Immunocell-array for Molecular Dissection of Multiple Signaling Pathways in Mammalian Cells*

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The knowledge of signaling pathways that are triggered by physiological and pathological conditions or drug treatment is essential for the comprehension of the biological events that regulate cellular responses. Recently novel platforms based on “reverse-phase protein arrays” have proven to be useful in the study of different pathways, but they still lack the possibility to detect events in the complexity of a cellular context. We developed an “immunocell-array” of cells on chip where, upon cell plating, growing, drug treatment, and fixation, by spotting specific antibodies we can detect the localization and state of hundreds of proteins involved in specific signaling pathways. By applying this technology to mammalian cells we analyzed signaling proteins involved in the response to DNA damage and identified a chromatin remodeling pathway following bleomycin treatment. We propose our technology as a new tool for the array-based multiplexed analysis of signaling pathways in drug response screening, for the proteomics of profiling patient cells, and ultimately for the high throughput screening of antibodies for immunofluorescence applications. *Molecular & Cellular Proteomics 6:939–947, 2007.

The misregulation or hyperactivity of intracellular and extracellular signaling cascades may represent the molecular signature of human diseases (1). Thus, the capability of monitoring the complexity of all biochemical networks involved in specific pathologies is essential for the realization of patient-tailored therapies and for the characterization of drug response (2, 3). Signaling pathways are complex, and they are regulated by specific spatiotemporal dynamics taking place in a cellular context. To deal with this complexity, approaches are required that can describe “when and where” (4, 5) a single event takes place. New genomics and proteomics technologies have recently been developed (6–8) allowing the simultaneous analysis of multiple events and the characterization of complex samples. Among them, reverse-phase protein microarrays (9) represent the most valid approach to probe tissue or cell lysates with a large number of antibodies or patient sera to allow the identification of new biomarkers, the analysis of protein expression profiles, and the efficacy and toxicity of drug candidates. These novel protein array platforms have proven to be very powerful in the study of different molecular pathways; however, although this technology represents an important tool for proteomics studies, it does not allow the detection of multiple events in the “cellular” context of compartments and organelles where phenomena have to be analyzed for the comprehension of biological networks.

We developed an “immunocell-array” of cells on glass slides where, upon fixation, we can detect simultaneously, with specific antibodies, the localization and state of hundreds of proteins involved in different signaling pathways, minimizing reagents and cell consumption and obtaining high quality images by high resolution microscopy. Using this tool we analyzed the kinetic response (protein post-transcriptional modification and profiling) of different target proteins upon DNA damage and identified a chromatin remodeling pathway in mammalian cells. We expect that this new cell-based technology will offer a powerful tool in proteomics studies for drug discovery and will introduce a new immunofluorescence methodology in life science applications.

EXPERIMENTAL PROCEDURES

Cells and Culture Conditions—All cell lines were grown in tissue culture plates (Falcon, BD Bioscience) at 37 °C in a humidified incubator with 5% CO₂. Human osteosarcoma U2OS (American Type Culture Collection (ATCC)) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS,1 1% l-glutamine, and 1% P/S. Human fibroblast Tg3-hTert (Tg3 fibroblasts immortalized with human telomerase reverse transcriptase, kindly provided by Dr. K-S, Kolmogorov-Smirnov.

1 The abbreviations used are: FBS, fetal bovine serum; P/S, penicillin/streptomycin; DAPI, 4',6-diamidino-2-phenylindole; p-, phosphorylated; VASP, vasodilator-stimulated phosphoprotein; PML, promyelocytic leukemia; ATM, ataxia-telangiectasia mutated; DAXX, death domain-associated protein 6; HDAC, histone deacetylase; CBP, CREB-binding protein (CREB)-binding protein; K-S, Kolmogorov-Smirnov.
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C. Moroni IFOM-IEO Campus, Milan, Italy) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS (origin, United States), 1% L-glutamine, and 1% P/S.

Primary human melanocytes were isolated in our laboratory according to the protocol developed by Meenhard Herlyn. Cells were cultured in McCoy’s 5A medium (Invitrogen) supplemented with 2% FBS (United States origin), 5 μg/ml recombinant human insulin (Roche Applied Science), 5 μg/ml human holotransferrin (Sigma), 0.5 μg/ml hydrocortisone (Sigma), 20 μM chola toxin from Vibrio cholerae (Sigma), 16 μM phorbol 12-myristate 13-acetate (Sigma), 10 nM endothelin 1 human, porcine (Sigma), 10 ng/ml recombinant human stem cell factor (Peprotech). NIH3T3 cells (ATCC) were cultured in McCoy’s 5A medium (Invitrogen) supplemented with 2%

PBS, and blocked for 1 h with 2% BSA. After blocking, the slides were washed, cells were permeabilized for 10 min with 0.1% Triton X-100, monoclonal anti-p-tyrosine (Upstate, clone 4G10), monoclonal anti-paxillin (Zymed Laboratories Inc., catalog number 33-2400), monoclonal anti-SC35 (Sigma, clone SC35), monoclonal anti-HDAC1 (homemade), monoclonal anti-p53 (Santa Cruz Biotechnology, DO-1), monoclonal anti-CDK2 (BioLegend), monoclonal anti-ERK1 (homemade), monoclonal anti-EpCAM (homemade), monoclonal anti-p-tyrosine (Upstate, clone 4G10), monoclonal anti-ALK (Sigma, clone SC54), monoclonal anti-E3B1 (homemade), monoclonal anti-Eps8 (Cell Signaling Technology, sc-8408), monoclonal anti-BMI1 (homemade), monoclonal anti-small ubiquitin-like modifier (Zymed Laboratories Inc., catalog number 33-2400), monoclonal anti-SC35 (Sigma, clone SC35), monoclonal anti-SC35 (Sigma, clone SC35), monoclonal anti-HDAC1 (homemade), monoclonal anti-p-CDK2 (Santa Cruz Biotechnology, sc-66), and monoclonal anti-Sirt6 (homemade). Secondary antibodies included: goat anti-mouse and rabbit Al exa Fluor 488 (Molecular Probes-Invitrogen) and goat anti-mouse and rabbit Cy3 (Jackson ImmunoResearch Laboratories). For control staining mouse IgG, isotype (BD Biosciences) was used at 1 μg/ml. Image Acquisition—Imaging of immunocell-arrays and immunostained coverslips was performed with an automated fluorescence microscope (Olympus IX81 Scan R microscope-based imaging platform for screening, Olympus Europa, Hamburg, Germany) equipped with a digital camera (ORCA-ER C4742-80, Hamamatsu) using 10×, 20×, 40×, and 60× UPlanApo magnification objectives. For the immunocell-arrays analyzed in Figs. 4 and 6, each antibody was spotted in a 10× replicate, and one 20× field per spot (containing ~35 cells) was acquired; population to be compared thus contained ~350 cells. For the immunofluorescence staining of the experiment shown in Fig. 7A, 25 images were acquired with a 20× magnification objective for each antibody at each time point.

Image Analysis—Images were processed and analyzed with automated routines written in Matlab programming language with Image Processing Toolbox. Background was removed by subtracting from each image its morphological opening with a circle of radius 20 pixels. After background subtraction, a binary mask was generated identifying nuclear regions by applying a threshold segmentation on DAPI staining mouse IgG, isotype (BD Biosciences) was used at 1 μg/ml. Image Acquisition—Imaging of immunocell-arrays and immunostained coverslips was performed with an automated fluorescence microscope (Olympus IX81 Scan R microscope-based imaging platform for screening, Olympus Europa, Hamburg, Germany) equipped with a digital camera (ORCA-ER C4742-80, Hamamatsu) using 10×, 20×, 40×, and 60× UPlanApo magnification objectives. For the immunocell-arrays analyzed in Figs. 4 and 6, each antibody was spotted in a 10× replicate, and one 20× field per spot (containing ~35 cells) was acquired; population to be compared thus contained ~350 cells. For the immunofluorescence staining of the experiment shown in Fig. 7A, 25 images were acquired with a 20× magnification objective for each antibody at each time point.

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2 M. Herlyn, unpublished data.
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RESULTS

Setup of an Immunocell-array Protocol on Glass Slides—

We set up a novel method for simultaneous staining with multiple antibodies spotted in array format on fixed cells grown directly on glass slide as described in Fig. 1. Tg3-hTert fibroblasts were plated on microarray-grade glass slides coated with gelatin, grown at the desired confluency, and fixed by 4% paraformaldehyde for 10 min. Cells were subsequently permeabilized, blocked by BSA incubation, and further processed for primary and AlexaFluor488-conjugated secondary antibody spotting. Antibodies were spotted by means of a non-contact spotter; the antibody dilutions were prepared in 96-well plates. For primary antibody spotting, fixed slides were gently washed and, after draining the excess washing solution, placed on the slide holder in 65% humidity.

To verify the specificity of staining in localized areas, we spotted a monoclonal antibody against PML, a nuclear protein localized in nuclear dots (PML bodies) (14), and a mouse 

IgG1 isotype in 2% BSA in alternate fashion in a 7 x 7 array format so that we could easily score eventual PML staining in adjacent IgG1 spots due to antibody cross-reactivity. After primary antibody spotting, the slide was incubated to allow antibody-antigen recognition at 4 °C, washed with PBS to remove the unbound antibodies, and placed on the spotter sample holder for the secondary antibody spotting. After incubation at 4 °C, slides were washed with PBS, stained with DAPI to allow the identification of nuclei, then mounted with Mowiol, and processed for image acquisition with an automated platform for fluorescence microscopy. The results are summarized in Fig. 2 and show that PML staining was specifically localized in the nucleus of cells belonging to confined regions without the occurrence of cross-contamination of cells in IgG, adjacent spots. We confirmed the PML nuclear localization and the IgG1 staining of the immunocell-array (Fig. 2, E and F, respectively) by conventional immunofluorescence on coverslips (Fig. 2, G and H, respectively). We also tested cross-reactivity by spotting in alternate fashion anti-PML antibody and 2% BSA in PBS; also in this case we did not observe cross-contamination among spots (data not shown).

Adapted Primary Melanocytes and NIH3T3 Cells Are Suitable Cellular Model Systems for Targeting Cytoplasmic and Nuclear Proteins by Immunocell-array—To further explore the potential of this technology, we decided to perform immunocell-array experiments on different cellular types targeting several nuclear, cytoplasmic, and membrane proteins. We thus tested NIH3T3 cells and adult human primary melanocytes. NIH3T3 cells represent a suitable model for cytoskeleton-associated signaling pathways. In Fig. 3A we show a 7 x 19 immunocell-array of NIH3T3 cells grown on slide for 3 days, fixed, and stained with a panel of different antibodies (17 different antibodies and two lanes of BSA as negative control; see Table I for the antibody list); Cy3-conjugated antibodies were used as secondary antibodies. In Fig. 3B higher resolution images show detailed staining of all the tested antibodies for which we obtained specific staining. In particular we detected actin-associated structures by anti-α-actinin (15), -cortactin (16), -Eps8 (17), -Abi1 (18), -VASP (19); phosphorylated proteins by anti-p-tyrosine; Golgi staining by anti-giantin (20); focal adhesions by anti-paxillin (21) and anti-vinculin (21); vesicles by clathrin (22) and caveolin (23) staining; and cytoplasmic and vesicle-associated Eps15 staining (24) and NPM nucleolar staining (25). Primary melanocytes represent important tools for the biological studies related to diseases like pigmentation disorders, tumors (melanoma), or other genetic abnormalities; the possibility to perform high throughput signaling studies on patient-derived samples, where the availability of cells is one of the major constraints, certainly represents a valuable resource. We performed a 10 x 20 immunocell-array (Fig. 3C) by staining with 18 different antibodies and BSA as negative control (see Table I); AlexaFluor488-conjugated antibodies were used as secondary antibodies. Fig. 3D shows images at 40 X magnification of
all the tested antibodies; we obtained specific staining for all the targets confirming the reproducibility of our technology and the capability of employing different cellular models.

Immunocell-arrays Detect Deacetylation of Chromatin in Bleomycin-treated U2OS Cells—To evaluate the capability of immunocell-arrays in dissecting molecular pathways, we studied the DNA damage response following bleomycin treatment of U2OS cells by analyzing a panel of selected antibodies (see Table I for the antibody list). To perform a quantitative analysis of signal intensity of the different antibodies among different slides, we performed an immunocell-array experiment by plating U2OS cells on three slides, treating them with bleomycin for 8 and 24 h, and comparing them with an untreated slide. We performed on each slide an immunostaining for lamin-B, p-ATM, p53, and γ-H2AX in a 10 × 4 array format. p-ATM, p53, and γ-H2AX are well known targets of DNA damage response and become activated or up-regulated upon treatment. Because lamin-B, a nuclear envelope structural protein, is not expected to undergo changes in expression/regulation upon bleomycin treatment (26), we considered it as a control over interslide staining consistency. As expected, no significant changes in lamin-B staining were detected, whereas we observed an increase in expression of p-ATM, p53, and γ-H2AX (p < 0.001). These results are summarized in Fig. 4 with the heat map reporting the K-S statistics with sign (see “Experimental Procedures” for image analysis) for the various antibodies. Red color corresponds to increasing levels with respect to the untreated population, green corresponds to decreasing levels, and black corresponds to constant levels; the magnitude of the shift equals the K-S statistics against the untreated population (see “Experimental Procedures”). The cumulative distribution func-
For the DNA damage experiment, as shown in Supplemental Fig. 1, three slides were plated with U2OS cells; two of them were treated with bleomycin for 8 and 24 h (a, untreated; b, 8 h; c, 24 h). The three samples were processed for staining with 22 different antibodies (plus two lanes of BSA samples as background control) in a 24 × 100 array format and analyzed through automated fluorescence microscopy. Image magnifications (40× magnification) for all the tested antibodies at time 0 and 24 h of bleomycin treatment are reported in Fig. 5.

Fig. 6 summarizes the results concerning the 22 antibodies used in the experiment. The heat map in Fig. 6A reflects the time course behavior of the various antibodies. Cumulative distribution functions for each antibody at the different time points, from which the K-S statistics were driven, are reported in Fig. 6B.

We observed and confirmed that known targets of DNA damage response, such as p-ATM (27), γ-H2AX (28), and p53 (27), were activated or up-regulated upon treatment. Moreover Ezh2, which is a putative target of p53 (29), was down-regulated. Interestingly another protein of the polycomb group, BMI1, appeared to be down-regulated upon damage, suggesting its possible involvement in the cell cycle response through functional interaction with pathways of cell cycle block (30). Other proteins like Sirt6 (31), the deacetylase HDAC1 (32), the proapoptotic DAXX (33), and the transcription...
down-regulation of histone acetylation (both H3 and H4) was observed at 48 h. We further confirmed these data by performing immunoprecipitation with T52 on the same lysates followed by silver staining of the SDS-PAGE gel (see “Experimental Procedures”); we observed specific down-regulation of acetyl-H3 and acetyl-H4 at the earlier time point (24 h), in accordance with our immunocell-array detection, probably due to the intrinsic higher sensitivity of the method. Previous work done in yeast (35) and recently in mammalian cells (36) has underlined a mechanism of localized chromatin remodeling activity triggered by the double strand DNA repair pathway. Our results, obtained by immunocell-array technology and validated by conventional immunofluorescence, Western blot, and immunoprecipitation, further strengthen the role of chromatin state in DNA repair and genomic stability but mainly characterize our technology as a novel tool for protein profiling, in the context of cellular complexity, in the field of drug discovery.

DISCUSSION

We set up a new cell-array-based method for multiplexed immunofluorescence analysis of target proteins in different cellular models that provides many advantages over existing immunofluorescence-based methods. 1) It decreases the cost of the assay in terms of antibodies because only 1 μl of the primary or secondary antibody is required for producing up to 1000 spots in one experiment so that only a few nanoliters of reagents can be used. Rare patient cells can be processed for profiling studies, opening interesting perspectives in the monitoring of human disease. 2) It provides a flexible “high content” approach “on chip” for multiple targets and different kinetics in the context of whole-cell analysis where multiparametric data can be obtained, 3) Data can be acquired by high resolution automated microscopy and other commercial automated platform for image acquisition and analysis. 4) Finally the technology ensures a high degree of reproducibility and no well-to-well variability.

We propose the immunocell-array technology as a powerful tool for the molecular dissection of signaling pathways (kinase response, post-translational modifications, protein localization, and protein profiling) upon drug response in normal and disease states, for the characterization of cancer signatures (protein overexpression and mislocalization) on patient cell samples, and ultimately for the screening of monoclonal and polyclonal antibodies for immunofluorescence applications. In particular, by using immunocell-array, we identified a recently described pathway of deacetylation in mammalian cells upon DNA damage (36) that probably involves specific chromatin-modifying enzymes. We validated this finding by conventional immunofluorescence, Western blot, and immunoprecipitation approaches. A further detailed biochemical analysis of this pathway, however, is beyond the scope of our work, which is mainly directed toward the development and validation of a new technological tool for protein profiling. Finally with the increased availability of highly specific and selective antibod-
FIG. 5. 8 × 24 immunocell-arrays of U2OS cells treated with bleomycin. 40× magnifications of small areas of spots representing all the immunostained proteins in untreated (0 h) and 24-h bleomycin-treated slides are shown. p-TYR, phosphotyrosine; SUMO, small ubiquitin-like modifier; GIANT, giantin; DNA-PK, DNA-dependent protein kinase.

FIG. 6. Immunocell-arrays detect a chromatin remodeling pathway in response to DNA damage in U2OS cells. A, heat map showing the response of the 22 proteins (and BSA as control) that were stained in the immunocell-array of Fig. 5. Colors are proportional to the K-S statistics of the empirical cumulative distributions at 8 and 24 h against the untreated (0 h) condition and reflect the extent of protein up-regulation (green) or down-regulation (red) as detected by a K-S test with \( p < 0.001 \). B, empirical cumulative distributions of fluorescence intensities of all the antibodies used in the immunocell-array experiment from which K-S statistics of \( A \) were calculated. The fraction of cells over the total population is plotted as a function of its maximum nuclear mean fluorescence intensity (arbitrary units). Dotted lines, untreated population (0 h); dashed lines, population 8 h after treatment with bleomycin; solid lines, population 24 h after treatment with bleomycin. Distributions shifted to the right correspond to increased fluorescence signals and vice versa. pTYR, phosphotyrosine; SUMO, small ubiquitin-like modifier; DNAPK, DNA-dependent protein kinase.
ies to cancer-relevant targets, the present technology could accelerate the drug development process and offer new tools to patient monitoring during disease therapy.

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