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Multiplexed Quantitation of Protein Expression and Phosphorylation Based on Functionalized Soluble Nanopolymers

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Supporting Information

ABSTRACT: We report here for the first time the multiplexed quantitation of phosphorylation and protein expression based on a functionalized soluble nanopolymer. The soluble nanopolymer, pIMAGO, is functionalized with Ti (IV) ions for chelating phosphoproteins in high specificity and with infrared fluorescent tags for direct, multiplexed assays. The nanopolymer allows for direct competition for epitopes on proteins of interest, thus facilitating simultaneous detection of phosphorylation by pIMAGO and total protein amount by protein antibody in the same well of microplates. The new strategy has a great potential to measure cell signaling events by clearly distinguishing actual phosphorylation signals from protein expression changes, thus providing a powerful tool to accurately profile cellular signal transduction in healthy and disease cells. We anticipate broad applications of this new strategy in monitoring cellular signaling pathways and discovering new signaling events.

Protein phosphorylation, one of the most ubiquitous post-translational modifications, has been implicated in the regulation of almost all aspects of a cell's life. Aberrant phosphorylation dynamics within the cell contribute to the onset and development of many malignances.[1] Therefore, considerable effort has been devoted to profiling protein phosphorylation under different cellular conditions. Currently, a majority of studies report phosphorylation events that fail to distinguish changes in phosphorylation from protein expression. Recent studies indicated that nearly 25% of what appears to be differential protein phosphorylation is actually due to the changes in protein expression.[2] Thus, more accurate measurements of actual phosphorylation changes normalized by protein expression changes are needed for the correct interpretation of comprehensive phosphorylation dynamics. Traditionally, methods such as Western blotting are used for the detection of specific proteins, including phosphoproteins. While it is possible to detect phosphorylation and total protein signals on the same blot by using two distinctive primary antibodies, it is often necessary to strip off the first primary antibody before the use of the second. This is not only time consuming but also inevitably causes protein loss during the stripping process. Similar to Western blotting, simultaneous quantification of phosphorylation and total protein amount on microplates requires adding two primary antibodies sequentially into the same wells of the microplate.[3−5] This process also is troublesome due to the fact that two primary antibodies from different sources are required (e.g., phospho-specific antibody from mouse and antibody against the whole protein from rabbit) and that binding of the first primary antibody to the target protein may prevent the second primary antibody from reaching its epitope because antibodies are quite bulky (~150 kDa). Finally, all of these methods rely heavily on the availability and quality of antibodies, which can be a major limitation, in particular for phospho-specific antibodies.

In this study, we introduce a novel multiplexed assay for the simultaneous quantitation of protein phosphorylation and total protein concentration on 96-well microplates. The use of a multifunctionalized, water-soluble nanopolymer termed "pIMAGO" (phospho imaging) and a protein antibody has allowed us to accurately and quantitatively measure levels of protein phosphorylation normalized by protein amount. In recent years, titanium ions (IV) have been demonstrated to exhibit outstanding selectivity toward binding phosphorylated residues.[6−9] In our lab, we successfully implemented a novel homogeneous platform based on functionalized water-soluble nanopolymers in the form of dendrimers conjugated with multiple titanium ions (IV) for either phosphopeptide enrichment prior to mass spectrometric analyses or phosphoprotein detection on microplates.[10,11] Here, we further modify the dendrimer with infrared (IR) fluorescent dyes to allow direct fluorescence detection with high sensitivity and coupled with general antibody detection for unique applications in multiplexed phosphorylation analyses (Figure 1A). Because of the much smaller size (~15 kDa) of the pIMAGO reagent as compared to that of a typical antibody (~150 kDa), competition for epitopes is reduced facilitating simultaneous detection of phosphorylation by pIMAGO and total protein amount by protein antibody in the same well of a 96-well microplate. Furthermore, the nanopolymer's multiple functional groups per molecule effectively amplify fluorescent signals and thus greatly improve the detection sensitivity for low abundant phosphoproteins.

In this strategy, as illustrated in Figure 1B, proteins are first immobilized onto a microplate. Then each well is incubated with the pIMAGO reagent for selective binding to the phosphoamino acid residues on a phosphorylated protein. After washing away unbound pIMAGO, the primary antibody

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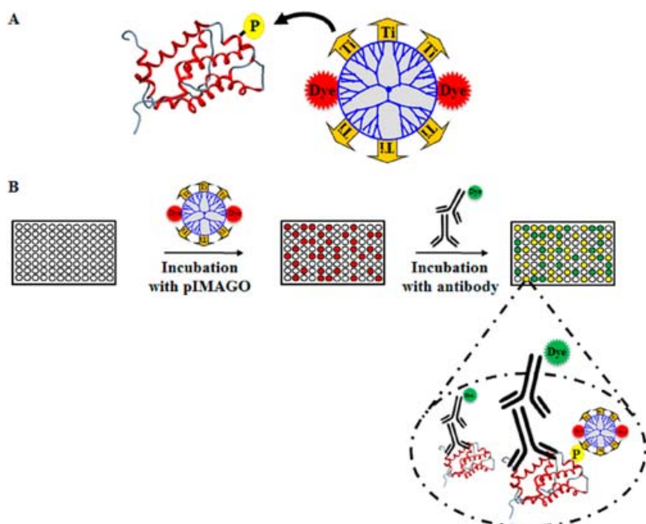

Figure 1. (A) A schematic representation of the pIMAGO reagent with fluorescent tags. (B) Experimental workflow for pIMAGO assay for multiplexed detection of both phosphorylation and protein concentration.

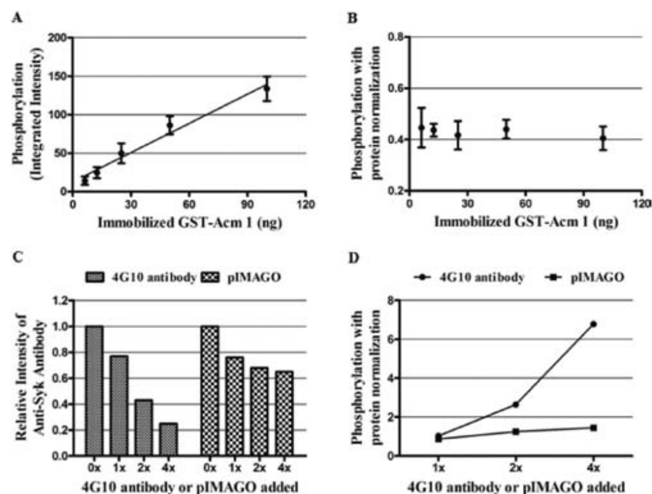

Figure 2. (A,B) Quantitative measurement of fluorescent signals for a yeast fusion protein, GST-Acm1, phosphorylation, and protein concentration. (C,D) pIMAGO and antiphosphotyrosine antibody (4G10) assay of Syk phosphorylation and protein concentration.

against the protein of interest is then added to the same wells to bind to the target protein, both phosphorylated and nonphosphorylated. Following further incubation and washing, a secondary antibody linked with an infrared dye that emits at a wavelength (800 nm) different from the dye on pIMAGO (700 nm) is added for fluorescence measurement. The final detection can be achieved using an imaging system (e.g., LI-COR Odyssey) equipped with two independent channels (i.e., one for 700 nm emission and the other for 800 nm emission) for multiplexed detection.

We first examined the sensitivity and specificity of pIMAGO for phosphoprotein detection using standard phospho- and nonphosphoproteins. When compared to 100 ng of the nonphosphoprotein bovine serum albumin (BSA), the signals generated from the detection of the same amount of the phosphoprotein α-casein were about 40-fold stronger, with high reproducibility (CV% < 10%, $n = 8$; data not shown). The specificity was further demonstrated with several nonphosphoproteins and phosphoproteins treated with or without phosphatase (Figure S1). Next, the quantitative range of the pIMAGO assay was demonstrated using standard phosphoproteins α- and β-casein and the phosphorylated yeast protein Acm1. Phosphoprotein levels could be quantified with high accuracy over a range of three orders of magnitude. As one example, quantitative detection of α-casein with serial dilutions ranging from 100 pg to 100 ng per well is shown in Figure S2.

We further explored the unique capability of the pIMAGO assay to quantify phosphorylation with protein normalization using the phosphorylated yeast fusion protein GST-Acm1. Following the scheme illustrated in Figure 1B, the pIMAGO reagent was added to wells containing phosphorylated GST-Acm1 immobilized on the microplate, followed by a further incubation with the primary anti-GST antibody in the same well to bind to total immobilized GST-Acm1, regardless of its state of phosphorylation. As shown in Figure 2A, the amount of phosphorylated GST-Acm1 detected by pIMAGO was proportional to the amount of immobilized GST-Acm1 protein. Importantly, the phosphorylation signal after protein normalization remained the same and was independent of the total amount of protein (Figure 2B, Figure S3). To further demonstrate the capability of the method to analyze a protein in a sandwich ELISA format, we coated the microplate with anti-GST monoclonal antibody from mouse to capture GST-Acm1 expressed in *E. coli* lysate. Then the pIMAGO reagent and a different anti-GST antibody (polyclonal antibody from rabbit) were consecutively applied to measure Acm1 phosphorylation and expression simultaneously (Figure S4). These results demonstrate the suitability of pIMAGO for use in multiplex assays to measure the extent of protein phosphorylation normalized by protein concentration.

Currently, multiplex measurement of phosphorylation against protein expression has been mainly performed using sets of two antibodies. We therefore performed multiplex analyses in parallel comparing the pIMAGO assay to the dual antibody method. We used spleen tyrosine kinase (Syk) as the model protein for the measurements and a widely used antiphosphotyrosine antibody, 4G10, for the detection of the extent of tyrosine phosphorylation of Syk. Syk plays a crucial role not only in adaptive immune receptor signaling but also functions as a tumor promoter in many hematopoietic malignancies and as a tumor suppressor in highly metastatic breast cancer and melanoma cells. A commercial active Syk was first immobilized onto a microplate. The antiphosphotyrosine antibody 4G10 and the pIMAGO reagent were then added to separate wells for the detection of Syk phosphorylation in a side by side comparison. In the next step, an anti-Syk antibody was added to the each well to measure the total amount of Syk protein. To evaluate whether the initial binding of 4G10 antibody or pIMAGO reagent would suppress the subsequent binding of the anti-Syk antibody, a series of increasing concentrations of 4G10 antibody or pIMAGO reagent was applied to the immobilized Syk. The signals derived from the reaction of the anti-Syk antibody with Syk were then measured and compared. As shown in Figure 2C, the signal from anti-Syk decreased sharply as a function of the amount of 4G10 antibody added. Doubling the amount of 4G10 antibody resulted in a dramatic decrease in the anti-Syk signal from 80% to 40% of the control (anti-Syk signal obtained in the absence of 4G10 antibody or pIMAGO reagent). The inhibitory effect of 4G10 antibody on the anti-Syk signal also was revealed by an incorrect assignment of the relative level of protein

phosphorylation when normalized for total protein level (Figure 2D). In contrast, the pIMAGO reagent, whose molecular size is about 10 times less than that of the 4G10 antibody, resulted in a much smaller inhibition of the subsequent binding of anti-Syk antibody to the target protein over a broad range of concentrations (Figure 2C, Figure 2D).

Finally, we applied the unique pIMAGO assay to measure differential phosphorylation of a protein in a complex biological system. We chose three breast cancer lines in which the amount of phosphorylated Syk could be modulated: MCF-7 cells lacking any Syk expression (Syk −/−), MCF-7 cells expressing exogenous Syk tagged by enhanced green fluorescent protein (Syk-EGFP) and then treated with or without trace hydrogen peroxide, and MCF-7 cells expressing exogenous Syk-EGFP with an added nuclear localization signal (NLS) at the C-terminus (Syk-EGFP-NLS).[12] The activity of Syk is a reflection of its own phosphorylation status, so its accurate measurement is of considerable interest. In cells expressing Syk-EGFP, the phosphorylation state of Syk can be greatly enhanced by treatment with hydrogen peroxide, a tyrosine phosphatase inhibitor.[13,14] In cells expressing Syk-EGFP-NLS, the kinase also is highly phosphorylated. To test whether the pIMAGO assay was capable of quantifying the extent of tyrosine phosphorylation of Syk-EGFP under these different cellular conditions, we immunoprecipitated Syk-EGFP protein from lysates of MCF-7 cells either lacking Syk, expressing Syk-EGFP and treated without or with hydrogen peroxide, or expressing Syk-EGFP-NLS. The beads/protein complexes were thoroughly washed to eliminate nonspecific binding and Syk-interacting proteins. After elution, Syk-EGFP was immobilized onto the microplate for the pIMAGO assay as described above. Samples derived from MCF-7 cells lacking Syk (Syk −/−) served as a negative control (Figure 3A). The level of Syk

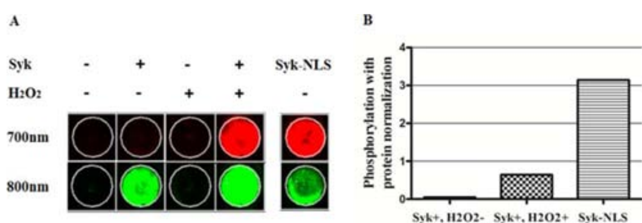

Figure 3. (A) Infrared fluorescence imaging of Syk isolated from MCF-7 cells expressing exogenous Syk-EGFP or Syk-EGFP-NLS. (B) Syk phosphorylation after protein normalization in different MCF-7 cells.

phosphorylation in the different cell states was calculated along with protein normalization (Figure 3B). Treatment of Syk-EGFP-expressing cells with hydrogen peroxide significantly increased the amount of the Syk phosphorylation by ∼10-fold, consistent with previous observations that Syk can be activated through hydrogen peroxide induced oxidative stress in cells.[13,14] More interestingly, the extent of phosphorylation of Syk-EGFP-NLS was found to be more than 60-fold higher than that of Syk-EGFP, indicating a significantly enhanced kinase activity of Syk when localized to the nucleus. This is interesting as it is thought that Syk localized to the nucleus represses invasive tumor growth in breast cancer, most likely through the substrates that it phosphorylates and regulates.[15] Thus, the pIMAGO assay provides a powerful and universal approach to quantify the extent of phosphorylation of a protein of interest normalized against its level of expression in different cell states.

In conclusion, we have presented dendrimer-based water-soluble nanopolymers functionalized with Ti (IV) ions and infrared fluorescent tags. Coupled with general antibody detection, the design led to unique applications in the simultaneous measurements of protein expression levels and phosphorylation. The technique, in theory, can measure absolute phosphorylation level on a protein provided that a calibration curve using known amount of the protein and phosphorylation can be constructed. Because it is difficult to construct such a calibration curve, the utilization of the method will be most likely in measuring changes in protein phosphorylation under different states. The technique may not be applicable to hyper-phosphorylated proteins in which only a specific single site changes its phosphorylation status, thus not significantly affecting overall phosphorylation level of the protein. The novel technique, however, is highly attractive to applications in which a good quality phospho-specific antibody is not available. The ability to facilitate the finding of new kinase and phosphatase substrates, screen kinase inhibitors, or profile changes in endogenous levels of phosphorylation without site microenvironment prejudice or safety concerns will be tremendously valuable for many research groups.

■ ASSOCIATED CONTENT

Supporting Information

Detailed procedures for the multiplex assay, the synthesis of the pIMAGO reagent, cell culturing, immunoprecipitation of Syk-EGFP and Supplement Figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Blume-Jensen, P.; Hunter, T. *Nature* **2001**, *411*, 355.
(2) Wu, R.; Dephoure, N.; Haas, W.; Huttlin, E. L.; Zhai, B.; Sowa, M. E.; Gygi, S. P. *Mol. Cell. Proteomics* **2011**, *10*, M111.009654.
(3) Gembitsky, D. S.; Lawlor, K.; Jacovina, A.; Yaneva, M.; Tempst, P. *Mol. Cell. Proteomics* **2004**, *3*, 1102.
(4) Grubb, R. L.; Calvert, V. S.; Wulkuhle, J. D.; Paweletz, C. P.; Linehan, W. M.; Phillips, J. L.; Chuaqui, R.; Valasco, A.; Gillespie, J.; Emmert-Buck, M.; Liotta, L. A.; Petricoin, E. F. *Proteomics* **2003**, *3*, 2142.
(5) Nielsen, U. B.; Cardone, M. H.; Sinskey, A. J.; MacBeath, G.; Sorger, P. K. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 9330.
(6) Iliuk, A. B.; Martin, V. A.; Alicie, B. M.; Geahlen, R. L.; Tao, W. A. *Mol. Cell. Proteomics* **2010**, *9*, 2162.
(7) Wang, F.; Song, C.; Cheng, K.; Jiang, X.; Ye, M.; Zou, H. *Anal. Chem.* **2011**, *83*, 8078.
(8) Zhou, H.; Ye, M.; Dong, J.; Han, G.; Jiang, X.; Wu, R.; Zou, H. *J Proteome Res* **2008**, *7*, 3957.

(9) Pinkse, M. W.; Uitto, P. M.; Hilhorst, M. J.; Ooms, B.; Heck, A. J. *Anal. Chem.* **2004**, *76*, 3935.
(10) Iliuk, A.; Martinez, J. S.; Hall, M. C.; Tao, W. A. *Anal. Chem.* **2011**, *83*, 2767.
(11) Iliuk, A.; Jayasundera, K.; Schluttenhofer, R.; Tao, W. A. *Methods Mol. Biol.* **2011**, *790*, 277.
(12) Zhou, F.; Hu, J.; Ma, H.; Harrison, M. L.; Geahlen, R. L. *Mol. Cell. Biol.* **2006**, *26*, 3478.
(13) Furlong, M. T.; Mahrenholz, A. M.; Kim, K. H.; Ashendel, C. L.; Harrison, M. L.; Geahlen, R. L. *Biochim. Biophys. Acta* **1997**, *1355*, 177.
(14) Ding, J.; Takano, T.; Gao, S.; Han, W.; Noda, C.; Yanagi, S.; Yamamura, H. *J. Biol. Chem.* **2000**, *275*, 30873.
(15) Wang, L.; Duke, L.; Zhang, P. S.; Arlinghaus, R. B.; Symmans, W. F.; Sahin, A.; Mendez, R.; Dai, J. L. *Cancer Res.* **2003**, *63*, 4724.