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Short communication

A multiplex assay based on encoded microbeads conjugated to DNA NanoBeacons to monitor base excision repair activities by flow cytometry

Guillaume Gines, Christine Saint-Pierre, Didier Gasparutto *

Laboratoire des Lésions des Acides Nucléiques, INAC/SCIB UMR_E3 CEA/UJF-Grenoble 1/CEA Grenoble, 17 rue des Martyrs, 38054 Grenoble cedex 9, France

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ABSTRACT

We reported here a new assay to detect base excision repair activities from purified enzymes, as well as in cell-free extracts. The multiplex format rests upon the encoding of magnetic beads with the fluorophore Alexa 488, thanks to a fast and original procedure. Fluorescently encoded microbeads are subsequently functionalized by lesion-containing DNA NanoBeacons labeled with the fluorophore/quencher pair Cyanine 5/BHQ2. Probes cleavage, induced by targeted enzymes leads to Cyanine 5 signal enhancement, which is finally quantified by flow cytometry. The multiplex assay was applied to the detection of restriction enzymes activities as well as base excision repair processes. Each test requires only 500 fmol of DNA substrate, which constitutes great sensitivity compared to other BER functional assays. The present biosensor is able to detect both uracil DNA *N*-glycosylase (UNG) and AP-endonuclease 1 (APE1) within few nanograms of nuclear extract. Additionally, we demonstrated that the corresponding assay has potential application in DNA repair inhibitor search. Finally, the current multiplexed tool shows several advantages in comparison to other functional BER assays with no need of electrophoretic separation, straightforward, easy and reproducible functionalization of encoded microbeads and a high stability of DNA probes in cell-free extracts.

1. Introduction

From prokaryotes to humans, cells are equipped with several DNA repair mechanisms to deal with the great diversity of nucleic damages produced by endogenous and exogenous agents (e.g. reactive oxygen species, UV and ionizing radiations, chemicals, etc.) (Friedberg et al., 2006; Gates, 2009; Schärer, 2003). Among them, base excision repair (BER) is a crucial DNA repair pathway that removes a large panel of DNA lesions including oxidized, alkylated, and deaminated bases as well as abasic sites and single-strand breaks by an excision/resynthesis mechanism (Almeida and Sobol, 2007; Baute and Depicker, 2008). First, the damaged base is recognized and eliminated by a DNA *N*-glycosylase that cleaves the *N*-glycosydic bond. The newly-formed AP-site is then incised by an AP-endonuclease generating a nick in the phosphate-sugar backbone of the DNA helix. Next steps involve the removal of the sugar moiety followed by the reincorporation of the correct nucleotide, mainly by the polymerase β. Repair is completed by a ligase that restores the strand continuity. BER is essential, exemplified by various pathologies clearly associated with DNA repair dysfunction (hyper IgM syndrome, MUTYH-associated polyposis, etc.) (Dunlop and Farrington, 2009; Kavli et al., 2005).

To better understand this central process, it is of paramount importance to develop fast, easy and sensitive assays to measure BER activities from purified enzymes or from cell extracts. Further, these assays must be suitable to guide diseases diagnostics and establish a screening method to identify DNA repair inhibitors (Madhusudan and Hickson, 2005). Numerous assays are based on the detection of the DNA cleavage induced at the damaged site by the joined action of a DNA *N*-glycosylase (excision of the altered nucleobase) and an AP-endonuclease (incision of the phosphodiester bond) – classical methods used lesion-containing oligonucleotide substrates labeled by a fluorescent dye or a radioactive marker (Swain and Subba Rao, 2011; Tchou et al., 1991; Vik et al., 2012). The enzymatic cleavage of such probes is detected by separative techniques, mainly by polyacrylamide gel electrophoresis which is not suitable for multiplexed and high-throughput assays. The use of molecular beacons has been extended to the detection of DNA repair activities based on the synthesis of selectively damaged probes (Chollat-Namy et al., 2005; Maksimenko et al., 2004; Matsumoto et al., 2010). Although they are easy to use in a continuous assay, the multiplexing potential of such probes is limited by the choice of spectrally resolved fluorophores for each molecular beacon. To circumvent these limitations, several biochips using immobilized oligonucleotide substrates have been

* Corresponding author. Tel.: +33 4 38 78 45 47; fax: +33 4 38 78 50 90.
E-mail address: didier.gasparutto@cea.fr (D. Gasparutto).

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developed in our laboratory (Corne et al., 2008; Sauvaigo et al., 2004); however their conception is time-consuming and required dedicated and expensive equipment.

Herein, we report an alternative on-support fluorescent multiplex assay. The principle rests upon the functionalization of fluorescently encoded magnetic beads (Alexa 488) with lesion-containing DNA NanoBeacons (NBs), labeled with the Cyanine 5/BHQ2 chromophores pair. BER activities at the damaged site induced quenched probes incision followed by dehybridization of the DNA fragment containing the quencher. The distance separation of the two dyes results in fluorescence enhancement at the bead surface. Microbeads are analyzed in two colors by flow cytometry, the fluorescence intensity of Alexa 488 corresponding to the bead barcode and the Cy5 signal intensity corresponding to the enzymatic activity.

2. Experimental section

2.1. Material and reagents

All solvents and chemicals reagents were purchased from Aldrich-Sigma (Saint Quentin-Fallavier, France) and used without further purification. Purified enzymes (Pst1, *Escherichia Coli* uracil *N*-glycosylase [UNG] and AP-endonuclease 1 [APE1]) and corresponding buffers were obtained from Trevigen and New England Biolabs (see detailed protocols from suppliers). Nuclear extracts from HeLa cells were purchased from Cilbiotech, aliquoted at 9 μg/μL and kept frozen at −80 °C. 4–4.5 μm biotinylated microspheres were provided by Kisker Biotech (Steinfurt, Germany).

2.2. DNA nanoprobes synthesis

Oligonucleotides (see Fig. S1 for sequences) were synthesized on an automated nucleic acid synthesizer *Applied Biosystems 392 DNA/RNA Synthetizer*, using the classical cyanoethylphosphoramite chemistry. Oligonucleotides were firstly purified by denaturing polyacrylamide gel electrophoresis and once again by reversed phase HPLC with a Hypersil HS C18 column heated at 50 °C. The integrity of the sequences was controlled by denaturing PAGE and Maldi-ToF mass spectrometry measurements. Finally, the fluorescent DNA probes were lyophilized and frozen at −20 °C until used.

2.3. Encoding of microbeads and nucleic acid probes immobilization

10 μL of biotinylated beads from the storage vial were withdrawn, washed three times with 1 × Binding and Washing buffer (B&W; 5 mM Tris–HCl pH 7.5, 0.5 mM EDTA, and 1 M NaCl). The desired ratio of streptavidin/streptavidin Alexa 488-conjugated (5 μL, 1 μg/mL in PBS) was added and the mixture was incubated 15 min at room temperature under gentle agitation. Beads were washed five times with 1 × B&W buffer to remove excess of streptavidin. Microspheres are resuspended in 25 μL of the same buffer. Before immobilization, biotin-modified oligonucleotides underwent a hybridization cycle in 1 × B&W buffer, 2 min at 90 °C and were allowed to cool slowly (1 h) to promote annealing. Probe (15 μL, 1 μM) was added on the microbeads suspension and agitated 1 h at room temperature. Functionalized beads were washed three times with 1 × B&W and resuspended in 40 μL of 1 × digestion buffer (10 mM HEPES/KOH (pH 7.8), 2 mM EGTA/KOH, 80 mM KCl, 0.1 mM $ZnCl_2$, 1 mM DTT, and 0.5 mg/mL BSA).

2.4. Enzymatic digestion and flow cytometry analysis

Enzymatic reactions were carried out from 1 μL of each beads batch (functionalized with either NB-Pst1 or NB-THF or NB-Ura). The enzyme or HeLa nuclear extract, buffer and ultrapure water were added and the incubation was pursued for 1 h at 37 °C. After incubation, 200 μL of PBS complemented with 0.02% of Tween20 was added to each sample. Flow cytometry analyses were conducted on a FACScalibur machine (Becton Dickinson). A minimum of 10^4 events for each bead populations were collected to calculate mean fluorescence intensities (MFI). Data were reprocessed with FCSexpress software (De Novo Software).

3. Results and discussion

As illustrated in Fig. 1, the multiplex assay comprises of four steps: (i) encoding of the biotinylated magnetic microbeads (4.5 μm in diameter) with several ratios of unlabeled streptavidin/Alexa 488-conjugated streptavidin, (ii) functionalization of streptavidin-bridged encoded particles with biotinylated hairpin-shaped DNA probes, labeled with Cy5/BHQ2 dyes, (iii) incubation of the resulting bead populations with the biological sample (purified enzymes, cellular extracts), and (iv) dual color flow cytometry analysis of samples. The cytometer measures the signal of Alexa 488 and Cy5 for each bead so that the fluorescence signature of the bead populations is attributed to a specific DNA probe which allows to distinguish the different targeted activities.

3.1. Microbeads barcoding

The preparation of optically encoded microbeads was achieved using biotinylated magnetic beads saturated with defined ratios of unlabeled streptavidin/Alexa 488-conjugated streptavidin. The adjustment of encoding conditions and probes immobilization is shown in ESI Fig. S2. We can obtain 8 bead populations that are perfectly resolved by flow cytometry and exhibit discrete fluorescent signals (Fig. 2A). We verified that the fluorescence signal for each bead cluster is proportional to the amount of fluorescent streptavidin (Fig. 2B).

3.2. Purified enzyme detection

As a proof of concept, three streptavidin-coated bead populations were functionalized with three different damaged NBs (see Fig S1 for sequences) that contain biotin at the 3'-end. NBs were double labeled with fluorophore cy5 proximal to the 3'-biotinylated end, and the BHQ2 quencher inserted at the 5'-extremity of stem-loop structures. The NB-Pst1 probe contains the restriction site of the enzyme Pst1 in the stem. An abasic-site analog, namely tetrahydrofuran, is introduced within the NB-THF probe. It has previously been shown that this analog is efficiently recognized and incised by APE1 (Wilson et al., 1995). The NB-Ura bears a single uracil as the deamination product of cytosine. This base in DNA is specifically excised by UNG (Lindahl, 1974; Slupphaug et al., 1995). After the action of UNG, the newly-formed AP-site can be processed and incised by APE1. Together, the three DNA functionalized bead populations constitute a triplex biosensor able to detect selectively Pst1, APE1 and UNG activities in a simultaneous manner. As shown in Fig. 3, the incubation of the biosensor with the restriction enzyme leads to the drastic increase in mean fluorescence intensity (MFI) of NB-Pst1 beads and, to a lesser extent, of NB-THF particles. It is noteworthy that the tetrahydrofuran moiety was introduced in the restriction site of Pst1 enzyme (Fig. S1) and so, does not totally prevent Pst1 activity. APE1 activity leads exclusively to the NB-THF cleavage resulting in a specific fluorescence enhancement of the corresponding functionalized microbeads. The co-incubation of the supported probes with APE1 and UNG induces fluorescence increase of both NB-THF and NB-Ura beads, as expected. The further addition of uracil glycosylase

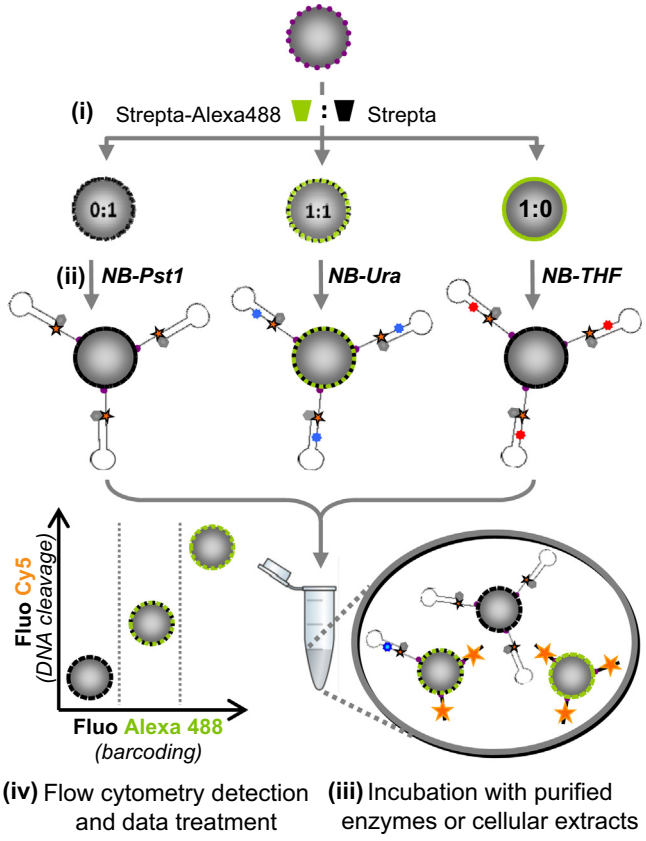

Fig. 1. Schematic illustration of the multiplex assay to monitor DNA repair activities by flow cytometry using fluorescently encoded magnetic beads functionalized with NanoBeacons (NBs).

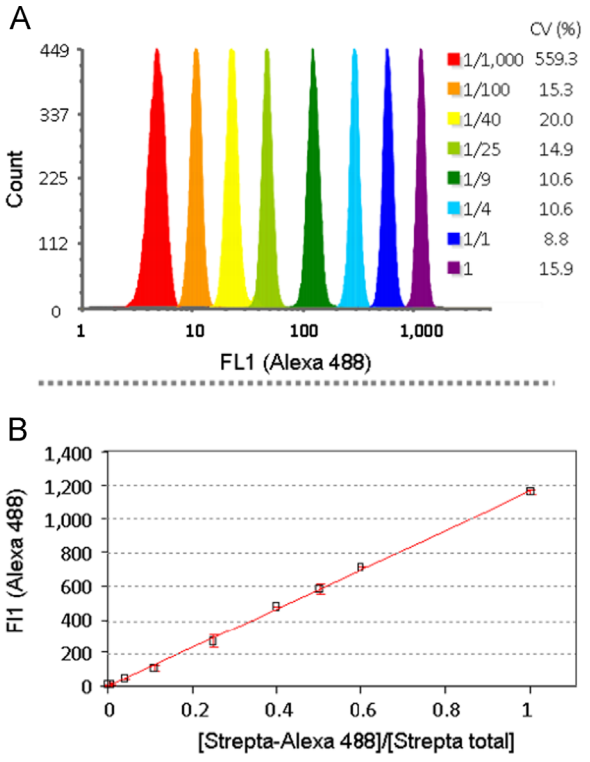

Fig. 2. Encoding of biotinylated microbeads with several ratios of streptavidin Alexa 488-conjugated/unconjugated streptavidin. (A) Overlays histograms obtained by flow cytometry analysis of encoded microbeads. (B) Mean fluorescence intensity of the 8 distinct bead populations is proportional to the amount of fluorescent streptavidin used for the encoding process.

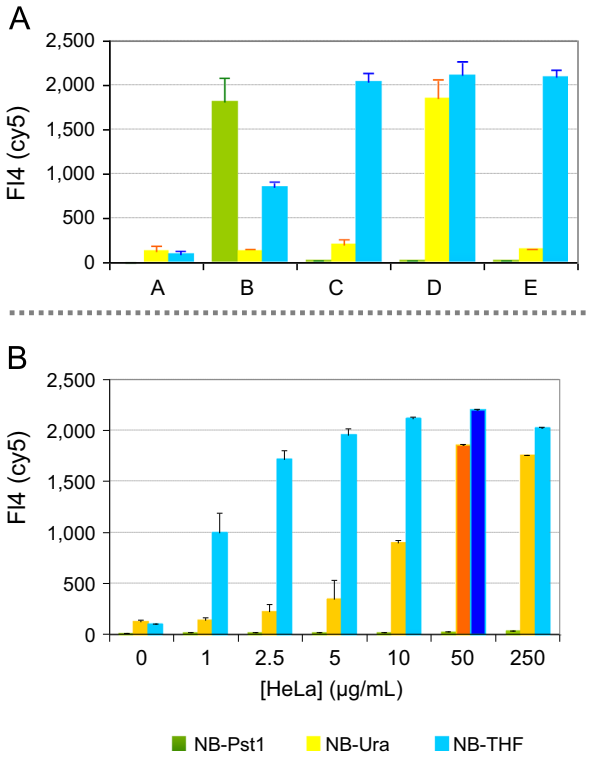

Fig. 3. Multiplex detection assay (A) of purified enzymes activities: A=buffer only; B=10 units (u) Pst1; C=1 u APE1; D=1 u APE1+0.005 u UNG; E=1 u APE1+0.005 u UNG+1 u Ugi. (B) APE1 and UNG acitivities in HeLa nuclear extract (commercial source). MFI is calculated for each bead population incubated at different nuclear extract concentrations.

inhibitor (Ugi) (Acharya et al., 2003; Wang and Mosbaugh, 1989) specifically inhibits UNG activity resulting in the selective fluorescence decrease of NB-Ura functionalized microbeads compared to NB-THF containing beads. 2D fluorescence histograms for each sample (A–E) are available in Supplementary information (Fig. S3). These results validate the specificity and reproducibility of our bioanalytical tool. It is noteworthy that our assay required only 500 fmol of DNA probe for each test, which is below previously reported assays (Georgiadis et al., 2012; Liu et al., 2007; Maksimenko et al., 2004; Svilar et al., 2012; Zhou et al., 2013).

3.3. Detection of BER enzymes' activity in cell-free extract

We also tested newly developed biosensor's ability to detect DNA repair activities in cell-free extracts. Microbeads functionalized with either the non-damaged NB-Pst1 (as a negative control), or NB-THF or NB-Ura were incubated with an increasing amount of HeLa nuclear extract. The main limitation of employing molecular beacons to assay cell extracts is their rapid non-specific degradation by nucleases (Gines et al., 2014). Fig. 3B demonstrates the great stability of our supported profluorescent NBs, as shown with the control beads (NB-Pst1) that remain non-fluorescent, even at the highest extract concentration. At the opposite, NB-THF and NB-Ura are cleaved with 1 µg/mL and 2.5 µg/mL of nuclear extract respectively. In other words, the assay needs only few tens of nanograms of HeLa nuclear extract to detect both APE1 and UNG activities. A recent study reported a detection limit of 500 ng of HeLa nuclear extract to detect APE1 with a molecular beacon in solution (Georgiadis et al., 2012), that proves the great effectiveness of our assay. Furthermore, the fluorescent signal increases in a concentration-dependant manner. We noticed that the quantitative cleavage of NB-Ura requires 50 µg/mL HeLa nuclear extract,

namely 10 folds more than for the on-bead NB-THF. This result is consistent with the fact that the cleavage of NB-THF needs only APE1 activity, while NB-Ura processing is based on the initial action of the UNG enzyme followed by the additional AP-site cleavage by the endonuclease.

4. Conclusions

In summary, the current encoding procedure expounded here makes possible a fast and economical preparation of fluorescently distinct bead populations. Moreover, the site specific conjugation of hairpin-shaped profluorescent DNA NanoBeacons, in a controlled and high reproducible manner, allows the development of an original and efficient nucleic acid-based bioanalytical platform. To our knowledge, this is the first time such an encoding strategy was exploited and was successfully applied to a multiplexed detection of DNA repair activities from purified enzymes or from cellular extracts, without DNA probe degradation. The current tool and the associated electrophoresis-free tests can be easily adapted to the detection of SNP or several enzymatic processings of biomolecules. Other promising applications include the development of DNA repair inhibitors screening assays to highlight potential anticancer compounds by using magnetic properties of the bead support (allowing easy handling and washing by a robot) coupled to an automated flow cytometry platform.

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Appendix. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.02.040.

References

Acharya, N., Kumar, P., Varshney, U., 2003. Microbiology 149 (7), 1647–1658.
Almeida, K.H., Sobol, R.W., 2007. DNA Repair 6 (6), 695–711.
Baute, J., Depicker, A., 2008. Crit. Rev. Biochem. Mol. Biol. 43 (4), 239–276.
Chollat-Namy, A., Gasparutto, D., Cadet, J., Favier, A., 2005. Chem. Nucleic Acid Compon. 7, 397–399.
Corne, C., Fiche, J.B., Gasparutto, D., Cunin, V., Suraniti, E., Buhot, A., Fuchs, J., Calemczuk, R., Livache, T., Favier, A., 2008. Analyst 133 (8), 1036–1045.
Dunlop, M.G., Farrington, S.M., 2009. Surg. Oncol. Clin. N. Am. 18 (4), 599–610.
Friedberg, E.C., Aguilera, A., Gellert, M., Hanawalt, P.C., Hays, J.B., Lehmann, A.R., Lindahl, T., Lowndes, N., Sarasin, A., Wood, R.D., 2006. DNA Repair 5 (8), 986–996.
Gates, K.S., 2009. Chem. Res. Toxicol. 22 (11), 1747–1760.
Georgiadis, P., Polychronaki, N., Kyrtopoulos, S.A., 2012. Mutat. Res.: Fundam. Mol. Mech. Mutagen. 736 (12), 25–32.
Gines, G., Saint-Pierre, C., Gasparutto, D., 2014. Anal. Chim. Acta 812, 168–175.
Kavli, B., Andersen, S., Otterlei, M., Liabakk, N.B., Imai, K., Fischer, A., Durandy, A., Krokan, H.E., Slupphaug, G., 2005. J. Exp. Med. 201 (12), 2011–2021.
Lindahl, T., 1974. Proc. Natl. Acad. Sci. USA 71 (9), 3649–3653.
Liu, B., Yang, X., Wang, K., Tan, W., Li, H., Tang, H., 2007. Anal. Biochem. 366 (2), 237–243.
Madhusudan, S., Hickson, I.D., 2005. Trends Mol. Med. 11 (11), 503–511.
Maksimenko, A., Ishchenko, A.A., Sanz, G., Laval, J., Elder, R.H., Saparbaev, M.K., 2004. Biochem. Biophys. Res. Commun. 319 (1), 240–246.
Matsumoto, N., Toga, T., Hayashi, R., Sugasawa, K., Katayanagi, K., Ide, H., Kuraoka, I., Iwai, S., 2010. Nucleic Acids Res. 38 (7), e101.
Sauvaigo, S., Guerniou, V., Rapin, D., Gasparutto, D., Caillat, S., Favier, A., 2004. Anal. Biochem. 333 (1), 182–192.
Schärer, O.D., 2003. Angew. Chem. Int. Ed. Engl. 42 (26), 2946–2974.
Slupphaug, G., Eftedal, I., Kavli, B., Bharati, S., Helle, N.M., Haug, T., Levine, D.W., Krokan, H.E., 1995. Biochemistry 34 (1), 128–138.
Svilar, D., Vens, C., Sobol, R.W., 2012. J. Vis. Exp. (66), e4168.
Swain, U., Subba Rao, K., 2011. Mech. Ageing Dev. 132 (89), 374–381.
Tchou, J., Kasai, H., Shibutani, S., Chung, M.H., Laval, J., Grollman, A.P., Nishimura, S., 1991. Proc. Natl. Acad. Sci. USA 88 (11), 4690–4694.
Vik, E.S., Alseth, I., Forsbring, M., Helle, I.H., Morland, I., Luna, L., Bjørås, M., Dalhus, B., 2012. DNA Repair 11 (9), 766–773.
Wang, Z., Mosbaugh, D.W., 1989. J. Biol. Chem. 264 (2), 1163–1171.
Wilson , D.M., Takeshita, M., Grollman, A.P., Demple, B., 1995. J. Biol. Chem. 270 (27), 16002–16007.
Zhou, D.-M., Xi, Q., Liang, M.-F., Chen, C.-H., Tang, L.-J., Jiang, J.-H., 2013. Biosens. Bioelectron. 41 (0), 359–365.