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A novel method for monitoring functional lesion-specific recruitment of repair proteins in live cells

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Abstract

DNA-protein relationships have been studied by numerous methods, but a particular gap in methodology lies in the study of DNA adduct-specific interactions with proteins *in vivo*, which particularly affects the field of DNA repair. Using the repair of a well-characterized and ubiquitous adduct, the abasic (AP) site, as a model, we have developed a comprehensive method of monitoring DNA lesion-specific recruitment of proteins *in vivo* over time. We utilized a surrogate system in which a Cy3-labeled plasmid containing a single AP-site was transfected into cells, and the interaction of the labeled DNA with BER enzymes, including APE1, Pol β , LIG1, and FEN1, was monitored by immunofluorescent staining of the enzymes by Alexafluor-488-conjugated secondary antibody. The recruitment of enzymes was characterized by quantification of Cy3-Alexafluor-488 co-localization. To validate the microscopy-based method, repair of the transfected AP-site DNA was also quantified at various time points post-transfection using a real time PCR-based method. Notably, the recruitment time kinetics for each enzyme were consistent with AP-site repair time kinetics. This microscopy-based methodology is reliable in detecting the recruitment of proteins to specific DNA substrates and can be extended to study other *in vivo* DNA-protein relationships in any DNA sequence and in the context of any DNA structure in transfectable proliferating or quiescent cells. The method may be applied to a variety of disciplines of nucleic acid transaction pathways, including repair, replication, transcription, and recombination.

Keywords

DNA-protein interaction; base excision repair; abasic sites; repair intermediate; co-localization

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1. Introduction

The study of DNA-protein relationships holds great biological significance and reveals previously unknown cellular phenomena. Understanding the intricacies of the interaction between important functional cellular proteins and the “blueprint” of the cell elucidates the mechanisms behind the regulation of cellular processes that may be important in understanding various diseases. DNA-protein interactions have been studied by numerous methods, but a particular gap in methodology lies in the study of DNA structure-specific interactions with proteins *in vivo*. Certain methods, including chromatin immunoprecipitation (ChIP) and ligation-mediated PCR (LM-PCR) are powerful methods to detect DNA-protein interactions *in vivo*. ChIP is useful for detecting interactions that are mediated by the sequence of the DNA while LM-PCR can determine the location and accumulation of certain DNA modifications in the DNA sequence, but information about the proteins interacting with those DNA modifications is, at best, indirect. Both methods are unhelpful for directly monitoring protein interactions that are mediated by a modification of the DNA structure, such as DNA adducts, DNA replication structures, or recombination structures (1-3). Notably, much of the interactions of interest in the DNA repair field are mediated by modified, adducted DNA. Currently, several methods are available to observe DNA structure-specific interaction with associated proteins *in vitro*, including DNA footprinting, electrophoretic mobility shift assay (EMSA), fluorescence anisotropy, and surface plasmon resonance (SPR), to name a few (4-8). These techniques are valuable for dissecting mechanisms and quantifying kinetic parameters of the enzymatic interaction between a protein and a modified DNA structure, such as a DNA adduct. However, all the aforementioned methods fall short in elucidating the interaction of interest in live human cells, which is necessary for a complete physiological understanding that can be translated into useful knowledge about basic mechanisms and even human disease. The goal of the method developed and described in the present study is to monitor functional and specific recruitment of DNA repair proteins to adduct-containing DNA *in vivo*. In order to accomplish this goal, we analyzed repair of the abasic (AP) site, as a model.

AP sites are one of the most frequent non-coding lesions generated in cellular DNA, either by spontaneous or enzyme-catalyzed processes (9,10). An abasic site is formed when the *N*-glycosyl bond between the base and deoxyribose sugar is hydrolyzed. This hydrolysis can occur during spontaneous depurination or as an intermediate step in the base excision repair (BER) pathway, which repairs small adducts in the cellular DNA. An AP-site is generated during BER after the initiating glycosylase excises a damaged base from the DNA structure. The resulting abasic site is subsequently cleaved at the first 5' phosphate by apurinic/aprimidinic endonuclease 1 (APE1), and repair is completed by gap-filling and ligation via other BER enzymes to fully restore the DNA sequence. The interaction of APE1 with abasic site DNA is well-characterized *in vitro*, both qualitatively and quantitatively (6,11-13). However, as previously noted for general DNA-protein interactions, *in vivo* interactions between APE1 and AP-site DNA remain to be elucidated. At best, a quantification of abasic sites in the genomic DNA can be performed using aldehyde reactive probe (ARP), but one limitation is that ARP also detects incised AP-sites (10). In fact, in a study where cleaved and intact abasic sites were quantified using a chemical method in human and rat tissues,

almost all of detected AP-sites were incised, indicating that the interaction between APE1 and abasic sites occurs rapidly and that the current methods for detecting AP-sites are unable to capture the interaction (14).

Using co-localization, a fluorescence-based technique commonly used for monitoring protein-protein relationships, we developed a method for monitoring adduct-specific DNA-protein interaction *in vivo* using the interaction of APE1 with AP-site DNA as a model. We further validated this method by monitoring the recruitment of downstream BER proteins, including Pol β , LIG1, and FEN1, in a lesion-specific manner. Finally, we determined the functionality of the observed BER enzyme-lesion DNA co-localization by modifying some *in vivo* DNA repair assays developed previously by our lab (15,16).

2. Materials and Methods

2.1 Construction of AP-site plasmid DNA

The preparation of the control and AP-site phagemid DNA was performed *in vitro* as previously described for 1,N⁶-ethenoadenine-containing phagemids with some modification (15). We engineered M13mp18 phagemids (7.2 kb) with a single tetrahydrofuran (THF) at the EcoRI restriction site in the multiple cloning site (MCS). Tetrahydrofuran is a stable analog of abasic site and is used in this study as a model AP-site. Typically, for each preparation of M13mp18-ctrl (no THF) or –AP (with THF) phagemid, 6 individual reactions were performed simultaneously according to the following protocol. Phosphorylation of the primers was performed by incubating 2 ug of THF-containing (5'-CCGAGCTCGXATTCGTAATC-3') or control (5'-CCGAGCTCGAATTCGTAATC-3') oligonucleotide (2 ul) with 1X PNK buffer, 400 nM ATP, 50 mM DTT, and 10 U of T4 polynucleotide kinase (New England Biolabs) in a 30 uL reaction volume at 37°C for 45 mins. The phosphorylated oligonucleotide was purified through a G-25 column (GE Healthcare Life Sciences) according to the manufacturer's protocol. Then 6 ul of this purified oligonucleotide was incubated with 2 ug of M13mp18 ssDNA in an annealing buffer containing 10 mM Tris-HCl, pH 7.5, and 50 mM NaCl in a 20 uL reaction volume. This annealing reaction was incubated at 80°C for 5 minutes and slowly cooled to room temperature with brief centrifugation when the reaction reached 50°C. Then the annealing reaction was incubated with an extension reaction mixture containing 12 mM Tris-HCl (pH 7.5), 6 mM MgCl₂, 0.9 mM ATP, 0.9 mM of each dNTP, 6 mM DTT, and 100 ug/mL BSA, 10 U of T7 DNA polymerase (New England Biolabs), and 400 U of T4 DNA ligase (New England Biolabs) in a final reaction volume of 50 uL for 5 mins on ice followed by 5 mins at room temperature. The extension reaction was subsequently incubated at 37°C for 1 h. After an hour, 50 nmol of ATP and 200 U of T4 DNA ligase were added to the extension reaction and incubated at 14°C overnight for efficient ligation to occur. The 6 individual reactions were then pooled and incubated with 1X Supercoil-It buffer (Bayou Biolabs) and 2 uL of Supercoil-It enzyme mixture at 37°C for 3 h. Phagemid DNA was recovered after the incubation by purification using Qiaquick PCR Purification kit (Qiagen). The DNA was eluted from the column using 50 ul of molecular grade water (DNase-, RNase-, and protease-free). Concentration of the eluted DNA was measured using a Nanodrop spectrophotometer, and the DNA was stored at -20°C. Eighty five femtomoles (fmol) of

each of the DNA constructs were treated with 20 nM APE1 (expressed and purified previously (17)) or 0.05 U of *EcoRI* and 10 U of *SacI*. Digestion products were evaluated by gel electrophoresis on a 1% agarose gel. For experiments in Fig. 2D, an oligonucleotide containing Cy3 in the *EcoRI* site was used for phagemid preparation (5'-CCGAGCTCGXATTCGTAATC-3'), where X = Cy3.

2.2 Construction of fluorescently labeled AP-site plasmid DNA

Similar to the previously described construction of AP-site phagemid DNA, fluorescently labeled AP-site or control phagemid DNA was prepared. During the polymerization step of the *in vitro* phagemid preparation, 1 nmol Cy3-dCTP (GE Life Sciences) was added to the dNTP pool generating a fraction of phagemids that were Cy3-labeled (control or AP-site-containing). In these constructs Cy3 was not placed at or around the assayed restriction site (*EcoRI*), whereas THF was placed in the *EcoRI* site. The quality of the constructs was evaluated as described in the above section. Quantification of Cy3 labeling of the phagemids was performed using an oligonucleotide containing a single Cy3 as a standard molecule to generate a standard curve and different dilutions of the phagemids. Quantification was carried out in a 96 well black plate with excitation at 480 nm and emission detected at 520 nm in a GloMax Multi-Detection system (Promega).

2.3 Transfection, harvest, and restriction digestion of AP-site plasmid DNA

Typically, 40 fmol of control or AP-site DNA was transfected into approximately 400,000 HCT116 cells in a well of a 6-well plate using 8 uL Lipofectamine 2000 (Life Technologies), according to manufacturer's protocol. At various time points post-transfection (5, 20, 30, 90, 150, and 300 min), the phagemid DNA was retrieved from the cells using the Qiagen Miniprep Kit (Qiagen) according to manufacturer's protocol. The retrieved phagemid DNA was aliquoted for 3 separate reactions: no digestion, *SacI* digestion, and *EcoRI* digestion. Digestions were carried out according to New England Biolabs recommended protocols for the respective enzymes in 20uL reaction volumes.

2.4 RNAi

Approximately 150,000 HCT116 cells were seeded on glass coverslips in the presence of gene-specific siRNA, Opti-mem low serum media (Life Technologies), and Lipofectamine 2000 (Life Technologies) according to manufacturer's protocol for reverse transfection. Twenty four hours post-siRNA transfection, media was replaced with growth DMEM (10% FBS, 1% antibiotic). Twenty four hours after the media change, cells were immunostained for APE1, Pol β , LIG1, or FEN1 as described in section 2.8. For APE1 KD 100 nM of each of two duplex siRNA sequences (Dharmacon) were used during transfection: 5'-CAAAGUUUCUUACGGCAUAUU-3' and 5'-ACAGCAAGAUCGUGUCCAAUU-3'. The other siRNA sequences and concentrations for transfection are as follows: 10 nM siPol β (5'-GAUCAGUAUUACUGUGGUGUU-3', Dharmacon), 50 nM siFEN1 (5'-GCAGCACAUGAUGAGUGCTT-3'; Ambion (18)), and 100nM siLIG1 (Ambion Silencer validated siRNA, proprietary sequence).

2.5 Western Blot

HCT116 whole cell extracts were prepared using M-PER Mammalian Protein Extraction Reagent (Pierce) according to manufacturer's protocol. Extracts were boiled in SDS-loading buffer and resolved on a 4-12% Bis-Tris polyacrylamide gel (Life Technologies) then transferred to nitrocellulose membrane. Western blotting was carried out with the affinity-purified anti-APE1 (1:1000; Novus Biologicals) and β -actin (1:1000; Sigma-Aldrich) monoclonal antibodies and a horseradish peroxidase (HRP)-linked anti-mouse secondary antibody (1:1000; Amersham Pharmacia Biotech) using enhanced chemiluminescent substrate (ThermoScientific) according to the manufacturer's protocol. Imaging of the bands was performed using X-ray film. For antibody specificity studies, Western blots were performed similarly using anti-Pol β (1:500; Abcam), anti-LIG1 (1:500; Novus Biologicals), and anti-FEN1 (1:1000; Abcam) antibodies and HRP-linked anti-mouse or rabbit secondary antibodies.

2.6 Real time PCR-based assay for AP-site repair

The real time PCR-based assay for repair was performed and analyzed as previously described with some modification and simplification (16,19). The control region was amplified using the following primers: forward 5'-GTGACGAACTCAGTGTTAC-3' and reverse 5'-TCGAGAGGGTTGATATAAG-3'. The MCS region was amplified using the following primers: forward 5'-TCCGGCTCGTATGTTGTGGAAT-3' and reverse 5'-AAGGCGATTAAGTTGGGTAACGCC-3'. Digested retrieved phagemid DNA samples were diluted 1:128 in molecular grade H₂O and served as templates in the PCR reactions. Each 20 μ L PCR reaction contained 1X Sybr green PCR mix (ABgene), 500nM of each primer, and 2 μ L of 1:128 diluted digested phagemid DNA samples. Reactions were carried out in duplicate in a 96-well plate under the following PCR conditions: 95°C for 10 min, 40 cycles of 95°C for 15 s, 53°C for 30 s, 72°C for 30 s, followed by a melt curve cycle to evaluate the specificity of the primers and overall reaction quality.

2.7 Analysis of real time PCR C_t values

The template samples used for PCR were the three digestion aliquots (undigested, *Sac*I, and *Eco*RI). For each sample a C_t value for the MCS region amplification (C_t-MCS) and a C_t value for the control region (C_t-control) were obtained. Then a ratio of C_t -MCS/C_t-control for each sample was calculated to determine the percent repair of AP-site DNA in cells. Therefore, for each sample, 3 ratios were obtained from the three digestion aliquots: undigested, *Sac*I, and *Eco*RI. The ratio of the undigested (uncut) sample for each time point was representative of unrepaired DNA while the ratio of the *Sac*I (completely linearized) sample for each time point was representative of completely repaired DNA. Recalling the amplification strategy described in part (c) of the general strategy outlined in the results section, the undigested ratio should therefore be the minimum ratio obtained while the *Sac*I ratio should be the maximum ratio for a given time point. The *Eco*RI ratio should lie between the maximum and minimum values. The percent repair at the *Eco*RI site was therefore calculated as:

$$Total\ repair = 100 \times \left[\frac{(\text{EcoRI ratio}) - (\text{undigested ratio})}{\text{SacI ratio}} \right]$$

2.8. Intracellular immunofluorescence staining

Approximately 400,000 HCT116 cells were seeded on glass coverslips in 6-well plates and allowed to attach overnight at 37°C followed by transfection with Cy3-labeled control or AP-site phagemids as described in section 2.3. HCT116 cells were fixed at various time points post-transfection (1, 2.5, 5, 20, 30 mins, 1.5, 2.5, and 5 h) with 4% paraformaldehyde for 10 min at room temperature. Cells were washed 3 times with 1X PBS, then permeabilized with 0.5% Triton X-100 in 1X PBS for 5 min at room temperature. The cells were subsequently blocked for 1 h at room temperature in 1% bovine serum albumin (BSA) in 1X PBS. Coverslips were incubated with anti-APE1 (1:2000; Novus Biologicals) and DAPI (600 nM; Life Technologies) for 1 h at room temperature. The cells were then washed 3 times in 1X PBS followed by incubation with anti-mouse secondary antibody conjugated to Alexafluor-488 (1:2000; Life Technologies) for 1 h at room temperature. The cells were again washed 3 times in 1X PBS followed by 2 washes with molecular grade H₂O. The coverslips were mounted onto glass slides with Tris-buffered fluorogel (Electron Microscopy Sciences) and allowed to set overnight at room temperature in the dark. Immunostaining after siRNA transfection as described in section 2.4 was performed similarly with anti-Polβ (1:1000; Abcam), anti-LIG1 (1:1000; Novus Biologicals), and anti-FEN1 (1:5000; Abcam) antibodies and anti-mouse or rabbit secondary antibody conjugated to Alexafluor-488 (1:2000; Life Technologies).

2.9 Imaging and co-localization analysis

After mounting the coverslips, the cells were imaged by confocal microscopy (Zeiss LSM510) using a 63X/1.4 N.A. oil lens. For detecting Cy3-labeled DNA, cells were excited at 543 nm and emission was detected using a 565-615 nm filter. For Alexafluor 488 fluorescence (APE1, Polβ, LIG1, or FEN1 detection), cells were excited at 488 nm and emission was detected using a 500 nm filter with a bandpass of 30 nm. For each time point, detector gain values were adjusted to maximize signal and minimize pixel saturation, and those settings were used to image both control and AP-site-transfected cells. Images were background subtracted, and the Pearson's correlation coefficient (r) of co-localization was determined by analyzing individual cells as selected regions of interest using the JACoP plugin from ImageJ (available from NIH). Cy3 fluorescence intensity for each image was quantified using ImageJ by measuring the mean gray value in the Cy3 channel for each image and normalized by dividing by the area quantified.

3. Results

3.1 Assay Design

We have established a new sensitive method for monitoring the recruitment of DNA repair proteins to AP sites in live human cells. The overall strategy is described in Fig. 1. We engineered a site-specific synthetic tetrahydrofuran (THF), which is a stable AP-site analog recognized by APE1 (11), on the M13mp18 phagemid (method described in detail below).

Of particular importance to understanding APE1 and other adduct-specific DNA-binding enzyme mechanisms is the ability to distinguish substrate DNA from a sea of non-specific DNA. In order to elucidate specific vs non-specific interaction, single damage and control (no damage) oligonucleotides were used to generate AP-site-containing or control (undamaged) double stranded phagemids via *in vitro* primer extension and ligation with a pool of dNTPs including Cy3-dCTP. The novelty of the described *in vitro* phagemid construction includes not only the incorporation of a single, site-specific damage but also the incorporation of a fluorescent marker, rendering the DNA visible under fluorescence microscope after transfection into cells. Quantification of Cy3 labeling of the phagemids indicated that approximately 5% of phagemid molecules were labeled with Cy3 as is shown in Fig. 1C. Assuming the Poisson distribution, the probability that a single phagemid contains more than one Cy3 molecule is 0.001. This indicates that the Cy3-labeled fraction of DNA contains mostly phagemids with 1 Cy3 molecule. Notably, transfection with this pool of labeled AP-site-containing phagemid results in labeling of the majority of cells in each well (Figure 1D). Untransfected cells imaged under the same conditions do not demonstrate measurable signal as shown in Figure 1E. Following transfection, the cells were analyzed in 2 ways: (a) by co-localization of Cy3-labeled DNA with Alexafluor-488-stained APE1 and (b) repair of transfected Cy3-labeled AP-site DNA to test the functionality of the interaction. It should be noted that the same reporter phagemid used in step (a) for detecting APE1 recruitment was used in step (b) for the repair study.

(a) Co-localization—After transfection with either Cy3-labeled control or AP-site DNA, the cells were fixed and stained for APE1 using an Alexafluor488-conjugated secondary antibody, so co-localization of Cy3 and Alexafluor-488 signals indicated APE1 is associated with the transfected DNA (Fig. 1B). Quantification of co-localization was achieved by determining the Pearson's correlation coefficient (r) in the nucleus, and the difference in r of APE1 for AP-site DNA vs. control DNA is compared over time post-transfection (Fig. 1A).

(b) Repair of AP-site DNA in cells—An important aspect of a robust method for detecting recruitment of repair proteins to a specific lesion is the quantification of functional interaction between the protein and DNA of interest. In the case of APE1, functional activity of the enzyme on the AP-site will result in complete repair of the DNA. We modified and simplified a previously described real time PCR-based method for assaying repair in cells to monitor repair of the engineered AP-site phagemid DNA (15,16,19). The strategy for the real time PCR-based AP-site repair assay is depicted in Fig. 2A. We have shown that the presence of adducts within a restriction site inhibits digestion by the restriction enzyme and is a reliable method of detecting lesions in a site-specific manner (15). The AP-site was placed in the *EcoRI* site on the M13mp18 phagemid rendering the phagemid resistant to *EcoRI* digestion (Fig. 2B). Thus, *EcoRI* treatment of the phagemids at various time points post-transfection into human cells can distinguish between unrepaired and completely repaired DNA. This strategy is similar to our previously developed *in vivo* plaque assay, which relies on the same principle, but we have changed the read-out from plaque number to real time PCR C_t values (15). The real time PCR-based assay for repair relies on the amplification of 2 regions of the M13mp18 phagemid, and is a modification of a previously described method (19). A control reaction amplifies a region not associated with the

multiple cloning site (MCS), serving as a template loading control, while a separate reaction amplifies the MCS, the target region where THF was placed. The principle of the assay is that target region amplification will occur if the DNA is undigested (THF at the *EcoRI* site restricts *EcoRI* from digesting the DNA), and amplification will not occur (or will be far less efficient) when the DNA is linearized by *EcoRI* digestion (*EcoRI* site only sensitive after THF repair). The phagemids are harvested post-transfection and aliquots are digested with *EcoRI*, *SacI*, or no enzyme. Thus, for each time point post-transfection, PCR is performed using 3 different digestions of the phagemid as template. The *SacI* site also lies in the MCS but is not modified, resulting in linearization regardless of repair status at the *EcoRI* site and making it an “expected maximum” C_t value for a given sample. The undigested phagemid (no enzyme) remains circular and the MCS region is amplifiable, making it an “expected minimum” C_t value for a given sample. These controls allow a repair percentage to be calculated from the resulting target region C_t values for the *EcoRI* digested aliquot.

3.2 Confirmation of *in vitro* plasmid construction and validity of the real time PCR-based assay for AP-site repair

The confirmation of AP-site presence in the M13mp18-AP DNA and not in the M13mp18-ctrl DNA and quality of the construct preparation are shown in Fig. 2B. APE1 cleaved the THF site (synthetic AP-site) converting nearly 100% of the covalently closed circular (CCC) DNA to nicked DNA (Fig. 2B). Restriction digestion of control DNA and modified DNA (containing THF at the *EcoRI* site) was performed to determine the effect of the AP-site on restriction digestion. The results for the *EcoRI* digestion demonstrated complete cleavage of the control DNA while the AP-site DNA was completely resistant. Both M13mp18-control and M13mp18-AP DNA are sensitive to *SacI* digestion.

In order to validate the real time PCR-based method of monitoring *in vivo* repair of abasic sites, a linear mixing experiment was performed *in vitro* to mimic a range of repair from 0-100%. M13mp18-AP phagemids containing a single AP-site were mixed with different amounts of M13mp18-control, which represented the repaired DNA. These standards were then assayed for repair, and the results were plotted against the expected repair values, such as 0, 10, 30, 50, 70, 90, and 100% based on the ratio of AP-site-containing DNA to undamaged DNA (Fig. 2C). The observed repair increased linearly with increasing amounts of undamaged DNA ($R^2 = 0.97$).

Cy3 appears to be a bulky adduct and may be repaired by the NER pathway. However, if Cy3 is repaired and removed from the phagemid DNA, it will defeat the purpose of the assay. Therefore, another construct was prepared by placing Cy3 on the *EcoRI* site and tested for repair in HCT116 cells by real time PCR along with the Cy3-labeled AP-site-containing phagemid. At 24 h post-transfection Cy3 was not repaired, while $\approx 95\%$ of AP-site DNA was repaired (Fig. 2D). The Cy3 itself was not repaired, but when it is present with the AP-site in the same DNA construct, it does not have any effect on AP-site repair in human cells. Taken together, the Cy3-labeled M13mp18-ctrl and -AP constructs were validated for use in the following *in vivo* microscopy study and functional validation of APE1-DNA interaction.

3.3 Co-localization of APE1 protein and Cy3-labeled DNA

Cy3-labeled control or AP-site DNA was transfected into HCT116 cells, and the cells were fixed and stained for APE1 and DAPI at several time points post-transfection (see Materials and Methods section). The Cy3-labeled DNA can be visualized as red dots in the cells while APE1 can be visualized by the green stain (Fig. 3A). The images in Fig. 3B are representative of key time points to demonstrate the specificity of the association between APE1 and AP-site DNA. To measure the extent of co-localization in the merge panels (Fig. 3A) we derived the Pearson's correlation coefficient (r) of co-localization at each timepoint post-transfection using the JACoP plugin in ImageJ (20) (Fig. 3B). Since the interaction of interest in this method is between a DNA-binding protein and DNA, it is necessary to distinguish between specific and nonspecific co-localization in order to validate the method. Importantly, the unmodified DNA is used in this analysis to demonstrate the “background” co-localization, which indicates the general non-specific association between APE1, a DNA-binding protein, and DNA. In the first 30 sec-1 min of transfection, the control DNA and APE1 have a relatively high r value (approximately 0.7), but the r value quickly decreases within 5 min post-transfection, reaching an r value of ≈ 0.3 -0.4, where it remains for the remainder of the time points, defining the r value of non-specific DNA association. On the other hand, from as early as 30 sec post-transfection, we obtained a high r of co-localization of AP-site DNA with APE1 protein (≈ 0.7), which remains until 30 min post-transfection when the r value steadily decreases until it reaches ≈ 0.4 , the non-specific r value of co-localization as revealed by the control-DNA transfected cells. An unpaired t-test of the time points between 5 and 30 min post-transfection demonstrated a significant difference between the control and AP-site DNA (p -value < 0.05). Of note, it may be argued that the difference in r values observed between the control and AP-site DNA could be due to a difference in the intensity of Cy3 fluorescence between the two samples possibly because of inefficient transfection or loss of the phagemid during the experiment. However, at 5 and 30 min post-transfection, when the difference between r values was greatest, there was no difference in Cy3 fluorescence intensity between control and AP-site DNA-transfected cells, nor was there a difference at the highest time point, 300 min (Fig. 3C). We noted that occasionally the transfected DNA (both AP-site and undamaged) would form aggregates in the cells, but we have found that these aggregates participate in repair since complete repair of AP-site-containing plasmid was observed (see Results Section 3.5). We also validated the primary antibody used to probe for APE1 by knocking down APE1 expression with gene-specific siRNA and immunostaining the cells in the same way as performed for the co-localization analysis (Figure 3D). Importantly, we found that the antibody was specific for APE1 since knockdown resulted in decreased signal (Figure 3D).

3.5 Functional validation of *in vivo* APE-AP-site interaction

In order to validate the functionality of the recruitment of APE1 to AP-site DNA observed by co-localization, the kinetics of AP-site repair *in vivo* were determined. Both the control and AP-site phagemids were transfected into colon cancer HCT-116 cells and harvested at 5, 20, 30, 90, 150, and 300 min post-transfection then assayed for repair. The time at which 50% AP-site DNA was repaired ($t_{1/2}$) was approximately 30 min post-transfection while 100% AP-site DNA was repaired at approximately 90 min post-transfection (Figure 4A).

These results were consistent with the co-localization results where the high r value for AP-site DNA decreased at 90 min to the r value of the undamaged DNA, indicating that repair was complete.

The rate determining steps of abasic site repair are post-AP-site incision, as determined *in vitro* (21), suggesting that the action of APE1 is rapid in the context of the repair pathway. This is interesting in relation to our results since APE1 was co-localized with the AP-site DNA for the entirety of the repair process, presumably after incision had taken place (Figures 3B and 4A). We asked whether similar fast kinetics of AP-site incision occurred in live cells as had been demonstrated *in vitro* by others. Therefore, we measured the rate of incised AP-site repair in human cells. Incised AP-site DNA was transfected into HCT116 cells and tested for repair at 5, 20, 30, 90, 150, and 300 min post-transfection (Fig. 4B). Of note, the incised AP-site is also resistant to *EcoRI* digestion, so the restriction enzyme digestion-based PCR method for monitoring repair of intact AP-sites can be utilized similarly. The $t_{1/2}$ for incised AP-site DNA was approximately 30 min post-transfection, which was similar to the $t_{1/2}$ observed for intact AP-site DNA, indicating that the APE1-directed incision step of AP-site repair was not rate-limiting, as had been previously determined *in vitro* (21).

3.6 Recruitment of downstream BER enzymes during repair of AP-sites *in vivo*

Since we had observed the recruitment of the initiating enzyme in the pathway, APE1, for the entirety of the repair process (Figure 3B), but its catalytic activity was not determining the rate of complete repair (Figure 4), we hypothesized that the other members of the repair pathway were being recruited during that time period. We decided to utilize our co-localization-based method to monitor the recruitment of the other repair enzymes and to determine the time kinetics of their arrival to the lesion. During single nucleotide base excision repair (SN-BER), natural AP-sites, like those produced after DNA glycosylase-mediated excision of an adduct, leave a 5'-deoxyribose phosphate (dRP) behind after incision by APE1. The resulting 1 nt gap is filled by DNA polymerase β (Pol β) via its catalytic activity as a DNA polymerase, and it removes the dRP via its dRP lyase activity (21). This results in a nick that is a substrate for DNA ligase nick sealing and the completion of repair. If the dRP is modified (oxidized or reduced), it cannot be removed by Pol β , and an alternative mechanism of repair occurs called long patch base excision repair (LP-BER), which involves the coordinated activities of Pol β polymerase activity and 5' flap incision by flap endonuclease 1 (FEN1) for multiple nucleotide incorporation (22-24). The action of FEN1 results in a nick that is ligated by DNA ligase 1 (LIG1). Specifically, the repair of the AP-site analog, THF, used in this study occurs by the LP-BER since the 5'-THF phosphate group generated after incision by APE1 mimics oxidized 5' dRP and cannot be removed by Pol β (25).

Using our co-localization method and immunostaining for the enzymes of interest, we followed the recruitment of APE1, Pol β , LIG1, and FEN1 to Cy3-labeled control or AP-site DNA in HCT116 cells. In our initial co-localization experiments with APE1 (Figure 3), we had observed an approximate 2-fold increase in r value for AP-site DNA over control for the duration of repair. Since each enzyme is expressed at different levels in the cells, which

affects the raw r value, we normalized the results for all the enzymes by examining the fold change of r for the AP-site DNA over the r for control DNA. We found that only APE1 was specifically co-localized with AP-site DNA 5 min post-transfection while the other enzymes exhibited no difference in r value for AP-site DNA over undamaged DNA (Figure 5A). At 15 min post-transfection, LIG1 was recruited to the AP-site DNA (1.8 ± 0.1 fold r value over undamaged DNA). Pol β demonstrated less co-localization than LIG1 and APE1 at this time point but exhibited increased co-localization (1.3 ± 0.017 fold r value over undamaged DNA) compared to 5 min post-transfection. FEN1, however, was still not recruited to the damaged plasmid at 15 min post-transfection (0.9 ± 0.2 fold r value over undamaged DNA) at which point APE1, Pol β , and LIG1 were recruited more significantly than FEN1 (Figure 5A; p -value < 0.05). Finally, at 30 min post-transfection, all four enzymes were co-localized with AP-site DNA, indicating that between 15 and 30 min, FEN1 was recruited to the repair site (Figure 5A) and there was no significant difference in recruitment among the four enzymes at 30 min post-transfection (Figure 5A). Representative images of co-localization of all the tested BER enzymes with AP-site DNA at 30 min are shown in Figure 5B. These results are consistent with results observed *in vitro* for LP-BER of a THF substrate. The incised THF has been shown to be a poor substrate for Pol β polymerase activity, but the presence of FEN1 stimulates Pol β -mediated multiple nucleotide insertion by the removal of the 5'-THF phosphate (24).

Importantly, at 90 min post transfection, no difference in r value was observed between AP-site plasmid and undamaged plasmid (Figure 5A). Thus, the timing of co-localization among the various repair enzymes was consistent with the time kinetics of repair (Figure 4A), an independent experiment from the microscopy-based technique. Again we also determined that there was no difference in Cy3 fluorescence intensity between control and AP-site DNA-transfected cells. Quantification of the 30 min post-transfection cells for each experiment is shown in Figure 5B. We also tested the specificity of the Pol β , LIG1, and FEN1 primary antibodies in the same manner as described for APE1 and found that the immunostain for each enzyme was specific (Figure 5C).

4. Discussion

The method presently described reliably detected and could monitor functional recruitment of repair proteins to AP-site DNA over time. Importantly, the method was able to successfully distinguish specific and non-specific co-localization between repair enzymes and lesion-containing DNA over time (Fig. 3 and 5). Moreover, this assay has the potential for broad applicability and may facilitate mechanism studies of initial steps in numerous nucleic acid transaction pathways *in vivo*, including replication, transcription, and recombination.

Specifically, the method described in the present study revealed valuable information about APE1 specific and non-specific DNA discrimination and abasic site repair kinetics *in vivo*. The dissociation constant (K_D) of the interaction between APE1 and AP-site DNA is lower than for undamaged DNA (0.87 nM vs. 5.3 nM, respectively) (6), which is reflected in the co-localization results by the longer residence time on AP-site containing DNA vs control DNA (Fig. 3 and 5). The 6-fold difference in K_D of APE1 for specific and non-specific

DNA determined *in vitro* (6) might have predicted that the present method may not be sensitive enough to detect a difference in co-localization of APE1 with control vs AP-site DNA, but we observed a significant difference in *r* value post-transfection. The initial high *r* value of co-localization between APE1 and both control and AP-site DNA (0.7) is expected since APE1 is a DNA-binding protein and is known to associate with DNA. However, the relatively shorter “high” association time (1 min) between the enzyme and undamaged DNA as compared to the longer “high” association time between APE1 and AP-site DNA (30 min) demonstrates substrate recognition and discrimination by APE1 (Fig. 3). These results show the interaction time between APE1 and abasic site DNA is longer than with undamaged control DNA, which not only shows the specificity of APE1 for AP-site DNA but is also consistent with the known *in vitro* kinetics of APE1-DNA interactions. These results demonstrate an amazing property of APE1, which is to recognize a single abasic site in a 7.2 kb phagemid. Importantly, the specific association can be compared to the non-specific association and reveals a significant difference in interaction time, suggesting that this described microscopy-based method is a useful and valid method of monitoring specific DNA-protein interactions inside cells.

Of note, our method confirmed a previously assumed property of APE1 *in vivo* as it had only been determined *in vitro*, which is that APE1 is an extremely fast enzyme as has been demonstrated by pre-steady state kinetics studies (11). The rate of AP-site incision by APE1 was so fast that a maximal single turnover rate could not be measured and was at least 850s^{-1} (11). The same study demonstrated that the rate of product dissociation by APE1 was relatively slower and was rate limiting in the entire catalytic cycle, making the steady state incision rate approximately 2s^{-1} . By utilizing incised AP-site DNA as a substrate, no difference in *in vivo* repair kinetics was observed compared to AP-site DNA (Fig. 4A and 4B), which correlates with an *in vitro* BER reconstitution experiment where a post-AP-site incision step was shown to be the rate-determining step of BER of abasic sites (21). APE1 rapidly binds the substrate and catalyzes hydrolysis, then dissociates from the product as indicated by the combined microscopy-based and real time PCR-based analyses of the full reaction.

Interestingly, the newly developed method could detect specific recruitment of downstream BER proteins as well, and recruitment time kinetics were consistent with AP-site repair time kinetics (Figures 4 and 5). Recruitment of the various enzymes was observed until 30 min post-transfection; then, at 90 min post-transfection, when repair is complete (Figure 4A), the enzymes are no longer differentially recruited to AP-site DNA over undamaged DNA (Figures 3B and 5A), which is consistent with the completion of repair, meaning the plasmid is no longer damaged.

Interestingly, APE1 and LIG1 were co-localized with damaged DNA simultaneously earlier during repair at the 15 min time point (Figure 5A), which is somewhat unexpected given the role of LIG1 in the last step of repair (26). This may be explained by the interaction between LIG1 and APE1, which has been demonstrated *in vitro* (27). It is possible that APE1 recruits LIG1 through protein-protein interaction even before its activity is needed. This explanation is consistent the results of the study by Ranalli et al. demonstrating APE1 as a far-reaching coordinator of LP-BER by stimulating the activities of downstream enzymes such as LIG1

and FEN1 (27). In fact, the same study reported that LIG1 alone demonstrated poor enzymatic activity on nicked DNA, but the addition of APE1 could stimulate its activity 10-fold. Those results indicate that APE1 may be a requirement for robust ligation and completion of repair, so it is possible that LIG1 is recruited more efficiently by APE1 protein rather than its nick substrate.

The recruitment of Pol β and FEN1 at approximately the same time point toward the end of repair (30 min time point) is expected and consistent with the “hit and run” mechanism of LP-BER described by Liu et al. (24) where Pol β and FEN1 alternate nucleotide insertion and flap cleavage activities, respectively, until the point at which LIG1 seals the nick and repair is complete. Traditionally, it had been thought that FEN1 cleaved the product of strand displacement by Pol β during repair synthesis (28). However, Liu et al. demonstrated that FEN1 cleavage of the blocked 5' end plus one nucleotide during repair is required before Pol β can insert the second nucleotide, meaning there is no strand displacement by Pol β (24). Notably, our results are consistent with those *in vitro* findings and support the “hit and run” LP-BER mechanism for repair since efficient recruitment of Pol β to the repair intermediate required the presence of FEN1.

Fluorescence microscopy-based strategies can be and are used to study the interaction between proteins and specific DNA adducts when an antibody is available to probe for the adduct of interest. For example, useful antibodies against cyclopyrimidine dimers (CPDs) and 6-4 photo products (6-4 PP) are used in the study of NER mechanisms in live cells (29). However, few powerful antibodies against adducts repaired by BER are available, with the exception of 8-hydroxydeoxyguanosine (8-oxo-dG), which has been used as a clinical prognostic tool (30). Even with the use of anti-CPD and anti-6-4 PP antibodies, studies of repair pathways are limited by the lack of information about repair intermediate structures. A previous published report on NER using these antibodies relied on the presence of repair proteins known to be involved at different repair intermediates since specific tools to monitor those intermediates in genomic DNA after UV damage were not available (29). By modifying our phagemid-based method to probe for various repair intermediates in addition to the DNA damage, the method can report the status of DNA damage and repair intermediates interacting with various repair proteins in a specific pathway.

DNA-protein interactions can be monitored by microscopy in genomic DNA using the fluorescent DNA stain Sytox Orange (31). However, at best, specificity of interaction at a certain binding site must be inferred with the use of a mutant protein that cannot bind its appropriate sequence. Even so, this type of assay cannot be used for the investigation of DNA adduct-protein interactions. A recent report by Lan et al. described a site-specific method of monitoring ROS-induced damage in genomic DNA by microscopy-based methods (32). This method is particularly powerful for understanding overall oxidative DNA damage repair. However, it is not lesion specific, and again, at best, only lesions with available antibody-based detection strategies can be studied. As previously stated, antibodies are often unavailable for the critical DNA adducts to be studied, and even the specificity of available antibodies is often questionable.

Interestingly, our results also supported those of another live cell study of LP-BER in which UV damage endonuclease (UVDE) was used to create a strand break 5' to a UV-induced photoproduct in NER-deficient cells. This strand break with a 5' photoproduct is repaired by Pol β –mediated LP-BER (33). The recruitment of GFP-tagged Pol β and FEN1 to the UV damage sites was monitored by microscopy, in which FEN1 demonstrated slightly slower recruitment kinetics than Pol β , which was observed in our study as well (33). Notably, our newly developed method provides greater substrate flexibility than the UVDE-based method since any commercially available adduct can be placed in the phagemid. Additionally, the phagemid-based method does not require the use of a specific cell line. A concern may arise that this phagemid DNA-based assay may not provide insight into biologically relevant DNA-protein interactions within chromatin. However, reporter plasmid DNA has been known to form a chromatin-like structure in cells and reliably mimic nuclear functions (34,35). Notably, promoter activity is often measured using the luciferase-based reporter assay, which is, in fact, the gold standard method used extensively in gene expression and transcription mechanism studies, and relies on a plasmid-based system to understand nuclear biology. Additionally, the LP-BER kinetics of complete THF repair in our plasmid-based study (≈ 90 min) was comparable to the results of the UVDE-based strategy for detecting LP-BER-mediated photoproduct clearance in nuclear DNA (1 h) discussed above (33).

In conclusion, the presently described techniques have been shown to be powerful in monitoring the recruitment of DNA-binding proteins to specific DNA structures *in vivo*. The microscopy-based methodology can be applied to other interactions of interest in a variety of disciplines of nucleic acid transaction pathways other than DNA repair, including replication, transcription, recombination, and others. In regard to versatility in the field of DNA repair, many modified bases of interest are commercially available for synthesis in an oligonucleotide sequence as described in this manuscript. The relatively slight modifications required for application to other disciplines may include the development of a structure-specific DNA substrate that is transfectable into cells and able to be fluorescently labeled. Furthermore, the real time PCR-based repair assay described in this manuscript can be widely applicable to other adducts of interest in the field of DNA repair, but for application to another field, an appropriate method for detection of enzyme functionality on the labeled substrate may need to be applied. However, the microscopy-based assay for monitoring specific DNA-protein relationships described here has the potential for broad applicability and may facilitate mechanism studies of initial steps in numerous nucleic acid transaction pathways *in vivo*.

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References

1. Pfeifer GP, Steigerwald SD, Mueller PR, Wold B, Riggs AD. Genomic sequencing and methylation analysis by ligation mediated PCR. *Science*. 1989; 246:810–813. [PubMed: 2814502]

2. Pfeifer GP, Chen HH, Komura J, Riggs AD. Chromatin structure analysis by ligation-mediated and terminal transferase-mediated polymerase chain reaction. *Methods in enzymology*. 1999; 304:548–571. [PubMed: 10372381]
3. Kuo MH, Allis CD. In vivo cross-linking and immunoprecipitation for studying dynamic Protein:DNA associations in a chromatin environment. *Methods*. 1999; 19:425–433. [PubMed: 10579938]
4. Adhikari S, Uren A, Roy R. Excised damaged base determines the turnover of human N-methylpurine-DNA glycosylase. *DNA repair*. 2009; 8:1201–1206. [PubMed: 19616486]
5. Adhikari S, Uren A, Roy R. N-terminal extension of N-methylpurine DNA glycosylase is required for turnover in hypoxanthine excision reaction. *The Journal of biological chemistry*. 2007; 282:30078–30084. [PubMed: 17716976]
6. Adhikari S, Uren A, Roy R. Dipole-dipole interaction stabilizes the transition state of apurinic/aprimidinic endonuclease--abasic site interaction. *The Journal of biological chemistry*. 2008; 283:1334–1339. [PubMed: 18025089]
7. Kneale, GG., editor. *DNA-Protein Interactions: Principles and Protocols*. Humana Press; Totowa, NJ: 1994.
8. Sauer, RT., editor. *Protein-DNA Interactions*. Academic Press Inc.; San Diego, CA: 1991.
9. Lindahl T, Nyberg B. Rate of depurination of native deoxyribonucleic acid. *Biochemistry*. 1972; 11:3610–3618. [PubMed: 4626532]
10. Atamna H, Cheung I, Ames BN. A method for detecting abasic sites in living cells: age-dependent changes in base excision repair. *Proceedings of the National Academy of Sciences of the United States of America*. 2000; 97:686–691. [PubMed: 10639140]
11. Maher RL, Bloom LB. Pre-steady-state kinetic characterization of the AP endonuclease activity of human AP endonuclease 1. *The Journal of biological chemistry*. 2007; 282:30577–30585. [PubMed: 17724035]
12. Adhikari S, Manthena PV, Kota KK, Karmahapatra SK, Roy G, Saxena R, Uren A, Roy R. A comparative study of recombinant mouse and human apurinic/aprimidinic endonuclease. *Molecular and cellular biochemistry*. 2012; 362:195–201. [PubMed: 22042551]
13. Wilson DM 3rd, Barsky D. The major human abasic endonuclease: formation, consequences and repair of abasic lesions in DNA. *Mutation research*. 2001; 485:283–307. [PubMed: 11585362]
14. Nakamura J, Swenberg JA. Endogenous apurinic/aprimidinic sites in genomic DNA of mammalian tissues. *Cancer research*. 1999; 59:2522–2526. [PubMed: 10363965]
15. Choudhury S, Adhikari S, Cheema A, Roy R. Evidence of complete cellular repair of 1,N6-ethenoadenine, a mutagenic and potential damage for human cancer, revealed by a novel method. *Molecular and cellular biochemistry*. 2008; 313:19–28. [PubMed: 18373235]
16. Woodrick J, Gupta S, Khatkar P, Sarangi S, Narasimhan G, Trehan A, Adhikari S, Roy R. Slow repair of lipid peroxidation-induced DNA damage at p53 mutation hotspots in human cells caused by low turnover of a DNA glycosylase. *Nucleic acids research*. 2014; 42:9033–9046. [PubMed: 25081213]
17. Adhikari S, Manthena PV, Sajwan K, Kota KK, Roy R. A unified method for purification of basic proteins. *Analytical biochemistry*. 2010; 400:203–206. [PubMed: 20109435]
18. Sampathi S, Bhusari A, Shen B, Chai W. Human flap endonuclease I is in complex with telomerase and is required for telomerase-mediated telomere maintenance. *The Journal of biological chemistry*. 2009; 284:3682–3690. [PubMed: 19068479]
19. Lee HW, Lee HJ, Hong CM, Baker DJ, Bhatia R, O'Connor TR. Monitoring repair of DNA damage in cell lines and human peripheral blood mononuclear cells. *Analytical biochemistry*. 2007; 365:246–259. [PubMed: 17449003]
20. Zinchuk V, Zinchuk O, Okada T. Quantitative colocalization analysis of multicolor confocal immunofluorescence microscopy images: pushing pixels to explore biological phenomena. *Acta histochemica et cytochemica*. 2007; 40:101–111. [PubMed: 17898874]
21. Srivastava DK, Berg BJ, Prasad R, Molina JT, Beard WA, Tomkinson AE, Wilson SH. Mammalian abasic site base excision repair. Identification of the reaction sequence and rate-determining steps. *The Journal of biological chemistry*. 1998; 273:21203–21209. [PubMed: 9694877]

22. Robertson AB, Klungland A, Rognes T, Leiros I. DNA repair in mammalian cells: Base excision repair: the long and short of it. *Cellular and molecular life sciences : CMLS*. 2009; 66:981–993. [PubMed: 19153658]
23. Dianov GL, Prasad R, Wilson SH, Bohr VA. Role of DNA polymerase beta in the excision step of long patch mammalian base excision repair. *The Journal of biological chemistry*. 1999; 274:13741–13743. [PubMed: 10318775]
24. Liu Y, Beard WA, Shock DD, Prasad R, Hou EW, Wilson SH. DNA polymerase beta and flap endonuclease 1 enzymatic specificities sustain DNA synthesis for long patch base excision repair. *The Journal of biological chemistry*. 2005; 280:3665–3674. [PubMed: 15561706]
25. Masaoka A, Horton JK, Beard WA, Wilson SH. DNA polymerase beta and PARP activities in base excision repair in living cells. *DNA repair*. 2009; 8:1290–1299. [PubMed: 19748837]
26. Prasad R, Singhal RK, Srivastava DK, Molina JT, Tomkinson AE, Wilson SH. Specific interaction of DNA polymerase beta and DNA ligase I in a multiprotein base excision repair complex from bovine testis. *The Journal of biological chemistry*. 1996; 271:16000–16007. [PubMed: 8663274]
27. Ranalli TA, Tom S, Bambara RA. AP endonuclease 1 coordinates flap endonuclease 1 and DNA ligase I activity in long patch base excision repair. *The Journal of biological chemistry*. 2002; 277:41715–41724. [PubMed: 12200445]
28. Klungland A, Lindahl T. Second pathway for completion of human DNA base excision-repair: reconstitution with purified proteins and requirement for DNase IV (FEN1). *The EMBO journal*. 1997; 16:3341–3348. [PubMed: 9214649]
29. Luijsterburg MS, von Bornstaedt G, Gourdin AM, Politi AZ, Mone MJ, Warmerdam DO, Goedhart J, Vermeulen W, van Driel R, Hofer T. Stochastic and reversible assembly of a multiprotein DNA repair complex ensures accurate target site recognition and efficient repair. *The Journal of cell biology*. 2010; 189:445–463. [PubMed: 20439997]
30. Sova H, Jukkola-Vuorinen A, Puistola U, Kauppila S, Karihtala P. 8-Hydroxydeoxyguanosine: a new potential independent prognostic factor in breast cancer. *British journal of cancer*. 2010; 102:1018–1023. [PubMed: 20179711]
31. Cremazy FG, Manders EM, Bastiaens PI, Kramer G, Hager GL, van Munster EB, Verschure PJ, Gadella TJ Jr, van Driel R. Imaging in situ protein-DNA interactions in the cell nucleus using FRET-FLIM. *Experimental cell research*. 2005; 309:390–396. [PubMed: 16040027]
32. Lan L, Nakajima S, Wei L, Sun L, Hsieh CL, Sobol RW, Bruchez M, Van Houten B, Yasui A, Levine AS. Novel method for site-specific induction of oxidative DNA damage reveals differences in recruitment of repair proteins to heterochromatin and euchromatin. *Nucleic acids research*. 2014; 42:2330–2345. [PubMed: 24293652]
33. Asagoshi K, Liu Y, Masaoka A, Lan L, Prasad R, Horton JK, Brown AR, Wang XH, Bdour HM, Sobol RW, et al. DNA polymerase beta-dependent long patch base excision repair in living cells. *DNA repair*. 2010; 9:109–119. [PubMed: 20006562]
34. Magin S, Saha J, Wang M, Mladenova V, Coym N, Iliakis G. Lipofection and nucleofection of substrate plasmid can generate widely different readings of DNA end-joining efficiency in different cell lines. *DNA repair*. 2013; 12:148–160. [PubMed: 23286905]
35. Mladenova V, Mladenov E, Russev G. Organization of plasmid DNA into nucleosome-like structures after transfection in eukaryotic cells. *Biotechnology & Biotechnological Equipment*. 2009; 23:1044–1047.

Abbreviations

AP-site	abasic site
APE1	apurinic apyrimidinic endonuclease 1
BER	base excision repair
Polβ	DNA polymerase β

LIG1	DNA ligase 1
FEN1	flap endonuclease 1

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Highlights

- A method of monitoring lesion-specific recruitment of proteins *in vivo* is described.
- Recruitment of repair enzymes to abasic sites is monitored by co-localization.
- Repair protein recruitment is consistent with known protein-protein relationships.
- Cells demonstrated complete repair of abasic sites by 90 minutes.

Fig. 1. Experimental strategy and fluorophore labeling of plasmid DNA

(A) Experimental strategy for detecting recruitment and functional interaction of DNA repair proteins with AP-site DNA. (B) Schematic diagram of Cy3-labeled AP-site DNA co-localizing with Alexafluor-488 –labeled APE1. (C) The percent of M13mp18-control or –AP plasmid DNA preparations containing Cy3 was quantified. Error bars indicate SEM of the measurement of 3 separate dilutions of the plasmids. (D) Representative images of HCT116 cells post-Cy3-labeled AP-site DNA transfection. Images acquired using 40 $\times$ /1.3 N.A. oil lens. (E) Representative images of untransfected HCT116 cells imaged with the Cy3 excitation and emission conditions described in the Materials and Methods section. Images acquired using 63 $\times$ /1.3 N.A. oil lens.

Fig. 2. Validity of plasmid preparation and real time PCR-based assay for repair of AP-sites *in vivo*

(A) Strategy for real time PCR-based assay for repair of AP-sites *in vivo*. (B) Validity of M13mp18-ctrl or -AP plasmid DNA preparations shown by APE1 and restriction enzyme treatments. Treated samples were resolved on a 1% agarose gel and imaged after ethidium bromide staining. CCC, covalently closed circular. (C) Mixing experiment demonstrating the linearity of the repair assay. (D) Two Cy3-labeled constructs were tested for repair in HCT116 cells, one containing an AP-site on the *EcoRI* site (Cy3-AP site)- and one containing Cy3 on the *EcoRI* site (Cy3-Cy3). Repair was assayed 24 h post-transfection. Error bars indicate SEM from 3 independent experiments. Expression of APE1 in HCT116 cells as determined by Western blot is shown in the inset.

Fig. 3. Nuclear co-localization of Cy3-labeled plasmid DNA and APE1

(A) Representative cell images from 1, 5, and 90 min post-transfection of either Cy3-M13mp18-Ctrl or Cy3-AP site plasmid DNA. Images acquired using 63X/1.4 N.A. oil lens. White dotted line indicates the nucleus. (B) Pearson's correlation coefficient (r) of co-localization quantified at the specified time points post-transfection of Cy3-M13mp18-Ctrl or Cy3-AP site plasmid DNA; values indicate the average r for each time point. Error bars indicate SEM from 3 independent experiments, $n = 15$ cells. Bracket indicates the range of time points in which a significant difference between two points was calculated by t-test, * p -value < 0.05 . (C) The average intensity of Cy3 fluorescence was measured at representative time points during the kinetic experiments. Graph indicates the average ratio of Cy3 mean gray value/area measured in the field. Error bars indicate SEM from 3 independent experiments, $n = 15$ cells. (D) Validation of APE1 antibody. HCT116 cells were transfected with non-targeting negative control siRNA (siCtrl) or APE1-specific siRNA (siAPE1) and analyzed for APE1 expression by Western blot using whole cell extracts and by immunostaining with anti-APE1 antibody and Alexafluor 488-conjugated secondary antibody. HDAC1 expression was used as a loading control in Western blot.

Fig. 4. Functional validation of APE1-AP-site interaction *in vivo*

(A) Kinetics of repair of AP-sites in HCT116 cells. (B) Kinetics of repair of incised AP-sites in HCT116 cells. Error bars in both (A) and (B) indicate SEM from 3 independent experiments.

Fig. 5. Recruitment of downstream BER enzymes during repair of AP-sites *in vivo*

(A) Pearson's correlation coefficient (r) of co-localization quantified at the specified time points post-transfection of Cy3-M13mp18-Ctrl or Cy3-AP site plasmid DNA; values indicate the fold change of average r for AP-site DNA over the average r for control DNA for each time point. Error bars indicate SEM from 3 independent experiments, $n = 15$ cells. Significance determined by t-test. * p -value < 0.05 , ns = not significant. (B) Representative images from 30 min post-transfection of either Cy3-M13mp18-Ctrl or Cy3-AP site plasmid DNA. Cells were immunostained for APE1, Polβ, LIG1, or FEN1 using specific primary antibodies and Alexafluor-488-conjugated secondary antibody. Images acquired using 63X/1.4 N.A. oil lens. Bar graphs show the mean Cy3 intensity of cells containing Cy3-M13mp18-Ctrl or Cy3-AP site plasmid DNA for each experiment. Error bars indicate SEM from 3 independent experiments, $n = 15$ cells. Significance determined by t-test, ns = not significant. (C) Validation of Polβ, LIG1, and FEN1 antibodies. HCT116 cells were transfected with non-targeting negative control siRNA (siCtrl) or gene-specific siRNA and analyzed for protein expression by Western blot using whole cell extracts and by immunostaining with the same primary antibody and Alexafluor 488-conjugated secondary antibody used in the co-localization studies. HDAC expression was used as a loading control in Western blot.