Electronic Supplementary Material (ESI) for ChemComm.
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Supporting Information

Direct digestion of living cells via gel-based strategy for mass spectrometric analysis

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Experimental section

Reagents and materials

The acrylamide/bisacrylamide solution (30%, 29:1, w/v) was purchased from Bio-Rad (California, USA). Sequencing grade trypsin was purchased from Promega (Madison, WI). Sodium dodecyl sulfate (SDS), ammonium bicarbonate (AMBIC), dithiothreitol (DTT), and 2-iodacetamide (IAA) were all purchased from Sigma. The complete protease inhibitors cocktail was purchased from Roche (Mannheim, Germa ny). Formic acid and acetonitrile were obtained from Thermo Fisher (USA).

Cell culture

The human breast cancer cell line MCF-7 (ER+/PR+/HER2-, Luminal A subtype) was obtained from the Cell Resource Center, Shanghai Institute for Biology Science, Chinese Academy of Science (Shanghai, China). They were cultured in DMEM (Gibco, USA) supplemented with 10% fetal serum (Gibco, USA) and Penicillin/Streptomycin (1:1000) at 37 ºC with 5% CO_2. The growth of cells was monitored by optical microscope. Nearly 48 h after reseeding, cells were collected

into a 15 ml tube and washed with ice-cold PBS twice. The cell pellet was transferred into a 1.5 ml tube for subsequent experiment. The cell number was counted.

In-gel digestion

For the cell-absorb method, 50 µl of polyacrylamide gel matrix was prepared firstly. In brief, 30 µl of 25 mM NH_4HCO_3, 16.7 µl of acrylamide/bisacrylamide solution (30%, 29:1, w/v), 2.5 µl of ammonium persulfate (1%), and 0.05 µl of TEMED were mixed in a tube. After polymerization, the gel was cutted into pieces. Thereafter, the gel pieces were washed by 50% acetonitrile/50 mM NH_4HCO_3 twice, and dehydrated with 100% acetonitrile twice. Then the dehydrated gel pieces were vacuum dried. For each experiment, the pellets of harvested cells, including ~10^5 cells, were redissolved in 30 µl of ice cold sample buffer (25 mM NH_4HCO_3, pH 8.0) supplemented with protease inhibitors. The sample was added into the tube containing vacuum-dried gel pieces on ice. After absorbed by the vacuum-dried gel pieces for approximately 10 min, cells were sharply cooling and heating twice. Subsequently, the sample was reduced and alkylated by adding 50 µl of 10 mM DTT and 30 mM IAA successively, and then washed and dehydrated twice as was described above. The dehydrated gel pieces were vacuum dried secondly. 50 µl of 0.01µg/µl Trypsin in 25 mM NH_4HCO_3 was added to the gels on ice. After tryptic digestion overnight, the resulted peptides were extracted by washing with a gradient of different concentration of acetonitrile in ddH_2O supplemented with 0.1% formic acid. The extracted peptides were vacuum dried and stored in -20 °C.

For the pro-absorb method, cells were broken in ice cold sample buffer (2% SDS in

25 mM NH_4HCO_3, pH 8.0) by ultrasound. The solution of proteins was added into the tube containing vacuum-dried gel pieces on ice. After absorbed by the vacuum-dried gel pieces, subsequent digestion steps were carried out as described above.

For the tube-gel method, cells were broken in ice cold sample buffer (2% SDS in 25 mM NH_4HCO_3, pH 8.0) by ultrasound. Then, the solution of proteins was mixed with 16.7 µl of acrylamide/bisacrylamide solution (30%, 29:1, w/v), 2.5 µl of ammonium persulfate (1%), and 0.05 µl of TEMED in a tube. After polymerization, the gel was cutted into pieces. And the subsequent digestion steps were carried out as described above.

For SDS-PAGE-based method, cells were lysed in ice cold sample buffer (2% SDS in 25 mM NH_4HCO_3, pH 8.0) by ultrasound. Then, the solution of proteins were loaded and concentrated into one stripe via SDS-PAGE. The gel bands including the protein sample were cutted and adopted for subsequent in-gel digestion as described above.

LC-MS/MS analysis

The vacuum dried peptide samples were resuspended with 0.1% of formic acid and loaded onto a Waters Symmetry C18 trapping column (300µm i.d. × 1 cm length) with Waters NanoAcquity UPLC system, and separated by a linear gradient from 2% to 35% B over 75 min (A = 0.1% formic acid in H_2O, B = 0.1% formic acid in acetonitrile) at 300 nL/min through a 75 µm i.d. × 15 cm column packed with 1.7 µm BEH C18 material (Waters, Milford, USA). The eluted peptides were analyzed by the Orbitrap Fusion mass spectrometry (Thermo Fisher Scientific) in the data-dependent mode. Full MS scan was acquired over an m/z range of 400-2000 with a mass

resolution of 120,000. MS/MS spectra were acquired in data-dependent mode using top-speed mode with 3-s cycles. Tandem MS was carried out using the ion trap mass analyzer with an isolation window of 1.6 Da by quadrupole mass analyzer. HCD fragmentation was adopted with normalized collision energy of 30%. The dynamic exclusion time was set at 30 s.

Data processing

The raw data file exported by mass spectrometry was converted to an .mgf file, and then processed with Mascot (v2.3.02) to reconstruct MS/MS spectra. The MS/MS spectra were searched against a Uniprot database for human (Homo sapiens, 20205 entries, 02-Dec-2015). Trypsin specificity with a tolerance of two missed cleavage sites was defined. The carbamidomethylation of cysteine was considered as a fixed modification, and the oxidation of methionine was set as a variable modification. The mass tolerance for peptides and fragment ions was set at 20 ppm and 1 Da, respectively. The identification result was filtered with a p-value < 0.05. Only peptide with a score above 10.0 was considered. And the false discovery rate (FDR) was calculated by a decoy database search (using a reverse sequence version of the reference data-base). The value of FDR was filtered at 1.0%.

Table S1. Comparison of the cost of time for different protocols. The value in the boxes refers to the length of time for each step.

	SDS-PAGE	Pro-absorb	Cell-absorb
Cell lysis & protein extraction	25 min	25 min	
SDS-PAGE & sample incorporation	~1 h	10 min	10 min
Wash	30 min	30 min	30 min
Reduction & alkylation	~2 h	~2 h	~2 h
Wash	30 min	30 min	30 min
Protein digestion	~16 h	~16 h	~16 h
Peptide extraction	~1 h	~1 h	~1 h

A

Missed cleavage	Cell-absorb	Pro-absorb	SDS-PAGE
0	14636	10496	11114
1	2716	1812	1788
2	325	166	149
3	46	11	14
>=4	10	5	4

B

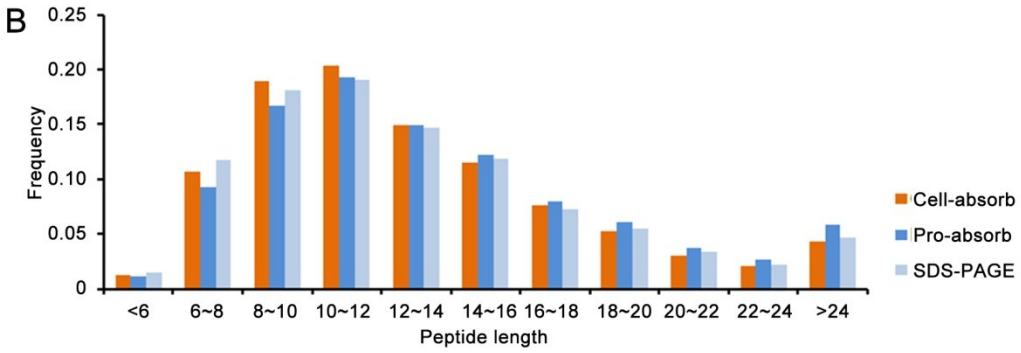
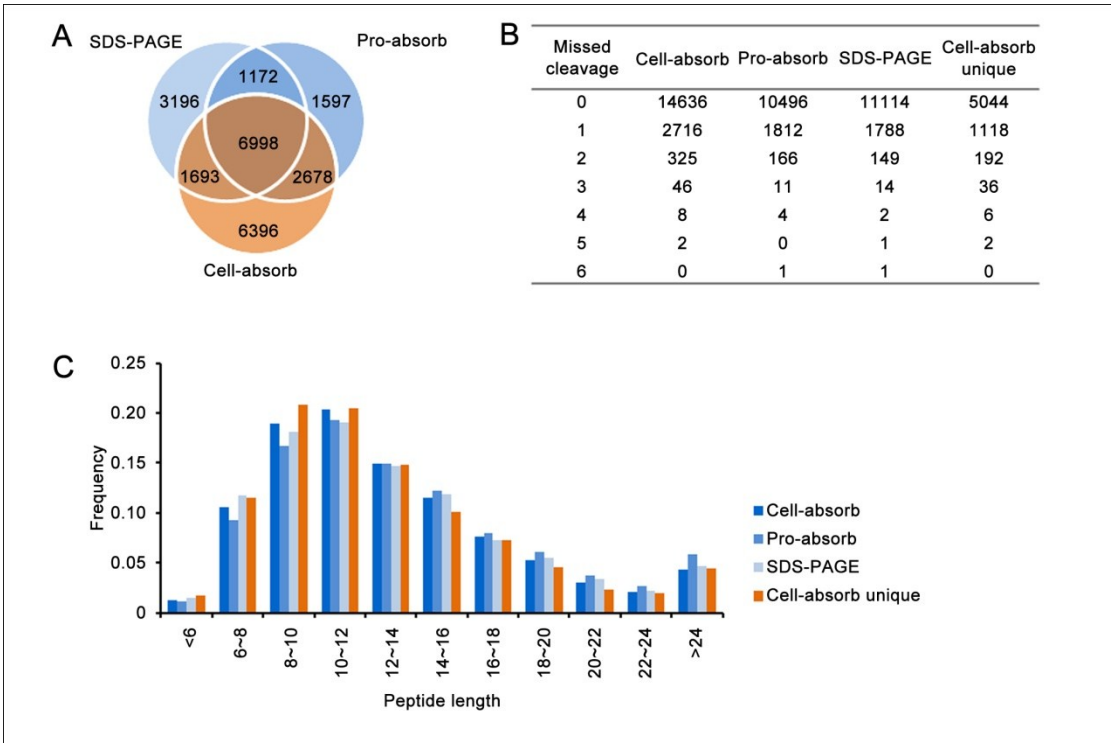


Figure S1. The properties of peptides identified in different methods. (A) statistical summary of the missed cleavage sites of peptides identified by three in-gel digestion methods; (B) the distribution of the length of peptides identified by three in-gel digestion methods.



Missed cleavage	Cell-absorb	Pro-absorb	SDS-PAGE	Cell-absorb unique
0	14636	10496	11114	5044
1	2716	1812	1788	1118
2	325	166	149	192
3	46	11	14	36
4	8	4	2	6
5	2	0	1	2
6	0	1	1	0

Figure S2. Analysis of the properties of peptides identified specifically by the cell-absorb method. (A) Venn diagram analysis of peptides identified by the SDS-PAGE based method, the pro-absorb method and the cell-absorb method; (B) statistical summary of the missed cleavage sites of peptides identified specifically by the cell-absorb method; (C) the distribution of the length of peptides identified specifically by the cell-absorb method.

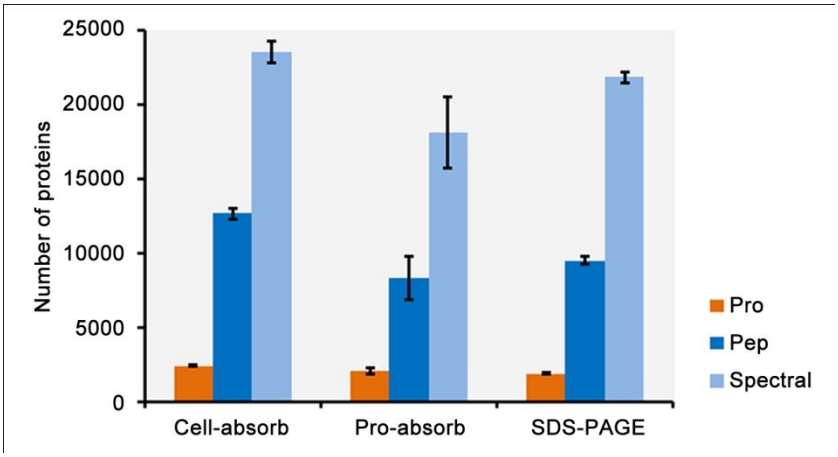


Figure S3. Analysis of the reproducibility of the SDS-PAGE based method, the cell-absorb method, and the pro-absorb method.