Benchmarks

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A rapid and efficient method to purify proteins at replication forks under native conditions

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Tools for studying replication fork dynamics are critical for dissecting the mechanisms of DNA replication, DNA repair, histone deposition, and epigenetic memory. Isolation of protein on nascent DNA (iPOND) is an elegant method for purifying replication fork proteins. Here, we present accelerated native iPOND (aniPOND), a simplification of the iPOND procedure with improved protein yield. Cell membrane lysis and nuclei harvesting are combined in one step to reduce washes and minimize sample loss. A mild nuclei lysis protocol is then used to better preserve DNA-protein complexes. aniPOND is faster than iPOND, avoids formaldehyde cross-linking, and improves protein yield 5- and 20-fold for the CAF1-complex or PCNA respectively. Moreover, using aniPOND, but not iPOND, we could detect the polycomb repressive complex 2 (PRC2) components SUZ12, EZH2, and RBBP4 at replication forks. This faster, higher-yield method will facilitate MS analysis of replication fork complexes.

iPOND (isolation of protein on nascent DNA) was developed by Sirbu et al. in 2011 (1) to study DNA replication. A similar procedure was presented in the same year by Kliszczak et al. (2). First, nascent DNA is labeled by a brief pulse of the thymidine analog 5-ethynyl-2′-deoxyuridine (EdU) (3). Then, biotin azide is covalently linked to the alkyne functional group on EdU in the presence of copper catalyst via a click reaction (4). Finally, biotinylated DNA is precipitated using streptavidin-coated beads and Western blotting is used to identify the associated proteins. Here, we build on this elegant method to generate a new protocol that is simpler, faster, and significantly improves protein yield (see Supplementary Material for a detailed protocol).

Limitations of iPOND include modest protein yield and the use of formaldehyde cross-linking to preserve DNA-protein complexes. Formaldehyde cross-linking could interfere with protein identification by mass spectrometry (MS) and detection of large molecular weight proteins by Western blotting if not fully reversed (5,6). In 2012, Sirbu et al. (6) proposed an alternate protocol, native iPOND (niPOND), for performing iPOND in the absence of formaldehyde, although results were not presented.

We were able to capture replication fork proteins using iPOND, but not with niPOND (data not shown). Here, we present accelerated native iPOND (aniPOND), which improves capture efficiency on average by an order of magnitude compared to iPOND and cuts the total time needed to perform the assay in half compared to niPOND (Figure 1A) (Supplementary Material).

In the iPOND and niPOND protocols, cells are labeled with EdU, scraped, spun, and washed, and then permeabilized or lysed prior to the click reaction (Figure 1A). For aniPOND, harvesting and lysis were performed in the flask simultaneously. Nuclear extraction buffer (NEB) was used to extract most of the soluble cytoplasmic and nuclear proteins and generate nuclei in one step (Figure 1A). Chromatin bound proteins were retained in the nuclear fraction (Supplementary Figure S1). This single-step procedure reduces manipulation of the sample, likely contributing to the enhanced recovery with aniPOND.

After a one-hour click reaction, biotinylated chromatin needs to be sheared and extracted from nuclei for the subsequent streptavidin capture step (Figure 1A). In the original iPOND protocol, chromatin is sonicated in a buffer containing the strong ionic detergent sodium dodecyl sulfate (SDS). This approach cannot be used in native preps as SDS or milder ionic detergents such as sodium deoxycholate disrupt protein complexes. The niPOND protocol suggests the use of micrococcal nuclease to digest chromatin, followed by overnight extraction in buffer containing 0.1% Triton-X. Using this approach we recovered <3 mg of protein from $6x10^7$ cells. In our new aniPOND approach, we first performed two single sonication/spin cycles on nuclei in a buffer with 1% NP40 to release more non-chromatin protein, then applied extensive sonication to shear and solubilize the chromatin in the remaining sample. This strategy yielded DNA fragments of ~150 bp (Protocol Figure 1B) and >7 mg of protein. The niPOND approach

Method summary:

We developed aniPOND to capture proteins at replication forks with improved efficiency under native conditions. Compared with the original approach, aniPOND increased protein yield 5–20 fold.

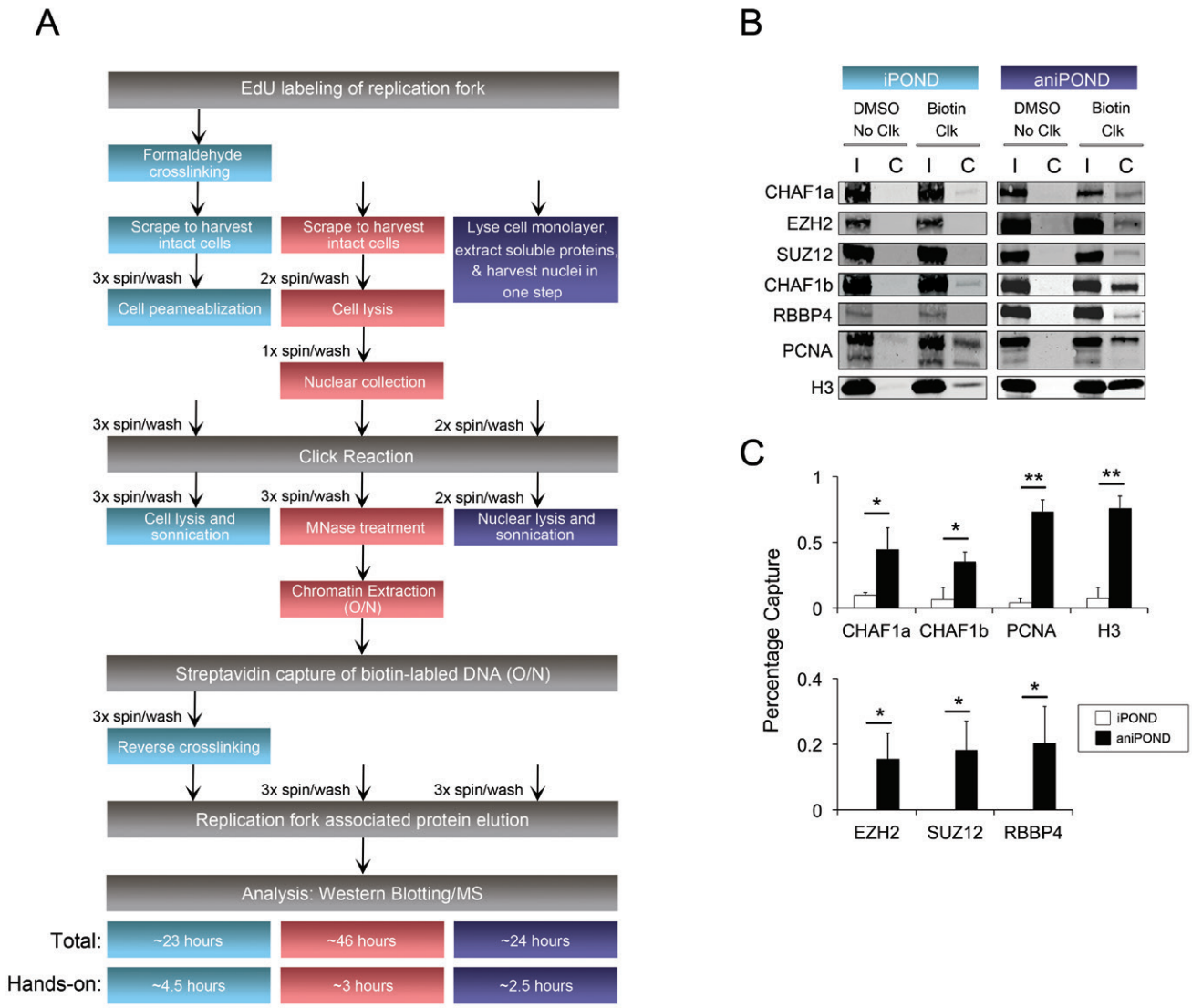

Figure 1. Enhanced capture efficiency of aniPOND over conventional iPOND. (A) Flow-chart showing an overview of the iPOND (blue), niPOND (red), and aniPOND (magenta) procedures. O/N = overnight. Total or hands-on time for each protocol is listed at the bottom of the flowchart. (B) Representative Western blot of input (I) or captured (C) proteins following iPOND or aniPOND on SW13 cells labeled for 10 min with EdU. (C) Quantification of Western blot results +/- SD (n = 4). Statistics were performed using Student's ***t***-test, * $P < 0.005$ and ** $P < 0.001$. In all experiments, a total of ~$6x10^7$ cells in 4 175 cm^2 tissue flasks was used. Cells were seeded at $1.5x10^7$ cells per flask. Input = 0.25% cell equivalent ($1.5x10^5$) cells. Capture = 25% cell equivalent ($1.5x10^7$) cells.

requires an overnight incubation, but the new aniPOND version of this step is complete in one hour.

Following chromatin extraction, the next step is to purify biotin labeled replication forks using streptavidin (Figure 1A). The protocols for iPOND, niPOND, and aniPOND are similar at this stage, except that iPOND requires an extra cross-linking reversal step. We ran Western blots to compare the capture efficiency of iPOND and aniPOND; niPOND data was not included because that technique was unsuccessful in our hands. Relative to iPOND, aniPOND significantly increased the yield of the CHAF1a and CHAF1b subunits of the CAF1 histone chaperone complex by 5-fold, and PCNA by 20-fold (Figure 1B, C). aniPOND not only improved the capture of these abundant replication fork proteins, but also allowed detection of rarer proteins such as PRC2 subunits (Figure 1B, C). A recent study using iPOND followed by MS described two new replication fork proteins, TFII-I and ZNF24 (7). We also detected these factors using aniPOND (Supplementary Figure S3). The replisome component MCM-3 was also recovered by aniPOND (Supplementary Figure S3). In addition, aniPOND was effective when performed on a different cell line (Supplementary Figure S2).

We also tested aniPOND in a more complex pulse-chase experiment. Similar to the above data, aniPOND captured replication fork proteins (PCNA and CAF1 complex) after a 10 min EdU pulse, and a subsequent 15 or 30 min chase period in which thymidine displaced replication forks from EdU-labeled DNA, diminishing the levels of PCNA and CAF1 complex (Figure 2A and B). PRC2 subunits and histone H3 levels remained constant (Figure 2A and B), indicating that these proteins remain on nascent DNA even after the replication fork passes. These observations are in agreement with published data (1,8).

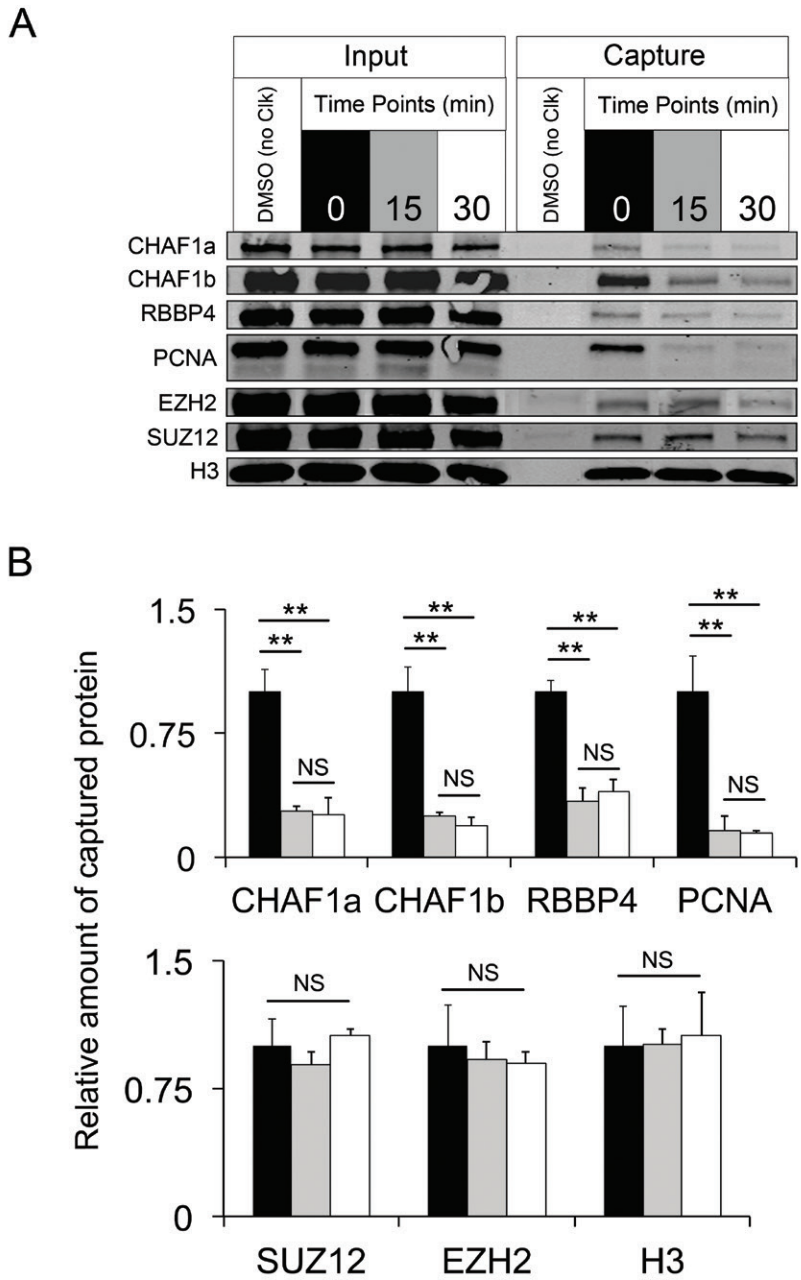

Figure 2. Application of aniPOND for pulse-chase experiments. (A) Western blot of input and captured proteins following aniPOND on SW13 cells incubated with EdU for 10 min and then chased in thymidine for the indicated times. (B) Quantification of Western blots +/- SD ($n = 3$). Statistics were performed with Student's *t*-test, ** $P < 0.001$, NS = not significant. The amount of capture at 15 and 30 min is normalized to the average at time 0. The amount of cells used and protein loaded was as in Figure 1B.

In summary, aniPOND provides a simpler, faster method to purify replication fork proteins under native conditions with higher yield. Coupled with MS, our approach may expand the list of replication fork proteins, given that it captures less abundant proteins such as members of the PRC2 complex. Comprehensive MS analyses will be required to determine whether aniPOND consistently detects more proteins than iPOND.

Author contributions

K.H.T.L. performed all experiments. K.H.T.L., M.A.E.H. and R.B. developed the protocol. K.H.T.L. and R.B. wrote the manuscript.

Acknowledgments

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Competing interests

The authors declared no competing financial interests.

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Accelerated native isolation of proteins on nascent DNA (aniPOND)

PROTOCOL FOR:

A rapid and efficient method to purify proteins at replication forks under native conditions

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Legend

⇨ ATTENTION

* HINT

REST

Reagents

- **EdU** (Invitrogen Canada, Burlington, ON, Canada)
- **Thymidine 99%-100%** (Sigma-Aldrich Canada, Oakville, ON, Canada)
- **(+)-Sodium L-ascorbate** (Sigma-Aldrich Canada)
- **Azide PEG4 biotin conjugate** (Click Chemistry Tools, Scottsdale, AZ)**$CuSO_4 \cdot 5H_2O$** (Sigma-Aldrich Canada)
- **Phosphate buffer saline (PBS)** (Invitrogen Canada)
- **Dimethyl sulfoxide (DMSO)** (Invitrogen Canada)
- **NaCl Chip Grade** (Invitrogen Canada)
- **EDTA** (Invitrogen Canada)
- **Tris-HCl pH 8.0** (Invitrogen Canada)
- **IGEPAL CA630** (Invitrogen Canada) – Similar to **NP40 detergent.**
- **Protease/Phosphatase Inhibitor** (Cell Signaling Technologies, Danvers, MA)
- **Streptavidin Agarose Resin 10 ml** (Thermo Fisher Scientific, Rockford, IL)
- **Laemmli Sample Buffer 2×** (BioRad Canada, Mississauga, ON, Canada)

Antibodies

- **PCNA** (Abcam, ab18797, Toronto, ON, Canada)
- **Histone H3** (Abcam, ab1791)

Equipment

- **Eppendorf Centrifuge 5810R (15ml conical tube)** (Thermo Fisher Scientific, Mississauga, ON, Canada)
- **Eppendorf Centrifuge 5424R (for 1.5 ml Eppendorf tube)** (Thermo Fisher Scientific)
- **Fisher Scientific Sonic Dismembrator Model 100** (Thermo Fisher Scientific)
- **Sarstedt SARMIX GM 1 Rotator** (Sarstedt Inc., Montreal, QC, Canada)
- **Odyssey Infrared Imaging System** (LI-COR Biotechnology, Lincoln, NE)

Recipes

Recipe 1. Nuclei extraction buffer (NEB)

20 mM Hepes pH7.2
50 mM NaCl
3 mM $MgCl_2$
300 mM sucrose
0.5% IGEPAL CA630
⇨ *Prepare fresh every time.*

Recipe 2. Click reaction mix

PBS
25 µM biotin-azide
10 mM (+)-sodium L-ascorbate
2 mM $CuSO_4$
* *To prepare 10 ml, for one sample:*
1. *Prepare 50 mM stock biotin-azide. Aliquot and store -20°C.*
2. *Prepare 100 mM stock (+)-sodium L-ascorbate. (Prepare fresh)*
3. *Prepare 100 mM stock $CuSO_4$. (Prepare fresh)*
4. *Add 8.8 ml ice cold PBS into a 50ml conical tube.*
5. *Add 5 µl stock biotin-azide, or DMSO for negative control.*
6. *Add 1 ml (+)-sodium L-ascorbate.*
7. *Add 200 µl $CuSO_4$.*

⇨ *Prepare the reaction mix in the order described (STEPS 4 to 7).*

Recipe 3. Buffer B1

25 mM NaCl
2 mM EDTA
50 mM Tris-HCl pH 8.0
1% IGEPAL CA630
Prepare fresh before use. Add protease inhibitor fresh before use.

Recipe 4. Buffer B2

150 mM NaCl
2 mM EDTA
50 mM Tris-HCl pH 8.0
0.5% IGEPAL CA630
Prepare fresh before use. Add protease inhibitor fresh before use.

Recipe 5. DNA extraction solution

**To prepare 7 µl, for one sample:*
1. *Prepare 0.5 M EDTA and 1 M Tris-HCl (pH 6.7).*
2. *Mix 2 µl of 0.5 M EDTA and 4 µl of 1 M Tris-HCl (pH 6.7) and 1 µl Proteinase K.*
3. *Prepare enough to apply 7 µl of this solution to sample to be analyzed.*

Procedure

1. Cell culture preparation

1.1. Amplify cells needed for the experiment. Each sample requires ~6–8 × 10^7 cells. For the current protocol, SW13 cells were seeded at a density of 1.5 × 10^7 cells in 175 cm^2 tissue culture flasks, 4 flasks per sample. The number of cells might differ between cell lines. MCF-7 cells were seeded at the same density as SW13 cells.

1.2. Cells are trypsinized and counted and then seeded at 1.5 × 10^7 cells in a volume of 20 ml media into 175 cm^2 tissue flasks one day before EdU pulsing.

⇨ *To optimize EdU uptake, cells must not be over confluent, as observed under the microscope. For SW13 and MCF-7 cells, seeding at 1.5 × 10^7 cells translates to just under 100% confluency the next day. If chase steps are required, prepare 37°C and CO^2 equilibrated media by incubating enough media overnight in the incubator.*

2. EDU labeling of replication forks

2.1. Prepare 1 ml of 200 µM EdU in pre-warm media to be added directly to each flask of 20 ml to a final concentration of 10 µM for EdU pulsing. From the 10 µM stock, dilute 5 µl into 1 ml media to make 200 µM EdU (i.e. each sample would require 10 µl diluted into 4 ml media, 1 ml per flask).

**Re-using media that are already in the culture flasks will minimize disruption to cells and maximize EdU uptake.*

➪*This step needs to be performed as fast as possible to minimize effect of temperature and CO_2 changes on cell cycle progression.*

2.2. Incubate cells at 37°C in the incubator for desired length of time: 10 min for the current protocol.

2.3. If thymidine chase is required, proceed to **3. Thymidine chase**.

2.4. If thymidine chase is not required, proceed to **4. Nuclei harvesting**.

3. Thymidine chase

3.1. Stop EdU uptake by discarding EdU containing media from the flasks.

3.2. Rinse the monolayer once with 10 ml 37°C and CO_2 equilibrated media to remove residual EdU.

3.3. Add 20 ml of media containing 10 µM thymidine and incubate at 37°C in the incubator for an additional period of chase time.

➪*This step needs to be performed as fast as possible to minimize the effect of temperature and CO_2 changes on cell cycle progression.*

4. Harvesting nuclei

4.1. Arrest all pulse/chase by discarding EdU/thymidine containing media.

4.2. Immediately add 5 ml ice cold nuclei extraction buffer (NEB, see **Recipes** above) to each flask to detach monolayer and extract nuclei. Incubate 10–15 min at 4°C. The NEB will solubilize the cytoplasm, permeabilize the nuclei, and remove all soluble nuclear proteins.

**Depending on the confluency of the monolayer, this step might take a little less or more time. If nuclei remain bound to the flask/plate, slightly tap the side to get them off. Alternatively, you can use a scraper to make sure you collect all nuclei.*

4.3. Combine two flasks of sample into one 15 ml conical tube, hence, for one sample (four flasks), there will be two 15 ml conical tubes. 15 ml conical tubes are preferable to 50 ml conical tubes because a tighter pellet forms after centrifugation. Keep the tubes on ice at all times.

➪*It is important to maintain the sample at 4°C, so this step should be done in the cold room. Alternatively, if there is a sliding door refrigerator nearby, keep the flasks in the sliding door fridge and you should handle only two flasks at a time and maintain other samples at 4°C.*

4.4. Centrifuge for 10 min at >2500× *g* at 4°C.

4.5. Discard the supernatant; wash the samples in 5 ml of ice cold PBS, pour samples from two 15 ml conical tubes into one 15 ml conical tube.

➪*Avoid excessive pipetting or you will risk losing sample to the wall of pipettes.*

**Take a small aliquot, dilute with trypan-blue, and check under a microscope to make certain your prep contains purely nuclei.*

4.6. Centrifuge for 10 min at >2500× *g* at 4°C. During this time, start preparing click reaction mix (see Recipes).

4.7. Completely aspirate PBS and put samples on ice. Proceed to **5. Click reaction**.

➪*Carefully aspirate the PBS to prevent removing the pellet.*

5. Click reaction

5.1. Resuspend the pellet with ice-cold click reaction mix. Completely resuspend the pellet to ensure efficient click reaction.

5.2. Each 10 ml click reaction mix is sufficient for one sample of ~6 × 10^7 cells. The negative control click reaction mix will contain DMSO instead of biotin azide.

5.3. Rotate samples at 4°C for 1 hour. During this time, prepare enough Buffer B1 and B2 (see **Recipes**), add protease inhibitor, and store on ice to make sure the buffers are at 4°C for use.

Each sample requires 1.5 ml of B1 and 500 µl of B2 to dilute. Each sample requires 100 µl of streptavidin-coated bead slurry (see **7. Streptavidine capture), 100 µl of streptavidin-coated beads requires 200 µl of B2 to wash before use.*

5.4. Centrifuge for 10 min at >2500× *g* at 4°C.

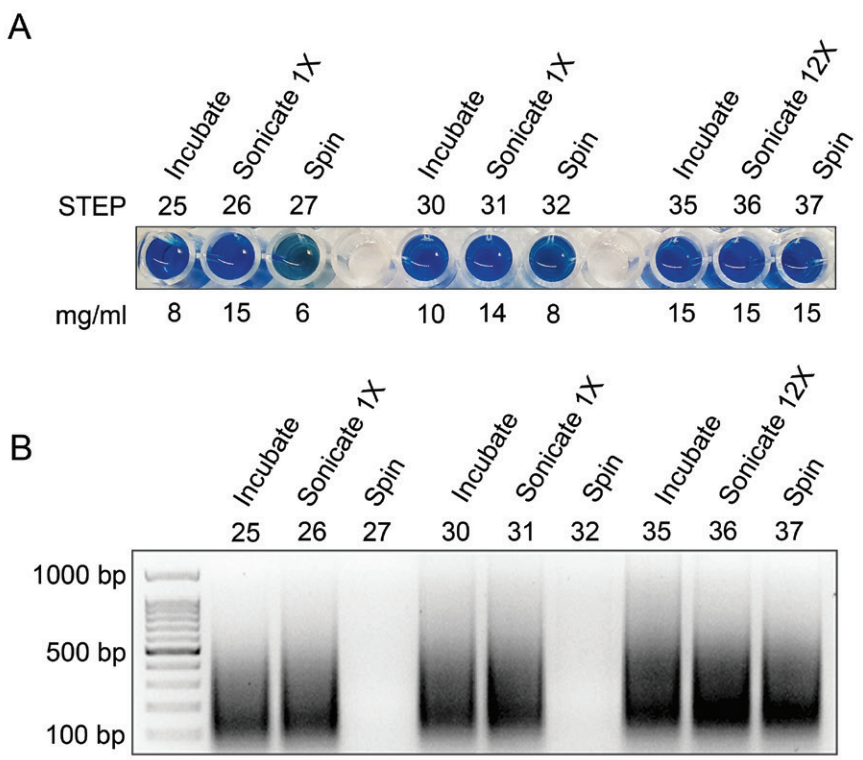
5.5. Discard click reaction mix and wash once with ice-cold PBS.

➪*Avoid excessive pipetting or you will risk losing sample to the wall of pipettes.*

5.6. Centrifuge for 10 min at >2500× *g* at 4°C. Note the pellet should be slightly blue because of the $CuSO_4$ in the click reaction mix.

5.7. Completely aspirate PBS and put samples on ice. Proceed to **6. Lysis and sonication**.

➪*Carefully aspirate the PBS to prevent removing the pellet.*



Supplementary Protocol Figure 1. Monitoring protein concentration and DNA fragment size during the Lysis and Sonication step. (A) Bradford assay of protein lysate at various stages was performed. 250 µl of 1:5 Bradford reagent was added to 9 wells of a 96 well plate. The numbers on top denote protocol steps at which 2 µl of sample was extracted and added to the well. Numbers on the bottom indicate protein concentration in mg/ml. Note how protein levels in the supernatant lower dramatically after the first and second spins (steps 6.4 and 6.9) because the chromatin is pelleted, but the third sonication solubilizes chromatin, so the supernatant contains ~all the protein (step 6.14). (B) 10 µl of sample was extracted at the steps indicated above and DNA was isolated to examine fragment size. DNA fragmentation was evident immediately after first incubation of sample with buffer B1, this is caused by $CuSO^4$ and the reducing agent Sodium Ascorbate. See: http://beng.soe.ucsc.edu/sites/default/files/project-reports/thesis_Final.pdf.

6. Lysis and sonication

⇨ *The first two incubation and brief (1×) sonication steps below (steps 6.1 -6.10) serve to remove soluble proteins and other non-chromatin proteins. The chromatin remains intact and is pelleted by centrifugation during these two incubation/sonication steps. However, after the third incubation and extensive (12×) sonication (steps 6.11 -6.14), we find that the chromatin is efficiently solubilized. This is evident from a dramatic reduction in pellet size once the prep is spun down.*

6.1. Add 500 µl of ice cold Buffer B1 with protease inhibitor to each sample. Completely resuspend by pipetting up and down and then transfer to a 1.5 ml Eppendorf tube.

6.2. Incubate for 15 min on ice.

**To visualize protein concentration changes that will occur in the subsequent steps:*

a) Set up a 96-well plate with 9 wells of 250 µl 1:5 Bradford reagent (Protocol Figure 1).

b) At the end of each step below indicated with ">", add 2 µl of the sample to one of the wells to visualize the protein concentration (Protocol Figure 1). At the end of lysis and sonication, you may take the plate to a spectrophotometer to quantify the protein concentration.

> Add 2 µl of lysate to well 1.

(optional hint) To analyze DNA fragmentation that will occur in the subsequent steps (adapted from Sirbu et al., 2013):

a) At the end of the step below indicated with ">", collect 10 µl of the sample and keep on ice until the end of Step 7.9

b) Add 90 µl of H_2O and 4 µl of 5 M NaCl to each sample.

c) Incubate the samples at 65°C overnight (16–20 hours).

d) Quick spin, add 1 µl of RNase A (20 mg/ml) to each sample.

e) Incubate samples in a 37°C water bath for 30 min.

*f) Prepare the DNA extraction solution (see **Recipes**). Note: if samples were taken at all ">" indicated, there will be a total of 9 samples, therefore prepare 9 × 7 µl of solution.*

g) Add 7 µl of solution to each sample. Incubate at 45°C for 2 hours.

h) During incubation, prepare a 1.5% (wt/vol) agarose/TAE gel with ethidium bromide.

i) Perform electrophoresis in 1× TAE buffer to resolve DNA fragments.

j) Visualize DNA fragments under UV light.

6.3. Sonicate 1 × 10 seconds on ice. Adjust sonicator power to between setting 3 to 4, which should give an output power of around 10 watts. (see **Equipment**)
> Add 2 µl of sonicated mixture to well 2.

6.4. Centrifuge max speed for 10 min at 4°C.
> Add 2 µl of supernatant to well 3.

6.5. Discard the supernatant; take note of the pellet size by drawing a line on the tube with a marker.

**You will observe that there is less protein in well 3 compared to well 1 and 2 (see Protocol Figure 1A). This is because chromatin bound proteins are trapped in the pellet. Also, if DNA fragmentation analysis is performed, you will observe that no chromatin is released to the supernatant after this first round of lysis-1× sonication-spin (see Protocol Figure 1B, lane 3). Lane 1 and 2 represent total fragments in the sample.*

6.6. Add 500 µl of ice cold buffer B1 with protease inhibitor to each pellet. Completely resuspend by pipetting up and down.

6.7. Incubate for 15 min on ice.
> Add 2 µl of lysate to well 4.

6.8. Sonicate 1 × 10 seconds on ice with same sonication settings.
> Add 2 µl of sonicated mixture to well 5.

6.9. Centrifuge max speed for 10 min at 4°C.
> Add 2 µl of supernatant to well #6.

6.10. Discard the supernatant; take note of the pellet size. The pellet size should be comparable to that you observed at step 6.5.

** You will observe that there is again less protein in well 6 compared to wells 4 and 5, although there is more compared to well 3 (see Protocol Figure 1). This is because chromatin bound proteins are still trapped in the intact nuclei in the pellet. Also, if DNA fragmentation analysis is performed, you will again observe that no chromatin is released to the supernatant after this second round of lysis-1× sonication-spin (see Protocol Figure 1B, lane 6). Lane 4 and 5 represent total fragments in the sample.*

6.11. Add 500 µl of ice cold buffer B1 with protease inhibitor to each tube. Completely resuspend by pipetting up and down.

6.12. Incubate for 15 min on ice.
> Add 2 µl of lysate to well 7.

6.13. To solubilize chromatin, sonicate 12 (twelve) × 10 seconds on ice with 10 seconds in between on ice with the same sonication settings as above. Leave all tubes on ice until sonication is completed.
> Add 2 µl of sonicated mixture to well 8.

6.14. Centrifuge max speed for 10 min at 4°C. Note the pellet size, it should be substantially smaller than than in STEP 6.5 and STEP 6.10 because the extensive sonication solubilizes the nuclei
> Add 2 µl of supernatant to well 9.

**You will observe that this time the protein concentration between wells 7, 8, and 9 are comparable (see Protocol Figure 1). This is because the first two incubation and brief (1×) sonication steps only served to release soluble and non-chromatin proteins. The third incubation followed by vigorous (12×) sonication solubilizes the chromatin efficiently. Also, if DNA fragmentation analysis is performed, you will now observe that chromatin has been efficiently released to the supernatant after this last round of lysis-12× sonication-spin (see Protocol Figure 1B, lane 9). Lanes 7 and 8 represent total fragments in the sample. You will also observe that the DNA fragment is around 150 bp in all samples. This is due to $CuSO_4$ mediated fragmentation of DNA in the presence of a reducing agent (sodium ascorbate). This is in agreement with previous observations by Sirbu et al. (2013) and in published materials (see figure legend).*

6.15. Keep the supernatant and transfer to another 1.5 ml eppendorf tube.

⇨ *If the supernatant appears cloudy, centrifuge again at max speed for 10 min at 4°C to remove insoluble material.*

6.16. Store supernatant on ice, add 500 µl of ice cold buffer B2 with protease inhibitor to bring NaCl concentration back up to physiological level.

6.17. Total sample size should be ~1ml. Take out 20 µl of sample and store at -20°C, label this tube as INPUT.

6.18. Proceed to **7. Streptavidine Capture**.

7. Streptavidine Capture

7.1. Prepare streptavidin-coated beads for overnight streptavidin-biotin capture. Each sample requires 100 µl of streptavidin-coated beads. The constitution of streptavidin-coated beads is 50% bead particles and 50% storage buffer. Take out enough streptavidin-coated beads into an eppendorf tube.

7.2. Centrifuge for 2 min at 500× *g* at 4°C

7.3. Let the streptavidin-coated beads settle down on ice for a minute. Use a micro-pipette and carefully remove the supernatant.

7.4. Wash streptavidin-coated beads with ice cold buffer B2 with protease inhibitor at the same

volume as the bead particle. For instance, if one sample requires 100 µl streptavidin-coated beads, 50 µl will be storage buffer, therefore wash with 50 µl of B2.

7.5. Centrifuge for 2 min at 500× g at 4°C.

7.6. Repeat STEPS 7.3 to 7.5 twice. Hence a total of three washes after removal of storage buffer.

7.7. Completely remove B2, add same volume of B2 as the volume of beads. Mix well.

**To facilitate mixing without trapping beads in pipette tip, cut the end of the pipette tip to enlarge the opening.*

7.8. Add 100 µl of washed streptavidin-coated beads, now resuspended in B2, to each sample. Use a P200 with pipette tip that is cut at the end to ensure correct delivery of 100 µl.

7.9. Rotate samples overnight (16–20 hours) at 4°C. Store B2 at 4°C for future wash steps. Each sample requires 4 ml B2 with protease inhibitor to wash.

7.10. Centrifuge for 2 min at 500× g at 4°C.

7.11. Let the streptavidin-coated beads settle down on ice for a minute. Use a micro-pipette and carefully remove the supernatant.

7.12. Wash streptavidin-coated beads with 1 ml ice cold B2 with protease inhibitor.

7.13. Centrifuge for 2 min at 500× g at 4°C.

7.14. Let the streptavidin-coated beads settle down on ice for a minute. Use a micro-pipette and carefully remove the supernatant.

7.15. Repeat STEPS 7.12 to 7.14 three times

Leave samples on ice, proceed to 8. **Protein elution**.

8. Protein elution for Western blotting

8.1. Prepare 2× Laemmli sample buffer by adding 50 µl 2-Mercaptoethanol (2-ME) to every 950 µl Laemmli sample buffer.

8.2. Add 50 µl of Laemmli sample buffer with 2-ME to each sample (50 µl of bead particles). Carefully pipette up and down with a P200 micropipette tip to ensure the sample buffer is mixed well with the beads.

8.3. Take out the INPUT stored at -20°C, add 20 µl of 2× Laemmli sample buffer with 2-ME to each INPUT.

8.4. Boil all tubes at 100°C for 15 min.

⇨ *Use safety caps to ensure the eppendorf tubes do not pop open during boiling.*

8.5. Leave tubes on ice for a short period of time to let vapor condense. Do a quick spin to bring down samples. Return all tubes on ice.

8.6. Use a gel-loading tip to completely collect all Laemmli sample buffer from tubes containing beads, which now contains CAPTURE.

8.7. Leave all samples on ice and proceed to **9. Western blotting**.

You can store all INPUT and CAPTURE samples at -20°C and proceed to western blotting the next day.

9. Western blotting

9.1. The entire 50 µl of CAPTURE is equivalent to 50% of all cells in one sample (3×10^7 cells). The entire 40 µl of INPUT (after 1:2 dilution in sample buffer) is equivalent to 2% of cells (1.2×10^6 cells). CE: cell equivalent.

9.2. Load 25 µl (25% CE) CAPTURE and 5 µl of INPUT (0.25% CE), including CAPTURE and INPUT for DMSO negative control.

9.3. Perform standard Western blotting.

9.4. Detect proteins of interest with antibodies of your choice. Positive controls for aniPOND should include PCNA and Histone H3 (see Antibodies above).

Troubleshooting

Positive control protein (i.e. PCNA or H3) not detected.

Cells exited cell-cycle, over-confluent cells – Make sure cells were seeded the day before and just confluent the next day for the experiment.

Proper cell-cycling was interrupted – Make sure STEP 2.1 was performed as quickly as possible to minimize disruption to cells.

Inefficient chromatin extraction – increase sonication times during STEP 6.13 to promote solubilization of chromatin.

Protein detected in the DMSO negative control lane.

Contaminants in the DMSO tube – Make sure the supernatant recovered at STEP 6.15 is clear, if not, repeat the centrifugation step to remove contaminants.

Supplementary Material For:

A rapid and efficient method to purify proteins at replication forks under native conditions

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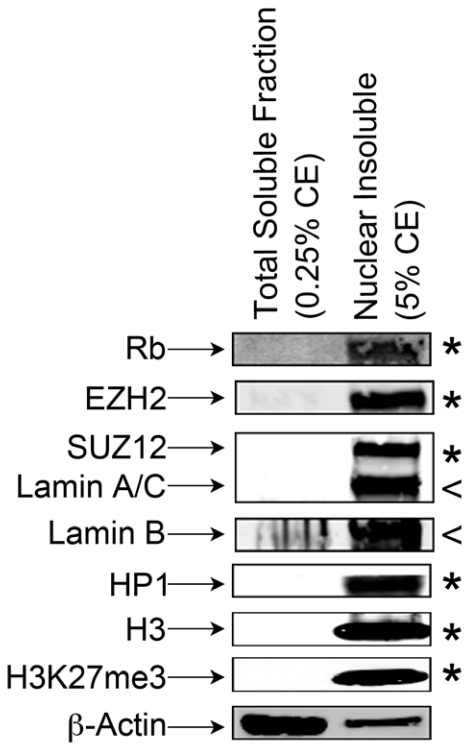
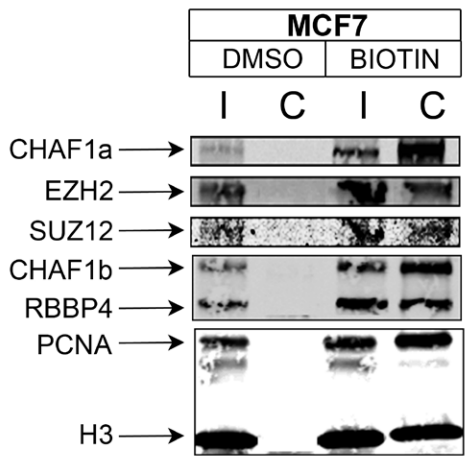
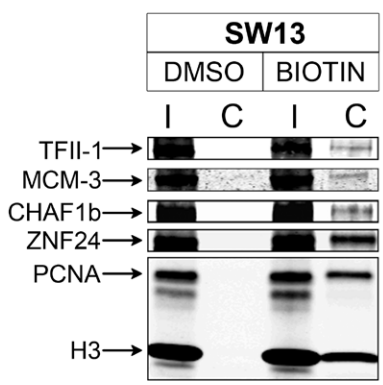
Keywords: native; iPOND; DNA replication; chromatin; EdU; click chemistry; mass spectroscopy



Supplementary Figure S1. Retention of insoluble nuclear protein after cell permeablization. NEB was applied to a monolayer of $1.5x10^7$ SW13 cells in a 175 cm^2 flask. After a 15 min incubation at 4°C and gentle tapping on the side of the flask, detached nuclei were spun, washed in PBS, lysed by boiling in sample buffer, and analyzed by Western blotting together with a portion of the soluble fraction. Chromatin of nuclear membrane bound proteins in the nuclear faction is indicated with * of <, respectively. β-actin was used as a control to shown cytoplasmic protein in the soluble fraction. CE: cell equivalents.



Supplementary Figure S2. aniPOND analysis in MCF-7 cells. MCF-7 cells were used instead of SW13 cells to perform aniPOND. DMSO: no click reaction; Biotin: click reaction performed; I: Input; C: Capture.



Supplementary Figure S3. Capture of TFII-I, MCM-3, and ZNF24 using aniPOND. aniPOND was performed as in Figure 1A. DMSO: no click reaction; BIOTIN: click reaction performed; I: input; C: capture.