JCB Journal of Cell Biology

SPOTLIGHT

SIRFing the replication fork: Assessing protein interactions with nascent DNA

Dana Branzei[1,2] and Michele Giannattasio[1,3]

Roy et al. (2018. *J. Cell. Biol.* https://doi.org/10.1083/jcb.201709121) describe an ingenious single-cell assay system, in situ analysis of protein interactions at DNA replication forks (SIRF), for the quantitative analysis of protein interactions with nascent DNA at active and stalled replication forks. The sensitive and accurate SIRF methodology is suitable for multiparameter measurements in cell populations.

DNA replication reactions are central for cellular proliferation and are critically implicated in the etiology of human diseases such as cancer, aging, and developmental disorders. The replication fork, the site of ongoing DNA synthesis, is both a crowded and dynamic place, with various proteins associating constantly or transiently, globally or at specific genomic regions, during normal replication conditions or specifically after replication stress cues (Branzei and Szakal, 2017). Besides well-known replisome components, various factors known for their function in DNA repair or in other cellular processes have recently been discovered to associate with the replication fork. The dynamics of these interactions can provide valuable information on the type of problems or reactions that occur at replication forks or proximal to sites of replication in specific contexts. Therefore, tools that enable sensitive and quantitative information on the process of replication and associated proteins are critical for the advancement of our understanding of replication-associated DNA metabolism. Pursuing this quest, revolutionary methods that allow genomewide monitoring of the replication process emerged. Especially worth mentioning are molecular combing, a single-molecule resolution assay involving genome combing and monitoring of replication fork speed and interorigin distance (Michalet et al., 1997), and chromatin immunoprecipitation (IP; ChIP)-on-chip/ChIP sequencing (ChIP-seq) combined with BrdU-IP on chip, techniques measuring the level of protein–DNA interaction versus the regions of ongoing replication revealed by the incorporation of the thymidine analogue BrdU in a genome-wide fashion using cell population experiments (Katou et al., 2003). These technologies continue to prove extremely useful for the understanding of the replication process and of other chromosome structural processes influenced by the dynamic binding of proteins to chromatin. However, as they are laborious, they are not suitable for large screens. Related to the ChIP-on-chip/ChIP-seq approach but focused on the discovery of proteins associated with the nascent DNA rather than in the precise mapping of their location on chromatin is identification of proteins at active, stalled, and collapsed replication forks using isolation of proteins on nascent DNA (iPOND), a ground-breaking technique in which proteins cross-linked with newly replicated DNA are isolated and resolved (Sirbu et al., 2011). In iPOND, newly replicated DNA is labeled with the thymidine analogue EdU and conjugated with biotin using click chemistry. After shearing of the chromatin and genome purification, proteins cross-linked to biotinylated DNA are resolved by Western blot analysis or by stable isotope labeling with amino acids in cell culture (SILAC) using mass spectrometry (Sirbu et al., 2013). Both ChIP-on-chip and iPOND are extremely valuable, but they are laborious, require a large amount of starting material, and have limited quantitation potential. The more evolved ChIP-seq and iPOND-SILAC techniques are quantitative, but they require high costs and specialized equipment. In this issue, Roy et al. describe a sensitive and accurate single-cell resolution technique that can readily identify proteins bound to the newly replicated DNA.

The new technique, called in situ analysis of protein interactions at DNA replication forks (SIRF), is the wedding of iPOND and a modified version of the proximity ligation assay (PLA) developed to detect and measure in situ protein–protein interactions (Söderberg et al., 2006). In SIRF, like in iPOND, newly synthesized DNA is labeled with EdU and then biotinylated by click chemistry between EdU and biotin-azide. In SIRF, cells are subsequently incubated with primary antibodies against biotin and the protein of interest (Fig. 1), and the protocol then follows the principles of a modified, highly sensitive, and accurate PLA assay (Söderberg et al., 2006). That is, cells are

[1]Italian Foundation for Cancer Research, the Fondazione Italiana per la Ricerca sul Cancro Institute of Molecular Oncology, Milan, Italy; [2]Istituto di Genetica Molecolare, Consiglio Nazionale delle Ricerche, Pavia, Italy; [3]Dipartimento di Oncologia ed Emato-Oncologia, Università degli Studi di Milano, Milan, Italy.

Correspondence to Dana Branzei: dana.branzei@ifom.eu.

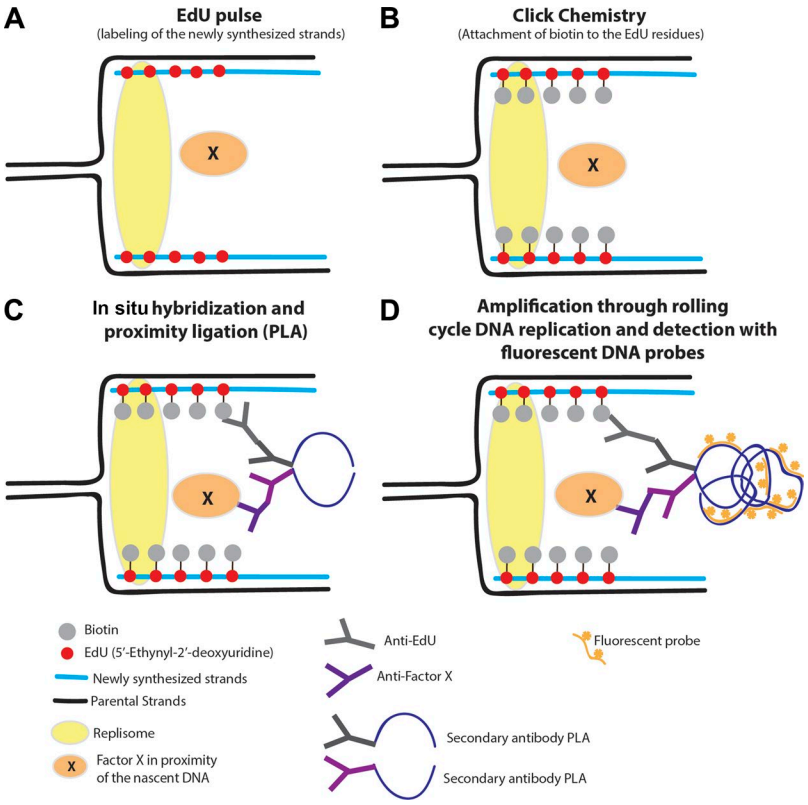

Figure 1. **SIRF workflow. (A)** Nascent DNA is labeled with EdU. **(B)** Nascent DNA is biotinylated using click chemistry. **(C)** In situ hybridization using primary antibodies against factor X and biotinylated DNA. Primary antibodies are recognized by secondary antibodies conjugated to sequence-specific DNA oligomers. If the factor X and nascent DNA are in proximity, the oligomers anneal and form a circular DNA molecule, which is then ligated. **(D)** Rolling cycle replication creates ∼100 circular DNA molecules for each epitope-to-epitope interaction. Circular DNA molecules are then recognized by fluorescent probes providing amplification and specificity to the resulting interaction signal.

incubated with secondary antibodies conjugated with oligonucleotides that function as proximity probes. If the secondary antibodies are in a proximity of <40 nm, indicative of direct interaction between the examined protein and biotinylated DNA, the DNA oligomers are able to anneal, guiding the formation of a nicked circular DNA molecule. After ligation, DNA circles serve as templates for localized rolling circle amplification. DNA sequence–specific fluorescence DNA probes are then annealed to the amplified DNA circles, allowing the signal to be visualized and quantified (Fig. 1).

In their study, Roy et al. (2018) provide validation data for sensitivity, proximity, and quantitation in SIRF. The authors smoothly sail the SIRF readers through several examples of proteins known or expected to be associated with the replication fork such as proliferating cell nuclear antigen (PCNA) and replication protein A (RPA), providing evidence for the much higher sensitivity of SIRF versus normal immunofluorescence (IF; Zellweger et al., 2015) and for how the investigated protein interactions with nascent DNA are reliably quantified. Roy et al. (2018) validate previous findings obtained with iPOND technology such as the requirement for MRE11 nuclease recruitment to the fork (Ray Chaudhuri et al., 2016). They additionally bring new insight into the consequences of 53BP1 loss on key protein interactions at the replication fork. The sensitive SIRF methodology works like a charm in all the tested scenarios, promising to enable research on the replication fork in the years to come.

The SIRF methodology is worth noting for several reasons. First, SIRF requires little starting material (∼10,000 cells per condition), but its high sensitivity allows detection of factors not visualized by IF under unperturbed conditions such as RPA and RAD52. Second, by changing experimental conditions, one can investigate the amount of the same protein at stalled or collapsed forks or the recruitment behind replication forks when EdU is chased away with low amounts of thymidine. Finally, SIRF can be combined with other IF parameters in a heterogeneous cell population such as markers of cell cycle, staining of early or late replicating cells, and the presence of specific receptors. This single-cell resolution feature, absent from methodologies measuring cell population averages, enables the understanding of how protein interactions with the replication fork are influenced or correlate with other parameters in a cell population environment.

The results presented with the new SIRF technology (Roy et al., 2018) open up the avenue to answer several important questions. For instance, replisome and DNA metabolism factors such as RPA, RAD51, RAD52, and MRE11 are not detected by conventional IF during unchallenged replication and at stalled forks, but they can be readily detected by SIRF, supporting results of other sensitive techniques relying on halogenated DNA pulldown and Western blot analysis of specific proteins (Petermann et al., 2010) and iPOND results (Ray Chaudhuri et al., 2016). Given the accuracy and high sensitivity of SIRF, it will be interesting to address panel changes in replication fork interactions with a set of proteins capable of binding single-stranded DNA (ssDNA) exposed at the tip of newly replicated DNA and to correlate those changes with DNA transitions reported to occur at the fork in the same experimental conditions and cell lines (Zellweger et al., 2015). As antibodies for detecting posttranslationally modified proteins proposed to mediate specific DNA transitions may become available, when used in combination with other methodologies,

SIRF could provide useful information on the dynamics of protein modifications, interactions with the nascent DNA, and DNA transitions at the replication fork. Moreover, because of its ability to detect factors that are not usual components of the replisome but later associate in the rear of the replication fork—possibly on the ssDNA region exposed proximal to the nascent DNA or on the nascent DNA itself—SIRF has the potential to help in uncovering factors that may facilitate postreplicative repair, a topic that has remained little understood especially in mammalian cells (Branzei and Szakal, 2017). When combined with epigenetic marks and IF of specific binders that give information on the chromatin status as well as certain genomic regions such as centromeres or telomeres, SIRF promises to bring answers to how DNA replication is regulated either globally or at specific genomic regions and in different chromatin states (Branzei and Szakal, 2017).

In summary, SIRF technology opens up new ways to understanding how local protein–replication fork interactions lead to the dynamic changes of the replication fork architecture and chromosome structure. These questions are important for understanding fundamental DNA metabolism processes associated with chromosome replication and to monitor key interactions at the replication fork in clinical settings.

Acknowledgments

Work in the Branzei laboratory is supported by grants from the Associazione Italiana per la Ricerca sul Cancro (IG 18976), the European Research Council (consolidator grant 682190), and a European Training Network grant (SYNTRAIN; 722729) to D. Branzei.

The authors declare no competing financial interests.

References

Branzei, D., and B. Szakal. 2017. Building up and breaking down: mechanisms controlling recombination during replication. *Crit. Rev. Biochem. Mol. Biol.* 52:381–394. https://doi.org/10.1080/10409238.2017.1304355

Katou, Y., Y. Kanoh, M. Bando, H. Noguchi, H. Tanaka, T. Ashikari, K. Sugimoto, and K. Shirahige. 2003. S-phase checkpoint proteins Tof1 and Mrc1 form a stable replication-pausing complex. *Nature.* 424:1078–1083. https://doi.org/10.1038/nature01900

Michalet, X., R. Ekong, F. Fougerousse, S. Rousseaux, C. Schurra, N. Hornigold, M. van Slegtenhorst, J. Wolfe, S. Povey, J.S. Beckmann, and A. Bensimon. 1997. Dynamic molecular combing: stretching the whole human genome for high-resolution studies. *Science.* 277:1518–1523. https://doi.org/10.1126/science.277.5331.1518

Petermann, E., M.L. Orta, N. Issaeva, N. Schultz, and T. Helleday. 2010. Hydroxyurea-stalled replication forks become progressively inactivated and require two different RAD51-mediated pathways for restart and repair. *Mol. Cell.* 37:492–502. https://doi.org/10.1016/j.molcel.2010.01.021

Ray Chaudhuri, A., E. Callen, X. Ding, E. Gogola, A.A. Duarte, J.E. Lee, N. Wong, V. Lafarga, J.A. Calvo, N.J. Panzarino, et al. 2016. Replication fork stability confers chemoresistance in BRCA-deficient cells. *Nature.* 535:382–387. https://doi.org/10.1038/nature18325

Roy, S., J.W. Luzwick, and K. Schlacher. 2018. SIRF: Quantitative in situ analysis of protein interactions at DNA replication forks. *J. Cell Biol.* https://doi.org/10.1083/jcb.201709121

Sirbu, B.M., F.B. Couch, J.T. Feigerle, S. Bhaskara, S.W. Hiebert, and D. Cortez. 2011. Analysis of protein dynamics at active, stalled, and collapsed replication forks. *Genes Dev.* 25:1320–1327. https://doi.org/10.1101/gad.2053211

Sirbu, B.M., W.H. McDonald, H. Dungrawala, A. Badu-Nkansah, G.M. Kavanaugh, Y. Chen, D.L. Tabb, and D. Cortez. 2013. 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.* 288:31458–31467. https://doi.org/10.1074/jbc.M113.511337

Söderberg, O., M. Gullberg, M. Jarvius, K. Ridderstråle, K.J. Leuchowius, J. Jarvius, K. Wester, P. Hydbring, F. Bahram, L.G. Larsson, and U. Landegren. 2006. Direct observation of individual endogenous protein complexes in situ by proximity ligation. *Nat. Methods.* 3:995–1000. https://doi.org/10.1038/nmeth947

Zellweger, R., D. Dalcher, K. Mutreja, M. Berti, J.A. Schmid, R. Herrador, A. Vindigni, and M. Lopes. 2015. Rad51-mediated replication fork reversal is a global response to genotoxic treatments in human cells. *J. Cell Biol.* 208:563–579. https://doi.org/10.1083/jcb.201406099