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Cell fractionation - an important tool for compartment proteomics

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ABSTRACT

In order to maximize coverage in proteome studies, a successful approach is the fractionation of cellular compartments. For providing evidence for the most reliable and efficient separation technique, we compared four different procedures for subcellular fractionation of Jurkat cells. The analysis of fractions by LTQ-Orbitrap yielded between 559 and 1195 unambiguously identified unique proteins. The assumed correct localization of the proteins was defined using Scaffold3 according to GO annotations, with the highest reliability (~80%) for the cytoplasmic fraction and the lowest (~20%) for the cytoskeletal fraction. This comparison revealed evidence for the efficiency of separating subcellular fractions and will thereby facilitate the decision on which procedure might be the best match to a specific research question and contribute to the emerging field of compartment proteomics.

Keywords: Subcellular compartments; Cellular fractionation; Protein localization; Mass spectrometry.

1. Introduction

In proteomics it is desired to obtain the largest possible coverage of the proteome of interest and especially to detect proteins of mediate or even minor abundance, too [1]. Beside the development of more and more sensitive mass spectrometers the most frequently applied approach for increased proteome coverage lies in the fractionation of the sample prior to analysis. This can be performed on the levels of subcellular compartments [2-4], proteins or peptides [5, 6] or a combination of different approaches [7]. The biologically most meaningful way is to separate subcellular compartments in order to preserve the linkage of proteins with the compartment in which they exert their activity. In many cases the biological relevance of a protein is closely linked to specific compartments and thereby it's influence on the whole phenotype of a cell.

Hence a great variety of methods for separating the subcellular compartments and subsequent proteome analysis have been developed (for review see [8]). Beside the coverage of the proteome, in praxis the hands-on time plays an important role for deciding in favor of a specific technique. Other criteria are reproducibility and in a few cases also high throughput

capacity.

A well-established technique for separation of organelles is solely based on two different types of centrifugation, density velocity and density gradient centrifugation making use of differences in sedimentation coefficients and densities. With endpoint centrifugation, the membrane fraction of a broken cell can be obtained, regardless of the origin of the membrane [8]. Pellets resulting from a centrifugation scheme will stem mainly from the cytoplasmic membrane and only to lower percentages from organelles. A further sub-fraction that can be highly enriched by centrifugation contains the nuclei [9]. Due to their similarity in size but differences in density the remaining organelles like mitochondria, microsomes and lysosomes are often separated by density gradient centrifugation [10-12]. The centrifugation steps can be performed in buffers preserving protein structure and that are compatible with proteomic techniques like 2D-gel electrophoresis or LC-MS shotgun proteomics [8]. In summary, centrifugation schemes can be seen as recommended for enrichment of nuclei and membranes or for specific organelles like mitochondria, lysosomes and microsomes. Unfortunately, due to the

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nature of centrifugation, it is also time consuming and prevents high throughput.

In a more chemical orientated approach one can use a sequence of detergents with increasing solubilisation efficiency. Thereby a detergent like digitonin will be used to extract cytoplasmic proteins from a cell extract. The subsequent centrifugation will yield a highly enriched fraction of cytoplasmic proteins in the supernatant, whereas proteins from the pellet will be extracted by a stronger detergent like Triton X-100 [13]. There is a great variety in the sequence and choice of detergents described in other studies [14, 15]. Regrettably, this approach suffers from the wide variety of proteins and their interactions in turn leading to a modest specificity of extraction steps for subcellular compartments. Nevertheless, there are also some biologically highly relevant subcellular compartments like the proteome of the lipid rafts that can be extracted with high specificity [16].

In order to obtain high specificity and reproducibility while being cost- and time efficient, various combinations of physical and chemical methods using centrifugation and detergents have been developed. In addition, many protocols have been designed that lack ultracentrifugation and can be performed in volumes that are suitable for most widely distributed bench-top centrifuges, thereby increasing the high throughput capacity significantly.

Here we focused on the comparison of four different methods ranging from a rather simple separation into a soluble, mostly cytoplasmic fraction and an insoluble, mainly membranous fraction up to separation schemes leading to more than five different fractions. For three separations commercially available kits from Fermentas (ProteoJet Membrane extraction kit), Qiagen (Qproteome Cell Compartment Kit [17]) and Pierce (Subcellular Protein Fractionation Kit) were used. A fourth procedure was adapted from literature [18]. Hence we provide evidence for the decision on the most suitable separation for different purposes. It is noteworthy that the results might be cell line or tissue specific, so this has to be tested for the sample of choice. Here we focused on Jurkat cells, which serve as a cellular model for T helper-cells. They mimic important changes that also occur in native T-helper cells once they become stimulated. These processes lead to differential protein expression which has consequences in the cytoplasm, the nucleus and also in the membrane compartment.

With the development of shotgun mass spectrometry and data bases with predictions and reports on the subcellular distribution of proteins, a fast and reliable tool became available for testing the efficiency of the separation procedures. Again, in order to achieve optimal coverage and high reproducibility, a subfractionation was applied. The obtained fractions were applied to a SDS-gel and after a short run each lane was cut into three parts which were subjected to in-gel digestion. Measurement of the peptides by modern mass spectrometry revealed up to 670 proteins per fraction. For validating the results of subcellular fractionation approaches the number of several hundreds of proteins can be assumed

to be sufficient to obtain a representative data set and for judging the success of the cellular fractionation.

In this study we provide evidence for the question which separation technique is the most favorable for a specific research question and approach. In addition to the achieved proteome coverage of subcellular compartments there are further requirements that need to be taken into account. For a specific research topic it might be helpful to use a combination of methods. The comparisons conducted here will help to facilitate proteomic research of subcellular compartments and organelles.

2. Material and methods

2.1 Cell culture

Jurkat T cells (clone E6-1, TIB-152, LGC Promochem, Wesel, Germany) were routinely maintained in RPMI-1640 medium (Biochrom AG., Berlin, Germany) containing 10% fetal bovine serum (Biochrom AG., Berlin, Germany), 1% L-Glutamine (Biochrom AG., Berlin, Germany), 1% streptomycin (100 mg/ml) / penicillin (100 U/ml) (PAA, Pasching, Austria) at an atmosphere of 5% CO₂, 95% humidity at 37 °C in a CO₂ incubator (MCO-18AIC, Sanyo Electric Co Ltd, Gunma-ken, Japan). Jurkat cells were cultured at 1 x 10⁶ cells per ml medium. Cell viability and cell numbers were recorded by trypan blue exclusion.

2.2 Cell lysis and fractionation

All steps of the different fractionation methods were performed on ice using pre-chilled solutions unless noted otherwise. Centrifugation and incubation were carried out at 4 °C. If the composition of a buffer is not given, no further information was provided by the supplier. All fractions obtained were stored at -20 °C until further use. The fractionations were performed at least three times per method and the protein estimations were carried out in triplicates.

Method 1 (see also Fig. 1): Buffer 2 and 3 were supplemented with protease inhibitor solution (Roche, Mannheim, Germany) before use. Jurkat cells (5 x 10⁶) were pelleted for 5 min at 250 x g and washed twice with 3 ml and 1.5 ml buffer 1, respectively. The cell pellet was resuspended in 1.5 ml buffer 2 by vortexing. The suspension was incubated for 10 min while continuously rocking. After 15 min centrifugation at 16,000 x g the supernatant 1 contained the cytosolic proteins. The pellet 1 was solved in 1 ml buffer 3 and the mixture was incubated for 30 min shaking at 1400 rpm in a thermomixer (Eppendorf, Hamburg, Germany). The suspension was centrifuged for 15 min at 16,000 x g. The supernatant 2 contained the membrane proteins, the cell debris containing pellet 2 was discarded. The protein determination for both fractions was carried out using the Bradford Quick Start Protein Assay according to the recommendations of the supplier (Bio-Rad Laboratories GmbH, München, Germany).

Method 2 (see also Fig. 1): All buffers were supplemented

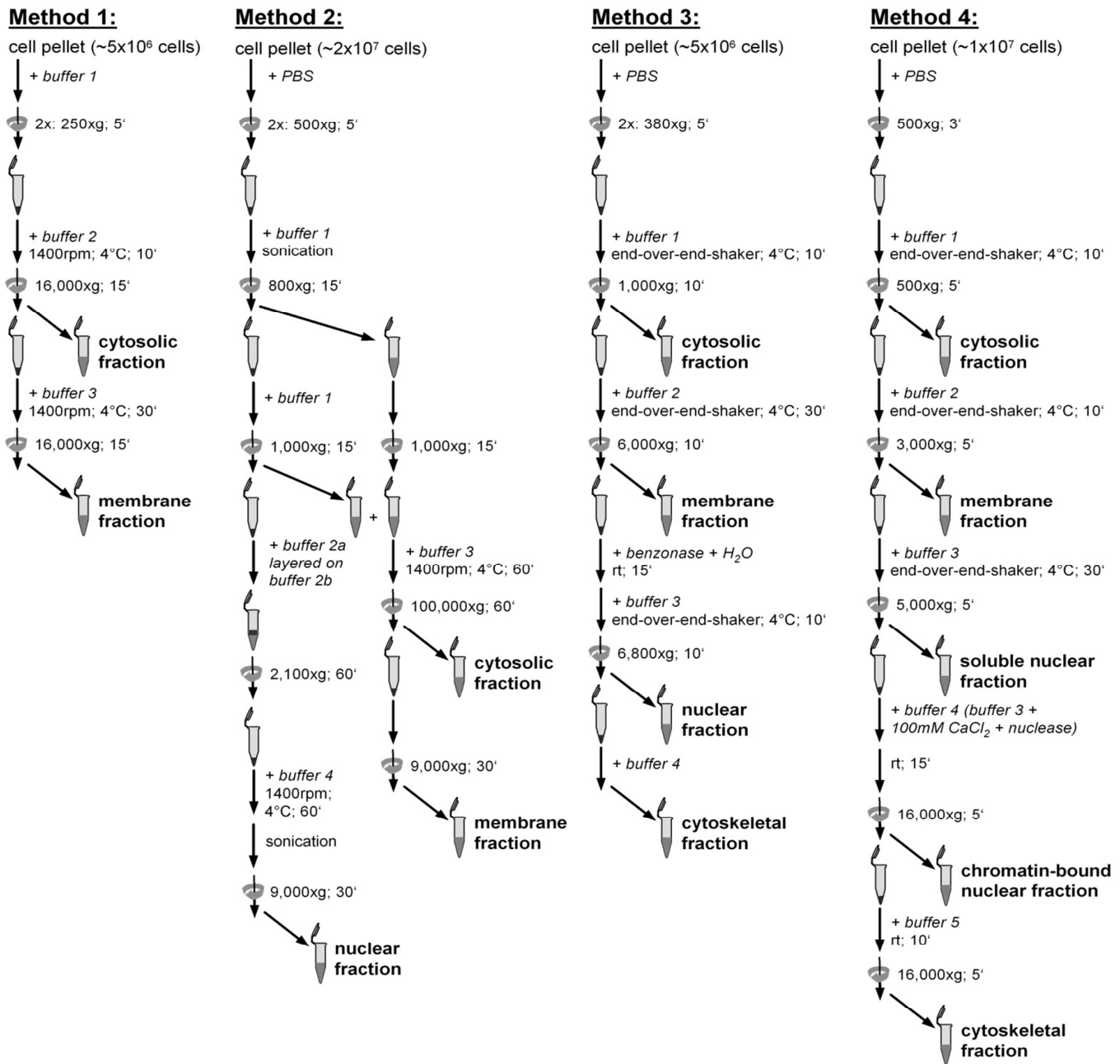


Figure 1. Schematic workflow. All centrifugation and incubation steps of the four different fractionation methods are shown (rpm is given for incubation in a thermomixer, x g for centrifugation).

with 1x protease inhibitor solution and 1 mM DTT directly before use. Jurkat cells (2×10^7) were washed twice with PBS and pelleted for 5 min at 300 x g. The cell pellet was resuspended in 1 ml buffer 1 (250 mM sucrose, 50 mM Tris-HCl, 5 mM MgCl₂) and cell lysis was performed by sonication on ice (3 times 10 s bursts with intensity ~40% and 30 s breaks). The suspension was centrifuged at 800 x g for 15 min and the pellet 1 was saved to isolate nuclei. The supernatant 1 was centrifuged again at 1,000 x g for 15 min. The obtained supernatant 2 was saved to isolate the cytosolic proteins, whereas pellet 2 was discarded.

The pellet 1 saved for isolation of the nuclei was dissolved in 1 ml buffer 1 and centrifuged at 1,000 x g for 15 min. The obtained supernatant 3 was added to the supernatant 2 for isolating cytosolic proteins and stored on ice until later. The pellet 3 was resuspended in 1 ml buffer 2a (1 M sucrose, 50 mM Tris-HCl, 5 mM MgCl₂) and layered onto a 3 ml cushion of buffer 2b (2 M sucrose, 50 mM Tris-HCl, 5 mM MgCl₂). Afterwards centrifugation at 2,100 x g for 1 h was carried out. The pellet 4 was taken up in 500 µl buffer 4 (20 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 0.5 M NaCl, 0.2 mM EDTA, 20% glycerol, 1% Triton X-100) and incubated 1 h

shaking at 1400 rpm and 4 °C in a thermomixer. Afterwards the suspension was sonicated again on ice (3 times 10 s bursts with intensity of ~40% and 30 s breaks) and centrifuged at 9,000 x g for 30 min. The supernatant 5 contained the nuclear proteins.

The pooled supernatants 2 and 3 were centrifuged for 1 h at 100,000 x g in an ultracentrifuge. The supernatant 6 contained the cytosolic proteins. The pellet 6 was solved in 0.5 ml buffer 3 (20 mM Tris-HCl, 0.4 M NaCl, 15% glycerol, 1.5% Triton X-100), incubated 1 h shaking at 1400 rpm and 4 °C and centrifuged at 9,000 x g for 30 min. The supernatant 7 contained the membrane proteins. The Lowry-DC-Protein Assay (Bio-Rad Laboratories GmbH) was used to determine the protein content of all fractions obtained with method 2.

Method 3 (see also Fig. 1): All buffers were supplemented with protease inhibitor solution before use. Jurkat cells (5×10^6) in a 1.5 ml reaction tube were pelleted for 5 min at 380 x g and washed twice with 1 ml PBS. The cell pellet was mixed with 1 ml buffer 1 and incubated for 10 min on an end-over-end shaker. The lysate was centrifuged at 1,000 x g for 10 min. The supernatant 1 contained the cytosolic proteins. The pellet 1 was resuspended in 1 ml buffer 2 and incubated for 30 min on an end-over-end shaker and centrifuged at 6,000 x g for 10 min. The newly gained supernatant 2 contained primarily membrane proteins. The pellet 2 was mixed with 20 µl distilled water containing 35% benzonase by gently flicking the bottom of the tube. After 15 min incubation at room temperature 0.5 ml buffer 3 was added and the suspension incubated for 10 min on an end-over-end shaker. The insoluble material was pelleted by centrifugation at 6,800 x g for 10 min. The supernatant 3 contained the nuclear proteins. The pellet 3 contained primarily cytoskeletal proteins and was resuspended in 250 µl room temperature buffer 4. The protein content of all fractions was determined using the BCA Protein Assay Macro Kit (SERVA Electrophoresis GmbH, Heidelberg, Germany).

Method 4 (see also Fig. 1): All buffers were supplemented with protease inhibitor solution before use. Jurkat cells (1×10^7) were washed with PBS and pelleted for 3 min at 500 x g in 1.5 ml reaction tubes. The cell pellet was solved in 1 ml buffer 1 and incubated for 10 min on an end-over-end shaker. The lysate was centrifuged at 500 x g for 5 min. The supernatant 1 contained the cytosolic proteins. The pellet 1 was mixed with 1 ml buffer 2, vortexed and incubated for 10 min on an end-over-end shaker. After centrifugation at 3,000 x g for 5 min, the obtained supernatant 2 contained primarily membrane proteins. The pellet 2 was dissolved in 0.5 ml buffer 3, vortexed and incubated for 30 min on an end-over-end shaker. Following centrifugation at 5,000 x g for 5 min the supernatant 3 contained soluble nuclear proteins. Buffer 4 was used at room temperature and prepared by adding 25 µl of 100 mM CaCl₂ and 15 µl of micrococcal nuclease to 0.5 ml buffer 3. 0.5 ml buffer 4 was added to the cell pellet 3, vortexed and incubated for 15 min at room temperature.

The mixture was vortexed 15 s and centrifuged at 16,000 x g for 5 min. The supernatant 4 contained chromatin-bound nuclear proteins. The pellet 4 was resuspended with 0.5 ml buffer 5, vortexed and incubated for 10 min at room temperature. After centrifugation at 16,000 x g for 5 min the supernatant 5 contained the cytoskeletal proteins. The protein content of all fractions was determined using the BCA Protein Assay Macro Kit following the manufacturer's instructions (SERVA Electrophoresis GmbH, Heidelberg, Germany).

2.3 1D-gel electrophoresis

20 µg protein of each fraction were precipitated 15 min at -20 °C by addition of a 5-fold volume of ice cold acetone. The precipitates were centrifuged at 16,000 x g and 4 °C for 10 min and the supernatant was discarded. The dried pellets were dissolved in SDS-sample-buffer (62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 5% mercaptoethanol, 0.05% bromophenol blue) and separated by SDS-PAGE on a 4% stacking gel and 12% separation gel run according to standard laboratory procedures. For visual control of successful separation the gels were stained with Coomassie Brilliant Blue G250 after electrophoresis. For protein analysis and MS identification the proteins were allowed to enter only for about 2-3 cm into the gel and cut into 3 gel slices per sample after short staining with Coomassie solution.

2.4 Trypsin digestion and analysis by LC-MS/MS

The gel slices were destained with 50% methanol containing 5% acetic acid. After reduction with 10 mM DTT, proteins were alkylated with 100 mM iodoacetamide and then digested overnight at 37 °C using sequencing grade trypsin (Roche Applied Science, Mannheim, Germany). All membrane fraction containing gel slices were digested in a trypsin solution containing 30% methanol (except method 4). The resulting peptides were extracted two times from the gel with 5% formic acid and 50% acetonitrile. The combined extracts were evaporated, the residual peptides were dissolved in 0.1% FA and the solution was desalted by using C18-StageTips (ZipTipC18, Millipore Corporation, Billerica, MA, USA).

A nano-HPLC system (nanoAquity, Waters, Milford, MA, USA) coupled to a an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) via a nano electrospray ion source (TriVersa NanoMate, Advion, Ithaca, NY, USA) was used for LC/MS/MS analysis. Chromatography was performed with 0.1% formic acid in solvents A (100% water) and B (100% acetonitrile). Samples were injected on a trapping column (nanoAquity UPLC column, C18, 180 µm×20 mm, 5 µm, Waters) and washed with 2% acetonitrile containing 0.1% formic acid and a flow rate of 15 µl/min for 8 min. Peptides were separated on a C18 UPLC column (nanoAquity UPLC column, C18, 75 µm×100 mm, 1.7 µm, Waters). Peptide elution was conducted using a gradient from 2 - 70% solvent B (0 min - 2%; 5 min - 6%; 45 min - 20%; 70 min - 30%; 75 min - 40%; 80 min - 70%) with a flow rate of 300 nl/min.

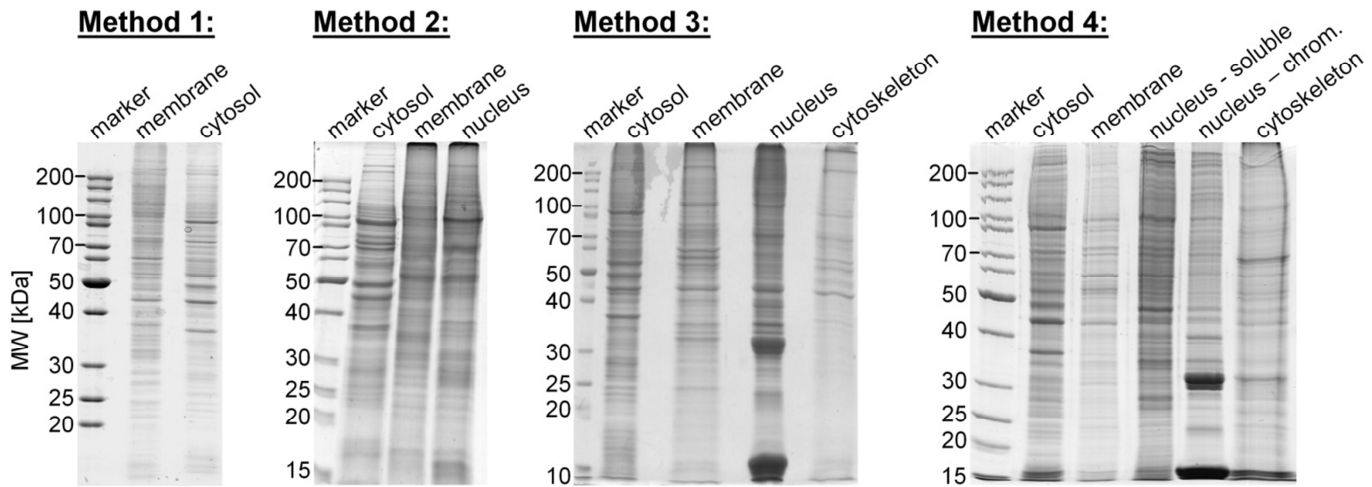


Figure 2. 1D-gels showing the different subcellular fractions. For initial evaluation of the fractions obtained by the four different methods, 20 µg of each protein fraction were separated in a 12% SDS-Gel and stained with colloidal Coomassie. The marker is located on the left hand side of each gel (nucleus-chrom. = chromatin-bound nuclear fraction).

Full scan MS spectra (from 400-1500 m/z , $R = 60000$) were acquired in positive ion mode in the LTQ-Orbitrap.

Peptide ions exceeding an intensity of 3000 were chosen for collision induced dissociation within the linear ion trap (isolation width 4 m/z , normalized collision energy 35, activation time 30 ms, activation $q = 0.25$). For MS/MS acquisition, a dynamic precursor exclusion of 2 min was applied.

2.5 Data analysis of the mass spectrometric results

MS/MS samples were analyzed by Proteome Discoverer (version 1.0; Thermo Fisher Scientific, San Jose, CA, USA) using the MASCOT search algorithm (version 2.2.06; Matrix Science, London, UK) [19]. Mascot was set up to search a reverse concatenated database of all human proteins annotated in the SwissProt database (version 10/07/2010) assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.5 Da and a parent ion tolerance of 5 ppm. Carbamidomethylation of cysteine was specified as a fixed modification. Oxidation of methionine and acetylation of the protein n-terminus were specified as variable modifications.

Scaffold 3 (version Scaffold 3_00_03, Proteome Software Inc., Portland, OR, USA) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they exceeded specific database search engine thresholds. Mascot identifications required at least ion minus identity scores of greater than -5 and ion scores of greater than 15. Protein identifications were accepted if they contained at least 2 identified peptides. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. False discovery rate of proteins was determined to be lower than 0.2% for all samples. Gene ontology annotations were obtained from the EBI GO database (www.ebi.ac.uk/GOA/, version 10/08/2010).

3. Results and Discussion

3.1 Fractionation of Jurkat cells

The workflow of the four different methods used to fractionate Jurkat cells into several cellular compartments is shown schematically in Fig. 1. In method 1, 3 and 4 commercially available kits were used, whereas method 2 uses an adapted protocol from Nature Protocols [18]. All methods rely on cell lysis through sequential addition of different buffers to the cell pellets followed by incubation and centrifugation at different speeds. In method 2 sonication is additionally used to lyse the cells. From method 1 only two different fractions, cytosol and membrane, were obtained. In addition to the three fractions prepared with method 2 – cytosol, membrane and nucleus, a fourth cytoskeletal fraction can be separated with method 3. With method 4 even five different subcellular fractions can be isolated: cytosol, membrane, cytoskeleton, with the nuclear fraction further split into soluble and chromatin-bound nuclear fraction. Method 1 is least time consuming, with about 1.5 hours needed for the fractionation. In approximately 2 hours a fractionation with method 3 or 4 is completed. With at least 3.5 hours of work method 2 is the longest protocol of all four. In addition, method 2 is the most complicated protocol because there are two lines of work steps which have to be performed in parallel while all other methods require only one straight workflow. Moreover, an ultracentrifuge with acceleration up to 100,000 $\times g$ is needed for method 2, while a normal table-top centrifuge with up to 16,000 $\times g$ is sufficient for all other methods used. Nevertheless, all buffers for method 2 can be prepared in the lab and no expensive kit is needed and the largest number of protein identifications was obtained.

The total amount of obtained protein differed for the various methods (Tab. 1) from 0.78 mg to 3 mg per 1×10^7 cells, ranging between 0.5 and 1.57 mg for the cytoplasmic fraction and 0.08 to 0.92 mg for the nuclear fraction. This shows that

Table 1. Protein amounts obtained per 1 x 10⁷ cells in each fraction.

Amount of protein obtained per 10 ⁷ cells [mg]				
Method Fraction	1	2	3	4
Cytosol	0.705	0.495	0.496	1.571
Membrane	0.630	0.208	0.135	0.362
Nucleus	–	0.079	0.183	–
Nucleus - soluble	–	–	–	0.520
Nucleus - chromatin-bound	–	–	–	0.401
Cytoskeleton	–	–	0.044	0.148
Total amount of protein	1.335	0.782	0.858	3.002

there is a rather wide variance in efficiency of the protein isolation. This should also to be taken into account when choosing the fractionation method combinable with the protein detection method used afterwards.

3.2 1D-gel electrophoresis

A first overview of the successful protein separation by the different fractionation methods was obtained by SDS-PAGE. All fractions gained using one method show clearly different band patterns, whereas the same subcellular fractions from different methods have some resemblance in their protein patterns (Fig. 2).

All cytosolic fractions show a comparable band pattern (e.g. five strong bands, of which one is at ~90 kDa, one slightly above 50 kDa, two between 40 and 50 kDa and one at ~38 kDa). Likewise the membrane fractions of method 1, 3 and 4 have a similar band pattern showing a more distinct band at approximately 60 kDa, whereas the separated membrane proteins of method 2 seem to run at slightly different heights. The nuclear fraction from method 2 has as well only partial similarities to the nuclear fractions of methods 3 and 4. The nuclear fraction from method 3 and the nuclear chromatin-bound fraction from method 4 show both two very prominent bands at ~15 and ~30 kDa. These bands are likely to represent histones. The soluble nuclear fraction from method 4 shares a stronger band at ~45 kDa with the nuclear fraction from method 3. As this band is also present in the chromatin-bound fraction, this protein might either be only loosely bound to the chromatin, or, more likely, is not completely separated from the chromatin-bound fraction.

3.3 Identification of proteins

The MS/MS data were analyzed by Proteome Discoverer using the MASCOT search algorithm. The MS/MS based peptide and protein identifications were validated by Scaf-

Table 2. Number of proteins identified in the subcellular fractions.

Method Fraction	1	2	3	4	Ø proteins identified / method
Cytosol	414	657	599	620	573
Membrane	249	458	352	523	396
Nucleus	–	603	258	–	431
Nucleus - soluble	–	–	–	670	670
Nucleus - chromatin-bound	–	–	–	370	370
Cytoskeleton	–	–	618	64	341
Total number of identified proteins	559	123 1	112 6	1195	1028

fold 3. For evaluation of method 4 the two nuclear fractions were combined.

In the cytosolic fractions an average of 573 proteins was identified by all methods (Tab. 2). In the membrane fraction the amount of identified proteins varies a lot between the different methods. With method 1 only 249 proteins were found, whereas 523 proteins were identified with method 4. With method 2 more than the double amount of proteins (603) could be identified in the nucleus compared to method 3 (258). The two different nuclear fractions, soluble and chromatin-bound, obtained with method 4 yielded in 670 and 370 identified proteins, respectively, leading to 750 identified proteins for the nucleus in total (Fig. 3). The amount of cytoskeletal proteins identified with method 3 and 4 ranges from 64 proteins identified with method 4 and up to 618 with method 3. The total numbers of identified proteins were in the same range (between 1126 and 1231) for method 2, 3 and 4 while for method 1 only 559 proteins could be identified in total. Altogether, only the amount of identified proteins in the cytoplasmic and the membrane fractions are comparable within all methods. All methods differ significantly in the amount of proteins identified per fraction as well as in the amount of protein isolated in total.

3.4 Enrichment factor of different fractionation methods

To get a deeper insight into how efficiently each fractionation method worked out, the overlap and intersections in cytosolic, membrane and nuclear fraction were determined and plotted in venn diagrams (Fig. 3). For this aim the two nuclear fractions of method 4, soluble and chromatin-bound, were combined. The most proteins identified in two overlapping fractions were found in cytosol and membrane for method 1 and 3, whereas method 2 and 4 show the biggest overlap in the membrane and nuclear fraction.

Disregarding method 1, because it only yielded two frac-

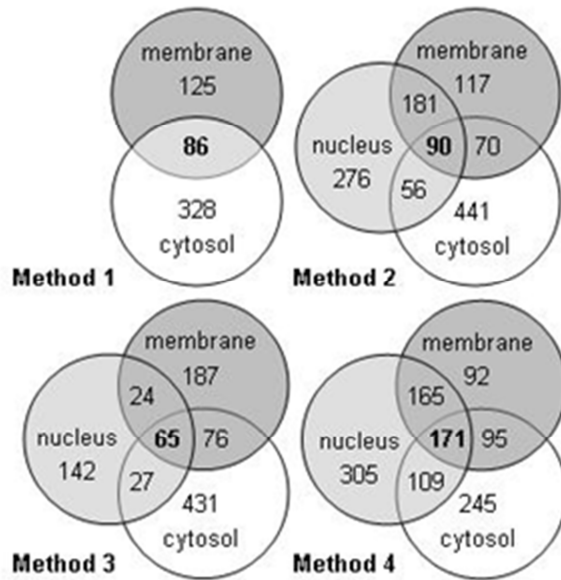


Figure 3. Overlap of proteins identified in the different subcellular fractions. For each of the fractionation methods used, a venn diagram was generated showing the overlap of the proteins identified in more than one fraction.

tions, the most proteins identified in only one fraction could be found with method 3 (80%). 68% of the identified proteins were found in only one fraction with method 2. Method 4 showed the smallest part of proteins identified in only one fraction (54%), while 46% of the identified proteins in this method were found in two or three of the fractions.

In this experiment Gene Ontology (GO) annotations were used by the evaluation program Scaffold 3 to analyze the subcellular localization of each protein identified in the samples. If the proteins identified in one fraction were supposed to be in that fraction according to the GO annotations, they were counted as proteins isolated in the 'correct' fraction. To compare how efficient each of the four fractionation methods fractionated the cells, the number of properly isolated proteins in each fraction was calculated. The percentage of the correctly separated proteins out of the total number of identified proteins in each fraction was calculated, too (Fig. 4). The cytosolic fraction was among all four methods the fraction with the most accurately isolated proteins (between 357 and 657 proteins) and comparable percentages about 80%. Between ~30 and 42% of the proteins found in the different membrane fractions where isolated correctly, leading to 74 till 188 isolated proteins in the 'correct' fraction in total. For the nuclear fraction 230 up to 345 nuclear proteins could be identified. The percentage of correct nuclear proteins from method 3 was very high with 90%, whereas method 4 showed a high amount of properly isolated proteins because of its two different nuclear fractions. Taking a closer look at transcription factors, there were 12 different ones detected using method 1 and 27 to 32 using method 2 to 4. With method 3 more appropriately isolated cytoskeletal proteins could be identified than with method 4, but the percentage is very low

for both methods. The high false positive rate is likely due to the solubilisation of most of the proteins of the last cell pellet, where surely proteins of not completely dissolved membranes or other cellular compartments were inside.

3.5 Discrepancies between the predictions of the evaluation program and the measurements

The Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a highly abundant protein, which accounts for 10 to 20% of the total cellular protein. It is commonly known as a glycolytic enzyme located in the cytoplasm with a key role in energy production [20]. By intensive research it became obvious that the GAPDH is in reality a multifunctional protein with diverse subcellular localizations in mammalian cells. The GAPDH can be found in the membrane, where it promotes endocytosis and membrane fusion and therefore vesicular secretory transport [21, 22]. Furthermore GAPDH is involved in the nuclear transport of RNA [23] and has the ability to activate the transcription in neurons [24]. Other functions in the nucleus are the assistance in DNA replication and DNA repair [25]. Due to the modulation of the cytoskeleton GAPDH can also be found in the cytoskeletal fraction [26, 27]. Thus the GAPDH can have not only a cytosolic, but also a membrane, nuclear and/or cytoskeletal localization.

According to the GO annotations the GAPDH is located only in the cytoplasm and membrane. This is contradictory to the various localizations described by the literature. In this experiment the GAPDH was found in all fractions obtained with method 2 and 4. With method 3 the enzyme was identified in the cytoplasmic, membrane and cytoskeletal fraction. For all of these three methods the localization in nucleus and

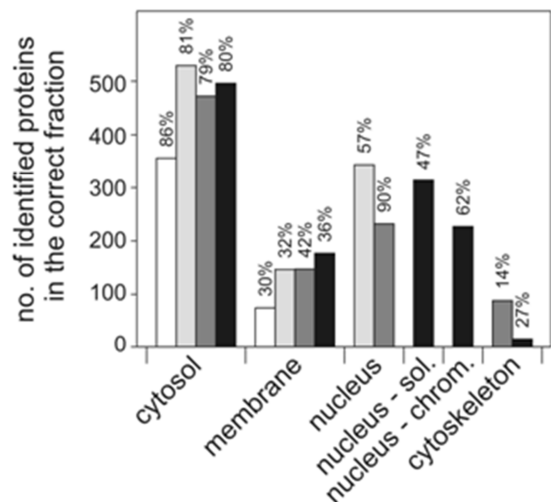


Figure 4. Evaluation of protein localization. For determination of the specificity of each method, the detected proteins in all fractions were analyzed in respect to their assumed localization according to GO terms using Scaffold 3. The bar chart shows the number of proteins identified in each fraction, which were expected to be in that cellular subfraction following Scaffold 3/GO annotations. On top of each bar the percentage of 'correctly' isolated proteins in the fractions is given (Method 1 = white bars; Method 2 = light grey bars; Method 3 = dark grey bars; Method 4 = black bars).

cytoskeletal fraction was validated as incorrect because of the incomplete GO annotations. So the GO annotations can only be used to get an overview of the subcellular localizations of a large dataset of proteins. If the localization of a distinct protein is of interest, then a literature search has to be made additionally.

3.6. Potential use of membrane proteins as markers for activation of Jurkat cells

Subcellular fractionation is an ideal tool to enrich and analyze different cellular compartments and low abundant proteins [28]. Due to the fractionation of the cells the less frequent membrane proteins, which otherwise are often covered by the numerous cytosolic proteins in MS measurement, can be identified and analyzed too. Surface proteins in the membrane are especially important for lymphocytes as they are needed for the recognition of antigens and cytokines and activation of other cells. Some of these surface proteins can be used as markers in the evaluation for different purposes. Activated lymphocytes express membrane proteins like CD25, CD69, CD71, and HLA-DR [29-32] which are absent or expressed only in low amounts on resting cells. These proteins are used as activation markers [33]. Similarly a number of known surface proteins like CD2, CD3 and CD5 were identified in the membrane fractions analyzed. In particular for CD2 and CD3 it is long known that they are involved in transmembrane signaling [34]. Despite the known marker, the analysis of the enriched membrane proteins gained by the subcellular fractionation could furthermore lead to the identification of new activation markers, when comparing the membrane proteome of resting and activated cells. Additionally, the identification and subcellular assignment of previously unknown proteins is conceivable. Newly identified membrane proteins may also be used to distinguish between the various T helper cell subpopulations and therefore assist in the process of revealing the different roles of T helper subsets.

4. Concluding remarks

The direct comparison between different methods allows an evidence-based decision on the method of choice for a specific research question. For some studies the mere separation of cytosolic and membrane proteins will be sufficient to perform subsequent analysis. Like for Western blotting method one provides a time-efficient solution of enrichment of certain proteins. When the analysis of the membrane fraction is of special interest the methods 2 or 4 might be favorable. If in the same instance also information about proteins with a nuclear localization it seems advisable to use method 4.

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