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Post-Translational Modification Profiling – a high-content assay for identifying protein modifications in mammalian cellular systems

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Abstract

Protein microarrays are extremely useful for detecting substrates of protein kinases, substrates of ubiquitin ligases and other post translational modifications. The ability to screen binding interactions as well as post-translational modifications of thousands of proteins at once has improved our ability to identify their targets. Utilizing such systems in combination with functional mammalian cell extracts that preserve enzymatic activity offers advantages in identifying semi-quantitative changes of these interactions in the context of specific cellular conditions. This article provides a detailed procedure for setting up an extract-based activity assay for high content detection of protein post-translation modifications. It also provides basic guidelines for data analysis.

Keywords

PTM profiling; post-translational modification; functional assay; ubiquitin (Ubl); protein microarray

Introduction

Recent advances in combining protein microarrays with biochemical and functional assays offer new ways for global comparative analysis of different types of samples across a wide array of protein targets¹⁻³. Protein microarray applications can be classified into two groups: abundance-based arrays that aim to describe and identify relative protein abundances and function-based assays that aim to identify binding interactions between macromolecules or between proteins and small molecules, enzyme-substrates interactions, and protein post-translational modifications (PTMs). In these two types of assays the type of

Internet Resources: <http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Protein-Expression-and-Analysis/Biomarker-Discovery/ProtoArray.html>
<http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Protein-Expression-and-Analysis/Biomarker-Discovery/ProtoArray/online-tools.html>
<http://www.mathworks.com/products/bioinfo/description3.html>
<http://www.bioconductor.org/>

proteins that are immobilized to the surface of the protein microarray can be very different (e.g. specific antibodies versus cellular proteins) and would yield different information⁴⁻⁶.

In this unit we will describe an extract-based functional assay for the detection of protein post-translational modifications in mammalian cell extracts^{6,7}. Specifically, the protocol focuses on the identification of substrates of ubiquitin and ubiquitin-like modifications (protocol 1) under conditions related to cell cycle regulation⁸. Though a specific cellular state is described, the basic guidelines and considerations herein provided could be adapted to different types of modifications as long as there are antibodies available for their detection. Since the activity of the extracts determines the context-specific interactions, our approach is applicable to the study of other cellular states such as differentiation, DNA damage, viral infection, nutritional changes, as well as cellular stimulation by growth factors or activation of other signaling cascades, provided that such extracts can be generated to faithfully recapitulate relevant events *in vitro*. Thus, the approach relies on the extract preparation step which is described in detail in the support protocol section.

Basic Protocol

BASIC PROTOCOL TITLE: “PTM Profiling using mammalian cell extracts and protein microarrays”

PTM Profiling using protein microarrays may be used to detect differential modifications of proteins under different cellular conditions. The protocol described herein will introduce this methodology and provide an illustration of how to conduct such an assay. In this protocol we focus primarily on protein microarrays that have cellular proteins absorbed to the surface and will address issues related to their use. Due to technical limitations and high production cost, currently there are only a few companies offering commercial protein microarrays. The protocol described here relies on the use of the human ProtoArray by Life Technologies. The protocol can be generally divided into two separate steps. The first step describes the generation of concentrated and functional mammalian cell extracts (support protocol) and the second step describes how to generate the modification profiles based on the activity exerted by these extracts using protein microarrays. In short, the protocol consists of generating active cellular extracts, incubating them on pre-blocked protein microarrays and allowing the enzymes in the extracts to modify the proteins on the surface of the microarray. Upon the completion of the reaction, the microarrays are washed extensively to remove non-covalent interactions, and the modified proteins are detected using modification-specific antibody (e.g. poly ubiquitin). The primary antibody is then detected by a fluorescently labeled secondary antibody. Thus, each protein is registered with an intensity value that reflects the level of ubiquitin or Ubl modification that was detected with that protein (see Figure 1 for a general illustration of the approach. When profiling modifications based on the activity of extract that are in different cellular states, a differential pattern in reactivity can teach us about the underlying processes.

Materials

Cell Culture: Cell lines: HeLa S3 (grow in suspension); HeLa cells or other cell line if using plates for treatment and preparation.

Spinner flasks/plates.

Dulbecco's Modified Eagle's Medium (DMEM)

Fetal bovine serum

Antibiotics/antimucotics

Trypsin (0.25%) with EDTA (1 mM)

PBS (for cell culture)

Synchronization: 200 mM Thymidine ; prepare fresh in sterile water and filter-sterilize it with 0.22 µm filter.

Nocodazole ; prepare 5 mg/ml stock in DMSO; store in small aliquots at –80°C.

Microarrays incubation and detection: ProtoArray (Invitrogen; Life Technologies); while other protein microarrays suppliers are available the protocol described herein is based on the use of this particular product.

TBS:

50 mM Tris-Cl

150 mM NaCl

Adjust pH to 7.6

TBS-T: TBS with 0.05% Tween-20

Blocking buffer (ArrayIt)

Extracts as described in the SupportProtocol

Detection antibody (anti-ubiquitin antibody; FK1, BioMol)

Secondary antibody (fluorescently labeled)

Equipment: Centrifuge (for 250 to 500 mL conical vessels)

Eppendorf centrifuge for 50mL tubes

Cover slips (24×60mm)

Forceps

GenePix 4000B microarray scanner (Molecular devices)

Gene Pix Pro software (Molecular Devices)

Optional: ProteinArray Prospector v.5 (available at <http://www.com/site/us/home/Products-and-services/applications/protein-expression-and-analysis/biomarker-discovery/protoarray/online-tools.html>)

Protocol steps: It is highly recommended that before you start with this protocol you read the user's manual of the human ProtoArray by Invitrogen/Life Technologies:

Blocking the array surface

1. Transfer the human ProtoArray from -20°C into 4°C for 15 minutes according to the manufacturer's instructions.

Important: handle microarrays with care. You should avoid touching the surface and handle the microarrays only by forceps to avoid damaging of the proteins or nitrocellulose membrane.

2. Block array at for 1 hour at 4°C with ArrayIt blocking buffer with gentle shaking. The cassette in which the microarrays are provided may be used for washing. Other blocking buffers such as 1% BSA may be used.

This step is meant to prevent non-specific binding and remove unabsorbed proteins from the surface. Uniform and effective blocking should be calibrated based on the specific material (extract/probes) one will use. Blocking time may also vary depending on the assay but should be done at 4°C.

3. Wash the microarrays three times with TBS at 4°C for 3 minutes each time. A volume of about 50mL should be used in order to cover the surface of the arrays. The volume may change depending on the washing container.

Probing the array

4. During the washes, thaw the cell extracts you wish to use and spin down at 16,000×g at 4°C, for 10 minutes. Thaw enough extract so that you will have 100µl per chip at the end. Take into consideration the loss of material in the pellet.

Avoid transferring any debris or lipids from the pellet as it may interrupt the interaction with the proteins on the surface of the microarray. Also, since the assay depends on the activity of the extracts, make sure you thaw them immediately before use.

5. Spread 100 µl of the thawed cell extract on the microarray and allow it to cover the whole surface by gently laying a cover slip on top of it.

This step should be done very gently. Avoid touching the surface with your pipette or tip and try to avoid bubbles. The cover slip should not be moved once it is placed on the nitrocellulose membrane to avoid scratching the surface.

6. Incubate the extract on the microarray for one hour at room temperature.

During this step the proteins on the microarray should be modified according to the state of activity in the extract. To promote or inhibit specific enzymatic activities such as ubiquitylation/SUMOylation/etc' one may supplement the extracts with the wanted

components or inhibitors. It is important that during this step the microarrays not dry; incubate in a closed container with a wet Whatman paper to keep a humid environment.

7. To stop the reaction, add a large volume of TBS into the container. Allow the coverslips to gently dissociate from the microarrays. Remove the coverslips without touching the microarray surface.

It is important that if you run an experiment on several microarrays the reactions will stop simultaneously so that the measured differences will not reflect different incubation times.

Washing the microarrays

8. Wash the microarrays twice with TBS-T and a third time with TBS. Washing should be carried out with gentle shaking and using large buffer volumes to allow complete coverage of the surface area. See Table 1 for common washing buffers recipes

Important: Note that the stringency of washes will allow for the detection of different interactions; to detect only covalent modifications you should add 0.5% SDS to the TBS buffer.

Probing the microarray with antibodies and drying

9. During the last wash prepare the solution for the primary antibody according to the modification of interest. For example, for detection of poly-ubiquitylation use 3 μ l of FK1 antibody (BioMol) in a 1:250 dilution in blocking buffer.

Incubate 100 μ l of the primary antibody for 1 hour at room temperature in a humidified chamber as described in the reaction incubation step (step 6). This step may be carried out overnight at 4°C.

10. Remove excess primary antibody by washing 3 times with TBS-T for 5 minutes each wash with gentle shaking.

If you are using a fluorescently labeled primary antibody proceed to step 13.

11. Incubate with fluorescently labeled secondary antibodies for 1 hour at room temperature.

Note that most microarray scanners will have only two excitation wavelengths at 532nm and 635nm so choose your secondary probes accordingly.

12. Dry the microarrays by sliding each array into a vertically-positioned 50 mL canonical tube (or in a flattened container in case you have a centrifuge that allows for plate rotation) and allow the excess buffer to collect at the bottom by spinning gently for 5 minutes at 200 \times g.

Proceed quickly with the centrifugation step so that residual wash solution will not dry unevenly on the slide surface. The slides are now ready for scanning.

Scanning, image acquisition and quantitation

13. Immediately after drying, the microarrays should be scanned with a suitable microarray scanner (e.g. GenePix 4000B). Adjust the gain and laser power to obtain the best dynamic range by avoiding saturation, yet maximizing the signal. Good starting parameters for scanning are: PMT gain value of 600, laser power of 100% and focus point of 0 μm .

A 10 μm resolution is usually good enough to obtain good images and generate a smaller file size than the 5 μm resolution scan.

14. The scan generates an image file that holds the signal intensity values for all the spots in the array as well as the local background level of each spot. Using quantification software (e.g. GenePix Pro), a reactivity level is assigned to each spot on the array and the signal intensity of each protein may be calculated. The dynamic range of a standard experiment may range from 500 to 65,000 signal intensity units (Figure 2A). However, the dynamic range and signal sensitivity may vary between different modifications and different detection antibodies for both biological and technical reasons, respectively. Yet, from our experiment we found the reproducibility of biological replicates to be around 80% (Figure 2B).

The signal to noise ratio (SNR) of a representative array is usually also given by the quantization analysis software. In DNA expression arrays a SNR value of 3 and above is considered to be sensitive enough to differentiate between background level and positive signal spots. We found in our assay that a signal intensity of 600 units (arbitrary units), may have an SNR value that is higher than 3 suggesting that even spots of low reactivity may yield meaningful information.

Microarray data analysis

15. Software (e.g. ProteinArray Prospector v.5) similar to the one used for mRNA expression analysis may be used to filter, normalize and analyze the data. In addition, other online tools or software packages such as 'R' Biconductor or Matlab (Math works) are available and provide scripts for analyzing large-scale microarray experiments.

Many of these tools were initially designed for gene expression data analysis with ratio measurements compared to a reference reactivity value (e.g. "Affymetrix" microarrays). Data analysis should be conducted according to standard statistical tests (see links for useful resources at the end).

Important: If different antibodies are used for detection or when trying to compare the reactivity levels of different proteins on the same microarray, one should make sure that there is no bias in the analysis towards proteins that are more abundant on the array. Thus, special attention should be given to normalization procedures. It is suggested that the signal intensity of the antibody of interest (e.g. poly-ubiquitin) will be normalized with the relative abundance of protein deposited on each spot. This can be done using the Relative Fluorescent Unit (RFU) value. For each ProtoArray batch production an RFU value is given for each spot on the array based on the spot's reactivity towards

angi-GST antibody. Since each protein on the chip is GST-tagged, the RFU of each spot represents the abundance of that protein on the array and this measure can be used for data normalization (see ProtoArray manual for more details). However, it should not be used as an absolute measure for protein quantitation (see ⁸ for an example of the use of this methodology).

More information about the RFU value may be found on line at: <http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Protein-Expression-and-Analysis/Biomarker-Discovery/ProtoArray.html>

Support Protocol Title: Preparation of functional mammalian cell extracts

Global PTM profiling using protein microarrays largely depend on the activity of the cellular extract and its ability to modify the proteins on the array. These activities are presumed to reflect the cellular program in the cell at the time the extract was prepared. Clearly, any cell-free system is going to differ from the natural cellular environment since cellular compartments have been lost and certain aspects of homeostasis with respect to the external milieu are no longer relevant, however, many aspects of regulation are retained. Previous studies have shown that mammalian cell extracts can recapitulate many cellular processes such as cell cycle progression ^{9, 10} and DNA replication ¹¹ and therefore, the use of functional extracts can be powerful in assaying biochemical activity and be used in a reproducible high-throughput manner. In this section we will describe how to generate extracts from synchronized human cells grown in suspension. The principles of preparation of extract should be applicable to adhesive cells, as well as to cell that were “synchronized” by other signaling events or cellular processes. Since the ability to detect PTMs and differential regulation of these modifications on the arrays depend almost completely on the activity of the extracts, it is highly recommended to validate this procedure prior to the use of protein microarrays. For example, when looking for poly-ubiquitylation modifications that may lead to degradation, it is possible to test the activity of the extracts by their ability to degrade an exogenous protein ¹², prior to performing the microarray experiment.

Materials

Extract Preparation: HeLa S3 cells - ATCC

DMEM with 10% (V/V) FBS and 1% (V/V) antibiotics.

Thymidine

5 mg/ml nocodazole in DMSO

PBS, see recipe.

Swelling buffer (SB), see recipe.

Energy mix, see recipe.

Sterile syringe needles: 20 1/2G

Recombinant proteins to supplement the extracts can be purified or bought commercially (Boston Biochem). For example in the case of ubiquitylation profiling use Ubiquitin (Boston Biochem; stock 10 mg/ml in SB). The extract may be supplemented with specific enzymes (e.g. E1-activating enzymes, 100 nM; or E2-conjugating enzymes, 500nM; to promote activity).

CAUTION: When working with human cells, all appropriate bio-safety practices must be followed.

NOTE: All solutions and equipment coming into contact with living cells must be sterile and aseptic techniques should be used.

Protocol steps

1. Inoculate a 2L spinner flask with four 80% confluent 15 cm plates of HeLa S3. Cells are grown in DMEM medium with 10% (V/V) FBS and 1% (V/V) antibiotics.
2. Add 1 liter of the pre-warmed medium into the spinner flask.
Spinner flask should be autoclaved prior to cell inoculation.
3. Collect the cells off the 4 plates (using trypsin). Aspirate medium off the plates and wash once with sterile phosphate Buffer Saline (for cell culture; Gibco). Add 2 mL of pre-warmed (37°C) Trypsine (Trypsin (0.25%)/EDTA (1 mM), Gibco) to each plate and incubate for a minute (37°C) or until you see cells are detached from the surface. Inhibit trypsin by adding fresh medium onto the plates (usually 5mL) and collect the cells to 15mL canonical tubes. Spin for 3 minutes 125×g, aspirate carefully the supernatant and resuspend with fresh medium (10 mL) and add them into the spinner flask.
4. Incubate at 37°C with 5% CO₂ for 3 days with slow stirring.
5. To synchronize cells add 11mL of thymidine (200 mM) to the cell suspension and incubate for 24 hours at 37°C with 5% CO₂.
Thymidine solution should be made fresh. Sterilize the solution under the hood using a 0.22um filter before addition to the medium.
6. Spin down cells at 125×g for 5 minutes in two 500mL tubes (autoclaved). Discard the supernatant and resuspend cells in 20 mL of pre-warmed DMEM. Wash again by spinning at 125×g for 5 minutes and carefully discard the supernatant.
7. Inoculate the cells into fresh medium and grow for 4 hours at 37°C and 5% CO₂ with slow stirring.

Note that the flask should be washed extensively to remove residual thymidine. Wash by adding 50mL PBS and shaking it. Repeat the same step again and before adding the cells repeat the wash with 50mL of DMEM. Discard the medium from the flask and inoculate cells.

8. Add 22 μ l of 5 mg/ml nocodazole in DMSO to the medium and incubate for 11 hours under the same conditions as above.
9. To harvest cells for extraction, centrifuge cells in 500 mL tubes at 125 \times g for 5 minutes. Remove the supernatant and resuspend cells in 30 mL PBS and spin again. Resuspend in 20 mL per tube and collect cells into one tube and centrifuge again. Remove supernatant carefully and place on ice.
10. Resuspend the cell pellet in ice cold PBS at a volume that is equal to the amount of pellet you got (usually around 3 mL). Redistribute the suspension in 2mL tubes and centrifuge at 16,000 \times g at 4°C for 5 minutes. Discard supernatant carefully.
11. Add ice-cold swelling buffer (see recipe below) to each tube at a volume equivalent to about 80% of the recovered pellet. Add 50 μ l energy mix (see recipe). Incubate on ice for 30 minutes and flip every few minutes to mix.
12. Freeze cells in liquid nitrogen and thaw at a 30C water bath. Repeat freeze-thaw cycles 3 times. Thawing should be done rapidly and the lysates should be thrown back into the liquid nitrogen immediately after thawing. After thawing put the tube on ice.

Repeated cycles of freezing and thawing disrupt cells through ice crystal formation and is used in order to break the cell membrane prior to DNA shearing.

13. Shear with 20 1/2G needle 7-8 times. Try to avoid bubbles.
14. Centrifuge at 2,300 \times g, at 4°C for 5 minutes. Collect supernatant and centrifuge it again at 16,000 \times g at 4°C for 30 minutes.

Collect all the samples into a pre-cooled 15mL tube and pipette up and down gently for mixing. Take an aliquot for testing the activity of the extract (see details of experimental procedures for testing the extract in Williamson et al ¹² and aliquot 200 μ l into 0.5 tubes. Freeze in liquid nitrogen and store at -80°C (Extracts can be usually kept up to 6 months).

Avoid freezing and thawing of the extract multiple times.

Reagents and Solutions

PBS—137 mM NaCl

2.7 mM KCL

8mM Na₂HPO₄

Adjust pH to 7.4

Store up to 1 year at 4°C

TBS—50 mM Tris-Cl

150 mM NaCl

Adjust pH to 7.6

Energy mix—375 mM creatine phosphate

50 mM ATP, pH8

50 mM MgCl₂

(Store in small aliquots at –80°C)

Swelling buffer—24.3 ml ice-cold Milli-Q water or equivalent

500 µl 1 M HEPES pH 7.5

37.5 µl 1 M MgCl₂

25 µl 1 M DTT

125 µl 1 M KCl

1 tablet EDTA-free protease-inhibitor (Roche)

Prepare fresh for each extract preparation

Commentary

Background Information

Our ability to probe biochemical reactions on a proteome-wide scale is still limited and lags behind corresponding developments in genome-wide analysis of genomics and transcriptomics. In recent years, functional mammalian cell extracts with protein microarrays allowed for the identification of physiologically relevant targets in a comprehensive manner. However, just like any other screening approach further validation and investigation is needed to decipher the mechanisms by which these PTMs regulate cellular activities. An advantage of using the approach detailed here is that it provides a cellular context to the modifications that are probed. Other functional assays using protein arrays are usually done with purified components and therefore lack the ability to analyze differential PTM regulation when profiling under different conditions^{13, 14}. Another advantage is that the complex biochemical environment in the extracts may provide important components (some of which we may not be aware of) that are needed for the proper activity of the relevant enzymes. Thus, although the approach is based on a cell-free system, many nuances of regulation may be retained and offer relevant insight to the complex cellular environment.

For background information on the development of the technique please see^{6, 15}

Critical Parameters and troubleshooting

The protein microarrays (ProtoArrays, Invitrogen/Life Technologies, Catalog #: [PAH052501](#)) currently have about 9000 purified human proteins spotted on them. The

spotted proteins include some alternatively spliced isoforms. The full gene list may be found online. While this is an impressive achievement, we are still far from having a full representation of the human proteome. Further, we cannot know for sure that the proteins that are deposited on the surface maintain their function or even fold properly. Based on kinase autophosphorylation it is estimated that about 60% of the proteins are still active on the array. However, we suspect that many of the interactions that are needed in order to perform a certain reaction will be limited when a protein is adsorbed to the surface. Yet, even with these limitations, there is still a lot to be discovered using this tool and just like any other screening approach, identifying the targets is merely the first step which must be followed by further *in vitro* and *in vivo* validation. Below we discuss a few important parameters and some problems that may arise when performing this assay. Further troubleshooting tips for both extract preparation and the protein microarrays may be found in ¹² and ¹⁵, respectively. See Table 2 for description of a few common troubleshooting tips.

While mammalian cell extracts have been proven useful in recapitulating several cellular biological processes such as a state of the cell cycle or response to damage, each condition must be validated with known functional properties or known substrate modifications. Proteins in their tethered state may be limited in their functionality, due to the surface attachment to or the tagging of the proteins with an N-terminus GST tag. Thus, false negative results may arise due to these technical reasons. Furthermore, the tethered protein may not mimic the compartmentalized cellular environment of the cell or its correct fold which may result in false positive hits that are reactive on the chip but would not be true substrates in the cell.

While the functional assay is essentially the same for different modifications, different detection antibodies for various modifications will require certain adjustments. Thus, the amount of antibody that is needed for detection may vary. This should be calibrated separately as a high concentration may cause saturation or non-specific signal and a low amount of antibody may result in weak signals and low sensitivity.

The handling of the microarrays is of key importance as well. When picking up, moving or washing the arrays it is crucial to avoid any contact between the surface and the hands, pipette tips and forceps. Work in a clean environment in order to avoid dust or other particles to come in contact with the surface. Scratching the surface of the membrane or having particles stick to it may cause local areas of complete non-reactivity or of non-specific binding which may cause high signal.

In some cases increased background may be due to insufficient blocking of the surface and this can be solved by longer incubation times or using synthetic blockers.

In addition, the washing conditions will determine the type of interactions (e.g. with or without SDS) one may detect. In case of covalent modifications one should use 0.5% SDS in the washes. However, make sure to remove it in the final wash since the SDS may quench the fluorescent probe.

Anticipated Results—Assays for the detection of alterations in PTMs suggest that about 1.5% of the proteins on the array may show significant changes in modifications when changing conditions. However, this number may change with different modifications and depending on the specific biological conditions that are tested. For example, the ISG15 ubiquitin-like modifier did not show any significant changes in target's reactivity upon changing of the extract conditions from being arrested in mitosis and then released into G1. This is consistent with its known biological functions that are not related to mitotic regulation. See Figure 3 for an example of a microarray with a uniform background signal (A, B) and an example of a non-uniform background (C, D).

Time Considerations—At least two replicates of an array are recommended to allow statistical determination of differential modifications. If the specific assay requires longer blocking or incubation times the duration of the experiment may change accordingly.

The functional assay procedure usually requires 4-6 hours for the detailed protocol. The analysis is expected to take longer depending on the quality of the data, amount of samples and the specific questions that one would like to address.

Acknowledgments

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Literature Cited

1. Zhu H, et al. Global analysis of protein activities using proteome chips. *Science*. 2001; 293:2101–2105. [PubMed: 11474067]
2. Zhu H, Qian J. Applications of functional protein microarrays in basic and clinical research. *Adv Genet*. 2012; 79:123–155. [PubMed: 22989767]
3. LaBaer J, Ramachandran N. Protein microarrays as tools for functional proteomics. *Curr Opin Chem Biol*. 2005; 9:14–19. [PubMed: 15701447]
4. Persaud A, Rotin D. Use of proteome arrays to globally identify substrates for E3 ubiquitin ligases. *Methods Mol Biol*. 2011; 759:215–224. [PubMed: 21863490]
5. Loch CM, Cuccherini CL, Leach CA, Strickler JE. Deubiquitylase, deSUMOylase, and deISGylase activity microarrays for assay of substrate preference and functional modifiers. *Mol Cell Proteomics*. 2011; 10:M110 002402. [PubMed: 20956615]
6. Merbl Y, Kirschner MW. Protein microarrays for genome-wide posttranslational modification analysis. *Wiley Interdiscip Rev Syst Biol Med*. 2011; 3:347–356. [PubMed: 20865779]
7. Merbl Y, Kirschner MW. Large-scale detection of ubiquitination substrates using cell extracts and protein microarrays. *Proc Natl Acad Sci U S A*. 2009; 106:2543–2548. [PubMed: 19181856]
8. Merbl Y, Refour P, Patel H, Springer M, Kirschner MW. Profiling of ubiquitin-like modifications reveals features of mitotic control. *Cell*. 2013; 152:1160–1172. [PubMed: 23452859]
9. Rape M, Reddy SK, Kirschner MW. The processivity of multiubiquitination by the APC determines the order of substrate degradation. *Cell*. 2006; 124:89–103. [PubMed: 16413484]
10. Ayad NG, Rankin S, Ooi D, Rape M, Kirschner MW. Identification of ubiquitin ligase substrates by in vitro expression cloning. *Methods Enzymol*. 2005; 399:404–414. [PubMed: 16338372]
11. Rankin S, Ayad NG, Kirschner MW. Sororin, a substrate of the anaphase-promoting complex, is required for sister chromatid cohesion in vertebrates. *Mol Cell*. 2005; 18:185–200. [PubMed: 15837422]
12. Williamson A, Jin L, Rape M. Preparation of synchronized human cell extracts to study ubiquitination and degradation. *Methods Mol Biol*. 2009; 545:301–312. [PubMed: 19475397]

13. Gupta R, et al. Ubiquitination screen using protein microarrays for comprehensive identification of Rsp5 substrates in yeast. *Mol Syst Biol.* 2007; 3:116. [PubMed: 17551511]
14. Del Rincon SV, et al. Development and validation of a method for profiling post-translational modification activities using protein microarrays. *PLoS One.* 2010; 5:e11332. [PubMed: 20596523]
15. Meng L. Functional assays on high-content protein microarrays. *Current Protocols in chemical biology.* 2012; 4:211–231.

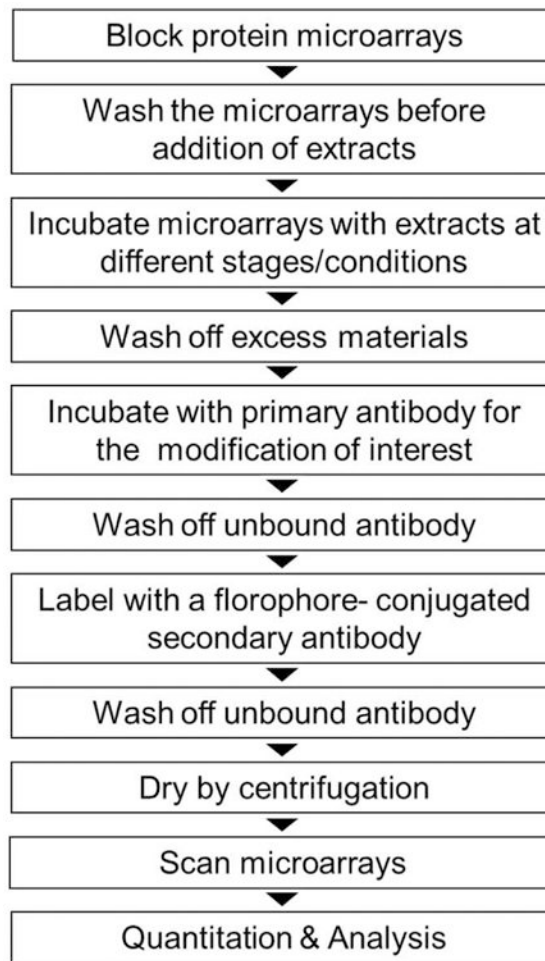


Figure 1. General depiction of the steps required to carry out PTM profiling for a certain modification using mammalian cell extracts and protein microarrays. Preparation of the extracts that are mentioned (Box 3) is detailed in the supplemented protocol.

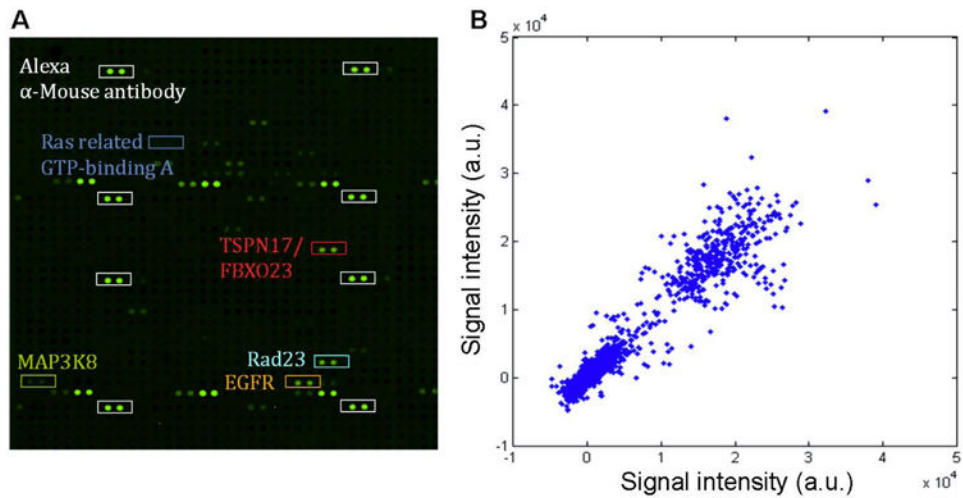


Figure 2.

A. An example of a portion of one developed microarray is depicted. Different proteins and their signal on the chip are presented. **B.** Reproducibility of the assay. Microarrays were incubated with mitotic extracts. The poly-ubiquitination signal (FK1 antibody, Biomol) per protein was calculated by the mean values of its spots reactivities. The reactivity level from two chips developed on different days were plotted in order to compare the variability between two biological replicates.

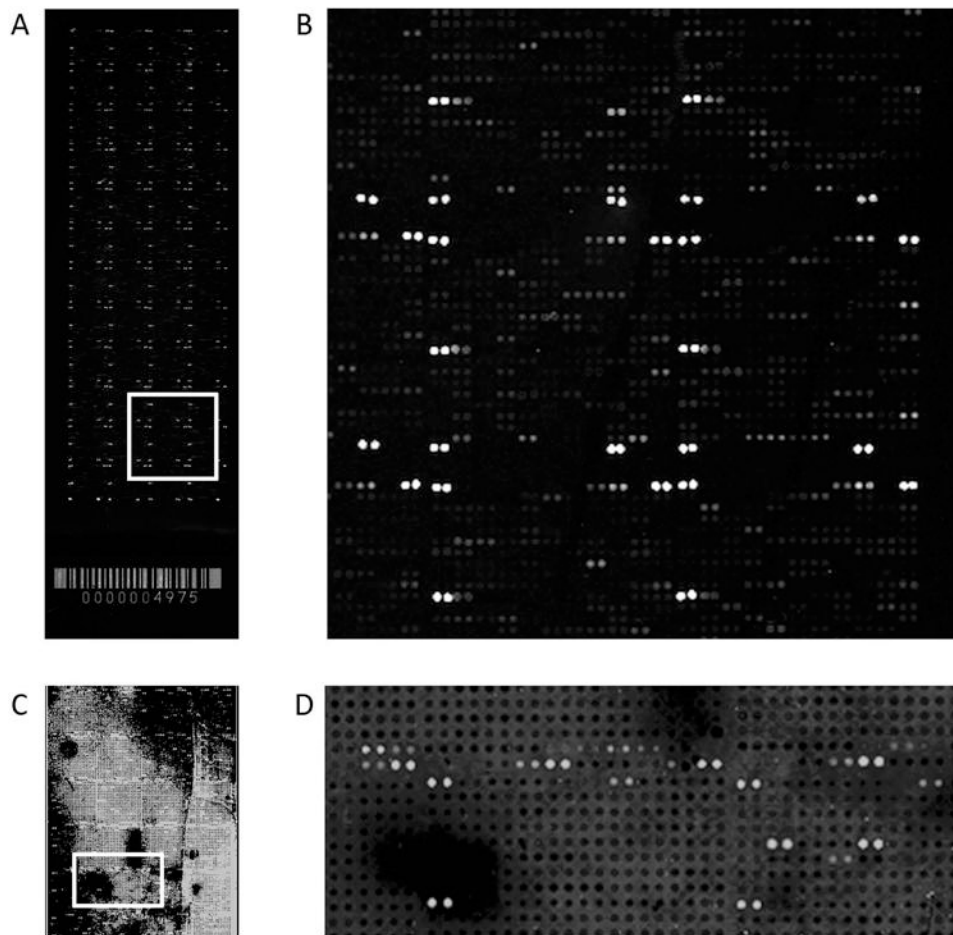


Figure 3.

Examples of expected outcomes after developing the microarrays. **A.** An image of a developed and scanned microarray with poly-ubiquitin antibody (FK1). Over 8000 unique human proteins on a ProtoArray human protein microarray are spotted on the surface and proteins that were modified by poly-ubiquitin were detected with a fluorescently-labeled secondary antibody. Control proteins help to orient the array grid in the data analysis step and serve as positive and negative controls for different applications. **B.** A portion of the microarray is enlarged (white box in A) to see the reactivity in a higher magnification. **C.** An example of a portion of a developed microarray with non-uniform blocking. The contrast is enhanced to show the differences in background reactivity. **D.** An enlarged portion of the white box in C in order to show the loss of signal reactivity in the spots that are located in the bad region.

Table 1

The table below provides some examples for commonly used compositions of washing buffers for different applications. As noted, it is recommended to make the buffers before use and to filter them with a 0.2 um filter before incubation with the array.

Washing Buffer	Reagents
ProtoArray washing buffer	Phosphate-buffered saline (PBS; see recipe) 1× synthetic block (e.g., Roti-Block, Carl Roth GmbH; http://www.carlroth.de) 0.1% (v/v) Tween 20
Protein- protein interactions	TBS 0.1% (v/v) Tween 20
Covalent modifications	TBS 0.1% (v/v) Tween 20 1% (v/v) SDS
Protein -DNA interactions (2×)	50 mM HEPES (pH 8.0) 100 mM L-glutamic acid potassium salt monohydrate 0.2% Triton X-100 16 mM magnesium acetate tetrahydrate 20% glycerol *Dilute with equal volume of H ₂ O

* Prepare fresh just before use.

** It is also recommended to filter sterilize the buffer before use with a 0.2 um filter.

Table 2

The table below provides some solutions to common problems in performing the assay.

Problem	Possible cause	Recommended solution
High background	Improper blocking/washing	Make sure the blocking buffer and washing reagents are fresh and clean.
	Array was allowed to dry	Do not allow the array to dry during probing or handling
	Array not dried properly before scanning	Make sure that the array is completely dry before scanning. You should not see any wet/humid parts on the membrane.
Low signal or low signal to noise ratio	Use of Alexa Flour 555 or Cy3 dyes for detection	These dyes give high background due to the intrinsic fluorescence of the nitrocellulose membrane. Avoid using them for detection.
	Antibody signal may not be specific	It is highly recommended to test the sensitivity of the antibody by performing in vitro studies and making sure an increase in signal is detected under the appropriate conditions. Making sure the antibody does not give non-specific background signal is crucial.
	Interaction conditions too stringent	Make sure the last wash before probing (addition of detection antibody or other binding interaction) is done with low concentration of salt/detergent.
	Incorrect scanning or imaging	Make sure the PMT gain and laser power were adjusted as required and that the positive spots (e.g. Alexa antibody) appear clearly with high signal intensity.
Uneven Background	Uneven blocking or washing	Make sure the array is completely covered by blocking/washer buffer during these steps.
	Improper handling of the arrays	Avoid touching the surface of the arrays and work in a clean environment. The membrane is very gentle and sticky and therefore dust or other particles should be limited from touching the surface.
	Array was allowed to dry	Do not allow the array to dry during probing or handling
	probe or other reagents contain precipitates	Make sure precipitates are removed by centrifugation prior to incubation on the array