DNA using real time PCR—The following day, all samples are cleaned up with the QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions and the purified DNA is eluted from the columns with 50 µl of 50°C DEPC-treated water. ChIP DNA is then quantified by RT-qPCR with input chromatin used for the standard curve. This is done using procedures that are in common use among labs in the field. We employ a Bio-Rad MyiQ instrument and use Bio-Rad iQ SYBR Green Supermix in combination with forward and reverse primer concentrations of 5 µM and 1 µL template. The samples obtained from biological replicates of each crosslinking time point are quantified in triplicate and inputs for the standard curve are run in duplicate. The qPCR

should be run using primers for the locus of interest with a standard protocol along with a melt curve according to manufacturer's instructions. For analysis of TF binding to loci with multiple binding sites in tandem, we have used primers specific for the unique sequence immediately flanking the binding site array. The difference between the immunoprecipitated sample and its corresponding mock sample (no antibody added) is then divided by the value obtained by quantitation of the input chromatin to obtain relative ChIP signals for each biological replicate. Values obtained for each biological replicate at a given time point are averaged and the standard deviation calculated for use in subsequent data analysis.

Representative ChIP data are shown in Figure 5, in which we partially recapitulated published results showing how the ChIP signal for the TATA-binding protein (TBP) interaction with the *URA1* promoter depends on formaldehyde incubation time. TBP ChIP was performed using commercially available TBP antibodies (Abcam) as described previously [21] and above. In this case, to facilitate handling of multiple samples, 300 ml master cultures at OD₆₀₀ ~ 1.0 were subdivided into 50 ml subcultures, which were incubated with 1% formaldehyde for various times, then quenched in glycine. Following formaldehyde treatment, chromatin isolation and fragmentation, IPs were performed using 2.5 µl of the TBP antibody per sample, or mock IPs were performed in the same way but without adding antibody, using 0.7 mg of chromatin protein for each sample. Real-time PCR was performed to quantify the *URA1* promoter DNA levels in the immunoprecipitates using a *URA1* promoter-specific primer set described previously [21].

Figure 5A shows the expected formaldehyde incubation time-dependent increase in the ChIP signal as reported in our earlier work [21]. Importantly, although the time-dependent data are somewhat noisy, all data are included in the subsequent analysis; we do not employ any kind of outlier analysis to exclude data. This necessitates the acquisition of data for at least 8 time points, and typically 12–18, in duplicate, in order to obtain sufficiently robust datasets whose final fits are not driven by one or two noisy data points. As described above, the ChIP signals used as input to the model are computed as the signal in the immunoprecipitate minus the mock signal, with the difference divided by the value obtained for the input DNA. Figure 5B shows that the percent of input DNA in mock IPs was similar to the level of DNA in the IP from the sample that was not treated with formaldehyde, and the mock levels did not vary systematically based on the formaldehyde incubation time. The total chromatin protein yields were also similar regardless of the formaldehyde incubation time (Figure 5C). These results indicate that the ChIP signals used as input to the model were not biased by formaldehyde-dependent changes in the mock or chromatin protein yields. Although this general behavior has been observed in many experiments we have performed using *S. cerevisiae*, mock IP and chromatin protein yield data should be carefully evaluated in other systems to ensure that this is the case.

Step 4: Analysis of ChIP data—In order to derive the normalized ChIP signal used in CLK data analysis described below, we estimate the saturated ChIP signal (IP_{sat}) as part of the CLK data fitting procedure and divide the ChIP signal by IP_{sat} at every crosslinking time point. Importantly, the normalized ChIP signal controls for antibody affinity and capture effects, which likely yield a proportional sampling but not all of the crosslinked TF-DNA for any given crosslinking time. We equate the normalized ChIP signal with the

fraction of “available” sites containing crosslinked TF. The CLK mathematical model is a derivation of the latter quantity as a function of crosslinking time and is used for estimation of kinetic parameters and interpretation of CLK data. A consequence of the irreversibility of crosslinking is that the ChIP signal at saturation is predicted to be crosslinking of all available sites by the TF. Available sites are those that are not crosslinked to other proteins that inhibit crosslinking of the TF of interest, or sites that are not effectively made unavailable to the TF because of formaldehyde treatment. More work is required to determine how such factors contribute to ChIP signals and their relative levels compared to total input DNA, but future iterations of the CLK approach could conceivably incorporate models of these phenomena once they are understood biochemically.

Prior work showed that ChIP signals did not depend on the formaldehyde concentration used to obtain CLK data, as 1% and 8% formaldehyde yielded similar time-dependent ChIP values [21]. To better understand the formaldehyde concentration dependence of the ChIP reaction, we measured TBP ChIP signals at the *URA1* promoter as in Figure 5, this time incubating various concentrations of formaldehyde with cells for ten minutes. The percent of immunoprecipitated DNA was at mock levels without formaldehyde incubation, and the signal increased with increasing concentrations of formaldehyde to 1% (Figure 6A). This formaldehyde concentration dependence is consistent with the formaldehyde concentration dependence observed over a similar range in *Drosophila* [30] as well as the formaldehyde concentration dependence predicted by the CLK mathematical model. The ChIP signal obtained using 3% formaldehyde appeared a little larger than the 1% value, although the difference was not significant. These results are consistent with the conclusion that 1% formaldehyde is not limiting in this system. Also, consistent with the results shown in Figure 5, neither mock ChIP signals nor chromatin protein yields were dependent on formaldehyde concentration (Figures 6B and C).

4. Data analysis and anticipated results

Once a locus-specific dataset spanning a wide range of hand mixing crosslinking times has been obtained, it can be evaluated as outlined in Figure 1 to determine how to proceed. By inspecting the data, an assessment must be made regarding whether or not kinetic parameters can be obtained by fitting hand-mixing data only. This requires a consistent, monotonic increase toward saturation and preferably a robust (i.e., above the variation in the data) separation of the OE data points over that of the WT as illustrated in Figure 7. If this is the case, then we recommend finalizing data collection (i.e., filling in obvious crosslinking time holes) by collecting a robust data set via manual crosslinking and extracting kinetic parameters by fitting the CLK model to the data using the MATLAB CLK analysis package (discussed more below). If the OE and WT data are essentially flat and not well separated over any sub-range of hand mixing times, the dynamics of the factor being studied could in principle be extremely fast (seconds) or very slow (hours) with high occupancy (> 0.9) and a relatively high native ChIP signal. In this case, we recommend collecting further ChIP data by quench-flow at sub-second to second crosslinking times from WT and OE strains. Relatively flat, overlapping WT and OE ChIP signals from sub-second to second crosslinking times, which are also comparable to native ChIP levels, likely indicate TF-DNA interactions that are extremely slow (hours); however, this would require further

investigation beyond the original CLK study [21]. A rapid rise in ChIP signal from the sub-second to second time scale followed by a kink and slower rise from the ~1 to ~30 second time scale with notable separation of the OE over WT ChIP signal indicates rapid dynamics. In this case, we recommend fitting the full OE and WT data set (i.e., quench-flow and hand mixing data) using the MATLAB CLK analysis package to determine kinetic parameters (see Figure 1). A rapid rise to saturation for both the OE and WT ChIP signal in the sub-second to second time scale indicates extremely rapid dynamics (i.e., ~seconds time scale) as a qualitative result. In this case, we do not recommend attempting to extract kinetic parameters via model fitting as the crosslinking dynamics, which we find tend to occur on the second time scale, are intermingled with factor-DNA dynamics that are also on the second time scale. However, in this case it is probably sufficient, and most likely of biological interest, to conclude that the binding dynamics occur on a very rapid time scale (seconds).

The fitting procedure is a multi-step process carried out in an automated fashion using a MATLAB pipeline [21]. The MATLAB CLK analysis package is freely available (<https://github.com/poorey/CLK-Analysis>) and is provided with detailed instructions for its implementation. Both WT and OE datasets are fit simultaneously, and in addition to time-dependent ChIP data, fitting requires only an initial guess for the ChIP saturation level at infinite time (IP_sat, obtained by inspection of a plot of the data), and the factor by which the TF is overexpressed in the OE strain compared to the WT strain (discussed in Section 3.2). A screenshot of the output from the MATLAB CLK analysis package used to fit a sample dataset is shown in Figure 8. It is important to note that the CLK analysis package performs non-linear regression in order to fit time-dependent ChIP data to the CLK mathematical model, and applying non-linear regression is inherently challenging. Depending on the data and initial parameter estimates supplied, non-linear regression can return suboptimal fits, unphysical parameters or both. We have taken great lengths to mitigate these challenges (e.g., performing linear regression using approximate models to arrive at good initial parameter estimates as input to non-linear regression; see [21] Supplemental Online Material). Next we discuss possible data profiles over different time scales and strategies for data fitting, as well as limitations.

When fitting either hand mixing only or hand mixing and quench flow data, the fitted curves should display a sharp rise followed by a kink and a slower rise toward saturation as shown in Figure 7. Along with checking the quality of the fits (i.e., relatively low deviations of the data about the fitted curves), the kinetic parameters should take physically reasonable values. We have found that crosslinking dynamics tend to occur on the second time scale [21]. Thus we expect $k_{xl} \sim 0.3 M^{-1} s^{-1}$ as this would correspond to ~10s crosslinking dynamics time scale at most for a reaction containing 360 mM formaldehyde. In other words, kinetic parameters that suggest crosslinking dynamics on the order of or slower than 10 seconds could yield good model fits to the data but unphysical or badly biased binding kinetic parameters. Parameters that suggest crosslinking dynamics are much faster including many orders of magnitude than the second time scale are likely unphysical artifacts of fitting either no or relatively noisy sub-second to second ChIP data. Importantly, extremely high crosslinking kinetic parameters will not affect binding kinetic estimates, but extremely slow

crosslinking dynamic parameters (i.e., 10 seconds or slower) could badly bias TF-DNA kinetic parameters. Regarding binding kinetic parameters, the knee of the second exponential associated with WT binding dynamics should roughly correspond to $1/k_a C_{TF}$ and the occupancy should roughly be given by the normalized ChIP signal associated with the kink in the WT curve. Poor model fits of the data or significant deviations of the parameters from these expected values could indicate problematic fits and unphysical (badly biased) estimates of TF-chromatin kinetic parameters. In these cases, we suggest re-running the MATLAB CLK package with different input saturation levels. If this does not solve these problems, we suggest either gathering more data at crosslinking times where the data is sparse and/or noisy or (for users experienced with non-linear regression and MATLAB) modifying the input parameters in the MATLAB CLK software.

5. Concluding remarks

To date, the CLK approach has been used to explore a relatively small number of TF-chromatin interactions. However, the method has captured dynamic behavior varying over a broad time scale, yielding estimates for binding kinetics that are within a factor of three of those obtained by live cell imaging, a completely independent approach [21]. As a practical matter, successful implementation of the method requires that the binding dynamics under study are at least an order of magnitude slower than crosslinking, placing a lower limit on the binding kinetics that are accessible by the method of around ~10 seconds. Extraordinarily slow dynamics could also be difficult to capture accurately if saturation (the ChIP signal at infinite time) is hard to estimate. Although the crosslinking reaction occurs rapidly compared to the chromatin interactions analyzed to date, overall ChIP recovery is relatively low compared to input DNA. The relatively low ChIP recovery values may be due to inefficiencies associated with antibody enrichment and/or crosslinking (e.g., crosslinking other TFs or histones to a given site). In addition, the model predicts that for many interactions, the ChIP signals are well below saturation after even 30 minutes of formaldehyde incubation, consistent with the observed reduced recovery of ChIP DNA after even long formaldehyde incubation times [21]. At the very least, we feel confident that the CLK method can provide rank-ordered estimates of occupancy (“high”, “medium”, “low” for example) given that the estimates of kinetic parameters obtained by the CLK method and FRAP agree within a factor of 2–3 and occupancy is derived from these same rate constants. Genome-wide analyses of TF binding in *Drosophila* have been used in combination with in vitro binding measurements to develop a thermodynamic model for TF binding in vivo [8]. Using the model, it was found that in vivo ChIP signals obtained by conventional ChIP assays correlate reasonably well with DNA binding affinity measured in vitro [8]. In agreement with this conclusion, a plot of the ChIP signal we obtained after a 20 min formaldehyde incubation (typical conventional ChIP assay conditions; [21]) versus the fractional occupancy (theta_bound) obtained by the CLK method showed that these values are surprisingly well correlated (Figure 9). In future work, a deeper understanding of the crosslinking reaction in cells will likely yield refinements in the CLK model that in turn can generate ever more accurate estimates of occupancy and dynamic behavior in vivo.

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Highlights

1. A method for measuring chromatin-binding dynamics in vivo is described.
2. A quench flow apparatus is adapted to obtain kinetic data on the sub-second scale.
3. The protocol includes strategies for key decision points.
4. Data analysis and interpretation are discussed.

Figure 1. Flow chart for implementation of the CLK method

The diagram indicates the main steps required for use of the method, along with key decision points that are factor- and system-dependent. See text for discussion.

Figure 2. Western blot to determine effect of formaldehyde and degree of overexpression compared to wild type

Soluble TBP levels present in whole cell extracts made from wild type or overexpression strains and treated with 1% formaldehyde as indicated were analyzed by Western blotting using a TBP-specific antibody. Formaldehyde incubation times are shown in minutes. A dilution series of the extract from the overexpression strain was used to compare to 15 µg of each wild type extract. Quantitation of the band intensities with ImageJ (NIH) showed that soluble TBP in the wild type extracts was not appreciably decreased by formaldehyde treatment as expected [21]; levels vary by 20% or less between extracts with and without formaldehyde. By comparison of the wild type extract band intensities to those from the dilution series, the TBP level is increased by 13-fold in this overexpression strain.

Figure 3. Outline for performing ChIP using samples obtained by hand mixing or quench flow crosslinking

ChIP is performed very similarly to experiments using 'standard' ChIP protocols with the exception that the formaldehyde incubation time is carefully monitored. Other details specific to the CLK ChIP procedure are described in the text.

Figure 4. Schematic of the quench-flow apparatus

The diagram illustrates the basic set-up of the KinTek RQF-3 instrument as used for CLK analysis. One syringe holds a cell suspension in growth medium whereas the other syringe holds a solution of formaldehyde at twice the desired final concentration. The lines from each syringe meet in a mixing chamber. The exit line is calibrated in length to achieve the desired reaction time, and the reaction is quenched by collecting the crosslinked sample in a tube containing a solution of glycine.

Figure 5. Representative formaldehyde incubation time-dependent ChIP data

Wild-type YPH499 yeast cells [31] were incubated with 1% formaldehyde for the indicated times, then chromatin was isolated and ChIP performed to quantify the association of TBP with the *URA1* promoter as done previously [21]. **(A)** Percent of input DNA in the anti-TBP immunoprecipitates. For a given crosslinking time, percent DNA recoveries can vary substantially depending on the TF, the locus and antibody. **(B)** Percent of input DNA in mock (no antibody added) immunoprecipitates of the same samples as in **(A)**. **(C)** Total protein yield from the chromatin isolation procedure that generated the input material for

(A) and (B). Error bars represent the standard deviation obtained from two biological replicates.

Figure 6. Dependence of ChIP signal on formaldehyde concentration

Wild-type YPH499 cells (as in Figure 5) were incubated with the indicated concentrations of formaldehyde for ten minutes, then chromatin was isolated and ChIP was performed as in Figure 5 to quantify the association of TBP with the *URA1* promoter. (A) Percent of input DNA in the anti-TBP immunoprecipitates. Asterisks indicate sample comparisons with p values < 0.05: ChIP signal obtained with 0.3% formaldehyde versus no formaldehyde (p = 0.02, two-tailed Student's T-test); 1% formaldehyde ChIP signal versus 0, 0.1% and 0.3% p values were all < 0.03. The difference between the 1% and 3% samples was not significant

(p value = 0.187). **(B)** Percent of input DNA in mock (no antibody added) immunoprecipitates of the same samples as in **(A)**. **(C)** Total protein yield from the chromatin isolation procedure that generated the input material for **(A)** and **(B)**. Error bars represent the standard deviation obtained from two biological replicates.

Figure 7. Simulation of CLK model fits

The blue curve shows the time-dependent increase in ChIP signal for a hypothetical TF-chromatin interaction occurring in WT cells, whereas the red curve shows the result obtained for the same interaction occurring in cells with a two-fold increase in the TF compared to WT.

Figure 8. Screenshot showing example of CLK data analysis

The figure shows what data analysis looks like using the CLK Analysis MATLAB Package. In this case, a sample data set has been analyzed, with the final fitted parameters (overall on-rate, k_aC ; off-rate, k_d , overall crosslinking rate, $k_{xL}C_{FH}$; and ChIP saturation value, IP_{sat}) shown in the lower central window and the best fits shown in the plot in the lower right (data from WT cells in blue; data from TF overexpression cells in red). Apart from the time-dependent ChIP data, the only other input parameters required are initial estimates of the ChIP saturation level (IP_{sat}) and the overexpression coefficient for the TF of interest in the overexpression strain compared to wild-type cells. These two initial estimates are added to the file “CLK_model_Script.m” (in the upper left window) using the MATLAB editor. The data analysis package is freely available upon request and is provided with a detailed ReadMe file for its implementation.

Figure 9. Comparison of CLK-calculated TBP occupancies and conventional ChIP signals at seven promoters

The plot shows how the ChIP signals obtained from a conventional ChIP experiment with 20 min formaldehyde incubation (x-axis) compare with CLK-calculated fractional occupancies (theta_bound values, y-axis) obtained for interaction of TBP with each of seven promoters analyzed previously [21]. Note the strong correlation of 0.88 associated with a linear fit to the data.

Table 1

Exit tube lengths and their corresponding time points. These serve as rough guidelines; all exit tubes require calibration (see text). I.D. = internal diameter

Exit tube length (cm)	Time point (msec) $\pm$ st. dev.	Exit tube I.D. (mm)
QF default at maximum setting	142 $\pm$ 52	0.8
21.5	264 $\pm$ 16	1.02
32	441 $\pm$ 30	1.6
60	814 $\pm$ 170	1.6
120	1365 $\pm$ 120	1.6