Integrated Microfluidics for Protein Modification Discovery*‡§

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Protein post-translational modifications mediate dynamic cellular processes with broad implications in human disease pathogenesis. There is a large demand for high-throughput technologies supporting post-translational modifications research, and both mass spectrometry and protein arrays have been successfully utilized for this purpose. Protein arrays override the major limitation of target protein abundance inherently associated with MS analysis. This technology, however, is typically restricted to pre-purified proteins spotted in a fixed composition on chips with limited life-time and functionality. In addition, the chips are expensive and designed for a single use, making complex experiments cost-prohibitive. Combining microfluidics with in situ protein expression from a cDNA microarray addressed these limitations. Based on this approach, we introduce a modular integrated microfluidic platform for multiple post-translational modifications analysis of freshly synthesized protein arrays (IMPA). The system’s potency, specificity and flexibility are demonstrated for tyrosine phosphorylation and ubiquitination in quasicellular environments. Unlimited by design and protein composition, and relying on minute amounts of biological material and cost-effective technology, this unique approach is applicable for a broad range of basic, biomedical and biomarker research. Molecular & Cellular Proteomics 14: 10.1074/mcp.M115.053512, 2824–2832, 2015.

Protein post-translational modifications (PTMs)† vastly diversify eukaryotic proteomes and are integrated in essentially all cellular processes (1). Proteomic approaches, such as mass spectrometry (MS), have been instrumental in monitoring global molecular dynamics for research and clinical applications (2–5). However, even in this modern era, large-scale analyses of PTMs by MS is challenging because of the limited number of modified peptides derived from proteins that, by themselves, may not be abundant. Moreover, comprehensive PTM analysis by MS often requires significant amounts of biological material that may not be available. PTM analysis using protein arrays can overcome these limitations because of the equimolar amount of the arrayed proteins (6, 7). Large-scale protein arrays have been successfully integrated into PTM research (8, 9). However, this technology relies on pre-purified proteins that are arrayed on a surface and thus, incompatible with biochemically challenging proteins, let alone insoluble proteins. Moreover, the production of recombinant protein arrays is impractical in-house. Therefore, such arrays cannot be used fresh, and they are inherently limited to certain designs, protein compositions, and model organisms of high commercial value. To overcome the abovementioned limitations, we designed a modular integrated microfluidic platform for PTM analysis (IMPA).

EXPERIMENTAL PROCEDURES

**Plasmids**—Human Securin ORF was amplified using the plasmid pCS2-FA-Securin as a template, and XhoI (5’) and BamHI (3’) flanked primers, and cloned into pEGFP-N1 vector. The Securin-eGFP ORF was then amplified using FseI flanked primer (5’) also carrying six repeats of His tag, and Ascl flanked primer (3’), and cloned into the pCS2-FA vector. For generating the Δ64 Securin mutant, we amplified Securin ORF encoding for amino acids 65–202 using Securin-eGFP as a template and FseI (5’)/Ascl (3’) flanked primers. p27 was amplified from the human full-length clone (Open Biosystems) using FseI (5’)/Ascl (3’) flanked primers, and cloned into pcDNA3 vector. Geminin cDNA was amplified from U2OS cDNA using FseI (5’)/Agel (3’) primers. The PCR product was cloned in-frame upstream to eGFP in pcDNA3 vector. The plasmids: pCS2-FA-Securin, pCS2-FA, pCS2-FA-Kifc1-eGFP, and Emi1 C terminus (amino acids 299–477) in pGEX, were described elsewhere (4,10,11). pCS2-GFP was kindly provided by Lior Appelbaum (Bar-Ilan University, Ramat Gan, Israel). Mcp-p27-His was generated by a two-step assembly PCR using human p27 ORF, a first primer set containing a c-Myc tag (5’) and a His tag (3’), and a second primer set containing a T7 promoter (5’) and a T7 terminator sequence (3’). A similar two-step assembly PCR was used to generate all other synthetic genes.

**Cell Culture and Synchronization**—HeLa S3 and HEK293 cells were maintained in tissue culture plates containing Dulbecco’s Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum.

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† The abbreviations used are: PTM, post-translational modification; IMPA, integrated microfluidic platform for PTM analysis; ORF, open reading frame; MS, mass spectrometry.

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(FBS), 2 mM l-Glutamine, and penicillin (100 μg/streptomycin (0.1 mg/ml) (all reagents were purchased from Biological Industries, Kibbutz Beit Haemek, Israel). Cells were maintained at 37 °C in a humidified 5% CO2 environment. For G1 extract preparation, HeLa S3 cells were grown in 1 l spinner flask. At a concentration of ~5 x 10⁵ cells/ml, cells were treated with 2 mm thymidine (Sigma, Israel) for 22 h, washed twice, released into fresh prewarmed media for 3 h, and incubated with 50 ng/ml nocodazole (Sigma, Israel) for 11 to 12 h. Cells were washed twice and released into fresh prewarmed media for 3.5 h to generate a G1 synchronous population. In order to generate S-phase extracts, HeLa S3 cells, at ~5 x 10⁵ cells/ml, were incubated 22 h with thymidine, washed twice, released into fresh prewarmed media for 3 h, and harvested.

Preparation of Cell Extracts—Preparation of cell extracts was performed as follows: cells were lysed in a swelling buffer [20 mM Heps pH 7.5, 2 mM MgCl₂, 5 mM KCl, 1 mM EDTA, 1 tablet of complete protease inhibitor mixture (Roche, Mannheim, Germany)] supplemented with energy-regeneration mix (1 mM ATP, 7.5 mM creatine phosphate, 70 mg/ml creatine phosphokinase, 0.1 mM EGTA), and homogenized by freeze-thawing and passage through 21 G needle successively. Extracts were cleared by subsequent centrifugations and stored at ~80 °C.

Degradation Assay—Degradation assay was performed in vitro in rabbit reticulocyte-coupled transcription and translation reaction (TNT-coupled reticulocyte system, Promega, Madison, WI) supplemented with ³⁵S-Methionine (PerkinElmer, Boston, MA). Unless otherwise indicated, degradation assays were performed in a final volume of 25 μl containing 20 μl HeLa S3 cell extracts, 1 μl of X20 energy-regeneration mix, 0.4 mg/ml Ub, 0.3 mg/ml His-tagged UbcH10 or UbcH10DN, 0.48 mg/ml C terminus-GST Eml1, and 1 μl radiolabeled IVT product. Samples were incubated at 23 to 30 °C. Aliquots were taken every 15 or 20 min, denatured, and quick-frozen in liquid nitrogen. Samples resolved by SDS-PAGE and visualized by autoradiography using GE phosphorimager, Typhoon-9500. Ub, radamine N terminus-labeled Ub (Rd-Ub), and all Ub mutants (K11-only, K48-only, K11R, and K48R), were purchased from Bio-techne Biochem, Cambridge, MA and supplemented to the reaction at a final concentration of 5:1 and 20:1, for the control and flow molds, respectively. The control layer was degassed and baked for 30 min at 80 °C. The flow layer was initially spin-coated (Laurell Technologies, North Wales, PA) at 2000 rpm for 60 s, and baked at 80 °C for 30 min. The control layer was separated from its mold, and control-channel-access-holes were then punched. Next, the flow and control layers were aligned manually under a stereoscope and baked for 1.5 h at 80 °C. The two-layer device (chip) was peeled from the flow mold, and flow-channels-access-holes were punched.

Surface Chemistry—Surface chemistry was performed to prevent nonspecific adsorption and achieve suitable binding orientation of expressed proteins, the entire accessible surface area within the microfluidic device was chemically modified. This surface chemical modification also facilitates the self-assembly of a protein array on the surface. Biotinylated-BSA (1 μg/μl, Thermo) was flowed for 30 min through the device, binding the BSA to the epoxy surface. On top of the biotinylated-BSA, 0.5 μg/μl of Stepavidin (Neutavidin, Pierce, Rockford, IL) was added for 30 min. The ‘button’ valve was then closed, and biotinylatedPEG (1 μg/μl, NanoS) was flowed over for more than 30 min, thus passivating the rest of the device. Following passivation, the ‘button’ valve was released and a flow of 0.2 μg/μl penta-His (Qiagen; #34440, Venlo, Netherlands) or GFP (Abcam; #ab6658, Cambridge, United Kingdom) Biotinylated antibodies was applied. The antibodies bound to the exposed Streptavidin, specifically to the area under the ‘button’, creating an array of anti-His- or anti-GFP tags. Heps buffer (50 mM) was used for washing between each surface chemistry step.

Production of a Human Synthetic Gene Library—Synthetic linear genes were generated by using two-step assembly PCRs. A library of human nuclear ORFs was cherry-picked from an Open Biosystem library of 15,500 full-length human ORFs, and used as a template for the first PCR step. Two epitope tags were added by using a 5′ primer carrying a c-Myc tag and a 3′ primer carrying a His tag. A PCR reaction mix (20 μl) contained 0.8 units of high-fidelity hot-start DNA polymerase (Phusion II, Finnzymes, Espoo, Finland) per reaction. Second-step PCRs were performed using the first PCR products as a template and a primer set containing T7 promoter (5′) and T7 terminator (3′). The reaction mixture (50 μl) contained 1.5 units of DNA polymerase. The PCR products were filtered in multwell 10 K filter plates (AcroPrep™, Pall Corporation, Port Washington, NY), and eluted with 40 μl DDW. The PCR products were distributed into UV-transparent 384-well dishes, and their concentration was determined by the Synergy™ 4 Hybrid Microplate Reader (BioTek, Winooski, VT). All PCRs were performed in 96-well plates.

DNA Arraying and Device Alignment—DNA arraying was performed using an array of anti-His- or anti-GFP tags. Heps buffer (50 mM) was used for washing between each surface chemistry step.

Protein Expression On-chip—A pre-mixed reticulocyte lysate supporting protein expression by T7 promoter (12.5 μl), was flowed into the DNA chambers, following surface chemistry. Next, the valves separating each unit cell were closed and the device was incubated on a hot plate for 2.5 h at 32 °C. The IIVT product in each unit cell was then diffused from the DNA chamber to the protein chamber. Proteins...
were immobilized on the anti-His antibodies under the ‘button’ through their C terminus His tag (C-terminal tagging ensures an array of full-length proteins). Unbound proteins, protein fragments, and reticulocyte lysate were mechanically washed and then discarded from the chip by 50 mM Hepes buffer.

The immobilized proteins were detected by immunofluorescence as follows: Cy3-coupled anti-Myc antibodies (1/100 dilution; Sigma C6594) were flowed into the device, and incubated with the immobilized proteins under the ‘button’ for 30 min at RT. Unbound antibodies were mechanically washed and then discarded from the chip (50 mM Hepes buffer). Protein expression levels were determined with a microarray scanner (LS Reloaded, Tecan, Männedorf, Switzerland) using a 532 nm laser and 575/50 nm filter.

**Mutant Ub Labeling**—K11R and K48R Ub mutants (Boston Biochem) were fluorescently labeled using DyLight™ 650 (Thermo; #62266). The dye-to-protein ratio was calculated by measuring optical absorption of the dyed proteins with NanoDrop™ (Thermo) at 280- and 655 nm wavelengths.

**On-chip ubiquitination and Ub-preference assays**—Unlabeled IVT products flowed into the chip and immobilized on the surface under the ‘button’ at the protein chamber through their C terminus His or GFP tags (see ‘Surface chemistry’). Next, the ‘button’ valves opened and G1 extract mixtures were flowed for 10 min (RT). The extracts contained 6 μM MG132 (Sigma, Israel), and either 0.04 mg/ml rhodamine-labeled Ub or Cy5-labeled K11R-Ub/K48R-Ub. The cell extract mixtures used for the ubiquitination assays depicted in figure 5E and 5F, right plot, also included 0.2 mg/ml unlabeled Ub-K11R or Ub-K48R. Unbound material was washed by Hepes buffer (50 mM). In the case of relatively high background, an additional washing step with Hepes buffer (50 mM) was applied. Protein expression levels were determined with a microarray scanner (LS Reloaded, Tecan, Männedorf, Switzerland) using a 532 nm laser and 575/50 nm filter.

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**Monitoring Abl-mediated Phosphorylation (Fig. 2D)**—Eight double-tagged proteins were expressed on-chip, immobilized on the protein chamber through their C terminus His tag, and incubated (45 min, 37 °C) with 200 nm human recombinant Abl (Abcam) in Abl buffer (Hepes 25 mM, SOV 10 mM, MgCl2 25 mM, EGTA 5 mM, EDTA 2 mM, ATP 10 mM). An equivalent reaction with no kinase was performed on the same chip. P-Tyr-to-protein ratio was determined as described for the Btk assay. Background signal was negligible.

**Monitoring Btk-mediated Phosphorylation**—On-chip Tyr kinase assay—Protein expression levels were determined with a microarray scanner (LS Reloaded, Tecan, Männedorf, Switzerland) using a 532 nm laser and 575/50 nm filter.

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affinity tag; and (3) quantified by a fluorescent tag or fluorescently labeled antibodies against the second affinity tag (Figs. 1A and supplemental Fig. S1). After washing, an immunodetectable- or fluorescently labeled-protein modifier, mixed with biologically active cell extracts and/or recombinant enzymes, is applied to all or some protein chambers (Fig. 1B); 10 to 12 μl of reagents are sufficient to cover a chip of 10,000 protein chambers or less.

The use of native cell extracts maintains the stoichiometric environment of a particular cell or tissue at a given physiological/pathological condition during the analysis; bridging the gap between in vitro to in vivo. Moreover, cell extracts are easy to manipulate, and well known for their capacity to recapitulate complex cellular processes and reduce promiscuous enzymatic activities associated with non-physiological environments (10, 11). Following incubation and washing, the PTM signal can then be normalized to total protein in each unit cell (Fig. 1C). This PTM-to-protein normalization is unique, with potential implications in studying stoichiometry of PTMs.

Phosphorylation is probably the most studied PTM, with critical implications in normal and malignant cellular processes (1). Tyrosine (Tyr) is one of three phosphorylatable amino acids in eukaryotes. Tyr phosphorylation (P-Tyr) can be visualized in situ by fluorescently labeled P-Tyr antibodies (Fig. 2A), and incubated on-chip with the depicted reagents. P-Tyr and protein levels were quantified (see C). The threshold value (red dotted line) corresponds to 2 S.D. above the P-Tyr-to-protein ratio of Btk (negative control). E and F, Btk target peptides (E), and Ptcg2 and Securin (F), were incubated on-chip with the depicted reagents. P-Tyr and protein levels were quantified (see B and C). G. A phosphorylated form of biotinylated Btk target peptides were deposited on the chip, and incubated in parallel with buffer (bar) or HEK293 cell extracts supplemented with increasing amounts of SOV. Peptides were immunolabeled on-chip with Cy5-coupled anti-P-Tyr antibodies. Data are normalized to maximum activity. Overall, average P-Tyr levels (B, E, and G) or P-Tyr-to-protein ratios (C, D, and F) were calculated from 15 ≤ n ≤ 30 dots, and plotted. * p value < 0.001. ** p value > 0.5.
matic activities, high protein density, and high viscosity of cell extracts pose a challenge for any in situ-based assay, let alone within micro-scale plumbing. We applied active HEK293 extracts to protein chambers deposited with Btk target peptide (Fig. 2E). At first, we could not observe unambiguous P-Tyr signals. A bright P-Tyr signal appeared when the Tyr phosphatase inhibitor sodium orthovanadate (SOV) was added to the reaction and, more so, when recombinant Btk was supplemented to the reaction. Plcg2, but not Securin, was phosphorylated upon incubation with HEK293 extracts with Btk and SOV (Fig. 2F). Altogether, these results demonstrate the capacity of our method to analyze Tyr phosphorylation in a quasicellular environment. Intrinsically, the potential of our platform for analyzing Tyr dephosphorylation is demonstrated as well (Figs. 2E, 2F). In fact, dose response of SOV performed on a phosphorylated form of Btk peptide (Fig. 2G) displays a typical inhibition curve in cell extracts (EC50 ~ 2.5 mM), emphasizing the quantitative nature of the system.

Fig. 3. Large-scale Tyr phosphorylation analysis. A, An array of 1024 nuclear proteins. Bars represent average quadruplicates of a single protein, as measured by Cy3-coupled anti-Myc antibodies. B and C, HEK293 extracts containing 10 mM SOV (B) or buffer (C), were applied to the chip. Fractions of the protein array showing Rad9 and Hck P-Tyr signal are shown. A magnified area depicting two dots is framed (B). D, Top 10 Tyr phosphorylated proteins identified on-chip (B). E, Fractions of a protein array showing P-Tyr signals of freshly expressed Hck and Rad9. See supplemental material for extended legend.
Next, we generated a 64 × 64 unit-cell array capturing quadruplicates of 1024 nuclear proteins (Fig. 3A). HEK293 extracts with SOV were applied to the chip, followed by in situ P-Tyr labeling (Fig. 3B, left). A fraction of the chip showing P-Tyr of the cell cycle checkpoint protein Rad9 and the Tyr protein kinase Hck, are shown (Fig. 3B, right). The bright and uniform P-Tyr signal appeared in all four Rad9 and Hck unit cells. This emphasizes the importance of multiple repeats in minimizing false hits, as well as the overall selectivity and accuracy of our method. Tyr phosphorylation was validated by applying phosphatase-active extracts (no SOV) to the chip (Fig. 3C). Seven out of the 10 top P-Tyr targets identified on-chip (Fig. 3D) are known substrates of Tyr kinases, an important indication of the low false-positive rate of the method. Tyr phosphorylation was validated by applying phosphatase-active extracts (no SOV) to the chip (Fig. 3D). Seven out of the 10 top P-Tyr targets identified on-chip (Fig. 3D) are known substrates of Tyr kinases, an important indication of the low false-positive rate of the method. Importantly, two of the 10 targets were non-receptor Tyr kinases (Hck and Frk). Relying on freshly synthesized protein arrays, we reasoned that Hck and Frk P-Tyr signals might result from autophosphorylation. P-Tyr labeling of freshly expressed arrays without further incubation with cell extracts, confirmed our hypothesis; a clear P-Tyr signal appeared for Hck but not for Rad9 (Fig. 3E). These results elegantly demonstrate the functionality of freshly arrayed proteins.

Phosphorylation is a single-step reaction involving one small molecule and a target protein. We wanted to test the potential of IMPA in analyzing a more complex PTM reactions. Ubiquitination is a common PTM, tightly linked to cancer, neurodegenerative- and other human diseases (24). Unlike phosphorylation, ubiquitination is a multi-enzymatic process that covalently binds one or more Ubiquitin (Ub) proteins to a target protein via Lysine (K) residues. The sequential binding of Ub to itself via one of its 7 K residues or its N-terminus, can generate distinct Ub-chains that differentially regulate proteins’ fate, most notably: degradation by the proteasome (25). As proof of concept, we focused on the anaphase-promoting complex/cyclosome (APC/C), a Ub ligase that targets Securin, mitotic cyclins, and other cell cycle proteins for degradation by the proteasome (4, 10, 26). We based our approach on rhodamine (Rd)-labeled-Ub (Fig. 4A) and a cell-free system generated from HeLa S3
cells, pre-synchronized to the G1-phase of the cell cycle (G1 extracts)\(^{(4,10,11)}\). Off-chip, \(^{35}\)S-labeled Securin-eGFP IVT product (Sec-eGFP), but not its stable mutant (\(\textmod{H}_{9004}64\) Sec-eGFP)\(^{(26)}\) and eGFP itself, was degraded in G1 extracts (Figs. 4B and \(\textsup{supplemental Fig. S2}\))\(^{11}\). This degradation was blocked in the presence of either the dominant negative version of the APC/C-activating E2 enzyme Ubch10 (\(\textmod{H}_{9004}^{10}\)Ubch10\(^{\text{DN}}\)) or the APC/C inhibitor Emi1 (11). The cell cycle protein p27, whose turnover is regulated by the Ub ligase SCF\(^{\text{Skp2}}\) in S-phase (supplemental Fig. S2)\(^{(27)}\), remained stable in G1 extracts (Figs. 4B and supplemental Fig. S2). Thus, our cell-free system can specifically detect APC/C-mediated ubiquitination. Importantly, Sec-eGFP turnover was accelerated whenever Ub or Rd-Ub was added, demonstrating an intact incorporation of Rd-Ub into ubiquitination reactions (Fig. 4C).

On-chip incubation with G1 extracts and Rd-Ub revealed bright Rd signals in chambers containing Securin, but not \(\textmod{H}_{4804}^{84}\)Ubch10\(^{\text{DN}}\) or Eμ1 (Fig. 4D). Importantly, these signals dropped in the presence of \(\textmod{H}_{2004}\)mg/ml Rd-Ub and \(\textmod{H}_{2004}\)mg/ml unlabeled K48R-Ub or K11R-Ub. F, On-chip ubiquitination of Kifc1-eGFP was assayed, as described in B (left plot) and E (right plot). Representative raw data are shown. G, Degradation of \(^{35}\)S-Kifc-eGFP was assayed in the presence of 0.4 mg/ml wt or mutant Ub. K11/K48-only Ub mutants carry a single K residue. Time-dependent degradation was quantified (\(n = 3\)). See supplemental material for extended legend. * \(p\) value < 0.001. ** \(p\) value < 0.005.

![G1 extracts](image.png)

**Fig. 5. Monitoring Ub-chain preference on chip.** A, Ub-chain preference can be visualized on-chip. K48R-Ub can form K11Ub-chains, but not K48Ub-chains. B, On-chip incubation of Sec-eGFP with G1 extracts containing Cy5-labeled K48R-Ub or K11R-Ub. Average ratios of Cy5-mutant-Ub/protein are plotted (\(n = 20\)). Representative raw data are shown. C, Degradation of \(^{35}\)S-Sec-eGFP in G1 extracts was assayed in the presence of wt or mutant Ub (see more details in Fig. 4B). D, A schematic illustrating the displacement of Rd-wt-Ub by an excess of unlabeled K48R-Ub, while monitoring Ub-chain preference of Securin. E, On-chip ubiquitination of Sec-eGFP was examined in the presence of 0.04 mg/ml Rd-Ub and 0.2 mg/ml unlabeled K48R-Ub or K11R-Ub. F, On-chip ubiquitination of Kifc1-eGFP was assayed, as described in B (left plot) and E (right plot). Representative raw data are shown. G, Degradation of \(^{35}\)S-Kifc-eGFP was assayed in the presence of 0.4 mg/ml wt or mutant Ub. K11/K48-only Ub mutants carry a single K residue. Time-dependent degradation was quantified (\(n = 3\)). See supplemental material for extended legend. * \(p\) value < 0.001. ** \(p\) value < 0.005.
rather than nonspecific ubiquitination or, alternatively, undesired protein-protein interactions with either Rd-Ub or any other ubiquitinated protein in the cell extracts. These experiments also emphasize a major advantage of our system in conducting parallel experiments per chip.

Securin degradation is mediated by K11 Ub-chains (18). We used it to test whether IMPA can also be utilized for monitoring Ub-chain preference. As proof of concept, we focused on K11- versus K48-linked ubiquitination, the two major Ub-chains responsible for protein degradation (25). Two strategies were developed. First, we coupled Cy5 to Ub molecules whose K11 or K48 was substituted with Arginine (R) (Fig. 5A). On-chip ubiquitination of Securin was significantly more profound when Cy5-K48R-Ub was added to the reaction (Fig. 5B). Differential labeling efficiency of the two mutant Ub molecules could, in principle, distort data analysis and interpretation. We, therefore, repeated the experiment using G1 extracts supplemented with Rd-Ub and an excess of unlabeled mutant Ub. An excess of K11R-Ub, but not K48R-Ub displaces endogenous Ub and delays Securin degradation (Fig. 5C). By the same token, an excess of unlabeled K48R-Ub, but not K11R-Ub, should displace Rd-Ub (wt) from Securin (illustrated in Fig. 5D). This strategy was informative; Rd signals in Securin chambers supplemented with K48R-Ub, were less than 25% of the equivalent Rd signal obtained with K11R-Ub (Fig. 5E). Similar results were obtained for Geminin, a K11-Ub target (18) (supplemental Fig. S5). Encouraged by these findings, we tested the Ub-chain preference of the newly discovered APC/C target Kifc1 (4). The two approaches (Fig. 5A and 5D) revealed a clear preference of Kifc1 to K11Ub-chains over K48-chains (Fig. 5F). Degradation assays in G1 extracts were confirmatory; Kifc1 degradation was inhibited by an excess of K11R-Ub or K48-only-Ub (single K Ub mutants), but nearly unaffected by K48R- or K11-only-Ub (Fig. 5G). Altogether, results in Fig. 5 demonstrate the potential of our system for studying ubiquitination as well as Ub-chain preference in cellular environments.

**DISCUSSION**

The modularity and flexibility of IMPA can open new research avenues; first, the method can be optimized for any type of PTM that can be fluorescently tagged or immunelabeled. Second, in combination with parallel DNA synthesis (28), the device, which is placed on a microarrayed ORF library, is readily available to study PTM fingerprints of any sequenced animal models in a large spectrum of physiological environments. Automated cherry picking allows the selection of any combination of proteins in a flexible number of repeats. The current version of our system enables a maximum of 8 parallel independent experiments per chip (e.g. Fig. 2G). This unique feature enables the simultaneous analysis of multiple PTM signatures in various biochemical settings on a single chip; it also lowers experimental complexity and cost, and simplifies normalization.

Peptide recognition by MS inherently provides information about PTM sites, which for the most part cannot be directly determined by colorimetric assays (unless they combine epitope specific recognition, which is not trivial). Nevertheless, PTM site information can be indirectly obtained by our method following site-specific mutagenesis, emphasizing the strength of cDNA platform over spotted protein arrays. Although informative, this approach is small-scale. It is, therefore, our great interest to devise a method that combines IMPA with MS, thus providing site information for each of perhaps dozens of different PTM types.

IMPA can be utilized for identifying targets of a specific enzyme in physiologically relevant contexts, as well as for elucidating PTM fingerprints of particular cells or tissues. The minute amount of biological material and reagents required for the analysis override the inherent limitation of PTM research in low-mass tissues, with relevance to basic and translational science.

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