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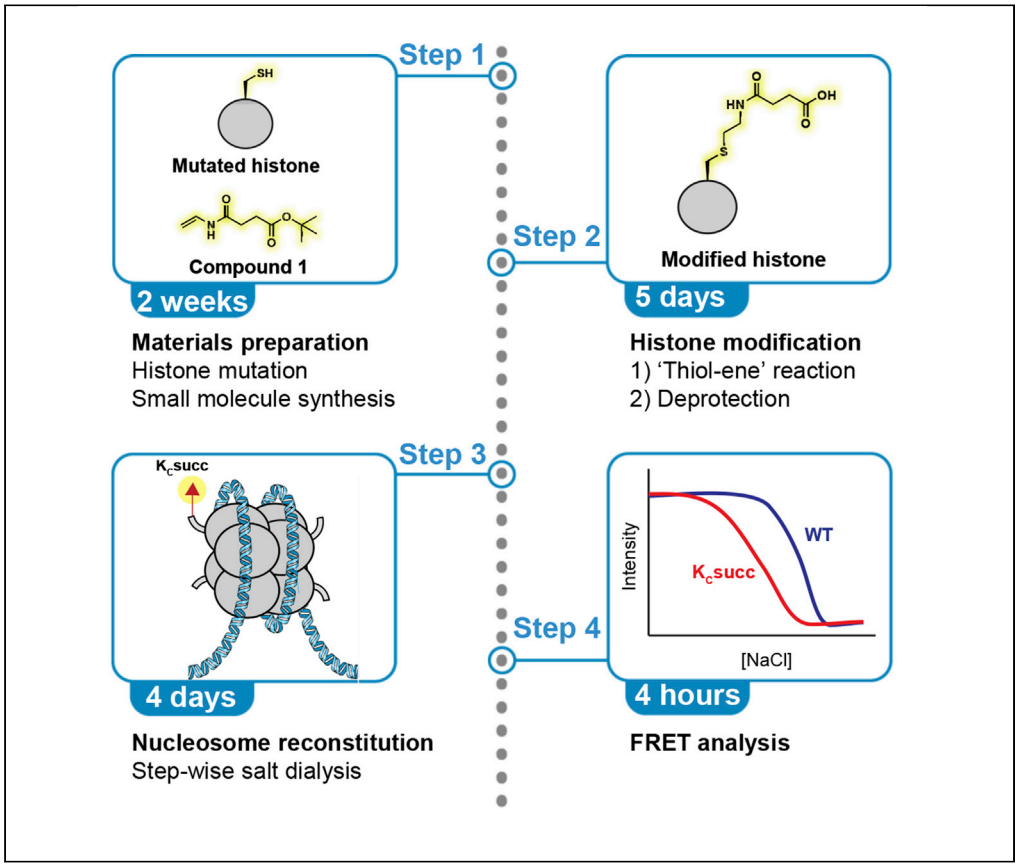
Protocol for the preparation of site-specific succinylated histone mimics to investigate the impact on nucleosome dynamics



Lysine succinylation is a recently discovered posttranslational modification that plays critical roles in metabolism, epigenetic signaling, and human diseases. To investigate the effects of site-specific histone lysine succinylation on nucleosome dynamics requires the generation of homogeneously modified histones, which is a significant challenge. Here, we report a protocol for the rapid site-specific installation of a succinyl lysine analog onto histone. We then use a Förster resonance energy transfer approach to characterize the impact on nucleosome dynamics.

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Highlights

Strategy for site-specific installation of a succinyl-lysine analog into histones

Strategy is generally applicable to all histones and other acid-resistant proteins

A FRET-based biophysical assay reveals that H2BK34succ destabilizes nucleosomes

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Protocol for the preparation of site-specific succinylated histone mimics to investigate the impact on nucleosome dynamics

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SUMMARY

Lysine succinylation is a recently discovered posttranslational modification that plays critical roles in metabolism, epigenetic signaling, and human diseases. To investigate the effects of site-specific histone lysine succinylation on nucleosome dynamics requires the generation of homogeneously modified histones, which is a significant challenge. Here, we report a protocol for the rapid site-specific installation of a succinyl lysine analog onto histone. We then use a Förster resonance energy transfer approach to characterize the impact on nucleosome dynamics.

For complete details on the use and execution of this protocol, please refer to Jing et al. (2018).

BEFORE YOU BEGIN

Primer design

Timing: 20 min

1. Primers for preparing the cysteine mutation on histone H2B lysine 34 (H2B K34C). The pET-histone expression plasmids were gifts from Professor Bing Zhu (Institute of Biophysics, Chinese Academy of Sciences, China). H2B K34C is introduced by site-directed mutagenesis using the following primers:
   a. Forward: 5′-GGAAAAAGCGCAGGAAGACAAGGTGCGAGAGTTATGCCATTTACG-3′
   b. Reverse: 5′-CGTAAATGGCATAACTCTCGCACCTTGTCTTCCTGCGCTTTTTCC-3′
2. Primers for preparing fluorophore-labeled DNA fragments. The fluorescein-labeled DNA fragments are prepared by PCR using primers labeled with Alexa 488 and Alexa 594 (IBA, Germany). Both fluorophores are attached *via* aminolink-C6 linkers. A template of 153 bp "Widom 601" DNA (Lowary and Widom, 1998) is synthesized and incorporated into pcDNA 3.1 vector using EcoRV (Tech Dragon Limited, HK). Below are the sequences of the 153 bp "Widom 601" DNA and fluorophore-labeled primers:
   a. DNA 601 (5′-3′):
      ATCGAGAATCCCGGTGCCGAGGCCGCTCAATTGGTCGTAGACAGCTCTAGCACCGC
      TTAAACGCACGTACGCGCTGTCCCCCGCGTTTTAACCGCCAAGGGGATTACTCCCTA
      GTCTCCAGGCACGTGTCAGATATATACATCCGAT
   b. Primers for inner fluorophore labeling:
      i. Forward: 5′-ATCGAGAATCCCGGTGCCGAGGCCGCT(Alexa 488)CAATTG-3′
      ii. Reverse: 5′-ATCGGATGTATATATCTGACACGTGCCT(Alexa 594)GGAGAC-3′
   c. Primers for end fluorophore labeling:

i. Forward: 5′-T(Alexa 488)ATCGAGAATCCCGGTGCCGAGGCCGCTCAATTG-3′
ii. Reverse: 5′-T(Alexa 594)ATCGGATGTATATATCTGAC-3′

Note: fluorophores were labeled at thymine via 5-C6-Amino-dT.

Preparation of fluorophore-labeled DNA fragments

Timing: 2 h

3. The fluorophore-labeled DNA fragments are prepared by PCR using the above-mentioned primers (Primer design) (Gansen et al., 2015, Bohm et al., 2011).
   a. Prepare the mix below:

Reagent	Amount
AccuPrime™ Pfx DNA Polymerase	8 μL
20 μM forward primer	20 μL
20 μM reverse primer	20 μL
Template (50 ng/μL)	10 μL
$MgSO_4$ (50 mM)	10 μL
10× Buffer	100 μL
dd H2O	up to 1000 μL

   b. Use the following thermocycler parameters for the PCR.

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	3 min	1
Denaturation	95°C	30 sec	30 cycles
Annealing	50°C	30 sec	
Extension	68°C	25 sec	
Final Extension	68°C	2 min	1
Hold	4°C	Forever	

   c. The PCR products are then precipitated by isopropyl alcohol, washed in ethanol, and dissolved in histone refolding buffer (2 M NaCl, 10 mM Tris-HCl, pH 7.5, 1.0 mM EDTA, 5 mM β-mercaptoethanol).
   d. The concentration of DNA is determined by UV-Vis spectrophotometer (A260 nm, NanoDrop 2000, Thermo).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
DH5α Competent Cells	Thermo Fisher Scientific	Cat#18265017
Rosetta (DE3) Competent Cells	Novagen	Cat#0954-3CN
Chemicals, peptides, and recombinant proteins		
Fmoc-protected amino acids	GL Biochem	N/A
Vinyl iodide	Alfa Aesar	Cat#L12656; CAS: 593-66-8
N^1,N^2-Dimethylethane-1,2-diamine	Alfa Aesar	Cat#L02204; CAS: 110-70-3

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
2,2′-Azobis[2-(2-imidazolin-2-yl) propane] Dihydrochloride (VA-044)	TCI	Cat# A3012; CAS: 27776-21-2
Recombinant proteins	This paper	N/A
Critical commercial assays		
Bradford assay kit	Bio-Rad	Cat#500-0006
QIAprep Spin Miniprep Kit	QIAGEN	Cat#27104
Slide-A-Lyzer™ MINI Dialysis Device, 3.5K MWCO, 0.5 mL	Thermo Fisher Scientific	Cat#PI88400
Oligonucleotides		
Primers for H2B K34C, see primer design	IDT	N/A
Primers for fluorophore-labeled DNA fragments, see primer design	IBA GmbH	N/A
Recombinant DNA		
pET-histone expression plasmids	Prof. Bing Zhu's lab	N/A
Software and algorithms		
OriginPro 8	OriginLab Corporation	http://www.originlab.com/
UniDec	University of Oxford	http://unidec.chem.ox.ac.uk/
GraphPad Prism 5	GraphPad Software Inc.	http://www.graphpad.com/
ImageJ	National Institutes of Health	https://imagej.nih.gov/ij/
Other		
Bioruptor® Sonicator	Diagenode	UCD-300
GENESYS 50 UV-Vis Spectrophotometer	Thermo Fisher Scientific	840-298000
NanoDrop™ 2000/2000c Spectrophotometers	NanoDrop	ND-2000/2000C
ÄKTA pure protein purification system	Cytiva	29383015
High-Performance Liquid Chromatography	Waters	Waters 2535 Quaternary Gradient Module; Waters 515 HPLC pump; Waters SFO system Fluidics Organizer; Waters 2767 Sample Manager.
Agilent 6130 Series Quadrupole LC/MS System	Agilent Technologies	G6130A
MaxQ™ HP Incubated Tabletop Orbital Shaker	Thermo Fisher Scientific	SHKE420HP
Mini Prep Cell	Bio-Rad	1702908

MATERIALS AND EQUIPMENT

Luria-Bertani (LB) media (storage: 22°C–25°C, make fresh)

Dissolve the following reagents in Milli-Q water to a final volume of 1 L and autoclave the media at 121°C for 30 min.

Reagent	Final concentration	Amount
Tryptone	10 g/L	10 g
Yeast extract	5 g/L	5 g
NaCl	10 g/L	10 g

Lysis buffer (storage: 4°C, make fresh)

To prepare 250 mL of lysis buffer, dissolve the following reagents and bring the volume to 240 mL using Milli-Q water. Adjust the pH to 7.5 using 10 M NaOH solution. Adjust the final volume to 250 mL using Milli-Q water.

Reagent	Final concentration	Amount
HEPES	25 mM	1.49 g
EDTA	1 mM	73.06 mg
NaCl	1 M	14.61 g
PMSF	1 mM	43.55 mg

HU-0/100/500/1000 buffer (storage: 4°C, make fresh)

To prepare 1 L of HU-0/100/500/1000 buffer, dissolve the following reagents and bring the volume to 980 mL using Milli-Q water. Adjust the pH to 7.5 using 10 M NaOH solution. Adjust the final volume to 1 L using Milli-Q water.

Reagent	Final concentration	Amount
Urea	6 M	360.36 g
HEPES	25 mM	5.96 g
EDTA	1 mM	292.24 mg
NaCl	0/100/500/1000 mM	0/5.84/29.22/58.44 g

"Thiol-ene" coupling reaction and deprotection reaction buffer (storage: 22°C–25°C, up to 2 months)

To prepare 50 mL of 'Thiol-ene' coupling reaction & deprotection reaction buffer, dissolve the following reagents and bring the volume to 45 mL using Milli-Q water. Adjust the pH to 7.0 using 10 M NaOH solution. Adjust the final volume to 50 mL using Milli-Q water.

Reagent	Final concentration	Amount
NaH_2PO_4	0.1 M	599.90 mg
Guanidinium HCl	6 M	28.66 g

Histone unfolding buffer (storage: 22°C–25°C, make fresh)

To prepare 50 mL of histone unfolding buffer, dissolve the following reagents and bring the volume to 45 mL using Milli-Q water. Adjust the pH to 7.5 using concentrated HCl solution. Adjust the final volume to 50 mL using Milli-Q water.

Reagent	Final concentration	Amount
Tris-HCl	20 mM	121.14 mg
EDTA	1 mM	14.61 mg
Guanidinium HCl	6 M	28.66 g
DTT	10 mM	77.13 mg

Histone refolding buffer (storage: 4°C, make fresh)

To prepare 1 L of histone refolding buffer, dissolve the following reagents and bring the volume to 980 mL using Milli-Q water. Adjust the pH to 7.5 using concentrated HCl solution. Adjust the final volume to 1 L using Milli-Q water.

Reagent	Final concentration	Amount
Tris-HCl	10 mM	1.21 g
EDTA	1 mM	292.24 mg
NaCl	2 M	116.88 g
β-mercaptoethanol	5 mM	390.65 mg

Histone dimer/tetramer storage buffer (storage: 4°C, make fresh)

To prepare 2 L of histone dimer/tetramer storage buffer, dissolve the following reagents and bring the volume to 1.9 L using Milli-Q water. Adjust the pH to 7.5 using concentrated HCl solution. Adjust the final volume to 2 L using Milli-Q water.

Reagent	Final concentration	Amount
Tris-HCl	10 mM	60.57 mg
EDTA	1 mM	14.61 mg
NaCl	2 M	5.84 g
β-mercaptoethanol	5 mM	19.53 mg
Glycerol	50% (v/v)	1 L

Nucleosome reconstitution buffer (storage: 22°C–25°C, up to 6 months)

To prepare 2 L of nucleosome reconstitution buffer, dissolve the following reagents and bring the volume to 1.9 L using Milli-Q water. Adjust the pH to 7.8 using concentrated HCl solution. Adjust the final volume to 2 L using Milli-Q water.

Reagent	Final concentration	Amount
Tris-HCl	10 mM	2.42 g
EDTA	0.1 mM	58.44 mg
KCl	2 M	298.21 g

Stepwise salt dialysis buffer (storage: 4°C, make fresh)

To prepare 500 mL of refolding buffer, dissolve the following reagents and bring the volume to 480 mL using Milli-Q water. Adjust the pH to 7.8 using concentrated HCl solution. Adjust the final volume to 500 mL using Milli-Q water.

Reagent	Final concentration	Amount
Tris-HCl	10 mM	605.70 mg
EDTA	0.1 mM	14.61 mg
KCl	1.6 M/1.4 M/1.2 M/1.0 M/0.8 M/0.5 M/0.2 M/10 mM	59.64 g/52.19 g/44.73 g/37.28 g/29.82 g/18.64 g/7.46 g/372.76 mg

Native loading buffer (storage: 4°C, up to 1 year)

To prepare 2 mL of native loading buffer, dissolve the following reagents and bring the volume to 1.9 mL using Milli-Q water. Adjust the pH to 6.8 using concentrated HCl solution. Adjust the final volume to 2 mL using Milli-Q water.

Reagent	Final concentration	Amount
Tris-HCl	62.5 mM	15.14 mg
Bromophenol Blue	0.01% (w/v)	200 µL 0.1% stock
Glycerol	40% (v/v)	0.8 mL

TBE buffer (storage: 22°C–25°C, up to 6 months)

To prepare 1 L of TBE buffer, dissolve the following reagents and bring the volume to 980 mL using Milli-Q water. Adjust the pH to 8.0 using concentrated HCl solution. Adjust the final volume to 1 L using Milli-Q water.

Reagent	Final concentration	Amount
Tris-borate	45 mM	8.23 g
EDTA	1 mM	292.24 mg

Elution buffer (storage: 4°C, make fresh)

To prepare 1 L of elution buffer, dissolve the following reagents and bring the volume to 980 mL using Milli-Q water. Adjust the pH to 7.8 using concentrated HCl solution. Adjust the final volume to 1 L using Milli-Q water.

Reagent	Final concentration	Amount
Tris-HCl	10 mM	1.21 g
EDTA	0.1 mM	29.22 mg
KCl	10 mM	745.51 mg
Glycerol	10% (v/v)	100 mL

FRET experimental high salt buffer (storage: 4°C, make fresh)

To prepare 500 mL of refolding buffer, dissolve the following reagents and bring the volume to 480 mL using Milli-Q water. Adjust the pH to 7.5 using concentrated HCl solution. Adjust the final volume to 500 mL using Milli-Q water.

Reagent	Final concentration	Amount
Tris-HCl	10 mM	605.70 mg
EDTA	0.1 mM	14.61 mg
NaCl	2 M	58.44 g
Ascorbic acid	1 mM	88.06 mg
BSA	0.1 g/L	50 mg

FRET experimental low salt buffer (storage: 4°C, make fresh)

To prepare 500 mL of refolding buffer, dissolve the following reagents and bring the volume to 480 mL using Milli-Q water. Adjust the pH to 7.5 using concentrated HCl solution. Adjust the final volume to 500 mL using Milli-Q water.

Reagent	Final concentration	Amount
Tris-HCl	10 mM	605.70 mg
EDTA	0.1 mM	14.61 mg
NaCl	10 mM	292.20 mg
Ascorbic acid	1 mM	88.06 mg
BSA	0.1 g/L	50 mg

Other buffers

Solution	Contents	Storage
10 M NaOH	20 g NaOH, fill up to 50 mL with Milli-Q water	22°C–25°C, up to 6 months
1 M DTT stock solution	771.27 mg DTT, fill up to 5 mL with Milli-Q water	−20°C, up to 6 months
HPLC Buffer A	1 mL TFA, fill up to 1 L with Milli-Q water	22°C–25°C, up to 2 months
HPLC Buffer B	1 mL TFA, fill up to 1 L with 90% acetonitrile (ACN) in Milli-Q water	22°C–25°C, up to 2 months

STEP-BY-STEP METHOD DETAILS

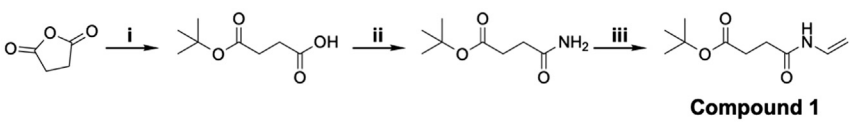
Synthesis of *tert*-butyl *N*-vinyl succinamate (compound 1)

Timing: 3 days

1. Synthesis of mono-*tert*-butyl succinate (Step i in Scheme 1) (Colombo et al., 2012)
   a. Dissolve succinic anhydride (3.00 g, 29.98 mmol), *N*-hydroxysuccinimide (0.3 eq, 1.04 g, 8.99 mmol), and DMAP (0.1 eq, 366.25 mg, 3.00 mmol) in 40 mL toluene.
   b. Add triethylamine (0.3 eq, 910.07 mg, 8.99 mmol) and *tert*-butyl alcohol (2.0 eq, 4.44 g, 59.96 mmol) into the mixture.
   c. Reflux the mixture for 24 h and let it cool to 25°C.
   d. Wash the upper organic phase with 1 M HCl solution and followed with brine.
   e. Dry the organic phase over Na_2SO_4 (s), filter, and concentrate under vacuum to give the crude product.
   f. Purify the crude product in a flash chromatography column eluted with 9:1 DCM/acetone to afford the product as a yellow oil (4.2 g, 24.11 mmol, 80% yield).
   g. Analyze the product by ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.63 (t, *J* = 6.1 Hz, 2H), 2.54 (t, *J* = 6.1 Hz, 2H), 1.44 (s, 9H).

   Note: Mono-*tert*-butyl succinate is commercially available (Sigma, cat#547352).

2. Synthesis of *tert*-butyl succinamate (Step ii in Scheme 1) (Grimm and Lavis, 2011).
   a. Dissolve mono-*tert*-butyl succinate (500 mg, 2.87 mmol), NH_4Cl (3.0 eq, 460.59 mg, 8.61 mmol), EDCI (1.5 eq, 825.38 mg, 4.31 mmol), and HOBt (1.5 eq, 659.35 mg, 4.31 mmol) in 10 mL DMF.
   b. Add DIEA (1.5 eq, 556.47 mg, 4.31 mmol) into the mixture above.
   c. After stirring for 6 h at 25°C, pour the reaction solution into 30 mL saturated $NaHCO_3$ solution and extract with ethyl acetate (3 × 20 mL).
   d. Wash the combined organic layers with brine, dry over Na_2SO_4, filter, and concentrate under vacuum.
   e. Purify the crude product in a flash chromatography column eluted with 9:1 DCM/MeOH to afford *tert*-butyl succinamate as a clear oil (497.18 mg, 2.87 mmol, 100% yield).
   f. Analyze the product by ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.43 (s, 1H), 6.30 (s, 1H), 2.39 (t, *J* = 6.5 Hz, 2H), 2.32 (t, *J* = 6.5 Hz, 2H), 1.27 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.18, 171.63, 79.94, 29.85, 29.60, 27.31; HRMS (EI): calculated for $C_8H_{15}NO_3$ (M^+): 173.1052, found 173.1057.
3. Synthesis of *tert*-butyl *N*-vinyl succinamate (Step iii in Scheme 1) (Compound 1) (Hansen and Skrydstrup, 2005).
   a. Dissolve *tert*-butyl succinamate (100 mg, 0.577 mmol), Cs_2CO_3 (1.0 eq, 188.10 mg, 0.577 mmol), CuI (0.2 eq, 21.99 mg, 0.115 mmol), vinyl iodide (0.8 eq, 71.10 mg, 0.462 mmol), and N^1,N^2-dimethylethane-1,2-diamine (0.4 eq, 20.36 mg, 0.230 mmol) in 5 mL anhydrous THF.
   b. Stir the reaction mixture for 17 h at 70°C under argon protection.
   c. Filter the reaction mixture and concentrate the filtrate under vacuum to afford the crude product.
   d. Purify the crude product in a flash chromatography column eluted with 50:50 hexane/ethyl acetate to afford Compound 1 as a yellow oil (60 mg, 301.13 μmol, 52% yield).
   e. Analyze the product by ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.02 (d, *J* = 8.9 Hz, 1H), 6.91 (m, 1H), 4.56 (dd, *J* = 15.9 Hz, 1H), 4.33 (dd, *J* = 8.8 Hz, 1H), 2.53 (t, *J* = 6.3 Hz, 2H), 2.44 (t, *J* = 6.3 Hz, 2H), 1.37 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.22, 169.61, 128.65, 95.30, 80.93, 30.91, 30.46, 27.97; HRMS (EI): calculated for $C_{10}H_{17}NO_3$ (M^+): 199.1208, found 199.1199.



Scheme 1. Synthesis of *tert*-butyl *N*-vinyl succinamate

Preparation of recombinant *Xenopus laevis* histones

Timing: 2 weeks

4. Wild type histones H2A, H2B, H3, and H4 are expressed in *Escherichia coli* Rosetta strain (DE3) and purified as previously described (Luger et al., 1999).
   a. Grow *E. coli* Rosetta cells harboring pET-histone expression plasmids in 1 L LB media containing ampicillin and chloramphenicol at 37°C until OD600 reaches 0.8.
   b. Induce protein expression by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.6 mM and incubate for 4 h at 37°C.
   c. Collect the cells by centrifugation at 3,500 × *g* for 25 min at 4°C.
   d. Resuspend the cells in lysis buffer (25 mM HEPES, 1 mM EDTA, 1 M NaCl, 1 mM PMSF, pH 7.5).

   ⚠ CRITICAL: For the lysis buffer, 1 mM PMSF is added just before use as it hydrolyses rapidly.
   e. Sonicate the cells (5 s ON, 10 s OFF, 30 min) and collect the pellet of the inclusion bodies containing the corresponding histone proteins by centrifugation at 4°C (12,000 × *g*, 15 min).
   f. Resuspend the pellet in 15 mL lysis buffer containing 1% (v/v) Triton X-100 and centrifuge at 12,000 × *g* for 15 min at 4°C, and then pour off the supernatant.
   g. Repeat step f twice and then wash the pellet twice with 15 mL lysis buffer. After final wash, pour off the supernatant.
   h. Add 250 μL DMSO to the pellet and incubate for 20 min at 25°C.
   i. Add 5 mL HU-500 buffer (6 M Urea, 25 mM HEPES, 1 mM EDTA, 500 mM NaCl, pH 7.5) to the mixture.
   j. Add HU-0 buffer (6 M Urea, 25 mM HEPES, 1 mM EDTA, pH 7.5) to the mixture to dilute the salt concentration to 100 mM with shaking.
   k. Keep shaking and mixing for another 2 h at 25°C.
   l. Centrifuge at 20,000 × *g* for 10 min at 25°C (***Note:*** Most of the pellet should be dissolved in the HU buffer).
   m. Collect the supernatant and load onto a HiTrap SP HP column (5 mL, GE) pre-equilibrated with HU-100 buffer (6 M Urea, 25 mM HEPES, 1 mM EDTA, 100 mM NaCl, pH 7.5).
   n. Elute histone proteins with a gradient of 0%–100% HU-500 buffer over 25 min at a flow rate of 2 mL/min.
   o. Analyze the peak fractions by 18% SDS-PAGE and pool the fractions containing the purified histone proteins.
   p. Load the combined fractions onto a HiLoad 16/60 Superdex 200 pg size-exclusion column (GE) pre-equilibrated with HU-1000 buffer (6 M Urea, 25 mM HEPES, 1 mM EDTA, 1000 mM NaCl, pH 7.5).
   q. Elute histone proteins using 100% HU-1000 buffer at a flow rate of 2 mL/min.
   r. Analyze the peak fractions by 18% SDS-PAGE and collect the corresponding fractions.
   s. Dialyze the histone proteins against *dd* H_2O containing 2 mM DTT at 4°C (at least three changes).
   t. Freeze dry the protein solution to obtain the lyophilized histone powder.

Note: Purification of mutated histone H2B K34C will be the same as the preparation of wild type histones.

Pause point: Purified histone proteins can be stored at −80°C for an indefinite length of time.

Preparation of modified histone H2BK_c34succ-tBu

Timing: 3 days

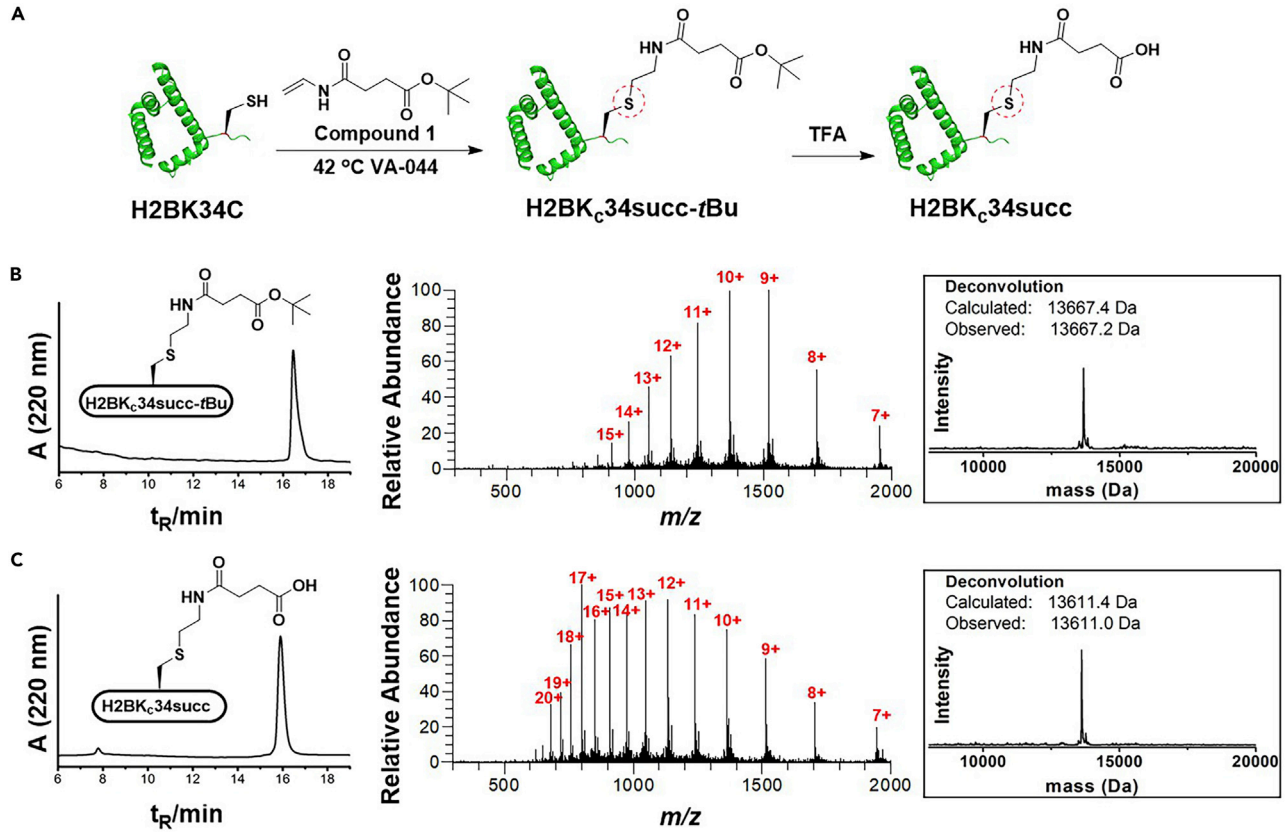


Figure 1. Installation of the succinyl lysine analog onto recombinant histone

(A) Scheme for the installation of the succinyl lysine analog ($K_c$succ) at position 34 of histone H2B.

(B and C) LC-MS and deconvolution results for the thiol-ene coupling product H2BK_c34succ-tBu (B) and the deprotected final product H2BK_c34succ (C).

Figure reprinted with permission from Jing et al., 2018.

The 'thiol-ene' coupling reaction of Compound 1 with mutated histone H2BK34C followed by HPLC purification generates the modified histone H2BK_c34succ-*t*Bu (Figure 1A) (Li et al., 2011).

5. The 'thiol-ene' coupling reaction:
   a. Dissolve mutated histone H2BK34C in 0.1 M phosphate buffer (pH 7.0) containing 6 M Gdn·HCl to a final concentration of 1 mM.
   b. Add DTT to a final concentration of 5 mM.

   ⚠ CRITICAL: DTT stock solution (1 M) should be freshly prepared.
   c. Add 50 equivalent (50 mM) of compound 1 into the reaction mixture.
   d. Add VA-044 (20 mM) and dimethyl sulfide (100 mM) separately into the reaction mixture.
   e. Initiate the reaction by incubating the reaction tube in a 42°C water bath.

   ⚠ CRITICAL: Normally, the 'thiol-ene' coupling reaction completes in 2 h.
   f. Analyze the reaction by LC-MS using a Vydac 218TP C4 column (4.6 mm × 250 mm, Grace Davison) and ESI-MS. The mobile phase is HPLC buffer A and HPLC buffer B. The deconvolution results are obtained using the UniDec software.

6. HPLC purification of the modified histone:

a. After the reaction finishes according to the HPLC analysis (histone H2BK34C should be fully converted to the modified histone H2BK$_c$34succ-*t*Bu), load the reaction mixture onto a semi-prep column (Vydac C4 column, Grace Davison).
b. Elute modified histone with a gradient of 20%–50% HPLC buffer B over 35 min.
c. Analyze the peak fractions by ESI-MS and obtain the deconvolution results of each peak in the UniDec software (Figure 1B).
d. Pool the fractions containing the pure modified histones, followed by lyophilization.

Pause point: Purified histone proteins can be stored at −80°C for an indefinite length of time.

Deprotection of the *tert*-butyl group on histone H2BK$_c$34succ-*t*Bu

Timing: 2 days

The *tert*-butyl group on histone H2BK$_c$34succ-*t*Bu is deprotected by treating the intermediate protein with TFA, followed by HPLC purification and lyophilization (Figure 1A).

7. Deprotection reaction in 70% TFA:
   a. Dissolve the intermediate protein H2BK$_c$34succ-*t*Bu in 0.1 M phosphate buffer (pH 7.0) containing 6 M Gdn·HCl to a final concentration of 5 mM.
   b. Keep the above protein solution on ice.
   c. Add TFA dropwise to the reaction mixture to a final concentration of 70% (v/v).
   d. Incubate the reaction at 25°C with shaking.
   e. Monitor the reaction by LC-MS using a Vydac 218TP C4 column (4.6 mm × 250 mm, Grace Davison) and ESI-MS. The mobile phase is HPLC buffer A and HPLC buffer B. The deconvolution results are obtained using the UniDec software.
8. HPLC purification of deprotected histone:
   a. After the reaction finishes according to the HPLC analysis (the intermediate protein H2BK$_c$34succ-*t*Bu should be fully deprotected), remove the TFA under a stream of compressed argon.
   b. Load the reaction mixture onto a semi-prep column (Vydac C4 column, Grace Davison) and elute deprotected histone with a gradient of 20%–50% HPLC buffer B over 35 min.
   c. Analyze the peak fractions by ESI-MS and obtain the deconvolution results of each peak using the UniDec software (Figure 1C).
   d. Pool the fractions containing the pure deprotected histones, followed by lyophilization.

Pause point: Purified histone proteins can be stored at −80°C for an indefinite length of time.

Preparation of histone H2A-H2B dimers and (H3-H4)$_2$ tetramers

Timing: 2 days

H2A-H2B dimers and (H3-H4)$_2$ tetramers are prepared by histone refolding (Figure 2) (Luger et al., 1999).

9. Preparation of H2A-H2B dimers:
   a. Mix equal molar quantities of H2A and H2B proteins in histone unfolding buffer (6 M Gdn·HCl, 20 mM Tris-HCl, pH 7.5, 1.0 mM EDTA, 10 mM DTT) to a final histone concentration of 1 mg/mL.
   b. Incubate the mixture for 30 min on ice.
   c. Dialyze the mixture in histone refolding buffer (2 M NaCl, 10 mM Tris-HCl, pH 7.5, 1.0 mM EDTA, 5 mM β-mercaptoethanol) for 4 h at 4°C, and then change the histone refolding buffer and dialyze for another 16 h.

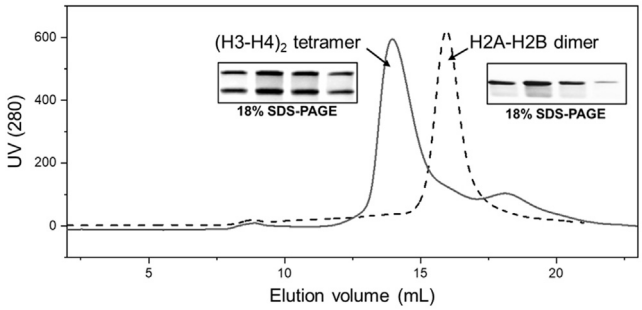


Figure 2. The elution profile of H2A-H2B dimers and $(H3-H4)_2$ tetramers in the gel-filtration chromatography detected at 280 nm

H2A-H2B dimers eluted out at around 16 mL, according to its molecular weight (26 kD), whereas $(H3-H4)_2$ tetramers eluted out at around 14.1 mL, according to its molecular weight (53 kD). The purity and stoichiometry of the fractions were verified by 18% SDS-PAGE.

d. Concentrate the refolded dimer solution using an Amicon® Ultra 15 mL centrifugal filter device (Membrane nominal molecular weight limit, 3 kDa) to less than 1 mL.

⚠ CRITICAL: When performing concentration with the centrifugal filter device, do not concentrate the total volume below 200 μL, or it may result in precipitation.

e. Load the concentrated mixture onto a HiLoad 16/60 Superdex 200 pg column (GE) pre-equilibrated with histone refolding buffer to remove any histone monomers and impurities.
f. Elute histone proteins with histone refolding buffer over 25 min at a flow rate of 1 mL/min.
g. Analyze the peak fractions by 18% SDS-PAGE to verify the quality of the purified dimers and pool the corresponding fractions.
h. Concentrate the pooled fractions using an Amicon® Ultra 15 mL centrifugal filter device (Membrane nominal molecular weight limit, 3 kDa).
i. For long-term storage, dialyze the concentrated fraction in storage buffer (2 M NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM β-mercaptoethanol, 50% glycerol).
j. Determine the protein concentration of the assembled H2A-H2B dimers by Bradford Protein Assay kit.
k. Store the assembled H2A-H2B dimers at −20°C.

10. Use the same method to prepare H2A-H2BK_c34succ dimers and $(H3-H4)_2$ tetramers.

Pause point: Purified histone dimers and tetramers can be stored at −20°C for up to 4 months.

Preparation and purification of fluorophore-labeled mononucleosomes

Timing: 2 days

Histone dimers and tetramers can be assembled into nucleosomes using salt deposition (Luger et al., 1997) (Figure 3).

11. Stepwise salt dialysis:
    a. Determine the protein concentration of the assembled histone dimers (modified H2A-H2BK_c34succ dimers or unmodified H2A-H2B dimers) and $(H3-H4)_2$ tetramers by Bradford Protein Assay kit.
    b. Determine the concentration of fluorophore-labeled DNA by a UV-Vis spectrophotometer (A260 nm, NanoDrop 2000, Thermo).
    c. Mix the purified histone dimers (modified H2A-H2BK_c34succ dimers or unmodified H2A-H2B dimers) and $(H3-H4)_2$ tetramers with the fluorophore-labeled "Widom 601" DNA (at a molar

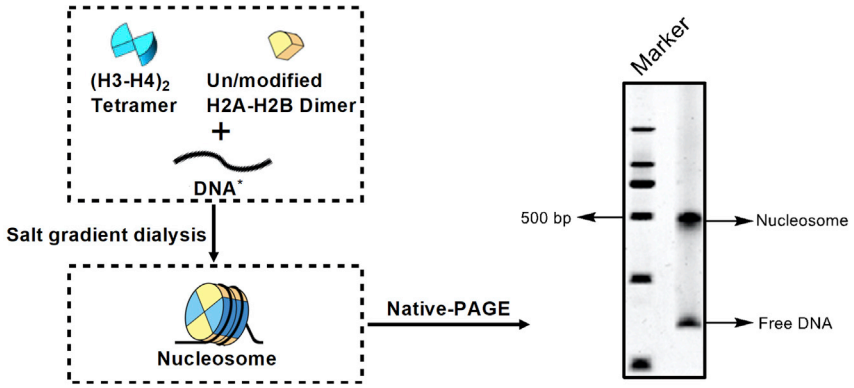


Figure 3. Schematics for the stepwise salt dialysis method to reconstitute nucleosomes
The quality and compaction of reconstituted nucleosomes were analyzed by Native-PAGE.

ratio of dimer : tetramer : DNA = 2 : 1 : 1.2) to a concentration of 2 μM in 50 μL reconstitution buffer (2 M KCl, 10 mM Tris-HCl, pH 7.8, 0.1 mM EDTA).

⚠ CRITICAL: Test the ratio of dimer, tetramer, and DNA at a much lower concentration (~30 nM in 30 μL) to confirm nucleosome formation so as not to waste components on a large preparation.

d. Transfer the mixture into a Slide-A-Lyzer MINI dialysis unit (Thermo) and sequentially dialyze at 4°C against reconstitution buffer containing 1.6 M KCl, 1.4 M KCl, 1.2 M KCl, 1.0 M KCl, 0.8 M KCl, 0.5 M KCl, and 0.2 M KCl for 50 min each, followed by 10 mM KCl 12 h.

Pause point: The final dialysis step in 10 mM KCl is left to proceed 12 h.

e. Check the quality and compaction of the reconstituted nucleosomes by Native-PAGE (5% TBE gel, acrylamide : Bis = 29 : 1).
f. Stain the gel with ethidium bromide (EB) for 15 min and visualize the nucleosomes under UV using a MyECL Imager system (Thermo Fisher Scientific).

12. Purification of reconstituted nucleosomes:
    a. Concentrate the reconstitution mixture to 75 μL using an EMD Millipore Amicon Ultra Centrifugal Filter (Cat#UFC500324, 3 kDa Ultracel, 0.5 mL).
    b. Add 25 μL native loading buffer (62.5 mM Tris-HCl, pH 6.8, 40% glycerol, 0.01% Bromophenol Blue) into the concentrated mixture.
    c. Purify the reconstituted nucleosomes by Native-PAGE (5% TBE gel, acrylamide : Bis = 60 : 1) using a mini prep cell (Mini Prep Cell, Bio-Rad 1702908). The elutant is monitored by a UV spectrometer (ÄKTA pure protein purification system).
    Elution buffer contains 10 mM KCl, 10 mM Tris-HCl, 10% glycerol, pH 7.8, 0.1 mM EDTA and running buffer contains 0.2× TBE.
    d. Analyze the peak fractions by Native-PAGE (5 % TBE gel, acrylamide : Bis = 29 : 1) and pool the corresponding fractions.
    e. The purity of the purified nucleosomes is verified by Native-PAGE (Figure 4).
    f. Concentrate the purified nucleosomes using an Amicon® Ultra 15 mL centrifugal filter device (Membrane nominal molecular weight limit, 3 kDa).
    g. Determine the concentration of the purified nucleosomes by UV-Vis spectrophotometer (A260 nm, NanoDrop 2000, Thermo).
    h. Store the purified nucleosomes at 4°C until use.

Note: Purified nucleosomes can be stored at 4°C for up to 1 week.

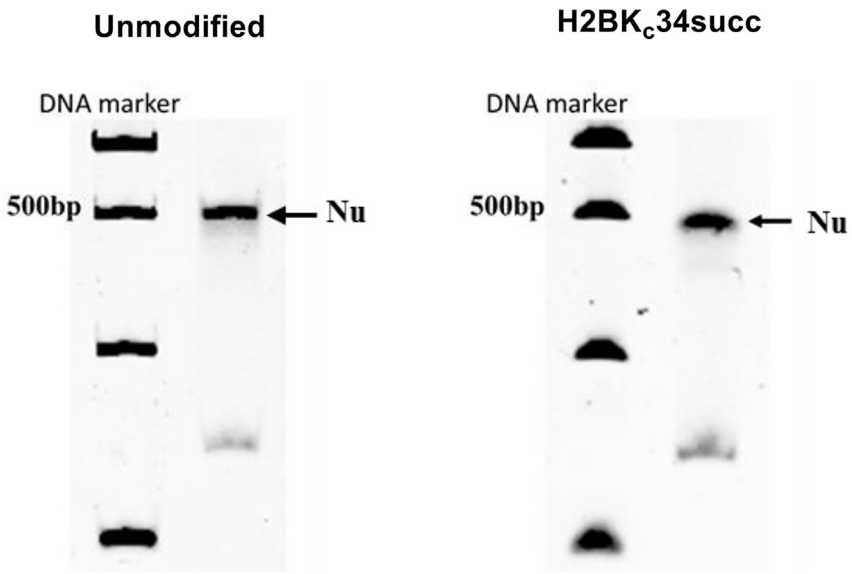


Figure 4. The final purified nucleosomes (unmodified and H2BK$_c$34succ, as indicated) used in the FRET experiments
After purification, the amount of free DNA was significantly reduced. Figure reprinted with permission from Jing et al., 2018.

Förster resonance energy transfer (FRET) analysis of nucleosome dynamics

Timing: 4 h

Salt-dependent dissociation of mononucleosomes can be quantified by a single-pair FRET-based approach as described in the literature (Bohm et al., 2011, Gansen et al., 2015). The donor and acceptor fluorophores used here are Alexa Fluor 488 (excitation at 490 nm and emission at 525 nm) and Alexa Fluor 594 (excitation at 590 nm and emission at 617 nm), respectively.

13. FRET sample preparation:
    a. Determine the concentration of the purified nucleosomes by UV-Vis spectrophotometer (A260 nm, NanoDrop 2000, Thermo).
    b. Dilute the purified nucleosomes in the elution buffer to a final concentration at 1 ng/μL nucleosome.

⚠ CRITICAL: Do not vortex during the FRET sample preparation step. Use a pipette to homogenize the mixture.

    c. Prepare the series of experimental buffers using FRET low salt buffer (10 mM NaCl, 10 mM Tris, 0.1 mM EDTA, pH 7.5, 1 mM ascorbic acid, 0.1 g/L BSA) and FRET high salt buffer (2 M NaCl, 10 mM Tris, 0.1 mM EDTA, pH 7.5, 1 mM ascorbic acid, 0.1 g/L BSA) at different salt concentrations from 0.01 M to 1.6 M (190 μL per sample).
    d. Add 10 μL diluted nucleosome solution into each experimental buffer (final total volume for each sample is 200 μL).

Final salt concentration/M	FRET experimental high salt buffer (V/μL)	FRET experimental low salt buffer (V/μL)	nucleosome solution (V/μL)
0.01	0	190	10
0.1	10	180	10
0.2	20	170	10

(*Continued on next page*)

Continued

Final salt concentration/M	FRET experimental high salt buffer (V/μL)	FRET experimental low salt buffer (V/μL)	nucleosome solution (V/μL)
0.3	30	160	10
0.4	40	150	10
0.5	50	140	10
0.6	60	130	10
0.7	70	120	10
0.8	80	110	10
0.9	90	100	10
1.0	100	90	10
1.1	110	80	10
1.2	120	70	10
1.3	130	60	10
1.4	140	50	10
1.5	150	40	10
1.6	160	30	10

e. Incubate the samples in a 384-well microplate (50 μL/well, 3 replicates for each sample) for 5 min at 25°C.

14. FRET measurements:
    a. Use a plate reader (Beckman Coulter, DTX 880) to measure fluorescence intensity in each well. Energy transfer changes due to salt-induced nucleosome dissociation can be measured by the emission of the acceptor (Alexa 594) upon donor excitation (Alexa 488). Fluorescence is detected in two spectral windows to yield the signal intensities of the donor (I_D^0) and transfer (I_T^0) channels.

15. FRET data processing and analysis:
    a. Correct the intensity of donor (I_D) and transfer (I_T) using the following equations:

$$I_T = \left(I_T^0 - B_T\right) - a_{DT}\left(I_D^0 - B_D\right) - f_{dir}$$

$$I_D = \left(I_D^0 - B_D\right)$$

Where, B_T and B_D are background intensities of the experimental buffer, a_{DT} is the spectral crosstalk from the donor to the transfer channel, and f_{dir} is the direct excitation of the acceptor dye. The formula for calculating a_{DT} and f_{dir} is described in Gansen *et al.* (Gansen et al., 2015).

    b. The FRET intensity (I_F) is a measure of energy transfer efficiency and is calculated using the following equation:

$$\text{FRET intensity } (I_F) = \frac{I_T}{I_T + I_D}$$

    c. Input the data into OriginPro 8 using salt concentration as the x-axis and FRET intensity as the y-axis. To measure the salt-dependent dissociation of the mononucleosomes, fit the following sigmoidal function to the experimental curve:

$$I_F(X) = I_F(0) + \frac{I_F(\infty) - I_F(0)}{1 + \exp\left(c_{1/2} - X\right)/b}$$

Stability of the mononucleosomes is quantified by the $c_{1/2}$ parameter and is the salt concentration where the intensity (Y axis) has dropped to half its initial value. Here, X is the salt concentration in M and b describes the slope of the curve at $X = c_{1/2}$. I_F (0) and I_F (∞) are the amplitude and offset of the salt-titration curve.

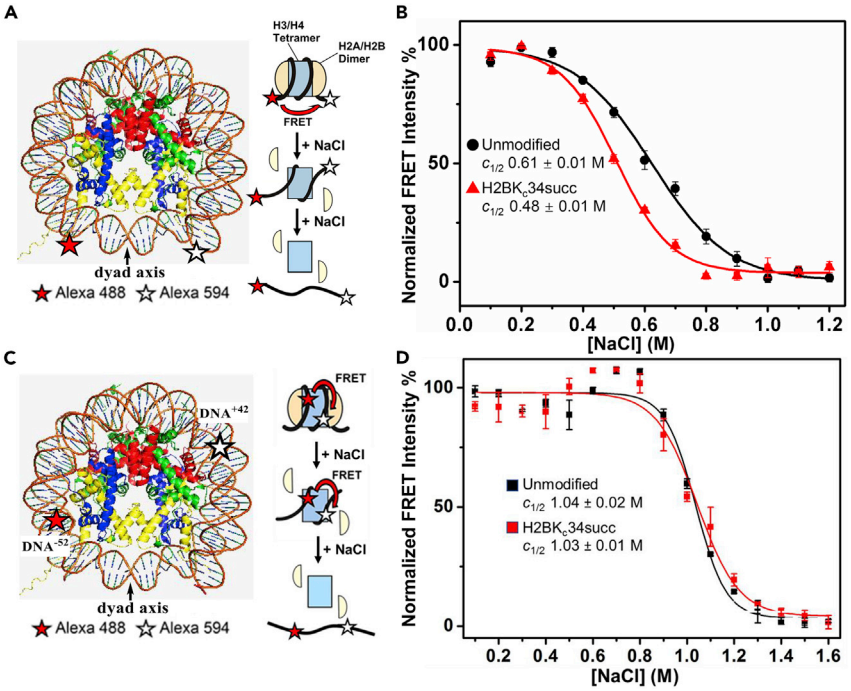


Figure 5. Expected outcomes of the FRET experiments

(A) Schematics for a FRET-based approach to study salt-induced stepwise nucleosome disassembly using end-labeled nucleosomal DNA.

(B) Plot showing the normalized FRET intensity as a function of salt concentration for the nucleosomes containing unmodified H2B (black trace) and H2BK$_c$34succ (red trace). The salt concentration at which the FRET has decreased by 50% is denoted as $c_{1/2}$ (n = 3, mean ± SD).

(C) Schematics for a FRET-based approach with internally labeled nucleosomal DNA.

(D) Plot showing the normalized FRET intensity as a function of salt concentration in nucleosomes containing unmodified H2B (black trace) and H2BK$_c$34succ (red trace). The salt concentration at which the FRET has decreased by 50% is denoted as $c_{1/2}$ (n = 3, mean ± SD). Figure reprinted with permission from Jing et al. (2018).

EXPECTED OUTCOMES

Examples of the expected outcomes of the FRET experiments in this protocol are given in Figure 5. The close proximity of the DNA ends in a fully assembled nucleosome enables efficient FRET, which is lost when the DNA ends are loosened from the nucleosome during dissociation of the H2A-H2B dimers (Figure 5A). Indeed, when titrating salt (NaCl) into the nucleosome solution, we observed a sigmoidal decay in the FRET intensity (Figure 5B). To quantify the nucleosome stability, we used the salt concentration at the transition midpoint of the titration curve ($c_{1/2}$ value). In addition to monitoring the release of the H2A-H2B dimers, we also examined the effect of H2BK34succ on the dissociation of the $(H3\text{-}H4)_2$ tetramer from DNA as the final step in the salt-induced nucleosome disassembly process. To this end, a pair of FRET dyes were placed at two internal sites of the nucleosomal DNA (Figure 5C). The FRET signal showed a similar decay with increasing salt concentration. However, no difference was observed between the unmodified and H2BK34succ nucleosomes (Figure 5D), indicating that succinylation does not destabilize the $(H3\text{-}H4)_2$ tetramer-DNA complex.

QUANTIFICATION AND STATISTICAL ANALYSIS

The deconvolution results in the MS analysis were obtained using UniDec software for Windows (University of Oxford, unidec.chem.ox.ac.uk/). All data fitting and statistical analyses were performed using OriginPro 8 for Windows (OriginLab Corporation, www.originlab.com), GraphPad Prism version 5 for Windows (GraphPad Software, www.graphpad.com), and Image J for Windows (National Institutes of Health, imagej.nih.gov/ij/). For the FRET experiments, the normalized FRET intensity was plotted as the mean ± s.d. (n=3). The sigmoidal function and equation used for the curve fit are specified in detail above.

LIMITATIONS

This protocol describes a method to generate site-specific succinylation histones mimics, which can be incorporated into mononucleosomes by stepwise salt dialysis. The effects of lysine succinylation on nucleosome stability and dynamics were investigated by a FRET approach. However, there are several limitations to this protocol. First, as *N*-vinyl-succinamic acid is not stable, we used *tert*-butyl *N*-vinyl succinamate (Compound 1) for the thiol-ene coupling reaction, followed by deprotection of the *tert*-butyl group to afford the desired succinyl lysine analog. However, the 'two-step' reaction strategy requires purification by HPLC to extract the modified histone after each step, which increases the workload and decreases the yield. Second, during nucleosome reconstitution, we used a Bradford Protein Assay kit to determine the concentration of assembled H2A-H2B dimers and $(H3\text{-}H4)_2$ tetramers. Although the Bradford Protein Assay kit is convenient, it is not very accurate. The accurate concentration and ratio of dimers and tetramers is crucial for nucleosome reconstitution. If available, we would recommend using nanodrop, Qubit™ Protein Assay Kit, or BCA Protein Assay for more accurate protein concentration quantification. Third, we performed the stepwise salt dialysis of the nucleosomal DNA with histone dimers and tetramers against a concentration gradient in a series of reconstitution buffers, with each dialysis step lasting 50 min. In practice, it is recommended to use a peristaltic pump to add low salt dialysis buffer (10 mM KCl) into the initial high salt dialysis buffer (1.6 M KCl) at a steady rate with stirring to gradually decrease the salt concentration. This will not only save you from having to prepare a series of dialysis buffers, but also allows the salt concentration to decrease linearly, which we believe is more favorable for the nucleosome assembly.

TROUBLESHOOTING

Problem 1

The 'thiol-ene' coupling reaction doesn't work (step 1).

Potential solution

Disulfide bonds may form between Cys-containing proteins, whereas the 'thiol-ene' coupling reaction requires free thiols. Increase the concentration of DTT to 10 mM in the reaction mixture and incubate with proteins for 30 mins at 37°C prior to adding compound 1.

Problem 2

After the 'thiol-ene' coupling reaction, the purification of the modified histone is difficult (step 2).

Potential solution

Besides the modified histones, DTT can also react with excess compound 1 to form a large number of byproducts. These small molecule impurities can make it difficult to purify the modified histone. Use dialysis to remove the small molecule impurities before HPLC purification. Alternatively, it is possible to use an Amicon® Ultra 15 mL centrifugal filter device (Membrane nominal molecular weight limit, 3 kDa) to remove small molecule impurities prior to HPLC purification.

Problem 3

The reconstitution of nucleosomes by stepwise salt dialysis fails and no clear bands are detected in the Native-PAGE (step 7).

Potential solution

The quantification of dimers, tetramers, and DNA may not be accurate. Repeat the quantification of the protein concentration using Bradford Protein Assay kit (use nanodrop, Qubit™ Protein Assay Kit, or BCA Protein Assay for more accurate quantification). Add an additional equivalent of histone dimers to ensure complete consumption of histone tetramers, as histone tetramers may interfere with the purification of nucleosomes. The concentration of DNA can be determined by UV-Vis spectrophotometer at A260 nm, as the ratio of DNA is crucial for nucleosome reconstitution. Normally, 1.2 equivalent of DNA is enough, however, the amount of DNA can be increased to 1.5 equivalent to ensure full assembly.

Problem 4

The FRET curve is not ideal, in particular, the FRET intensity drops quickly at higher salt concentrations (steps 9 and 10).

Potential solution

The nucleosomes may disassemble during long-term storage. It is highly recommended to use Native-PAGE (5 % TBE gel, acrylamide : Bis = 29 : 1) to verify the quality of the purified nucleosomes before starting the FRET experiments. After preparing the FRET samples, use a plate reader to measure the fluorescence intensities as soon as possible, as nucleosomes may not be stable after prolonged incubation in the FRET buffer, particularly in the high salt experimental buffer.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to the lead contact, Xiang David Li (xiangli@hku.hk).

Materials availability

All reagents generated in this study are available from the lead contact upon completing a Materials Transfer Agreement.

Data and code availability

No unique datasets or codes were generated in this study.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

Y.J., Z.L., and X.D.L. conceived and designed the project. Y.J. and Z.L. developed and optimized the protocol. Y.J. synthesized the small molecule compounds. Y.J. and Z.L. performed the protein chemistry, nucleosome reconstitution, and FRET experiments. Y.J. and Z.L. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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