doi:10.1017/S1431927616005924
Microsc. Microanal. 22 (Suppl 3), 2016

Single Molecule Localization Microscopy of DNA Damage Response Pathways in Cancer.

Donna R. Whelan[1], Yandong Yin[1], Keria Bermudez-Hernandez[1], Sarah Keegan[1], David Fenyö[1] and Eli Rothenberg[1].

[1.] Department of Biochemistry and Molecular Pharmacology, Perlmutter Cancer Center, New York University School of Medicine, New York, NY 10016.

The collective DNA Damage Response (DDR) network of cellular pathways serves to prevent, recognize, and repair DNA damage resulting from both endogenous and exogenous sources. Unchecked or misrepaired, DNA damage causes genomic instability and mutagenesis, cell death, and various types of cancer [1]. DNA replication during S phase is a process particularly prone to endogenous damage because of the requirement for the entire genomic DNA to be unwound and duplicated over a relatively short time period (minutes in yeast, hours in mammals). To achieve this, any impediments or corruptions to the DNA template such as bound transcription complexes, small molecules, or unusual secondary structures, must be removed. If these impediments are not easily removed they can cause replication fork (RF) slowing, stalling, and eventual regression or collapse. In the latter case, the single sided double strand break (DSB) that occurs must be repaired with high fidelity to ensure the viability of the cell. To do this, the homologous recombination (HR) DSB repair machinery completes a number of sequential operations: first to resect the DSB to generate a ssDNA overhang, then to generate a Rad51/ssDNA nucleofilament which conducts a homology search of the genomic DNA, and then synthesis of new DNA using the homologous sequence [2].

Despite the importance of HR, a well-defined and comprehensive model detailing the processes and kinetics of this pathway—or of the overarching DDR network—has not been achieved. This is in part due to *in vitro* assays that are limited by the number of proteins and pathways that can be examined simultaneously, as well as the diffraction limit of light, which has restricted *in vivo* visualization and foci analysis to spatial resolutions of approximately 250 nm. Single molecule localization microscopy (SMLM) is a powerful super resolution technique capable of imaging cellular structures with spatial resolutions of approximately 15 nm (Fig. 1A) [3]. By inducing RF stress *in vivo* and using multicolor SMLM to image the resulting structures and protein interactions, we have developed a novel visual proteomics approach for elucidating S-phase DDR pathways.

Specifically, human cancer cells U-2 OS were synchronized using serum starvation before nonlethal damage during mid-S phase with 100 nM camptothecin (CPT) for one hour. CPT is a small molecule that captures Topoisomerase I as a single strand break cleavage complex a short distance ahead of active RFs. This has previously been demonstrated to generate both regressed and collapsed RFs *in vivo* [4] often resulting in single sided DSBs in much the same way as rare endogenous spontaneous DSBs are formed [2]. To visualize these events, nascent DNA was labeled during the same hour as damage using ethylene deoxyuridine incorporation, which was then fluorescently identified after fixation using the copper-catalyzed 'click' reaction. Using indirect immunofluorescence the RF (proliferating cell nuclear antigen (PCNA)), DSB DNA resection (MRE11), and DNA damage (phosphorylated histone H2A (γH2A.X)) were also visualized. SMLM imaging was carried out as previously described [5].

Fig. 1B-E shows representative SMLM renderings of different structures identified in cells allowed to recover from CPT damage for one hour. In Fig. 1B DNA synthesized during the damage period is shown in red and a nearby PCNA localization is shown in blue while no green γH2A.X is observed.

This shows a replication fork that has neither regressed nor collapsed due to damage but continued synthesizing unlabeled DNA. This is assumedly due to removal of any impeding cleavage complex prior to fork collision, or a lack of impediment at this particular RF at the 100 nM treatment concentration. In contrast, Fig. 1C shows PCNA still localized at the end of the nascent DNA labeled during the drug treatment indicating RF stalling and possible regression or collapse. The colocalized γH2A.X is further evidence of DNA damage but is not prescriptive of the fate of the RF. To differentiate between RF stalling, regression and collapse, MRE11 was colabeled with PCNA/γH2A.X. In Fig. 1D a stalled or regressed RF is identified by the lack of MRE11 at the γH2A.X labeled site whereas Fig. 1E shows a collapsed RF with MRE11 stimulating resection for HR repair of the DSB. These structures were regularly observed in fixed cells and confirm the hypothesized action of CPT on replicating DNA and the relatively slow resection process required for HR. Furthermore, the elucidation of these structures using SMLM demonstrates the future potential of similar experiments examining the more complex aspects of HR and the DDR network. Finally, Fig. 1F shows the quantifiable interactions of DNA with γH2A.X, PCNA and MRE11 at stressed RF sites demonstrating a significant number of DSBs generated by collapsed replication forks (MRE11/DNA overlaps: 9.05 ± 0.43%) as well as the persistent presence of PCNA after damage (9.69 ± 0.49% without damage, 7.84 ± 0.96% with damage).

Unquestionably, SMLM is a new and powerful approach to examining the complicated pathways and interactions involved in DDR. In particular it allows for decades of hypothesized protein and DNA interactions to be tested within the context of the full cellular environment and will allow fast visualization of the *in vivo* roles of new DDR proteins that are identified by genetic experiments [6].

References:

[1] A Ciccia and SJ Elledge, Molecular Cell **40** (2010), p. 179.
[2] N Saleh-Gohari, *et al*, Molecular and Cellular Biology **25** (2005), p. 7158.
[3] DR Whelan and TDM Bell, Journal of Physical Chemistry Letters **6** (2015), p. 374.
[4] AR Chaudhuri, *et al*, Nature Structural & Molecular Biology **19** (2012), p. 417.
[5] DA Reid, *et al,* Proceedings of the National Academy of Sciences of the United States of America **112** (2015), p. 2575.
[6] Work in E.R.'s laboratory is supported by National Institutes of Health Grants GM057691, CA187612, and GM108119; a Shifrin–Meyer Breast Cancer Discovery award; and the Ralph S. French Charitable Foundation Trust.

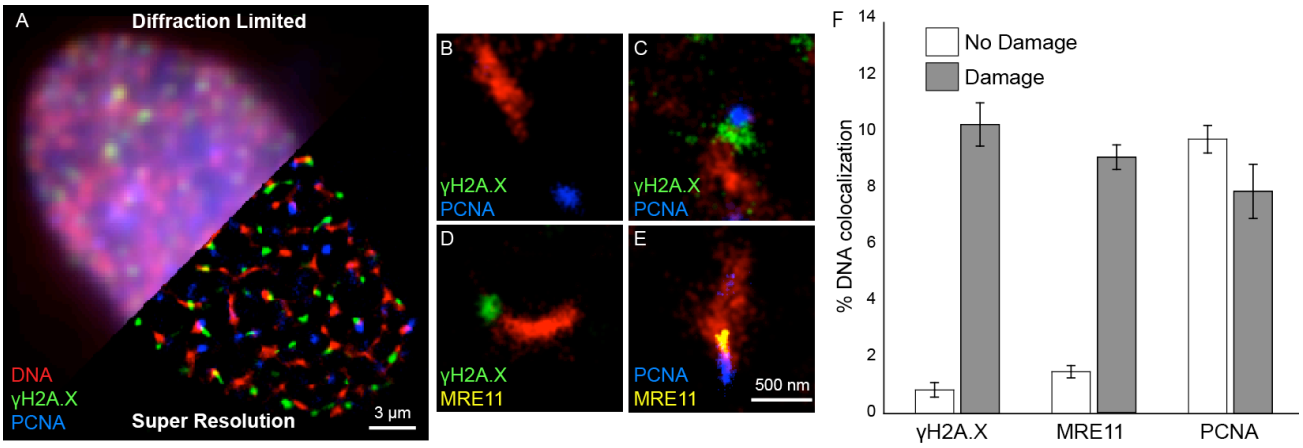

Figure 1. (A) Comparison of diffraction limited and super resolution images. **(B-E)** Representative protein/nascent DNA structures identified in cells after 1 hour recovery from damage with 100 nM CPT. **(F)** Quantification of protein interactions with nascent DNA with and without CPT damage (no recovery time).

https://doi.org/10.1017/S1431927616005924 Published online by Cambridge University Press