

Targeting the Proteome of Cellular Fractions: Focus on Secreted Proteins

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Abstract

The high complexity of the total cellular proteome underscores the need for a more targeted investigation of particular subcellular fractions as a means to detect the changes at the level of low abundance proteins. However, this approach requires the application of an enrichment strategy. In this chapter, we present the protocols, which have been used for the analysis of secretome from cell lines, targeting the investigation of protein expression changes.

Key words Subcellular proteomics, Cell lines, Secretome, Secretopeptidome, Endoplasmic reticulum, Golgi apparatus

1 Introduction

The cell proteome is characterized by a wide dynamic concentration range which extends over seven orders of magnitude [1]. As a result, the low-copy number proteins, which can serve as putative biomarkers for diseases, are extremely difficult to detect [1]. To overcome this limitation, application of a pre-fractionation strategy is necessary to decrease sample complexity. Analysis of secreted proteins is particularly interesting in the context of biomarker discovery. As an example, proteins secreted by cancer cells can be detected in body fluids such as blood or urine. Moreover, investigation of secretome along with the analysis of extracellular matrix proteins may help to elucidate molecular mechanisms underlying cancer aggressiveness. The theoretical basis and summary of different methods allowing for enrichment of secreted proteins are presented in this chapter.

The secretome refers to the proteins released by cells through conventional (i.e. signal peptide-dependent) and non-classical ways for secretion. The latter include vesicular (e.g. autophagy-based

secretion) and non-vesicular pathways (e.g. ATP-binding cassette (ABC) transporters, which belong to transmembrane proteins and utilize the energy from ATP hydrolysis to transport cargo) [2]. In order to investigate the secretome, various *in vitro* (cell culture), *in vivo* (tissue samples), and *ex vivo* (animal models) model systems have been used. However, the majority of the studies have employed *in vitro* systems and conditioned medium (CM) as a source of secreted proteins. This is mainly attributed to a more straightforward sample collection, allowing for collection of sufficient material to support downstream analysis. In addition, experimental conditions are more easily controlled in *in vitro* versus *in vivo* or *ex vivo* systems. For example, *in vivo* secretome profiling relies on the analysis of interstitial fluid (component of interstitial space outside parenchymal cells, lymphatic and blood vessels), which is not readily accessible. Even though various strategies have been described to extract interstitial fluid, the process is demanding and may require freshly collected (and not frozen) tissue specimens [3].

The classical procedure requires culturing the cells in serum-free medium (SFM) prior to the collection of conditioned medium. Bearing in mind the broad dynamic range of protein concentration in plasma ($3.5\text{--}5.0 \times 10^{10}$ pg/mL to 0–5 pg/mL for serum albumin and interleukin-6, respectively), this is a critical step in order to avoid masking effect and contamination of secretome profile by the highly abundant serum proteins [4]. Nevertheless, it should be noted that in some cases serum starvation may cause a series of undesirable effects to the cell homeostasis such as metabolic stress, reduced growth or enhanced autolysis. Therefore, a fine balance should be targeted with the cells being cultured in serum-supplemented medium until a sufficient confluence is achieved (70–80 %), followed by sequential washes of the cell layer with phosphate-buffered saline (PBS) and SFM to remove the excess of serum proteins. It has been shown that these washing steps have a great impact on the purity of collected material [5]. Cell confluence and incubation time with SFM depends mainly on the cell type and has to be optimized in order to minimize cell lysis and/or cell death. Usually, SFM incubation time varies from 12 to 48 h [6–8]. However, in the case of cells that are strongly dependent on the presence of serum proteins, optimization of the minimum FBS (fetal bovine serum) concentration or adaptation of cells to gradually decreasing serum concentration may be required [9]. After SFM incubation, it is highly recommended to observe the cellular morphology, shape, assess the percentage of dead-necrotic cells (Trypan blue exclusion dye), or evaluate the contamination level from cytoplasmic proteins (e.g. via western blotting of conditioned medium for actin or tubulin) [10]. Conditioned medium is collected and centrifuged to remove dead cells and cellular debris. Due to the low concentration of secreted proteins (ng/mL scale) [11], the medium has to be concentrated prior to analysis. Manifold

strategies have been described in the literature toward that end, including ultrafiltration [12], precipitation [13, 14], and dialysis followed by lyophilization [15, 16]. Depending on the downstream analysis, further desalting may also be required (e.g. gel filtration chromatography using desalting columns or ultrafiltration filter units following buffer exchange protocols).

In parallel to the analysis of proteins collected from conditioned medium, the naturally occurring peptides can be also investigated. In this case, the collection of the CM (conditioned medium) is performed in the same way as for the analysis of secreted proteins. However, due to the high sample complexity, a fractionation step is required in order to deplete the high molecular weight compounds. In this case, a strategy based on the use of filter devices (e.g. molecular weight cut off (MWCO): 20 kDa) is adopted to separate larger molecules ($MW > 20$ kDa) from smaller compounds ($MW < 20$ kDa, flow through). Therefore, for the peptidomics analysis, the latter (flow through) is collected. Following this step, samples have to be purified by desalting (e.g. PD-10 columns, 5 kDa MWCO). In this way, the final fraction is enriched for molecules between 5 and 20 kDa. The extracted peptides can then be analyzed by capillary electrophoresis coupled to mass spectrometry (CE-MS [17]) or LC-MS/MS.

Since the conventional approach of secretome analysis, based on collection of conditioned medium, requires serum starvation (i.e. SFM), which in some cases may result in changes of the secretome profile, application of alternative methodologies is also tested. An interesting approach relies on targeting the secretory pathway organelles (endoplasmic reticulum (ER), Golgi apparatus) and enables investigation of the “secretory cargo” (proteins “on their way” to be secreted). This approach was developed by Sarkar et al. [18] by utilizing human embryonic stem cells and mouse embryonic fibroblasts. The enrichment strategy is based on differential centrifugation. Briefly, after cell lysis, the suspension is centrifuged at $3,000 \times g$ to remove the nuclei. The sample is then depleted of mitochondria via affinity (e.g. Sarkar et al. [18], utilized magnetic microbeads (anti-Tom22)) or centrifugation, as shown in our protocols below. This strategy enriches for the group of putatively secreted proteins, not regularly detected using standard approaches [18]. As such, analysis using the aforementioned different methodologies in combination can lead to better secretome coverage.

In this chapter, we present a protocol for secreted proteome enrichment established and regularly used in our laboratory. The presented cell culture conditions refer to the bladder cancer cell line models T24 and T24M. An overview of the major steps of the analytical workflow is presented in Fig. 1. The selected strategies are characterized by high reproducibility (Fig. 2a, b) and optimum extraction yield (Fig. 2b). The efficiency of enrichment is estimated via comparison of results to the total cell extract (Table 1).

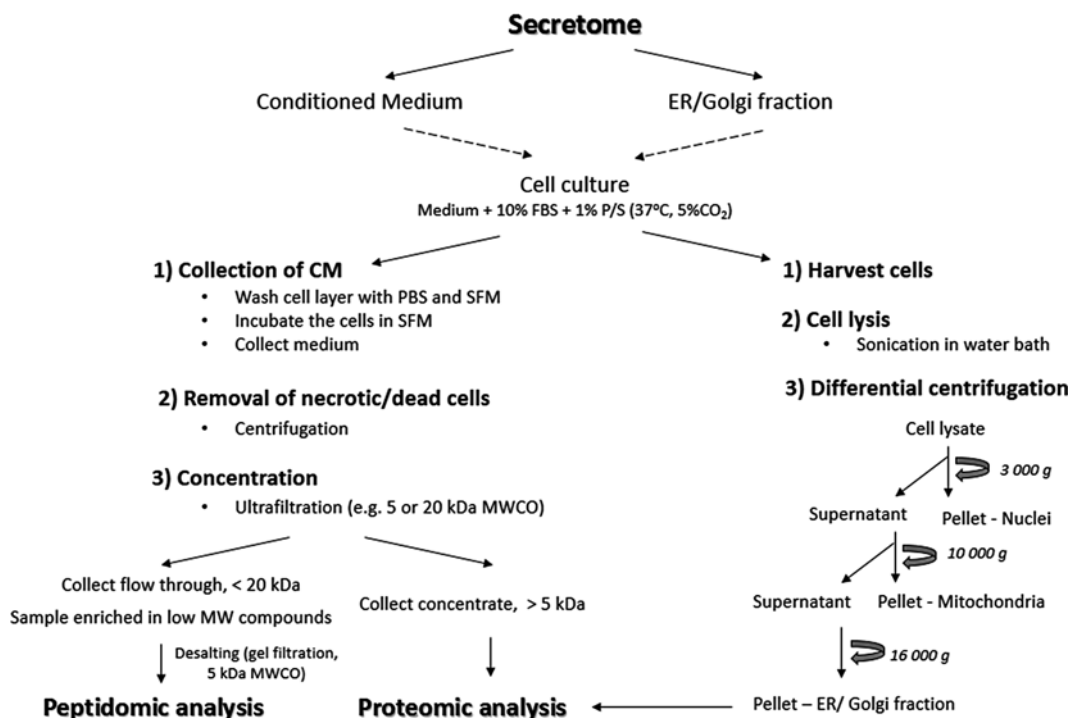


Fig. 1 Workflow of classical and non-conventional analysis of secreted proteins in cell line models. The standard approach includes analysis of secreted proteins collected from conditioned medium, whereas the non-conventional method is based on the investigation of protein components derived from secretory pathway organelles (ER/Golgi Apparatus)

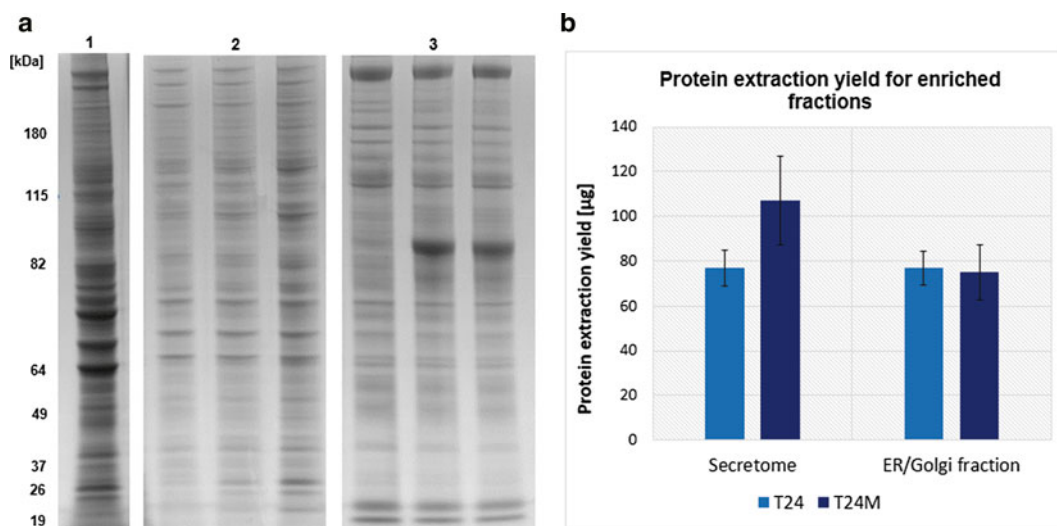


Fig. 2 Evaluation of sample preparation procedures for secreted proteins. Representative gel images of total cell extract [1], ER/Golgi fraction [2], and secretome (CM) [3] are shown (a). Three biological replicates were analyzed in order to confirm the reproducibility of applied procedures. In addition, graphical representation of the extracted proteins from conditioned medium and secretory pathway organelles (endoplasmic reticulum/ Golgi apparatus) is presented (b). The secreted proteins from CM were collected from 10 million cells resulting in extraction of 92 µg of protein whereas 20 million cells were used in order to enrich for the ER/Golgi fraction yielding in 76 µg of protein. Regularly, approximately 900 µg of protein (total cell extract) from 4 million cells can be extracted

Table 1**Evaluation of enrichment efficiency for secreted proteins extracted using classical and non-conventional (ER/Golgi) approaches**

	Secretome	ER/Golgi fraction	Cell extract
Average number of identifications	994	1,412	1,723
Number of unique identifications	1,613	2,048	2,036
Prediction of signal peptide	379 (24 %)	279 (14 %)	168 (8 %)
Prediction of non-classical secretion	461 (29 %)	632 (31 %)	715 (35 %)
Secreted according to UniprotKB subcellular localization	248 (15 %)	98 (5 %)	70 (3 %)
Extracellular according to Gene Ontology annotation ^a	332 (21 %)	181 (9 %)	149 (7 %)

The average number of proteins identified using LC-MS/MS techniques (Orbitrap Velos, 8 h LC run in all cases) from five biological replicates per preparation (two for the case of total cell extracts) is reported (5 % FDR). The number of total unique identifications (specifically: sum of unique identifications from all biological samples per preparation) is also shown. In silico analysis of the latter was performed using software for the prediction of signal peptides (SignalIP [19]) and non-classical secretion (SecretomeP [20])

^aThe following Gene Ontology annotations were included in the analysis: extracellular space (GO:0005615), extracellular matrix (GO:0031012), extracellular region (GO:0005576), proteinaceous extracellular matrix (GO:0005578), and extracellular vesicular exosome (GO:0070062). All annotations were reported in UniProt-GOA annotation database [21]

Classification of proteins as secreted was based on the UniprotKB, Gene Ontology as well as tools for prediction of the classical (SignalIP [19]) and non-conventional secretory pathways (SecretomeP [20]). Detailed comparison of identified proteins following high-resolution LC-MS/MS analysis of all fractions is presented in Fig. 3. Even though contamination of the secretome by intracellular proteins cannot be avoided (Fig. 3a, b), it is clearly shown that the applied procedures enable enrichment in secreted proteins (Table 1 and Fig. 3c, d) in a complementary fashion (Fig. 3e). In parallel, protocols for the peptidomic analysis of secretome via CE-MS are presented. This strategy allowed for the detection of approximately 700 peptides included in 0.9 mL of conditioned medium collected from 4 million bladder cancer cells.

2 Materials

2.1 Cell Culture

1. Serum-supplemented growth medium: DMEM (Dulbecco's modified Eagle medium), High Glucose, GlutaMAX™, Pyruvate, supplemented with 10 % (v/v) FBS and 1 % (v/v) Penicillin/Streptomycin (P/S) (*see* Note 1). Store at 4 °C (*see* Note 2).
2. Phosphate-Buffered Saline: 1× in sterile Ultra Pure Water.

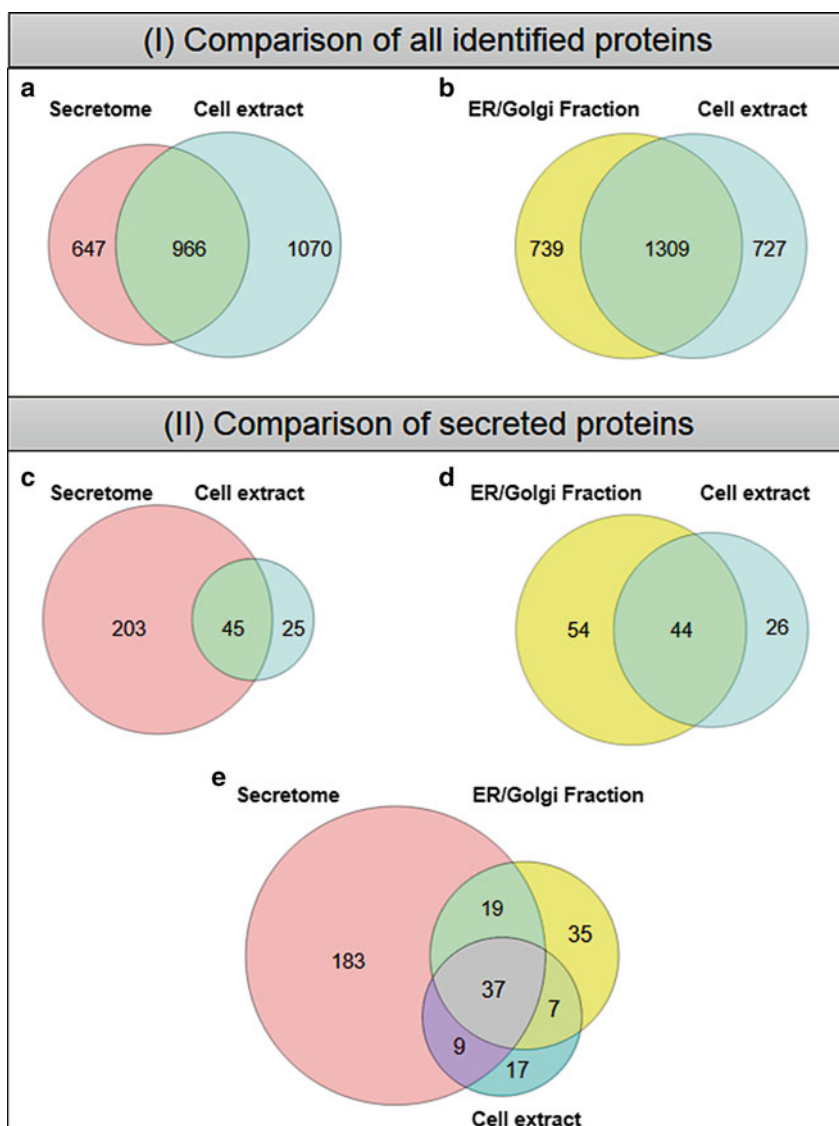


Fig. 3 Comparative analysis of identified proteins from secretome (classical and non-conventional approach) and total cell extract. (I) Comparison of all identified proteins. Venn diagrams representative of all identified proteins from conditioned medium (**a**) and ER/Golgi fraction (**b**). In both cases, total cell extract was used as reference. (II) Comparison of secreted proteins. Comparison of the proteins annotated as “secreted” according to UniprotKB in conditioned medium (**c**) and ER/Golgi fraction (**d**) compared to secreted proteins identified in total cell extract (Table 1—Secreted according to UniprotKB). Complementarity of the different methods; Overview of common and unique secreted proteins identified in the secretome, ER/Golgi fraction and total cell extract (**e**). Venn diagrams were prepared using Venn Diagram Plotter (Pacific Northwest National Laboratory, <http://omics.pnl.gov/>)

2.2 Enrichment of Secreted Proteins

1. Serum-free medium: DMEM, High Glucose, no Glutamine, no Phenol Red, no HEPES containing medium, supplemented with 4 mM Glutamine and 1 % (v/v) Penicillin/Streptomycin (*see Note 3*). Store at 4 °C (*see Note 2*).
2. Amicon Ultra Centrifugal Filter Units, 5 kDa MWCO (*see Note 4*).
3. Benchtop centrifuge.

2.3 Enrichment of Secreted Peptides

1. Centrstart 1 ultrafilter tubes (20 kDa MWCO) (*see Note 5*).
2. PD-10 desalting Columns (*see Note 6*).
3. Equilibration/Elution buffer for PD-10 column: Prepare 1 L of 0.01 % v/v ammonium hydroxide solution in distilled water. The pH should be about 10–11. Usually, there is no need for pH adjustment. Store at 4 °C.
4. Lyophilizer.

2.4 Enrichment of Endoplasmic Reticulum and Golgi Apparatus: Secretory Pathway Organelles

1. *Lysis buffer*: 250 mM sucrose, 25 mM KCl, 5 mM MgCl₂, 10 mM triethanolamine, 10 mM acetic acid, 1 mM β-glycerophosphate disodium salt, 1 mM sodium orthovanadate, pH 7.6 adjusted using triethanolamine or acetic acid. The buffer can be prepared as a 5× concentrated stock solution. Store at 4 °C.
2. *Solubilization buffer*: 7 M urea, 2 M thiourea, 4 % CHAPS, 100 mM DTE, 1 % ampholytes (*see Note 7*). Store buffer at –20 °C (*see Note 8*).
3. Bath sonicator (*see Note 9*).
4. Benchtop centrifuge.

2.5 Total Protein Extraction

1. *Lysis buffer*: 7 M urea, 2 M thiourea, 4 % CHAPS, 100 mM DTE, 1 % ampholytes. Store buffer at –20 °C (*see Note 8*).
2. Bath sonicator.
3. Benchtop centrifuge.

3 Methods

3.1 Cell Culture

1. Cultivate cells (37 °C, 5 % CO₂ in a humidified atmosphere) in DMEM (High Glucose, GlutaMAX™, Pyruvate) supplemented with 10 % FBS and 1 % Penicillin-Streptomycin until cells reach 80–90 % confluency (0.5–1 × 10⁶ cells/mL), then apply the protocol for CM collection and/or harvest the cells (*see Note 10*).

3.2 Collection of Proteins from Conditioned Medium

1. Remove the medium and rinse the cell layer three times with 1× PBS following by additional wash with serum and phenol red free medium.

2. Incubate cells with SFM for 24 h (37 °C, 5 % CO₂ in a humidified atmosphere) (*see Note 11*).
3. Collect the conditioned medium and centrifuge at 2,000×*g* for 10 min at room temperature (*see Note 12*); the supernatant should be stored at –80 °C until further processing.
4. Concentrate the conditioned medium with Amicon Ultra Filters up to 50–70 µL (4,000×*g*, 12 °C) (*see Note 13*).
5. Estimate protein concentration, e.g. using Bradford assay.
6. Samples were subjected to LC-MS/MS analysis (*see Note 14*).

3.3 Enrichment of Secreted Peptides

1. Collect the conditioned medium from approximately four million cells as described in Subheading 3.2 (“Collection of proteins from conditioned media”, **steps 1–3**) (*see Note 11*).
2. Fractionate the supernatant with Centriscart filters and collect the filtrate (unconcentrated material <20 kDa) (*see Note 5*).
3. Desalt the sample using PD-10 columns according to the manufacturer’s instructions (*see Note 6*).
4. Lyophilize the eluate.
5. Samples were subjected to CE-MS analysis (*see Note 15*).

3.4 Enrichment for Endoplasmic Reticulum and Golgi Apparatus: Secretory Pathway Organelles

1. Resuspend the cell pellet in 1× lysis buffer (*see Note 16*) by gently pipetting and sonicate the suspension in water bath for 10 min.
2. Centrifuge the sample at 3,000×*g* for 10 min at 4 °C and transfer the supernatant to a new tube (*see Note 17*).
3. Centrifuge the supernatant at 10,000×*g* for 15 min at 4 °C. Keep the supernatant and perform another centrifugation at 16,000×*g* for 30 min at 4 °C (*see Note 18*).
4. Dissolve the pellet which contains the fraction of ER/Golgi Apparatus in appropriate buffer, e.g. solubilization buffer (*see Note 19*).
5. Sonicate the sample for 15 min in bath sonicator to improve the solubilization process.
6. Centrifuge the sample to remove undissolved materials.
7. Determine protein concentration, e.g. using Bradford assay.
8. Samples were subjected to LC-MS/MS analysis (*see Note 14*).

3.5 Total Protein Extraction

1. Resuspend the cell pellet in lysis buffer (*see Note 20*) by gently pipetting and sonicate the suspension in water bath for 10 min.
2. Centrifuge the sample at 16,000 *g* for 15 min at room temperature (*see Note 21*) and transfer the supernatant to a new tube.

3. Determine protein concentration, e.g. using Bradford assay.
4. Store the samples in -20°C until analysis.
5. Samples were subjected to LC-MS/MS analysis (*see Note 14*).

4 Notes

1. Selection of the cell culture conditions varies according to the type of cells. Cultivating conditions have to be optimized for each cell line based on the relevant literature and preliminary experiments addressing cell tolerance in serum-free conditions.
2. Cell culture reagents such as fetal bovine serum, antibiotics, or trypsin aliquots are stored at -20°C . PBS and medium are kept at 4°C . Trypsin can also be stored at 4°C for a short period of time.
3. Substances contained in the growth medium can interfere with downstream analysis, e.g. HEPES which is included in some media is not compatible with CE-MS analysis. Therefore, application of HEPES free medium is recommended for peptidomics analysis.
4. The volume (4 or 15 mL) of the Amicon centrifugal devices has to be selected based on the initial volume of the analyzed sample.
5. For the peptidomics analysis, the molecules of interest ($\text{MW} < 20 \text{ kDa}$) are contained in the filtrate (inside the insert when using Centriscart columns). Amicon filters can be alternatively used, but in this case the flow through has to be collected, as this contains the low molecular weight components.
6. The basic principle of the desalting procedure with the PD10 columns is gel filtration chromatography. Molecules are separated according to their size in the porous matrix, which forms a steric barrier for the particles (for PD-10 column the matrix is Sephadex G-25). Therefore, elution of larger molecules initially occurs followed by molecules of gradually decreasing size.
7. Composition of the protein solubilization buffer depends on the type of samples under analysis and further downstream proteomics methodology (e.g. 2DE, LC-MS/MS, MALDI, etc.).
8. Thawed aliquots can be reused as long as they are stored again at -20°C .
9. Application of the suitable homogenization strategy is crucial for the subcellular proteomics analysis. Harsh homogenization can disrupt the integrity of cellular organelles and it may affect the fractionation process (mainly through contamination). Therefore, mild homogenization methods are required (e.g. Potter-Elvehjem homogenizer, gentle sonication).

10. Description of detailed culture conditions and associated protocols is beyond the scope of this chapter, since this material is readily available from existing literature and, as mentioned above, is cell type-dependent.
11. The volume of the SFM is the same as the volume of the growth medium regularly used for the particular cell line. This includes (the type of cell culture flask is given): T-25—4 mL, T-75—10 mL, T-175—20 mL. The volume can be decreased, if applicable, in order to obtain a more concentrated starting material. However, the cell layer has to be totally covered by growth medium. As highlighted in the introduction, the incubation time has to be estimated experimentally and it regularly varies between 24 and 48 h depending on the cell type. After incubation, the cellular morphology and shape have to be examined. High percentage of dead and necrotic cells results in increased cytoplasmic contamination in the secretome. If cell lysis and contamination problems are observed, shorter incubation times have to be applied.
12. The remaining cell pellet (following the removal of conditioned medium) reflects cell death and apoptosis. Usually, when the percentage of dead cells is low, the pellet should be almost invisible or very small at this step.
13. Concentration of secreted proteins can be also performed at room temperature (RT). However, at RT the time of the procedure is regularly two times longer. Alternatively, protein precipitation with TCA-NLS can be applied to concentrate the secreted proteins [14]. Specifically this involves:
 - (a) Incubate the conditioned medium with 7.5 % (w/v) TCA, 0.1 % (v/v) NLS at -20°C overnight.
 - (b) Centrifuge the sample $10,000\times g$ for 10 min at 4°C and discard the supernatant.
 - (c) Wash the pellet with 0.5 mL of ice-cold THF and repeat the centrifugation.
 - (d) Dry the final pellet in the air followed by solubilization in appropriate buffer.
14. In all cases of proteomics analysis, protein identification was conducted using FASP method and LC-MS/MS as follows; Tryptic digests were analyzed on a Dionex Ultimate 3000 RSLC nano flow system (Dionex, Camberly, UK) [22]. Peptides were fractionated by reverse-phase chromatography using a $75\text{ }\mu\text{m}\times 50\text{ cm}$, $2\text{ }\mu\text{m}$, $100\text{ }\text{\AA}$ C18 nano column with a linear gradient of solvent A, 0.1 % formic acid and acetonitrile (98:2) against solvent B, 0.1 % formic acid and acetonitrile (20:80) starting at 1 % B for 5 min rising to 30 % at 400 min then to 50 % B at 480 min. The samples were ionized in positive

ion mode using a Proxeon nano spray ESI source (Thermo Fisher Hemel, UK) and analyzed in an Orbitrap Velos FTMS (Thermo Finnigan, Bremen, Germany). The MS was operated in a data-dependent mode (top 40) to switch between MS and MS/MS acquisition at 60,000 and 7,500 resolution, respectively. Parent ions were fragmented at an energy of 40 by higher energy collision-induced dissociation (HCD). Raw data files were searched against the Human Swiss-Prot Database using X!Tandem (by using Trans Proteomic Pipeline [23]) with trypsin as enzyme specificity. The following search engine parameters were applied: (a) precursor mass tolerance 10 ppm, (b) fragment mass tolerance: 0.05 Da, (c) fixed modification: carbamidomethylation of cysteine, (d) variable modification: oxidation of methionine, and (e) allowing one missed cleavage site. Only protein identification with the protein false-discovery rate below 5 % was kept.

15. Peptidomic analysis was conducted by CE-MS using a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, USA) on line coupled to a MicroTOF MS (BrukerDaltonic, Bremen, Germany) [24]. Sheath liquid interface was used for coupling of CE to MS. Spectra were accumulated over a range of mass-to-charge ratios from 50 to 3,000. MosaiquesVisu was used to analyze the CE-MS data [25]. Peptides are initially characterized by their molecular mass, CE-migration time, and ion signal intensity (amplitude) value.
16. The volume of the lysis buffer should be adjusted to the amount of the starting material for example; for 20 million cells, a 700 μ L of lysis buffer is regularly employed. After the resuspension, cell aggregates/clumps should not be visible. If the pellet is not well resuspended, add more buffer and repeat the procedure.
17. After the centrifugation, a large pellet is present. Keep the pellet on ice until finishing the extraction. In the case of obtaining low amount of protein in the ER/Golgi fraction, repeat the lysis procedure.
18. The final pellet (enriched in the ER/Golgi fraction) is regularly very small and almost transparent.
19. To avoid diluting the sample, the minimal amount of solubilization buffer is required. We recommend to solubilize initially in low volume, for example, 25 μ L and increase the volume if necessary. In the end, the pellet has to be well dissolved.
20. The volume of the lysis buffer depends on the initial amount of starting material (e.g., for four million cells, 200 μ L of buffer is regularly used). After the resuspension, cell aggregates/clumps should not be visible.
21. Samples containing urea buffer have to be centrifuged at room temperature, since urea tends to precipitate at 4 °C.

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