JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Communication

pubs.acs.org/JACS

Photocleavable DNA Barcode–Antibody Conjugates Allow Sensitive and Multiplexed Protein Analysis in Single Cells

Sarit S. Agasti,[†,§] Monty Liong,[†,§] Vanessa M. Peterson,[†] Hakho Lee,[†] and Ralph Weissleder*[,†,‡]

[†]Center for Systems Biology, Massachusetts General Hospital/Harvard Medical School, 185 Cambridge Street, CPZN 5206, Boston, Massachusetts 02114, United States

[‡]Department of Systems Biology, Harvard Medical School, 200 Longwood Avenue, Boston, Massachusetts 02115, United States

Supporting Information

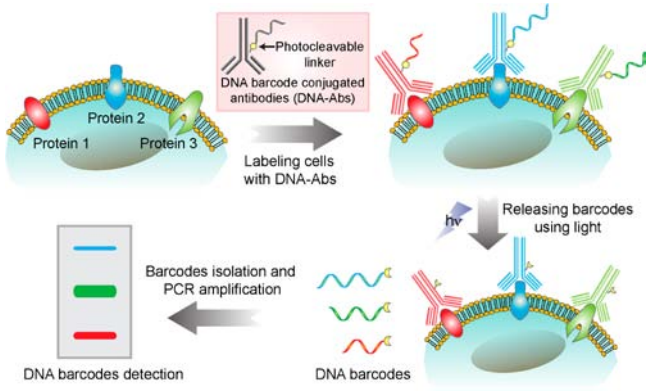
ABSTRACT: DNA barcoding is an attractive technology, as it allows sensitive and multiplexed target analysis. However, DNA barcoding of cellular proteins remains challenging, primarily because barcode amplification and readout techniques are often incompatible with the cellular microenvironment. Here we describe the development and validation of a photocleavable DNA barcode–antibody conjugate method for rapid, quantitative, and multiplexed detection of proteins in single live cells. Following target binding, this method allows DNA barcodes to be photoreleased in solution, enabling easy isolation, amplification, and readout. As a proof of principle, we demonstrate sensitive and multiplexed detection of protein biomarkers in a variety of cancer cells.

The ability to detect scant proteins and antigens in single cells is becoming increasingly important in biological research, forensic science, and clinical diagnostics. Analyzing protein signatures at single-cell resolution would aid studies of the role of cellular heterogeneity in disease progression, stem cell differentiation, response to drugs, and other cellular signaling processes.[1] Clinically, accurate molecular profiling and proteomic analysis of rare cells (e.g., circulating tumor cells) holds considerable promise for early disease detection and monitoring of treatment response.[2] Thus, sensitive, reliable, and multiplexable protein detection technologies are currently in great demand.[3]

To date, several platforms for analyzing cellular proteins have been described.[4] Although some recently developed methods have shown promise for single-cell analysis,[5] the majority of current methods are limited either by their need for large numbers of cells or by their ability to detect only few proteins simultaneously. One enticing approach is DNA barcoding, since a single DNA barcode can be detected through PCR amplification; infinite numbers of DNA barcodes can be easily discriminated on the basis of their sequence and/or size.[6,7] Although DNA barcoding technology has been applied to the detection of soluble proteins via several different formats,[6,8,9] the successful application of this technology to live cells has been rare.[10]

We hypothesized that DNA barcoding could be applied to live-cell analysis by using a light-mediated barcode releasing technique, which would enable barcode amplification and readout to be readily carried out following target binding. We describe here the synthesis and validation of a DNA-barcoding-based cellular protein detection method, which we term "light-mediated cellular barcoding" (LMCB). The LMCB method relies on the use of antibodies conjugated to specific DNA barcodes through a photocleavable linker molecule for initial target recognition and subsequent barcode amplification following cleavage of the DNA barcode–antibody conjugate (DNA–Ab). The generic concept of the LMCB method is shown in Scheme 1. Cells are first labeled with DNA–Abs targeted to specific protein biomarkers. Irradiation of the labeled cells with light (~365 nm) cleaves the linker between the antibodies and the barcodes, causing the barcodes to be released into the solution for easy isolation. Barcode amplification by polymerase chain reaction (PCR) and subsequent gel electrophoresis analysis of the amplified barcodes allows simultaneous detection and quantification of multiple protein analytes from single cells.

Scheme 1. Schematic Illustration of the Light-Mediated Cellular Barcoding Strategy[a]



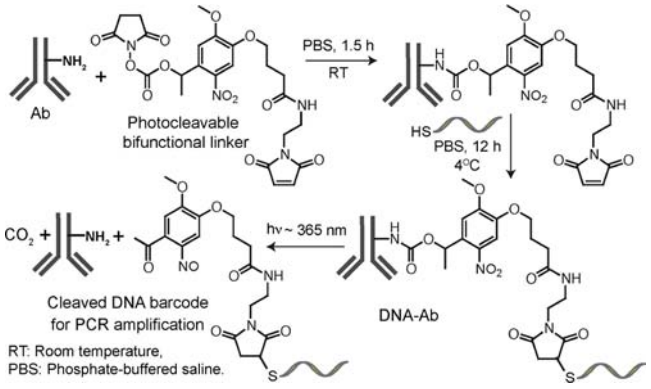
[a]Protein targets are labeled with DNA–Abs and then photocleaved to release DNA barcodes. Amplified barcodes are analyzed using gel electrophoresis for multiplexed detection of protein biomarkers from single cells.

Scheme 2 summarizes the synthetic approach used for the preparation of DNA–Abs. Figure S1 in the Supporting Information shows the characterization of the photocleavage

Received: August 9, 2012
Published: October 23, 2012

   dx.doi.org/10.1021/ja307689w | J. Am. Chem. Soc. 2012, 134, 18499–18502

Scheme 2. Synthetic Scheme of the Antibody–DNA Conjugation and the Photocleavage Reaction Leading to Barcode Release



reaction of the bifunctional linker by UV–vis spectroscopy. For cancer cell analysis, antibodies against epidermal growth factor receptor (EGFR), epithelial cell adhesion molecule (EpCAM), and human epidermal growth factor receptor 2 (HER2/*neu*) were conjugated with 55-, 70-, and 85-base DNA barcodes, respectively (Figure S2). We verified that after barcode conjugation, the barcode-modified antibodies still efficiently recognize their specific targets in various cell lines (Figure S3).

To demonstrate that the barcodes are indeed released upon light irradiation in intact live cells, we initially used fluorescent dye (FAM)-labeled, DNA-barcode-conjugated anti-HER2 antibody (FAM–DNA–HER2). A strong fluorescence signal emanated from the cell surface following incubation of SK-BR-3 cells (overexpressing the surface marker HER2/*neu*) with FAM–DNA–HER2 (Figure 1 left), while our control experiment using HER2/*neu*-negative MDA-MB-231 cells showed negligible fluorescence (Figure S4), indicating specific binding of the barcode-conjugated antibodies to the target molecular markers. A microscope image taken after 10 min of light exposure of the stained SK-BR-3 cells showed a significant decrease in the fluorescence signal from the cells (Figure 1 right) resulting from release of the fluorescent barcodes from the cells following photocleavage. DNA barcode release was complete within 15 min of light exposure, as measured by flow cytometry (Figure S5a). A control experiment performed with a non-photocleavable anti-HER2 antibody showed a minimal decrease in fluorescence over time (Figure S5b), indicating that the barcode release was a consequence of the photolytic cleavage of the linker molecule.

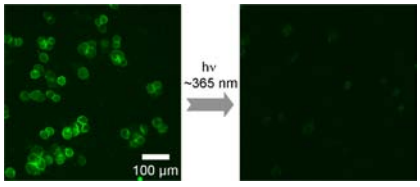


Figure 1. Fluorescently labeled DNA barcodes conjugated to anti-HER2 antibodies were used to stain SK-BR-3 cells. A decreased fluorescence signal after light irradiation demonstrated that barcodes were released from the labeled cells. See Figure S4 for additional images, including bright-field images.

We subsequently applied the LMCB method to both SK-BR-3 (HER2/*neu*^{high}) and control fibroblast 3T3 (HER2/*neu*^{low}) single cells using the 85-base DNA-barcode-conjugated anti-HER2 antibody (85bDNA–HER2). Cells were labeled with 85bDNA–HER2 against HER2/*neu* and then photocleaved for 15 min. Released barcodes were separated from the cells via centrifugation (300*g*, 3 min). PCR was then performed to amplify the 85-base DNA barcodes. The target marker (HER2/*neu*), which was previously undetectable, could be readily detected from single SK-BR-3 cells after ~25 cycles of PCR; as shown in Figure 2a, a band corresponding to the 85-base DNA barcode was seen on the gel. In contrast, even after 25 cycles of PCR amplification, 3T3 cells failed to show a significant band for the 85-base DNA barcode, which is consistent with the very low expression of HER2/*neu* in these cells. Another control experiment with HER2/*neu*-negative MDA-MB-231 cells likewise failed to show a significant barcode band following PCR amplification (Figure S6). Omitting the light-irradiation step resulted in no detectable signal (Figure 2b), confirming the importance of photocleavage in the LMCB method. To determine the expression level of different markers quantitatively, we next performed SYBR Green-based quantitative LMCB (or qLMCB) on the DNA barcodes. Figure 2c shows the HER2/*neu* expression level in both SK-BR-3 and 3T3 cells, whereas Figure 2d shows the EGFR expression level in human epithelial carcinoma A431 and 3T3 cells. Expression levels determined using the qLMCB method were consistent with those obtained by flow cytometry, with the difference being that flow cytometry required much larger cell numbers for analysis (~10^5 cells for flow cytometry vs ~1 cell for qLMCB). Overall, these results demonstrate that the protein signatures of single cells can easily be transformed into a detectable, quantifiable, and reliable signal using the LMCB method.

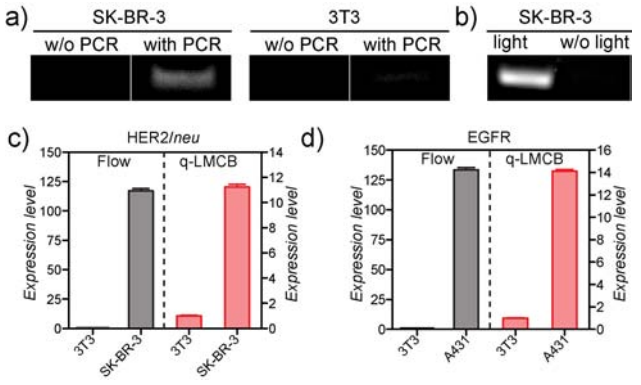


Figure 2. (a) Detection of HER2/*neu* in SK-BR-3 cells. After 25 cycles of PCR, a DNA band corresponding to the 85-base DNA barcode was visible. Control 3T3 cells, consistent with their low expression of HER2/*neu*, had a minimal 85-base DNA band even after PCR amplification. (b) No DNA band was detected in the absence of light irradiation, demonstrating the critical role of light in the assay method. (c, d) Expression of (c) HER2/*neu* and (d) EGFR (both relative to the control 3T3 cells) from qLMCB correlated well with results from standard flow cytometry-based detection. Error bars represent variations between duplicate measurements.

To determine the detection threshold of the LMCB method, we performed dilution experiments with SK-BR-3 cells. Cells were targeted using 85bDNA–HER2 and photocleaved as previously described. Figure 3a shows the gel analysis following 25 cycles of PCR amplification of DNA barcodes from different numbers of cells. A clear 85-base DNA band (varying in intensity depending on cell concentration) was observed for all samples, whose sizes ranged from 10^4 cells to single cells. To

dx.doi.org/10.1021/ja307689w | J. Am. Chem. Soc. 2012, 134, 18499–18502

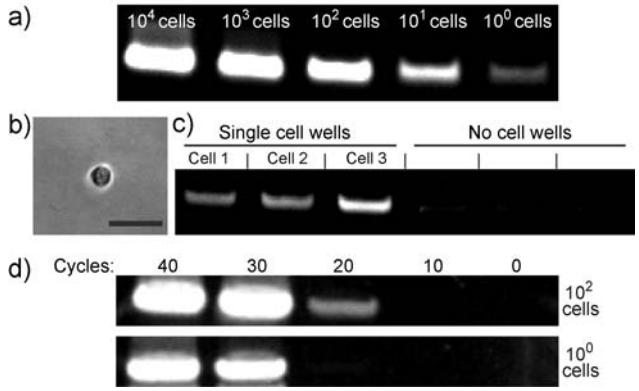


Figure 3. Detection sensitivity of the LMCB method. (a) After 25 cycles of PCR, the DNA barcodes from samples containing varying numbers of SK-BR-3 cells were detected. (b) Image showing a single SK-BR-3 cell inside a microplate well for digital analysis (scale bar 50 μm). (c) Analysis of a single SK-BR-3 cell using the LMCB method in digital format. Following PCR amplification, an 85-base DNA barcode could be detected in individual wells containing single cells ("single-cell wells"). In contrast, wells in which cells were absent ("no-cell wells") failed to produce a significant band following amplification. (d) Gel electrophoresis results showing the detection sensitivity of the LMCB method as a function of PCR cycle number.

verify further the single-cell sensitivity of the LMCB method, we performed cellular analysis in digital format. A dilute SK-BR-3 cell solution was distributed between many wells of a 384-well microplate in such a way that each well contained on average either a single cell or no cells. The wells were then imaged to identify the ones containing a single cell (Figure 3b). Barcodes isolated from the wells containing single SK-BR-3 cells produced a barcode band on the gel, whereas wells containing either no cells or control MDA-MB-231 cells (HER2/*neu*-negative) failed to show a significant band following amplification (Figure 3c and Figure S6). This result indicates that the LMCB method provides analysis of individual cells to characterize heterogeneities among a cell type (Figure S7). We also determined the effect of the number of PCR cycles on the detection sensitivity. As shown in Figure 3d, HER2/*neu* expression from 10^2 cells could be easily detected after 20 cycles of PCR, whereas for samples containing only single cells, ~30 cycles were required to obtain a detectable signal. This suggests that adjusting the number of PCR amplification cycles could increase the detection sensitivity, allowing even relatively low abundance biomarkers to be detected in cells.

Commonly used multiplexing methods are largely reliant on the use of fluorescently labeled antibodies; however, this allows only limited numbers of labels to be discriminated. In contrast, the use of DNA barcodes as labels for multiplexing represents an ideal platform because infinite numbers of DNA barcodes can easily be discriminated on the basis of their sequences and/or sizes. To test the LMCB method in a multiplexed format, we used barcode-conjugated antibodies for the simultaneous detection of EGFR, EpCAM, and HER2/*neu* in four different cell lines. Cells were incubated with a cocktail of antibodies for respective target binding and then photocleaved for barcode release and isolation. Since all of the barcodes have similar sequences toward the 5′ and 3′ ends, the barcodes could then be simultaneously amplified by PCR using a single set of primer pairs (Figure S2).[9b] Following amplification, individual barcodes were separated by gel electrophoresis on the basis of size. Figure 4a shows that the signals from individual biomarkers can be clearly distinguished from each other on the basis of barcode size. Varying band intensities, corresponding to the expression levels of biomarkers in different cells, are clearly evident in the gel. To verify the reliability of the multiplexed LMCB method, we compared the results to standard flow cytometry data obtained using 10^5 cells/experiment. As shown in Figure 4b, there was a good correlation between the LMCB and flow cytometry results.

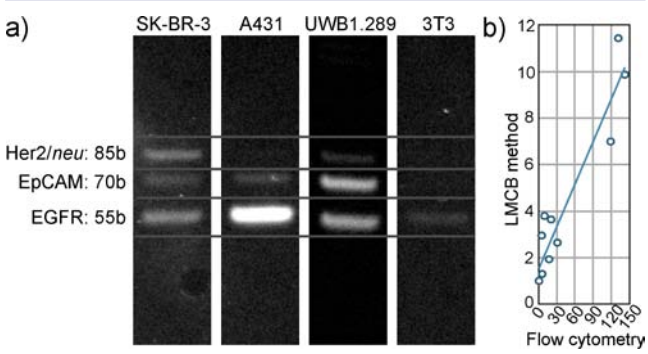


Figure 4. (a) Multiplexed protein detection using the LMCB method. Individual biomarker signals (corresponding to their expression levels) can be clearly distinguished from one other on the basis of barcode size. (b) Comparison of the LMCB results from (a) and data acquired using flow cytometry. For each biomarker, band intensities (normalized to control 3T3 cells) from the gel were plotted against fluorescence intensities (normalized to control 3T3 cells) from flow cytometry (R^2 = 0.90).

In summary, we have developed a novel method, light-mediated cellular barcoding (LMCB), that enables rapid, quantitative, multiplexed detection of protein expression in single live cells. The approach is robust and could be adapted to the analysis of other targets of interest, such as soluble proteins and pathogens. It is also a relatively simple technique that does not require complex purification steps, during which analytes are often lost. In this work, differently sized DNA barcodes were separated using size chromatography. While this provided a clean and simple model system for validating the technology, we ultimately anticipate either sequencing of the DNA barcodes to enable greater diversity or the use of imaging approaches with digital color-coded barcodes for multiplexing.[7a]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthesis of materials, experimental methods, and supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

rweissleder@mgh.harvard.edu

Author Contributions

[§]S.S.A. and M.L. contributed equally.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank H. J. Chung, K. S. Yang, K. Tran, and J. A. Hendricks for helpful discussions and Y. Fisher-Jeffes for reviewing the manuscript. This work was supported in part by the National

Institutes of Health (Grants 2P50CA086355-12 and R01EB010011 and TPEN Contract HHSN268201000044C).

REFERENCES

(1) (a) Shackleton, M.; Quintana, E.; Fearon, E. R.; Morrison, S. J. *Cell* **2009**, *138*, 822−829. (b) Tay, S.; Hughey, J. J.; Lee, T. K.; Lipniacki, T.; Quake, S. R.; Covert, M. W. *Nature* **2010**, *466*, 267−271. (c) Abbas, H. A.; Pant, V.; Lozano, G. *Cell Cycle* **2011**, *10*, 3257−3262.

(2) (a) Yu, M.; Stott, S.; Toner, M.; Maheswaran, S.; Haber, D. A. *J. Cell. Biol.* **2011**, *192*, 373−382. (b) Issadore, D.; Chung, J.; Shao, H.; Liong, M.; Ghazani, A. A.; Castro, C. M.; Weissleder, R.; Lee, H. *Sci. Transl. Med.* **2012**, *4*, 141ra92.

(3) (a) Wu, M.; Singh, A. K. *Curr. Opin. Biotechnol.* **2012**, *23*, 83−88. (b) Altelaar, A. F.; Heck, A. J. *Curr. Opin. Chem. Biol.* **2012**, *16*, 206−213.

(4) (a) Koob, A. O.; Bruns, L.; Prassler, C.; Masliah, E.; Klopstock, T.; Bender, A. *Anal. Biochem.* **2012**, *425*, 120−124. (b) Homola, J. *Chem. Rev.* **2008**, *108*, 462−493. (c) Thurer, R.; Vigassy, T.; Hirayama, M.; Wang, J.; Bakker, E.; Pretsch, E. *Anal. Chem.* **2007**, *79*, 5107−5110. (d) Ellerbee, A. K.; Phillips, S. T.; Siegel, A. C.; Mirica, K. A.; Martinez, A. W.; Striehl, P.; Jain, N.; Prentiss, M.; Whitesides, G. M. *Anal. Chem.* **2009**, *81*, 8447−8452. (e) Haun, J. B.; Castro, C. M.; Wang, R.; Peterson, V. M.; Marinelli, B. S.; Lee, H.; Weissleder, R. *Sci. Transl. Med.* **2011**, *3*, 71ra16. (f) Rosi, N. L.; Mirkin, C. A. *Chem. Rev.* **2005**, *105*, 1547−1562. (g) Rotem, D.; Jayasinghe, L.; Salichou, M.; Bayley, H. *J. Am. Chem. Soc.* **2012**, *134*, 2781−2787.

(5) (a) Bendall, S. C.; Simonds, E. F.; Qiu, P.; Amir, E.-a. D.; Krutzik, P. O.; Finck, R.; Bruggner, R. V.; Melamed, R.; Trejo, A.; Ornatsky, O. I.; Balderas, R. S.; Plevritis, S. K.; Sachs, K.; Pe'er, D.; Tanner, S. D.; Nolan, G. P. *Science* **2011**, *332*, 687−696. (b) Shi, Q.; Qin, L.; Wei, W.; Geng, F.; Fan, R.; Shin, Y. S.; Guo, D.; Hood, L.; Mischel, P. S.; Heath, J. R. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, 419−424.

(6) Nam, J. M.; Thaxton, C. S.; Mirkin, C. A. *Science* **2003**, *301*, 1884−1886.

(7) (a) Geiss, G. K.; Bumgarner, R. E.; Birditt, B.; Dahl, T.; Dowidar, N.; Dunaway, D. L.; Fell, H. P.; Ferree, S.; George, R. D.; Grogan, T.; James, J. J.; Maysuria, M.; Mitton, J. D.; Oliveri, P.; Osborn, J. L.; Peng, T.; Ratcliffe, A. L.; Webster, P. J.; Davidson, E. H.; Hood, L.; Dimitrov, K. *Nat. Biotechnol.* **2008**, *26*, 317−325. (b) Venkatesan, B. M.; Bashir, R. *Nat. Nanotechnol.* **2011**, *6*, 615−624.

(8) (a) Wacker, R.; Ceyhan, B.; Alhorn, P.; Schueler, D.; Lang, C.; Niemeyer, C. M. *Biochem. Biophys. Res. Commun.* **2007**, *357*, 391−396. (b) Fredriksson, S.; Dixon, W.; Ji, H.; Koong, A. C.; Mindrinos, M.; Davis, R. W. *Nat. Methods* **2007**, *4*, 327−329.

(9) (a) Sano, T.; Smith, C. L.; Cantor, C. R. *Science* **1992**, *258*, 120−122. (b) Hendrickson, E. R.; Truby, T. M.; Joerger, R. D.; Majarian, W. R.; Ebersole, R. C. *Nucleic Acids Res.* **1995**, *23*, 522−529.

(10) (a) Kazane, S. A.; Sok, D.; Cho, E. H.; Uson, M. L.; Kuhn, P.; Schultz, P. G.; Smider, V. V. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, 3731−3736. (b) Konry, T.; Smolina, I.; Yarmush, J. M.; Irimia, D.; Yarmush, M. L. *Small* **2011**, *7*, 395−400.