

Chapter 3

Isolation of Proteins on Nascent DNA in Hypoxia and Reoxygenation Conditions

Monica M. Olcina, Amato J. Giaccia, and Ester M. Hammond

Abstract During S-phase both DNA replication and histone deposition must be co-ordinated at and around the replication fork. Replication stress can interfere with the fidelity of this process and can result in genomic instability. The study of proteins associated with DNA replication forks is important for a detailed understanding of DNA replication and chromatin assembly both under basal as well as replication stress conditions. iPOND (isolation of Proteins on Nascent DNA) allows the temporal study of proteins and protein modifications associated with replication forks in a variety of conditions, allowing the ‘tracing’ of protein association and histone deposition and maturation at active, stalled and damaged replication forks. Importantly, low oxygen (hypoxic) conditions, found in tumours, can result in replication stress. Here we describe the adaptation of the iPOND technique allowing the isolation of proteins and protein modifications specifically with replication forks undergoing hypoxia-induced replication stress. Furthermore, we describe the adaptation of this method for the study of factors associated with replication forks recovering from hypoxia-induced replication stress following periods of reoxygenation. These adaptations are important in order to study proteins associated with replication forks undergoing replication stress in physiologically relevant conditions.

Keywords DNA replication • Replication stress • iPOND • Hypoxia • Reoxygenation • Chromatin • Click chemistry • 5-Ethynyl-2'-deoxyuridine (EdU)

M.M. Olcina (✉)

CR-UK & MRC Oxford Institute for Radiation Oncology, Department of Oncology, University of Oxford, Oxford, UK

Department of Radiation Oncology, School of Medicine, Stanford University, Stanford, CA 94305, USA

e-mail: molcina@stanford.edu

A.J. Giaccia

Department of Radiation Oncology, School of Medicine, Stanford University, Stanford, CA 94305, USA

E.M. Hammond

CR-UK & MRC Oxford Institute for Radiation Oncology, Department of Oncology, University of Oxford, Oxford, UK

3.1 Introduction

During DNA replication both DNA and the histones around which DNA is wrapped must be duplicated. To this end, the replisome is composed of factors typically associated with the replication machinery (such as helicases and DNA polymerases) as well as a variety of ‘chromatin-associated factors’. The study of proteins associated with the replisome is important for a detailed understanding of a process so central to cell division [1].

Furthermore, replication is tightly controlled in order to prevent genomic instability. Fidelity of the process, however, can be compromised when replication occurs under conditions of stress, such as when nucleotide pools are depleted or when the replication machinery encounters an obstacle (such as the transcriptional machinery) [1]. Studying which proteins are associated with forks under conditions of replication stress is very relevant for the cancer field since cancer cells are frequently exposed to continuous replication stress. For instance, nucleotide depletion resulting from aberrant oncogene activation or low oxygen (hypoxic) conditions often encountered in tumours can result in replication stress [2–4]. Interestingly, the replication stress associated with severe hypoxia (<0.1 % O₂) has recently been found to be distinct from kinetically (and mechanistically) similar types of replication stress (such as replication stress induced by agents also leading to nucleotide pool depletion such as hydroxyurea) [2, 5, 6].

The iPOND technique (isolation of Proteins on Nascent DNA), originally described in 2011, has been central to the characterisation of the replisome and replisome-associated proteins, facilitating the detailed study of replication fork dynamics [7–9]. iPOND allows the spatial and temporal study of proteins and protein modifications associated with replication forks [7]. Importantly this technique enables such studies to be carried out in a variety of conditions, allowing the ‘tracing’ of protein association and histone deposition and maturation at active, stalled and damaged replication forks [10]. As a result of these advances, our understanding of the crosstalk between DNA replication, repair, histone deposition and epigenetic memory during DNA replication has been greatly enhanced [7–9].

In iPOND, nascent DNA is labelled with a nucleoside analogue of thymidine called 5-ethynyl-2'-deoxyuridine (EdU). EdU contains an alkyne group that can be covalently linked to biotin-azide by click chemistry (copper-catalysed cycloaddition). This allows single-step purification of the EdU-labelled DNA-protein complexes with streptavidin-coated beads. Following an elution step, proteins and their modifications can be analysed by immunoblotting or mass spectrometry [8].

Here we describe an adaptation of the iPOND technique that allows the isolation of proteins and protein modifications specifically with replication forks undergoing hypoxia-induced replication stress. Furthermore, we describe the adaptation of this method for the study of factors associated with replication forks recovering from hypoxia-induced replication stress following periods of reoxygenation. It is important to note that once cells are exposed to reoxygenation following acute periods (<8 h) of severe hypoxia (<0.1 % O₂) stalled replication forks may recover but will

do so in a context of increased DNA damage caused by increased reactive oxygen species [2]. When normoxia, hypoxia and reoxygenation conditions are performed alongside, this protocol can provide valuable information regarding the differences in proteins (or complexes) associated with replication forks under these three different conditions (Figs. 3.1 and 3.2).

As part of the iPOND protocol, chase experiments, where EdU labelling is combined with increasing culture times without label (referred to as the chase period), can be carried out (Figs. 3.1 and 3.2). These experiments can provide a useful control when trying to validate whether a protein is strictly associated with active replication forks or whether it is simply carried alongside other chromatin components. Proteins truly associated with the replisome will not be detected in the chase sample. Furthermore, thymidine chase experiments can be used for studying the association of factors in relation to distance from the replication fork. This might be particularly relevant when studying processes such as histone deposition and maturation at elongating forks [1, 7].

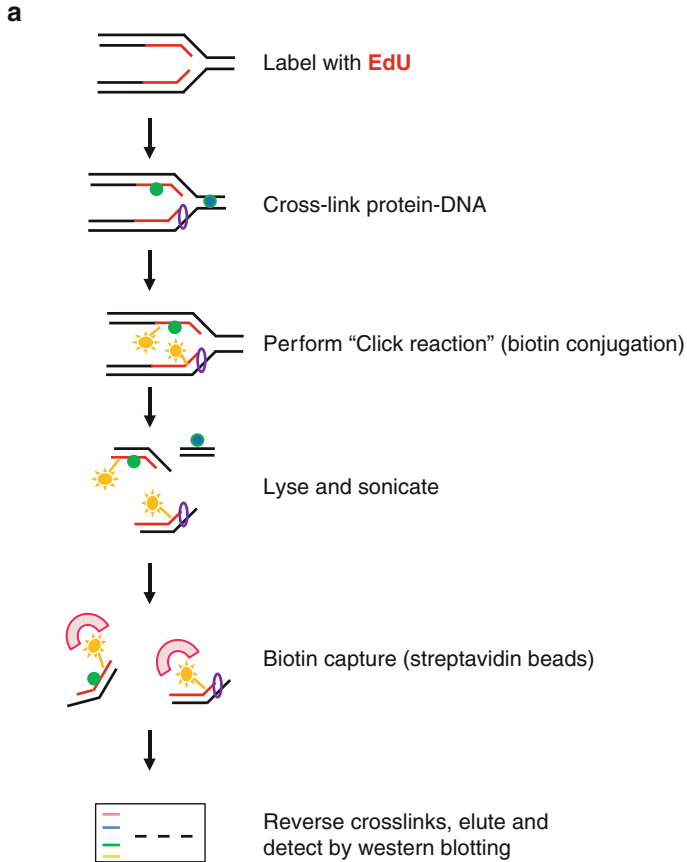
3.2 Reagents

- EdU (5-ethynyl-2'-deoxyuridine) (Invitrogen, cat. No. E10187)
- Thymidine (Sigma, cat. no. T1895)
- 37 % (w/v) formaldehyde solution (Sigma, cat no. 1635)
- Phosphate Buffered Saline (PBS)
- Glycine (Fisher, cat. no. BP 381)
- Triton X-100 (Sigma, cat. No. T8787)
- Bovine serum albumin (BSA) (Sigma, cat. No. A7030)
- Dimethyl sulphoxide (DMSO)
- Copper (II) sulphate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) (Fisher, cat. No. C489)
- (+) Sodium L-ascorbate (Sigma, cat. No. B10184)
- Sodium Dodecyl Sulphate (SDS) (Sigma, cat. No. L4390)
- Sodium Chloride (NaCl)
- Tris, pH 8.0 and pH 6.7
- Dithioerythritol (DTT) (Sigma, cat no. D-8255)

3.2.1 Reagent Setup

EdU: Dissolve EdU in DMSO for a final concentration of 10 mM. Aliquot and store at $-20\text{ }^\circ\text{C}$ protected from light. Stable for up to 1 year. Before use thaw at $37\text{ }^\circ\text{C}$. MW: 252.23.

Thymidine: Dissolve in water at a final concentration of 10 mM. Aliquot and store at $-20\text{ }^\circ\text{C}$. Stable for up to 1 year. Use at a final concentration of 10 μM .



Conditions:

Normoxia: 21% O₂, **Hypoxia:** 6 h <0.1% O₂, **Reoxygenation:** 6 h <0.1% O₂ + 2 h 21% O₂

b

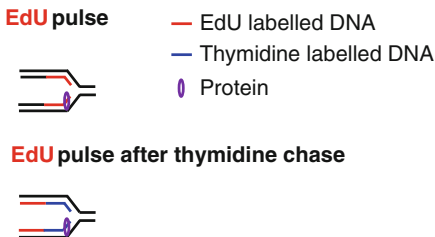


Fig. 3.1 Schematic representation of an overview of the iPOND experiment. **(a)** Schematic representation of the iPOND protocol performed under three different conditions: normoxia, hypoxia and reoxygenation. Adapted from [5, 8]. **(b)** Schematic representation of the thymidine chase experiment used to distinguish proteins specifically associated with the replication fork from non-specific chromatin binding factors. Thymidine chase displaces nascent EdU-labelled DNA away from the replication fork. Adapted from [5, 8]

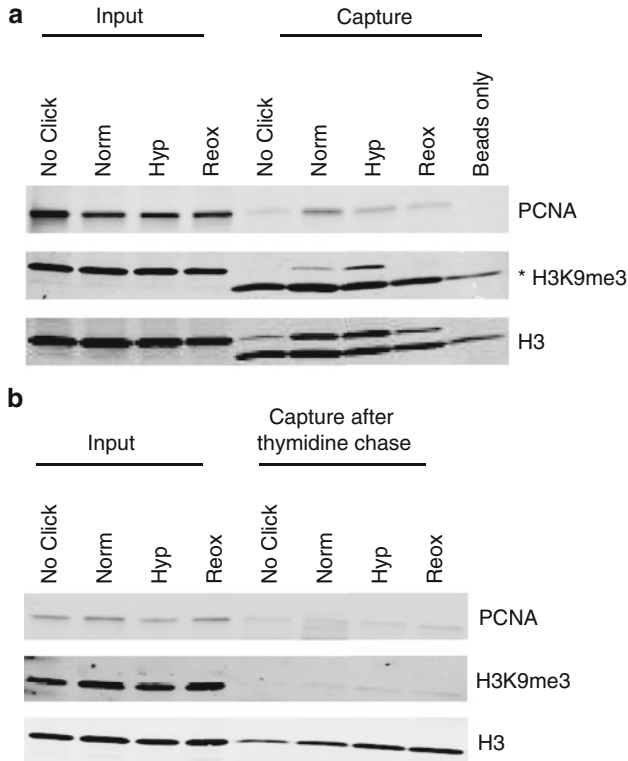


Fig. 3.2 Example of results that can be obtained when the iPOND protocol is used following exposure of cells to normoxia, hypoxia and reoxygenation. **(a)** iPOND was carried out following exposure of RKO cells to either Norm (21 % O₂), Hyp (6 h, <0.1 % O₂) or Reox (6 h, <0.1 % O₂ followed by 2 h of 21 % O₂). EdU labelling times: Norm (1 h), Hyp (6 h), Reox (2 h, during reoxygenation period). Asterisk denotes a non-specific band observed in all lanes at the end of the gel [5]. **(b)** Example of a thymidine chase experiment. iPOND was carried out following exposure of RKO cells to either Norm (21 % O₂), Hyp (6 h, <0.1 % O₂) or Reox (6 h, <0.1 % O₂ followed by 2 h of 21 % O₂). EdU labelling times: Norm (0.5 h), Hyp (3 h), Reox (1.5 h, during reoxygenation period). The chase into thymidine containing medium was performed for the last 30 min of each condition in Norm and Reox or for the last 3 h in Hyp. No click control represents a sample where the click reaction was carried out without biotin-azide [13]

1 % Formaldehyde/PBS: Dilute 37 % formaldehyde 1:37 with PBS (freshly prepared for each experiment). Keep at room temperature (RT) until cell fixation.

1.25 M Glycine: Prepare 1.25 M glycine stock solution. Store at RT. Use at 1:10 dilution for a final concentration of 0.125 M glycine.

Permeabilisation buffer: Make stock 20 % Triton X-100 and keep at RT. Dilute to 0.25 % Triton X-100 in PBS and store at 4 °C.

0.5 % BSA in PBS wash buffer. Prepare 0.5 % BSA in PBS. Filter and store at 4 °C.

Table 3.1 Click reaction cocktail components

Reagent	[Stock]	[Final]	Control reaction volume	Experimental reaction volume
1× PBS	–	–	3.9 ml	3.9 ml
DMSO	–	–	50 µl	–
Biotin-azide	1 mM	100 µM	–	50 µl
Sodium ascorbate	100 mM	100 mM	500 µl	500 µl
CuSO ₄	100 mM	2 µM	100 µl	100 µl
Total volume (includes approximately 0.4 ml of cell pellet volume)			5 ml	5 ml

Click reaction stock solutions: Dissolve biotin-azide in DMSO to a final concentration of 1 mM. Aliquot and store at -20°C . Prepare a stock 100 mM CuSO₄ solution in H₂O and store at RT. Prepare 20 mg/ml of sodium L-ascorbate (reducing agent) in H₂O and store on ice until needed. Prepare freshly and limit exposure to air (see also Table 3.1).

Lysis buffer: Prepare 1 % SDS in 50 mM Tris, pH 8.0. Store at RT. Prior to use add protease inhibitors.

Salt wash: Prepare 5 M NaCl and store at RT. Dilute to 1 M NaCl prior to use.

2× SDS Laemmli sample buffer (2× SB): Mix 0.4 g SDS, 2 ml 100 % Glycerol, 1.25 ml 1 M Tris 6.8 and 0.01 g Bromophenol blue in 8 ml H₂O. Prior to use add 1 M DTT to a final concentration of 0.2 M.

Note: For a full list of reagents and full instructions on crosslinking reversal and DNA fragment size analysis see full protocol [8].

Hypoxic chamber: For experiments where replication stress is desired a chamber that can be set $<0.1\%$ O₂ (or at least $<0.5\%$ O₂) is required.

Reoxygenation samples refer to conditions where cells have been placed in a hypoxic chamber for several hours before being returned to a standard tissue culture incubator (21 % O₂). See schematic for further details (Fig. 3.1).

3.3 Method

• Cell culture preparation—Timing 1–10 days

1. Each sample requires at least 1×10^8 cells at the time of EdU pulse. The required number of cells may vary depending on the application and cell type. For iPOND in hypoxia, RKO colorectal cancer cells have been used successfully. Other cell lines (including HEK293T, HCT116, U2OS and mouse embryonic fibroblasts and have been used in the original iPOND protocol) [1, 8].

NOTE: Theoretically any cell that can incorporate EdU during DNA replication can be used in this protocol.

2. Use typically 8–10 × 150 mm dishes per condition for RKO cells: Plate 6–7 × 10⁶ cells per dish 1 day before EdU pulse. Ideally plate 1 day before EdU incubation to ensure cells are growing optimally. Always remember that you need a no click control in addition to your experimental samples

CRITICAL STEP: Need to include one extra dish for counting the cell number in step 3.

Cells must be in log phase of growth and cannot be overgrown. It is useful to monitor appropriate incubator temperature and CO₂ content; otherwise EdU incorporation will not be maximal.

(If performing chases, media may need to be equilibrated to 37 °C and appropriate CO₂ content overnight.)

Ideally cells should be plated in glass dishes for experiments performed <0.1 % O₂. If this is not feasible the incubation times under hypoxic conditions may need to be prolonged to account for delayed replication stress in the presence of residual oxygen present in plastic dishes.

3. On the day of EdU incorporation: determine the cell number in the extra dish of cells. The number will be used to calculate the amount of the reagents for each sample in step 29.
4. Plan out times to pulse (chase), fix, quench, collect and wash samples.

CRITICAL STEP: Stagger samples ensuring that each sample is treated equally throughout all steps.

5. EdU incorporation: Add 23 µl of the 10 mM EdU stock into 23 ml of cell culture media in each dish to achieve a final EdU concentration of 10 µM. (Or alternatively make aliquots of 23 ml of media with 23 µl EdU mix in falcons, replace media and add these 23 ml of media to each dish. This is sometimes more convenient for the reoxygenation samples or if you are doing chase experiments).

If not performing chase experiments proceed to formaldehyde crosslink once EdU incorporation time has ended.

For normoxic samples: Return to the incubator for the desired pulse time (typically 1 h, this may need to be optimised to ensure equal EdU incorporation between different conditions).

For hypoxic samples: Add EdU and immediately place cells into the hypoxic chamber (typically for 6 h).

For reoxygenation samples: Place dishes in the hypoxic chamber and add EdU at the time of reoxygenation (typically as soon as they come out of the chamber and for 2 h).

6. **To perform thymidine chase or addition of drug:** Remove plates from incubator and decant media.
7. Carefully wash cells with 5 ml of chase media (pre-equilibrated).
8. Add 20 ml of chase media containing 10 µM thymidine or desired concentration of desired drug. Return to the incubator for the desired length of time. It is important that these steps are performed as fast as possible to prevent pH and temperature changes in the media that can affect replication rates:

For this experiment typically, **Normoxia**= 30 min thymidine chase, **Hypoxia**= 3 h EdU3 h thymidine chase, **Reoxygenation**= 30 min chase at the end of the reoxygenation timecourse.

NOTE: To allow the experiment to be feasible a decreased number of dishes (e.g. 4 × 150 mm dishes) may need to be used for thymidine chase experiment controls in order to allow the experimental samples and control samples to all fit into the chamber at once. This may limit the strength of the signal achieved at the end of the experiment.

- **Formaldehyde crosslinking and collection of cells—Timing 1 h/time point**

9. Decant media after EdU pulse and or chase.
10. Immediately fix cells on the dish by adding 10 ml of 1 % Formaldehyde in PBS (made fresh) and incubate for 20 min at RT. Rock gently at the start and then leave them to incubate.
11. Quench crosslinking by adding 1 ml of 1.25 M glycine.
12. Remove glycine and add 5 ml of PBS + Protease inhibitor cocktail (Pi) per plate.
13. Rock/wash plate for 5 min.
14. Collect samples by scrapping and transfer to a 50 ml falcon tube. Note the volume. The same volume will be used for the PBS washes.
15. Centrifuge for 5 min at $900 \times g$ 4 °C.
16. Decant supernatant.
17. Wash pellets 1× with PBS and centrifuge for 5 min at $900 \times g$ 4 °C. Vortex to resuspend pellets in PBS.
18. After last wash decant PBS.

PAUSE POINT—Samples can be flash frozen and stored at –80 °C for several weeks.

- **Cell permeabilisation—Timing 1 h**

- *TIP: For permeabilisation, click reaction and lysis, prepare the necessary buffers before/during permeabilisation (see Table 3.1 for full click reaction cocktail components):*

- *Permeabilisation buffer: 125 µl of Triton X-100 in 50 ml PBS*
- *0.5 % BSA in PBS*
- *PBS + Pi*
- *Copper sulphate: 0.025 g in 1 ml*
- *(+) Sodium 1 -ascorbate: 0.06 g in 3 ml (make this as close to the end as possible)*
- *Thaw rest of the reagents for click reaction.*

19. Resuspend cells in permeabilisation buffer at a concentration of 1×10^7 cells/ml
20. Incubate cells at RT for 30 min.
21. Spin down for 5 min at $900 \times g$, 4 °C.
22. Carefully decant supernatant.

23. Wash cells 1× with cold 0.5 % BSA in PBS.

CRITICAL STEP: BSA prevents cell pellet from detaching from wall of 50 ml falcon tube.

24. Centrifuge 5 min at $900 \times g$, 4 °C and decant supernatant.

25. Wash cells 1× with PBS.

26. Spin down 5 min at $900 \times g$ 4 °C.

27. Decant supernatant and place pellets on ice while finishing click reaction cocktail preparation.

• **Click reaction—Timing 2 h**

28. Thaw an aliquot of stock biotin-azide by placing on 37 °C heat block or incubator

29. To calculate click reaction volumes follow Table 3.1 (actual volumes should be adjusted based on the cell number measured per sample (step 3)):

NOTE: Two click reaction cocktails should be prepared: one for the control (no click) and one for the experimental samples (this one should contain biotin-azide).

30. Combine click reaction cocktail ingredients on ice (in a falcon before adding to the pellets) in the order listed in Table 3.1.

31. Resuspend cell pellets in click reaction cocktail by vortexing and transfer to 15 ml falcon.

32. Rotate reaction at RT for 1–2 h protected from light.

33. Centrifuge samples 5 min at $900 \times g$, 4 °C and decant supernatant.

34. Wash cells 1× with cold 0.5 % BSA in PBS using the same volume as used in click reaction for one sample.

35. Centrifuge samples 5 min at $900 \times g$, 4 °C and decant supernatant.

36. Wash cells 1× with PBS using the same volume as used in click reaction for one sample.

37. Decant PBS and invert tubes on paper towel to facilitate removal of all PBS.

PAUSE POINT—The samples can be flash frozen and stored at –80 °C for a few days.

• **Cell lysis and sonication—Timing 1 h**

38. Add Pi to lysis buffer before use and place on ice. (Make extra since this buffer will also be required to wash beads at capture stage.)

39. Resuspend samples at a concentration of 1.5×10^7 cells per 100 µl of lysis buffer and transfer to 1.5 ml Eppendorfs on ice. If a sonication control is necessary, take 5 µl of each sample before sonication.

40. Sonicate cells using a microtip sonicator:

– Pulse 15 s constant pulse, 40 s pause at 13–16 W (or standard settings (10–20 %)).

– Repeat pulse 1× for every 200 µl of cell lysate.

– Total pulse 4–5 min per sample.

- **CRITICAL STEP:** Lysate should appear clear/opaque after sonication and not cloudy. Cloudiness is an indicator of improper ratio of SDS to protein in lysate or insufficient sonication (see Table 3.2 for troubleshooting).
 - Prevent overheating during sonication by placing samples on ice.
41. Centrifuge samples for 10 min at 16,100 × g, RT.
 - CRITICAL STEP:** Lysates should appear clear after sonication. The presence of white precipitate on top of the lysate indicates insufficient lysate clearing (see Table 3.2 for troubleshooting).
 42. Note the lysate volume (LV).
 43. Take 5 µl of sonication control at this step if DNA fragment size analysis is desired.
 44. Dilute lysate 1:1 (v/v) with cold PBS containing Pi.
 - CRITICAL STEP:** Samples have been diluted to contain 0.5 % SDS, 25 mM Tris because biotin capture appears to be less efficient in lysates containing 1 % SDS.
 45. Note the final capture volume (FCV).
 46. Remove 15 µl of lysate as the sample input and place on ice. Add 2× SB in 1:1 (v/v) and freeze at –80 °C.

Table 3.2 Potential problems, reasons and solutions are highlighted to facilitate troubleshooting

Problem	Possible reason	Solution
Limited cell recovery	Insufficient pelleting during centrifugation	Increase time and speed of centrifugation. Add BSA to wash solution
Cloudy cell lysate following sonication	SDS-protein complexes have precipitated from solution or samples were not sonicated sufficiently (resulting in incomplete lysis)	Increase sonication times ensuring minimal sample foaming. Appropriate volumes of lysis buffer must be used
White precipitate after biotin capture centrifugation	Following sonication, cell membrane lipids failed to be properly pelleted	Lysate should be clear following sonication and centrifugation. If white precipitate is observed, centrifuge lysate again to ensure clearing
Control (no click) sample has high background	Non-specific binding to streptavidin beads	Include pre-clearing step. Increase the number of washes
Low control proteins signal	Limited EdU incorporation	Increase cell numbers to ensure optimal growth conditions. May need to increase EdU labelling times or duration of hypoxic treatment depending on oxygen concentrations used
Detection of protein of interest is low in input samples	Formaldehyde crosslinking may hinder epitope detection or antibody used is of poor quality	Optimise immunoblotting and potentially consider increasing boiling time for full formaldehyde crosslinking reversal

47. Pre-clear samples with protein A agarose beads to remove non-specific background for 1 h (wash beads as described below first). After pre-clearing remove 15 μl as pre-clearing input samples. This step is not essential but it may help when high background is detected in the no click control (see Table 3.2 for troubleshooting).

• **Biotin capture of biotin-tagged nascent DNA and associated proteins—Timing 16–20 h**

48. Incubate each sample with streptavidin-agarose beads at a concentration of 100 μl bead slurry (50 μl packed volume) per 1×10^8 cells.
49. Wash beads for all samples together by centrifuging bead slurry at $1800 \times g$ for 1 min, RT.
50. Carefully aspirate storage buffer off beads.
51. Wash beads $2 \times$ with 1:1 (v/v) of lysis buffer containing Pi.
52. Wash beads $1 \times$ with 1:1 (v/v) of PBS containing Pi.
53. Resuspend beads $1 \times$ with 1:1 (v/v) of PBS containing Pi.
54. Add equal volume of beads to each sample using pipet tip that is cut at the end.
55. Rotate biotin captures in cold room for 16–20 h.

• **Elution of proteins bound to nascent DNA—Timing 1–4 h**

56. Take down captures after 16–20 h of capture.
57. Centrifuge the streptavidin-agarose beads with captured DNA and associated proteins for 3 min at $1800 \times g$ RT.
58. Carefully aspirate most of the supernatant
CRITICAL STEP: Supernatant should be light blue/clear with no precipitate (see Table 3.2 for troubleshooting).
59. Wash beads with 1 ml of cold lysis buffer (no additives needed).
60. Rotate at RT for 5 min.
61. Centrifuge for 1 min at $1800 \times g$ RT.
62. Wash $1 \times$ with 1 ml of NaCl.
63. Rotate and pellet beads as in steps 60–61.
64. Repeat Lysis Buffer washes (steps 59–61) $\times 2$.
65. After last wash, aspirate all of the supernatant. Protein-DNA complexes isolated on beads are the Capture sample.
66. To elute proteins bound to nascent DNA, add $2 \times$ SB to packed beads 1:1 (v/v of packed beads. E.g. 100 μl SB: 100 μl packed beads—typically use 50 μl packed beads/ 1×10^8 cells).
67. Incubate capture and input samples for 25 min at 95°C .
68. Centrifuge for 1 min at $1800 \times g$ RT. The supernatant is ready to use in standard SDS-PAGE and immunoblotting procedures.

• **Analysis of proteins using western blotting**

69. Prepare standard SDS-PAGE gels in order to analyse both the proteins of interest and positive control (typically a known replication protein and a histone). Generally each sample from (1×10^8 cells) can provide enough

samples to be analysed in two to three immunoblots. It is important to note that depending on the proteins to be analysed and the antibody quality different amounts of proteins may be required.

Both the input and the iPOND purified samples should be examined concurrently. Typically load 2 μ l of input and 20 μ l of capture samples.

70. Perform standard electrophoresis, transfer, and immunoblotting using desired antibodies.
71. Mass spectrometry analysis can also be performed at this stage. Native iPOND (performed without formaldehyde crosslinking as described in [8]) facilitates mass spectrometry analysis.

3.4 Concluding Remarks

The protocol described above is an adaptation of the original iPOND technique that can be feasibly carried out under hypoxic conditions. Importantly, this protocol can be used to study both proteins associated with the replication forks undergoing hypoxia-induced replication stress and those associated with forks undergoing replication stress recovery following periods of reoxygenation (Figs. 3.1 and 3.2) [5]. This protocol can be combined with the use of drugs or can be carried out in cell lines containing mutations or deletions in genes of interest providing an easy way to interrogate the role of different factors in the recruitment of proteins to or around the replication fork under hypoxia/reoxygenation conditions.

Limitations to this protocol include the length and the high number of cells required. Modifications to the original iPOND protocol, however, have now been described that shorten the protocol by combining the cell lysis and nuclei harvesting steps. This reduces the number of washes resulting in improved yields due to reduced sample loss. Formaldehyde crosslinking is also omitted in this protocol, a modification that may facilitate mass spectrometry analysis [11].

This protocol also does not provide resolution at the single replication fork level; rather the data obtained should be interpreted as an average representation of replication forks present in that particular sample [8]. However, the original iPOND protocol and the adaptation described here provide important spatial and temporal information regarding the association of proteins bound directly or indirectly to DNA at or around replication forks. It is important to note that the level of spatial and temporal information that can be obtained by this method primarily depends on the EdU labelling time and the DNA synthesis rates under varying conditions. Under hypoxic conditions significantly different from those described in this protocol, EdU labelling times might need to be altered in order to account for changes in DNA replication rates under different conditions. The ultimate experimental goal will also determine the most appropriate EdU labelling times. Shorter times are recommended when studying proteins specifically associated with the replisome (or at the replication fork) whilst longer labelling periods should be used for the identification of proteins involved in chromatin deposition or maturation [5, 8].

While other established protocols such as chromatin fractionation and chromatin immunoprecipitation allow DNA-protein complex purification and could potentially be used to isolate complexes present at the replication fork, these techniques cannot provide information about the spatial location of a particular protein (or its modifications) in the genome with respect to replication. Such spatial information may be obtained by the use of certain imaging techniques such as super-resolution immunofluorescence or proximity ligation assays; however, these techniques require specific antibodies and are, therefore, not suitable for the identification of novel unknown replication associated factors [1].

Coupling iPOND to unbiased approaches such as LC-MS/MS allows the use of this powerful technique in the identification of novel players at active, stalled or damaged replication forks [12]. It would be interesting to carry out the iPOND in hypoxia/reoxygenation protocol coupled to LC-MS/MS in order to isolate novel factors associated with the replication fork under conditions of hypoxia-induced replication stress as well as during reoxygenation. Given that hypoxia-induced replication stress is different from replication stress induced by other agents typically used in the study of DNA replication (such as hydroxyurea), it is important to have the necessary tools to study factors associated with replication forks under these conditions [6].

Acknowledgements We are very grateful to Bianca Sirbu and David Cortez for help and advice on the iPOND procedure when we were adapting it for use under hypoxia and reoxygenation conditions. This work was supported by a Cancer Research UK grant (awarded to E.M.H.) and an MRC Centenary award (awarded to M.M.O.).

References

1. Dungrawala H, Cortez D. Purification of proteins on newly synthesized DNA using iPOND. *Methods Mol Biol.* 2015;1228:123–31.
2. Pires IM, et al. Effects of acute versus chronic hypoxia on DNA damage responses and genomic instability. *Cancer Res.* 2010;70(3):925–35.
3. Bester AC, et al. Nucleotide deficiency promotes genomic instability in early stages of cancer development. *Cell.* 2011;145(3):435–46.
4. Hammond EM, et al. The meaning, measurement and modification of hypoxia in the laboratory and the clinic. *Clin Oncol (R Coll Radiol).* 2014;26(5):277–88.
5. Olcina MM, et al. Replication stress and chromatin context link ATM activation to a role in DNA replication. *Mol Cell.* 2013;52(5):758–66.
6. Hammond EM, Green SL, Giaccia AJ. Comparison of hypoxia-induced replication arrest with hydroxyurea and aphidicolin-induced arrest. *Mutat Res.* 2003;532(1–2):205–13.
7. Sirbu BM, et al. Analysis of protein dynamics at active, stalled, and collapsed replication forks. *Genes Dev.* 2011;25(12):1320–7.
8. Sirbu BM, Couch FB, Cortez D. Monitoring the spatiotemporal dynamics of proteins at replication forks and in assembled chromatin using isolation of proteins on nascent DNA. *Nat Protoc.* 2012;7(3):594–605.
9. Lopez-Contreras AJ, et al. A proteomic characterization of factors enriched at nascent DNA molecules. *Cell Rep.* 2013;3(4):1105–16.

10. Cortez D. Preventing replication fork collapse to maintain genome integrity. *DNA Repair (Amst)*. 2015;32:149–57.
11. Leung KH, Abou El Hassan M, Bremner R. A rapid and efficient method to purify proteins at replication forks under native conditions. *Biotechniques*. 2013;55(4):204–6.
12. Sirbu BM, et al. Identification of proteins at active, stalled, and collapsed replication forks using isolation of proteins on nascent DNA (iPOND) coupled with mass spectrometry. *J Biol Chem*. 2013;288(44):31458–67.
13. Olcina del MM. Hypoxia-induced chromatin changes and ATM signalling. 2014, DPhil. University of Oxford.