Single-Cell Proteomics

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Single-Cell Chemical Proteomics with an Activity-Based Probe: Identification of Low-Copy Membrane Proteins on Primary Neurons**

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Abstract: *We propose a novel single-cell chemical proteomics (SCCP) strategy to profile low-abundance membrane proteins in single cells. In this approach, the membrane protein GB1 and its splicing variants were targeted on cultured cell lines and primary neurons using a specifically designed activity-based probe. The functionally labeled single cells were encapsulated in individual buffer droplets on a PDMS microwell array, and were further picked up one at a time and loaded into a capillary electrophoresis system for cell lysis, separation, and laser-induced fluorescence detection of the targeted proteins. The results revealed the expression of GB1 splicing variants in HEK and MEF cells, which was previously only suggested at the transcriptional level. We further applied this method to investigate single primary cells and observed significant heterogeneity among individual mouse cerebellar granule neurons. Interference experiments with GB1 antagonist and agonist validated this observation.*

With the completion of the Human Genome Project, "-omic" profiling of nucleic acids, proteins, metabolites, and their interaction networks has become one of the major approaches for revealing the chemical foundations of biology, and thus speeding up our understanding of the molecular mechanisms of life. In recent years, such investigation has even been extended to the single-cell level.[1] For example, single-cell genome sequencing has been recognized as the next-generation platform for genome-wide research, showing great potential for personalized medical applications.[2] Compared to single-cell genomics, however, single-cell proteomics (SCP) proposed by Dovichi,[3] Nolan,[4] and their respective co-workers, seems much more difficult because of the huge number of species that have a high variation in amount inside the proteome.[5] Conventionally, there are two major approaches for SCP analysis, namely, flow cytometry[6] and chemical cytometry.[7] Flow cytometry based on multiple parameters shows great potential in mapping cellular signal transduction networks and investigating molecular mechanisms of diseases.[8] It is function-oriented, thus avoiding interference from a large amount of uncorrelated proteins at the cost of tedious antibody-binding procedures and consumption of millions of primary cells. Meanwhile, chemical cytometry is mainly conducted by two-dimensional capillary electrophoresis, which has been successfully applied to single-cell proteomic profiling.[9] The heterogeneity among single cells can be readily discriminated, which is definitely meaningful for neuroscience or cancer biology where cells show high heterogeneity. Recently, Shi et al.[10] developed a new microchip-based SCP strategy, providing a comprehensive picture of protein signaling networks of tumor cells.

Herein, we propose a novel single-cell chemical proteomics (SCCP) approach based on the activity-based probe (ABP) technique[11] to identify the membrane proteins on single primary neurons. Transmembrane receptors are a group of low-copied (<1000 copies per cell) membrane proteins with fundamental functions such as signal transduction, gene regulation, and mass transportation.[12] Although they play an important role in cell function, their low abundance complicates the investigations at the single-cell level. We chose GB1, one of the subunits of the $GABA_B$ receptor,[13] as the low-abundance target membrane protein to perform SCCP on a single cell. The $GABA_B$ receptors are involved in numerous physiological processes through the regulation of both GABAergic and glutamatergic synapses at either the pre- or postsynaptic level, as well as in various pathological changes including nociception, cognitive impairment, epilepsy, spasticity, and drug addiction.

In SCCP, the target proteins on single cells were firstly labeled with an activity-based trimodular probe (termed as ABP), which was synthesized by a method based on "click" chemistry (Figure S1 in the Supporting Information).[14] The ABP molecule consists of three main parts, a "warhead" sharing the similar pharmacophore with the known GB1 receptor antagonists for targeting the active domain of the GB1 subunit, a photolabile diazirine group that can effectively generate a covalent, irreversible linkage between the

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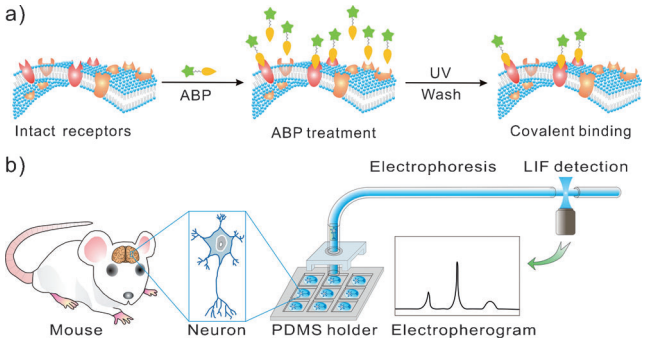

Figure 1. Illustration of the SCCP strategy. a) ABP labeling procedure: ABP was applied to functionally label the specific receptor; after UV irradiation, excessive ABP molecules were washed away. b) Single neuron extracted from a mouse was injected into a capillary for CE-LIF analysis to study a specific receptor protein functionally labeled with ABP.

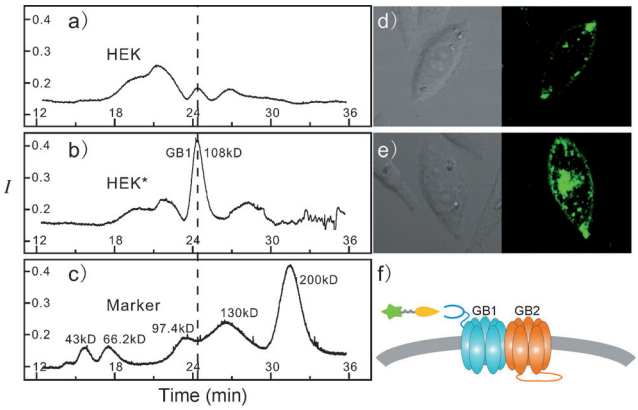

Figure 2. SCCP analysis of a HEK293 cell. a) Electropherogram of GB1 receptor and GB1-like proteins in a single HEK293 cell; b) electropherogram of a single HEK293 cell overexpressing GB1 subunits at the cell surface (HEK*); c) electropherogram of protein molecular weight markers; d) TIRF (total internal reflection fluorescence) image of a HEK293 cell after incubation with ABP; e) TIRF image of a HEK* cell labeled by ABP; f) schematic of ABP-labeled $GABA_B$ receptors.

probe and the GABAB receptors after UV irradiation, and a fluorescent BODIPY tag for fluorescent detection. A linker keeps the fluorescent tag far enough away from the "warhead" so that its activity is not altered. The cells were incubated with ABP for several minutes for the specific binding of probe to the GB1 subunits. The UV light was then applied to covalently crosslink the probe to the receptor (Figure 1a). Afterwards, the labeled cells were washed with the buffer to remove residual ABP molecules.

The functionally labeled single cells were subsequently encapsulated in buffer droplets stored in a PDMS chip holder for further capillary electrophoresis–laser induced fluorescence (CE-LIF) detection. As the employment of ABP significantly reduced the number of uncorrelated proteins, a one-dimensional CE-LIF system was constructed. CE is an ideal tool for single-cell analysis,[15] since the inner diameter of the capillary is compatible with a normal cell, and LIF provides the best detection sensitivity even down to the single-molecule level.[16] By using this home-built system, baseline resolution of the protein markers could be accomplished with a detection limit of 0.1 pM (ca. 8.55×10^{-22} mole) and a reproducibility of root-square deviation (RSD) < 0.1% for the migration time (Figure S2 in the Supporting Information). To facilitate single-cell injection, a unique microfluidic injector was developed (Figure 1b). Droplets encapsulating single cells were deposited into a PDMS microwell array. A PDMS rider fixed with a capillary could easily move among these microwells to pick up the droplet. Thus, a single cell could be injected into the capillary by negative pressure. Cell lysis and protein denaturation were then realized by heating for subsequent CE-LIF analysis.

For proof-of-concept validation of SCCP, analysis of GB1 receptors and GB1-like proteins on single cells from the cultured HEK293 cell line was initially performed. GB1 has many isoforms or splicing variants, namely GB1a, GB1b, GB1c, GB1e, GB1j, GB1k, GB1l, GB1m, and GB1n. Those isoforms are expressed in different tissues. For examples, Gb1a and GB1b are mainly expressed in the central nervous system, while GB1e is mainly expressed in peripheral tissues. As shown in Figure 2a, a series of peaks belonging to GB1-like proteins were separated and detected. By analyzing HEK cell overexpressing GB1a ($GB1a_{asa}$ mutant) at the cell surface,[13g] the peak for GB1a (108 kD) could be readily identified among those peaks as shown in Figure 2b, which could be further validated by the molecular markers (Figure 2c). The other peaks might be other GB1 splice variants encoding a truncated receptor, which still contain the same ligand binding sites to the ABP.

We next compared the intrinsic protein level of GB1 in different cell lines such as HEK293, MEF, and CHO cells by using the SCCP strategy. Interestingly, these cell lines gave different electropherogram patterns when our strategy was used (Figure 3a). As mentioned above, HEK293 cell expressed endogenous GB1a and other GB1 variant splices. However, MEF cells solely expressed GB1a, while there was no GB1a or its splice variants expression in the CHO cells. An electropherogram obtained from CHO cells overexpressing GB1a at the cell surface verified the labeling specificity of the ABP molecule. These results provided the first reported

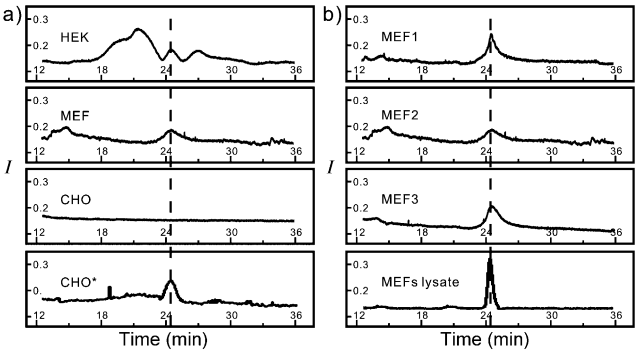

Figure 3. SCCP analysis of the different cell lines. a) Electropherograms of GB1 receptors in single HEK293, MEF, and CHO cells, and a CHO* cell overexpressing GB1 subunits at the cell surface. b) Electropherograms of three single MEF cells and purified GB1 sample from the ABP-labeled MEF cell population (MEFs lysate).

evidence at the protein level to reveal the existence of endogenous GB1a and other splicing variants in HEK293 cell and MEF cell, which was previously suggested by studies of GB1 in HEK293 cell line at the transcriptional level.[17] We further investigated the heterogeneity of GB1a levels in different MEF cells. Apparently, all the MEF cells expressed GB1a, but the protein levels were quite different (Figure 3b), thus suggesting that GB1a levels in MEF cells were heterogeneous. A comparison analysis of a purified GB1 sample from the ABP-labeled MEF cell population is also given in Figure 3b. As no GB1 variant was detected in splices in CHO cells, it is suggested that functional analysis of $GABA_B$ receptors by overexpressing this receptor in CHO cells were better than those in the HEK293 cells or the MEF cells.

We finally applied our strategy to identify the GB1 receptors on mouse cerebellar granule neurons (CGNs). Figure 4a showed the results from five CGNs as examples.

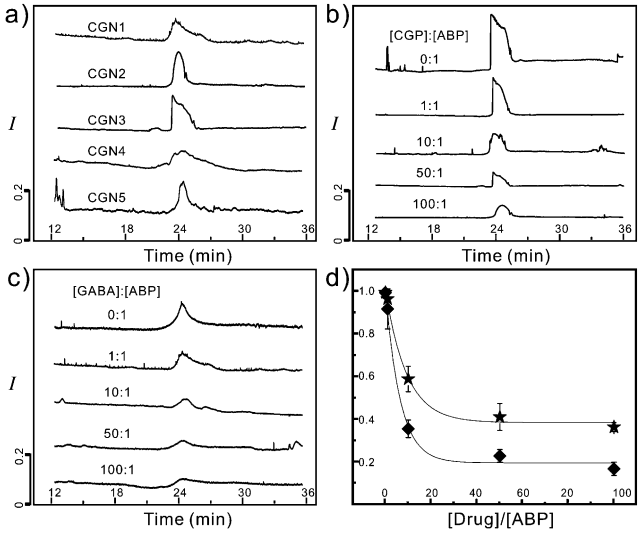

Figure 4. SCCP identification of mouse primary CGNs. a) Electropherograms of GB1 in five single CGNs. b) Electropherograms under competitive binding of ABP by CGP64213 (antagonist, ◆ in Figure 4d) on single CGNs. c) Electropherograms under competitive binding of ABP by GABA (agonist, ★ in Figure 4d) on single CGNs. d) Relative quantitation of competitive binding experiments. The error bars represent the RSD values ($n=3$).

The peaks in the electropherograms exhibited different areas representing the heterogeneity in the amount of GB1 expression. It is worth noting that the peak shapes also varied greatly. The reason for this phenomenon was unknown. It could result from the occurrence of two major GB1 variant splices (GB1a and GB1b, two major splicing variants in the central nervous system)[18] or specific posttranslational modifications.[19] As comparisons, results of SDS-PAGE analyses from CGNs, MEF, CHO, and HEK293 lysates are given in Figure S3 (see the Supporting Information). Functional competition experiments provided valuable information that the peaks were the GB1 variant splices such as GB1a and GB1b, but not other species. As shown in Figure 4b, the antagonist of $GABA_B$ receptors, CGP64213, reduced the electrophoretic peak, thus suggesting the specificity of ABP labeling towards the GB1 ligand-binding site. A higher concentration of the antagonist led to smaller peak areas (an average RSD of 5.17% for the peak height; $n=3$ for each competitive experiment). The same trend was observed with the addition of GABA, an agonist of $GABA_B$ receptors, as shown in Figure 4c (an average RSD of 4.91% for the peak height; $n=3$ for each competitive experiment). Figure 4d summarizes the relative quantitation and the tendency with normalized data. Meanwhile, electropherograms of ABP-labeled CGN lysates with competitions of CGP64213 by CE-LIF were given in Figure S4.

In conclusion, we have developed a single-cell chemical proteomic strategy based on ABP to identify endogenous membrane protein variant splices such as GB1 variant splices in a single cell line or neuron. It largely avoids tedious labeling procedures encountered in flow cytometry and the peak capacity problem in chemical cytometry, thus providing an alternative approach for proteomic analysis at the single-cell level.

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