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SUPPORTING INFORMATION：Experimental section, Fig. S1−S3 and Tables S1-S2

Fully Integrated On-line strategy for Highly Sensitive Proteome Profiling of 10-500 Mammalian Cells

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Materials and methods

NanoLC-MS/MS Analysis on Q Exactive HF-X and Orbitrap Fusion

The nanoLC and MS conditions on Q Exactive HF-X and Orbitrap Fusion were the same as previously reported.[1] The data of **Fig. 2** and **Fig. 3** were collected on a Q Exactive HF-X, while the data of **Fig. S1** were collected on a Orbitrap Fusion.

NanoLC-MS/MS Analysis on timsTOF Pro

Pierce™ HeLa digest standard (PN 88329) was purchased from Thermo Fisher Scientific. A nanoElute UHPLC system was online coupled to a timsTOF Pro mass spectrometer with a CaptiveSpray nano-electrospray ion source (Bruker Daltonics). All columns were heated at 50°C using an integrated column oven (Sonation GmbH, Germany). Mobile phases A and B were water and acetonitrile with 0.1% FA, respectively. The flow rate was 250 nL/min and a segmented 110-min gradient was: 2 %–22 % (v/v) buffer B in 75 min, 22%–35% (v/v) buffer B for 15 min, 35%–80% (v/v) buffer B for 10 min, followed by a 10 min wash from 36% to 80% (v/v) buffer B. RCPR after processing hair cells was online connected to conventional 100 μm I.D. x 20 cm capillary columns via a zero dead volume PicoClear union (New Objective).

Optimized DDA parameters on timsTOF Pro are the following: MS range m/z 300–1 500 was scanned in positive electrospray mode; The mass spectrometer collected ion mobility MS spectra over $1/k_0$ of 0.75 to 1.30, and then performed 4 cycles of parallel accumulation-serial fragmentation (PASEF) MS/MS with a target intensity of 5 000 and a threshold of 500. The ramp time was 200 ms and total cycle time was 1.03 s. Only doubly or triply charged features were selected to trigger MS/MS scans; Peaks were dynamically excluded within 0.30 min except that when current intensity/previous intensity was over 3.0.

Table S1. Major published proteomic methods for profiling 10-2 000 mammalian cells.

Cell number	Cell type	Number of identified protein groups	Sample preparation method	Year published	References
10	HeLa	1 500	nanoPOTS[a]	2018	2
	HeLa	1 461	autoPOTS[b]	2021	3
~25	HeLa	~ 1 800	μPOTS[c]	2018	4
50	MCF-7	1 017	In-solution	2014	5
~56	HeLa	~ 2 200	μPOTS	2018	4
100	HeLa	1 360	OAD chip	2018	6
	DLD-1	635	iPAD-100[d]	2015	7
	THP-1	549	In-solution	2017	8
	Jurkat T	1 226	DMF-SP3[e]	2019	9
	HeLa	644	FAST[f]	2021	10
	MCF-7	1 895	micro-FASP[g]	2020	11
~130	B or T lymphocytes	1 095	autoPOTS	2021	3
140	HeLa	3 000	nanoPOTS	2018	2
~150	HeLa	2 679	autoPOTS	2021	3
91-454	human pancreatic cell	average: 2 676	nanoPOTS	2018	2
500	DLD-1	1 060	High temperature trypsin digestion	2015	7
	MCF-7	187	Acetone precipitation	2010	12
	HeLa	905	FASP[h]	2011	13
	Jurkat T	2 467	on-chip SP3	2019	9
	HeLa	1 673	FAST	2021	10
~500	HeLa	3 347	autoPOTS	2021	3
650	HeLa	5 805	nanoPOTS and prefractionation	2018	14
1 000	MCF-7	271	Acetone precipitation	2010	12
	HeLa	1 536	FASP	2011	13
	Human macrophage	1 000	In-solution	2014	15
	THP-1	911	In-solution	2017	8
	U2OS osteosarcoma	829	In-solution	2019	16
	MCF-7	1 895	micro-FASP	2020	11
~1 440	MCF-7	3 402	AFA[i] assisted cell lysis	2015	17
1 900	Rat brain tissue	1 500	On-tissue micro-digestion	2013	18

2 000	HEK 239T	1 270	SISPROT[j]	2016	19
~2 000	Human breast cancer	1 426	CAAR[k]-based procedure	2019	20

[a.] nanoPOTS: nanodroplet processing in one-pot for trace samples

[b.] autoPOTS: automated preparation in one pot for trace samples

[c.] µPOTS: microdroplet processing in one pot for trace samples

[d.] iPAD-100: integrated proteome analysis device for 100 cells

[e.] DMF: digital microfluidics

[f.] FAST: fully automated sample treatment

[g.] micro-FASP: miniaturized filter-aided sample preparation

[h.] FASP: filter-aided sample preparation

[i.] AFA: Adaptive Focused Acoustics™

[j.] SISPROT: simple and integrated proteomics sample preparation technology

[k.] CAAR: citric acid antigen retrieval

Table S2. Major changes of DDA parameters on timsTOF Pro during optimization.

Parameters	Original	Optimized
Mobilogram peak detection-intensity threshold	5 000	4 000
Mass range	100-1 700	300-1 500
$1/k_0$	0.60-1.60	0.75-1.30
Ramp time	166	200
Charge range	0-5	2-3
No. of PASEF MS/MS scans	10	4
Total cycle time	1.89 s	1.03 s
Target intensity	20 000	5 000
MS2 intensity threshold	1 000	500
Release after (min)	0.40	0.30
Current intensity/previous intensity	4.00	3.00

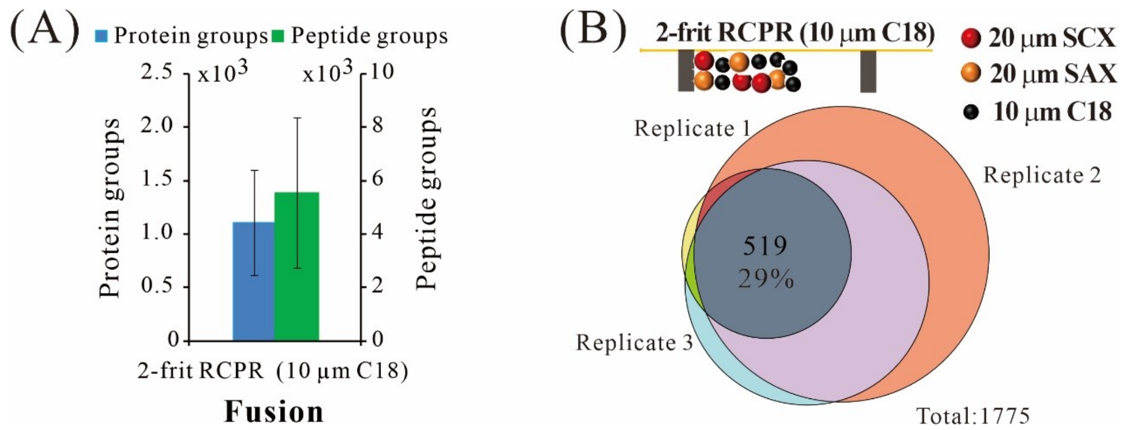

Fig. S1. (A) Identified protein groups and peptide groups from 10 ng of 293T total cell lysate processed by 2-frit mixed-mode RCPR (10 μm C18). Error bars indicate standard deviations from three technical replicates. (B) Venn diagram of protein groups identified by this type of mixed-mode RCPR.

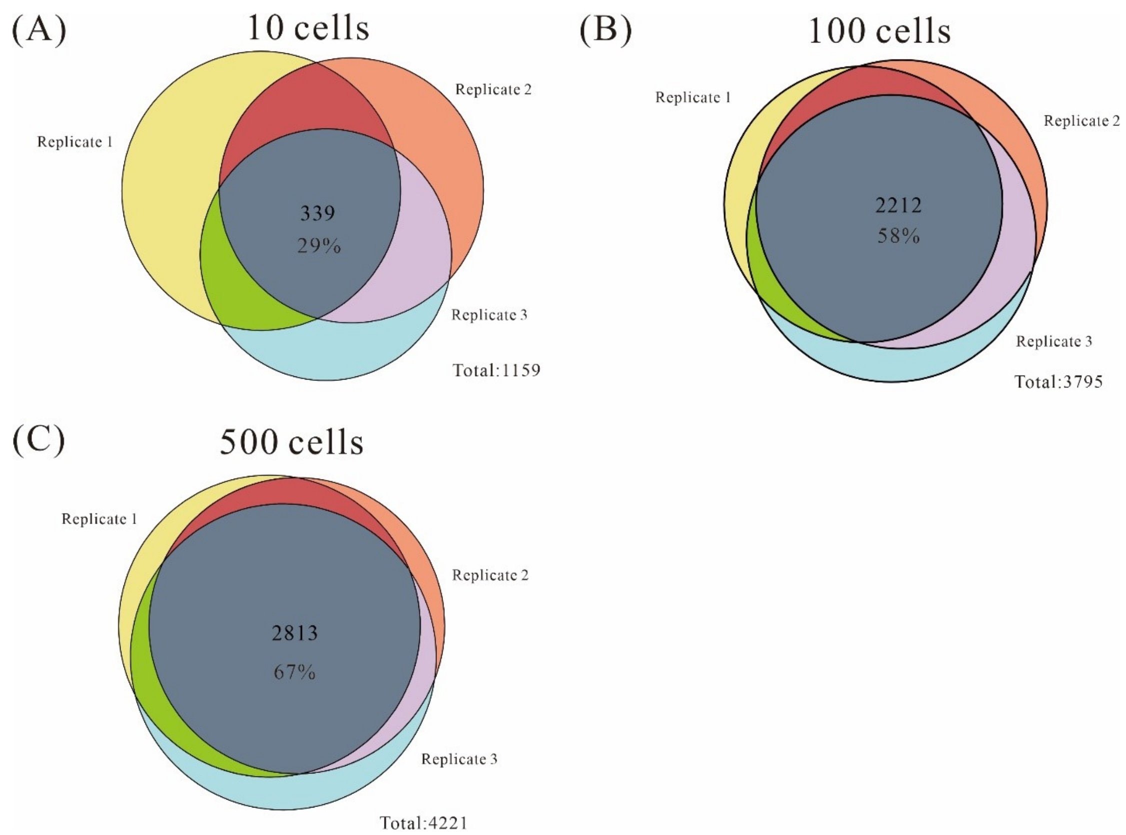

Fig. S2. Venn diagrams of label-free quantified protein groups from 10 (A), 100 (B), and 500 (C) FACS-sorted 293T cells.

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