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Imaging DNA double-strand breaks — are we there yet?

Jake Atkinson[1], Eva Bezak[2,3] and Ivan Kempson[1]

DNA double-strand breaks (DSBs) are fundamental to cell biology, from evolution to the latest gene-editing technologies. Yet, does an assay exist that truly quantitatively visualizes DSBs? Over-reliance on DSB detection by proxies can misguide interpretation of conventional assays and faithful DSB representatives await development.

DNA double-strand breaks (DSBs) can arise spontaneously from nuclease activities or through insults from reactive species, radiation and cytotoxic agents. DSBs are the most deleterious form of DNA damage, causing genome instability and chromosomal aberrations; DSB repair responses are thus rapid and stringently regulated[1]. By contrast, prescribed cleavage of DNA at targeted loci facilitates gene editing technologies such as CRISPR–Cas.

Quantifying DSBs is essential for identifying relationships between DNA cleavage and repair, which are pertinent to aging, mutagenesis, genotoxicity and therapeutics, both established (chemo- and radio-therapies) and emerging (gene editing, particle and FLASH radiotherapy, etc.). Currently, our most powerful tools to spatially observe DSBs rely on immunofluorescence labelling of DNA damage response (DDR) proteins or histone modifications such as 53BP1 and γH2AX, respectively. These well-established markers typify the DDR[2]. However, interpretation of these downstream proxies is often extended upstream, through a range of assumptions, to DSB formation in a directly proportional, quantitative manner. These interpretations can be problematic: repair can be partial, thereby skewing the proportionality between DSBs and 53BP1 or γH2AX 'repair' foci; initiation of the repair response does not equal repair completion; and 'repair' foci can exist in the absence of DSBs[3]. Thus, detecting DSBs with a high degree of reliability is still challenging, and more sensitive markers are required for accurate DSB quantification. In this context, we highlight sources of potential misinterpretation with common assays, along with novel approaches that may reshape our approach to measuring DNA damage.

[H1] Endogenous markers of DSBs

DSB sensors, including PARP1, the MRE11–RAD50–NBS1 complex, the Ku70–Ku80 complex and SIRT6 detect DSBs and initiate the DDR[2,4]. H2AX phosphorylation (γH2AX) is a pivotal early step in the repair process, allowing the remodelling of chromatin surrounding DSBs and the recruitment of repair proteins[2]. One such protein is 53BP1, which recruits additional DSB repair proteins and regulates repair-pathway choice. Through immunofluorescence, 53BP1 and γH2AX are widely used, often dogmatically, as DSB markers, as they form discrete foci at or in proximity to DSBs.

[H1] Biases of current assays

The only conventional method for in situ DSB detection is terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL), but it does not quantify discrete DSB foci. Consequently, other markers are used, but considering many reports in the literature, it is necessary to remember that DDR markers generally reflect repair, not DSBs. An excellent example is perturbation of the DDR with a TGF-β inhibitor prior to DNA insult. Consequently, fewer γH2AX foci are detected compared to control, which correlates with decreasing clonogenicity, i.e., repair is impaired, leading to reduced proliferative capacity[5]. This highlights the crux of assays based on DDR factors: they are ambiguous. Do foci correlate with DSB accumulation, increased repair capacity and/or efficiency, or expression of DDR factors? Other misconceptions when using DDR proteins as DSB markers may arise.

Regarding γH2AX, it is generally true that DDR induces H2AX phosphorylation, but H2AX phosphorylation is not always correlated with DNA damage (Ref.[3] and references therein). Although γH2AX is an excellent tool, especially for monitoring DDR kinetics, quantifying the relationship between γH2AX foci production and DSBs is challenging, especially due to the rapid progression of the DDR. Highly variable repair kinetics and H2AX phosphorylation at sites other than where DSB repair is occurring complicate the picture. For example, loss of shelterin complex functionality at short telomeres leads to chromosomal ends also being recognised by

DDR machinery[6]. Regarding 53BP1, biases lie in its dominance in the G1 phase of the cell cycle, where it mediates recruitment of non-homologous end-joining factors; inversely, homologous recombination dominates DSB repair in other cell cycle phases.

[H1] Novel assays

A few novel techniques to overcome the lack of highly specific diagnostic biomarkers of DSBs are emerging. For example, the DSB-binding protein Gam, derived from bacteriophage Mu, was fused to GFP[7]. Gam's normal function is to inhibit host exonucleases by binding to exposed ends of linear phage DNA, thereby preventing its degradation. GamGFP is capable of binding to DSBs, thereby allowing the observation of discrete DSB foci. A major drawback of this technique is that the efficiency of GamGFP binding to DSB sites in mammalian cells is challenged by its orthology with Ku[7]. Thus, although GamGFP is useful in specifically marking DSBs, its competition with Ku to bind to DSBs makes it difficult to utilize quantitatively without first knocking-out Ku.

Similar to GamGFP, a fluorescently conjugated SIRT6 was used to validate SIRT6 function in DSB detection[4]. One of its key attributes is its ability to localise to DSB sites independently of PARP1, MRE11 and Ku80 and initiate the DDR. It is believed that SIRT6 binds open-ended DNA as a homodimer, and its dimerization was suggested to be cooperative with binding[4]. Although there is evidence supporting SIRT6's capacity to bind to DSBs, it is also predicted that the SIRT6 monomer is capable of binding to single-stranded DNA breaks. Additionally, SIRT6 is involved in other processes including cellular metabolism and inflammation[8]. Thus, use of SIRT6 for DSB detection is likely to produce substantial off-target background signalling, complicating its establishment as a DSB-specific biomarker. GamGFP and SIRT6 highlight the challenges in using fluorescently tagged proteins, which may compete with DDR machinery or possess multiple functions that reduce foci specificity to DSBs. Exogenous markers are likely to be less ambiguous.

A technique more specific for DSB detection is sensitive recognition of individual DNA ends (STRIDE)[9]. In STRIDE, the nucleotide analogue BrdU is enzymatically conjugated to free 3'-OH DNA ends. This facilitates recognition through two sets of primary and secondary antibodies, with each secondary antibody being conjugated with its own set of oligonucleotides. Amplification of the fluorescence signal is then performed through rolling circle amplification (RCA). Labelling was demonstrated for DSBs induced by Cas9, chemical and biological agents, ionizing radiation and spontaneous formation[9].

At the other end of the DDR, persistent repair foci are uniquely identified by DNA damage *in situ* ligation followed by proximity ligation assay (DI-PLA)[10]. DI-PLA, which has broad utility and can be applied also to DDR factors, involves the blunting of DSB ends, ligation of biotinylated oligonucleotides, and incubation with antibodies against biotin and against a protein of interest, and is also followed by RCA. Able to discriminate between persistent DSBs and active repair or other chromatin modifications, DI-PLA provides an accurate measure of foci resistant to repair machinery, which threaten chromosomal integrity.

Although further use of these cutting-edge techniques is required to properly assess their practicality and efficacy compared to conventional and other novel techniques, STRIDE and DI-PLA vastly improve fluorescent targeting of DSBs, in a bid to eliminate the ambiguity conferred by labelling of the currently used endogenous protein proxies.

[1] Conclusion

The development of new and innovative fluorescence-based DSB detection techniques is pushing the envelope in the quantification of DSBs. A long road lies ahead of establishing these newer methodologies, which have the potential to eliminate the reliance on using endogenous DDR factors as DSB proxies. Due to their relative novelty, information on their reliability is sparse and they require additional refinement, optimisation and improved affordability prior to widespread use. Continual development of accurate DSB detectors is essential to improving our understanding of mutagenesis, cancer and aging-related diseases and for the development of suitable therapies.

1 Scully, R. et al. DNA double-strand break repair-pathway choice in somatic mammalian cells. *Nat Rev Mol Cell Biol* **20**, 698–714 (2019).
2 Mah, L. J. et al. γH2AX: a sensitive molecular marker of DNA damage and repair. *Leukemia* **24**, 679–686, (2010).
3 Rybak, P. et al. Low level phosphorylation of histone H2AX on serine 139 (γH2AX) is not associated with DNA double-strand breaks. *Oncotarget* **7**, 49574–49587 (2016).
4 Onn, L. et al. SIRT6 is a DNA double-strand break sensor. *eLife* **9**, e51636 (2020).
5 Bouquet, F. et al. TGFbeta1 inhibition increases the radiosensitivity of breast cancer cells in vitro and promotes tumor control by radiation in vivo. *Clin Cancer Res* **17**, 6754–6765 (2011).
6 Maciejowski, J. & de Lange, T. Telomeres in cancer: tumour suppression and genome instability. *Nature Reviews Molecular Cell Biology* **18**, 175–186 (2017).
7 Shee, C. et al. Engineered proteins detect spontaneous DNA breakage in human and bacterial cells. *Elife* **2**, e01222 (2013).
8 Yang, X. et al. Roles of SIRT6 in kidney disease: a novel therapeutic target. *Cell Mol Life Sci* **79**, 53 (2021).
9 Kordon, M. M. et al. STRIDE—a fluorescence method for direct, specific in situ detection of individual single- or double-strand DNA breaks in fixed cells. *Nucleic Acids Research* **48**, e14-e14 (2019).
10 Galbiati, A. et al. A novel single-cell method provides direct evidence of persistent DNA damage in senescent cells and aged mammalian tissues. *Aging Cell* **16**, 422–427 (2017).

Competing interests
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[1] *Future Industries Institute, University of South Australia, Mawson Lakes, 5095, South Australia, Australia*
[2] *Cancer Research Institute, University of South Australia, Adelaide, South Australia, Australia*
[3] *Department of Physics, University of Adelaide, North Terrace, Adelaide, South Australia, Australia*
** e-mail of corresponding author: Ivan.Kempson@unisa.edu.au*

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