Insights on maturation-associated alterations in the proteome: Novel approaches and developments

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Is Proteomics Necessary?

A number of biological processes are reflected exclusively on the protein level:

- *Secretory events*
- *Molecular transport events*
- *Activation or deactivation of signalling cascades,*
- *Release of individual proteins from depositories*
- *All post-translational modifications*

Translation is a post-transcriptionally regulated process

- *mi RNAs*
- *Regulation occurring at the ribosome*

Proteomic strategies

2D DIGE	Nano-LC-MS/MS
Minimal [amount of dye] labeling Saturation [amount of dye] labeling Protein detection / quantification from only 500 ng total protein	Separation using hromatography Efficient protein identification
Quantification on the protein level	Quantification on the peptide level „on the fly“
Quantification of protein isoforms and post-translational modifications	Fast procedure High degree of automation possible
200-500 µg protein necessary for identfication	No information on the protein level

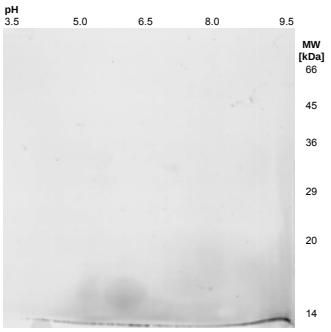
Challenges in Bovine Oocyte Proteome Analysis

- Protein content of a single bovine oocyte is about 90 ng
- For one single 2D DIGE “Minimal Labeling” gel containing 150 µg protein, about 1700 oocytes would be necessary - corresponding to about 100 cows
- In experiments based on ovum pickup, only about 5 high quality oocytes, corresponding to 0.45 µg protein, can be obtained per session /per cow

Dedicated protocols for highly reproducible protein extraction
High sensitivity in quantificaion

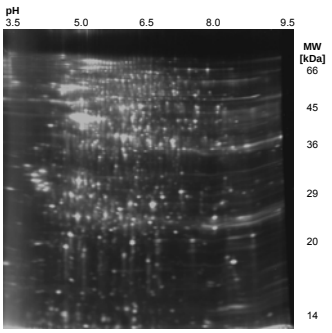
Sensitivity of Saturation Labeling

- Bovine blastocysts
- Cy5 saturation labeled
- 0.25 µg loaded on Gel
- Post-stained with silver



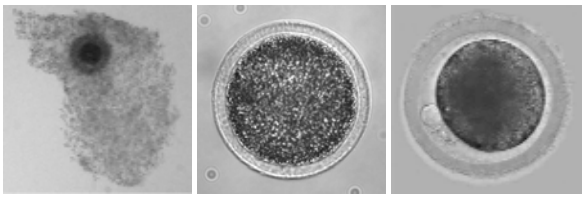
Sensitivity of Saturation Labeling

- Bovine blastocysts
- Cy5 saturation labeled
- 0.25 µg loaded on Gel
- Cy5 fluorescence readout



Oocyte Preparation and Maturation

Cumulus Oocyte Complex | Unmature Oocyte | Mature Oocyte



Extraction of cumulus oocyte complexes from 2 – 10 mm follicles (vacuum 100 mm/Hg)
Unmature Oocytes: Denudation –> Proteome analysis
Mature Oocytes: 22 h *in vitro* maturation (IVM) – Denudation –> Proteome analysis

Maturation medium: TCM-199 with Earle salts, 22 µg/ml FSH, 8 µg/ml LH
5% v/v Oestrus cow serum (OCS)

Maturation conditions: 22h, 39˚C, 5% CO_2

Experimental Design

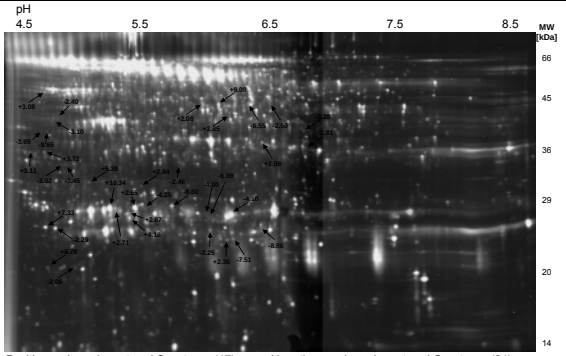
Scheme for 1 one out of 6 biological replicates

1. Gel
↓
Unmature Oocytes (Cy5) + IPS (Cy3)
↓
633 nm (Cy5) and 532 nm (Cy3)
↓
Control | IPS of 6 replicates

2. Gel
↓
IPS (Cy3) + Matured Oocytes (Cy5)
↓
532 nm (Cy3) and 633 nm (Cy5)
↓
IPS of 6 replicates | Sample

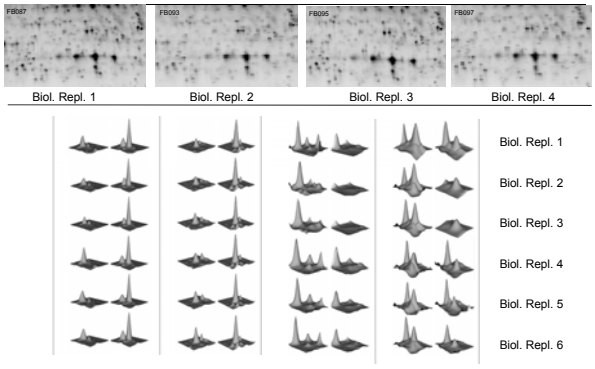
↓
Quantitative comparison of sample vs control and normalisation by IPS

Saturation DIGE Overlay Image, 0.5 µg protein per gel
(merged pH 4 – 7 und 6 – 9)



Positiv numbers: in matured Oozyten ▲ (17) Negative numbers: in matured Oozyten ▼ (21)

Reproducibility of Protein Separation and Spot Quantification



MS-MS-Identification

Disadvantage::

MS-identification is orders of magnitude less sensitive than quantification (300 vs 0,25 µg): ***3500 Oocytes necessary for a preparative master gel***

Identification criteria

2 peptides per protein

Ion Score > 30

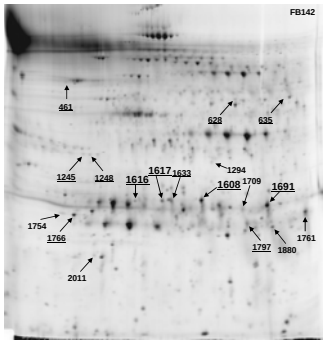
Search against bovine IPI using MASCOT

Fixed Modification: Cy3 Saturation label

Variable modifications: oxidation (M)

Number of „Miscleavages“: 2

Identification of Proteins: Maturation factors, Redox Enzymes and new Isoforms of GST



200 µg, Cy3 markiert, 18 Spots gepickt

		Ratio	SSE
461:	Tubulin beta chain Protein disulfide isomerase UV excision repair protein	+3,08	+1,22
628:	Dihydrolipoyllysine-residue succinyltransferase	+2,08	+1,02
635:	Elongation factor 1 gamma	-6,55	+1.09
1245:	Clusterin	-2,02	-1,14
1248:	14-3-3 ε	-3,45	+1,02
1608:	Glutathione S-transferase Mu5 NADH-ubiquinone oxidoreductase	-8,60	+1,06
1616:	Glutathione S-transferase Mu5	+10,34	+1,04
1617:	Glutathione S-transferase Mu5	+2,65	+1,06
1633:	6-phosphogluconolactonase	-4,25	+1,13
1691:	Glutathione S-transferase Mu5	-4,10	-1,04
1766:	Translationally-controlled tumor prot.	-2,29	+1,03
1797:	Thioredoxin-dependent peroxide red.	-2,25	+1,09

Identification of Protein Polymorphisms

1. Gel based 2D DIGE „Saturation labeling“, facilitates an elegant detection of protein polymorphisms: three GST-Mu5 isoforms were identified and quantified.
2. New protein isoforms so far not listed in databases could be detected.
3. Abundancies of different isoforms are clearly dependent on the oocyte maturation stage, demonstrating the importance of protein isoforms in biological processes as well as the advantage of proteome analysis on the on the protein (rather than the peptide) level.

Key steps in nano-LC-MS/MS Proteomics

Fractionation of protein lysate (e.g., by 1D PAGE)

Enzymatic cleavage of proteins to peptides

Separation of peptide fractions by nano-LC

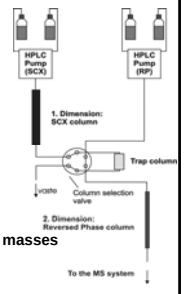
Apply column eluate to Tandem MS

Scan for peptide masses

In following scans, select one peptide for MS/MS

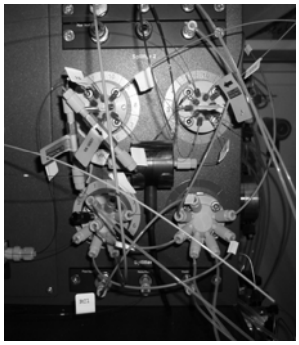
Probability based database search for matching MS/MS masses

Statistical analysis of dataset [Scaffold or TPP]

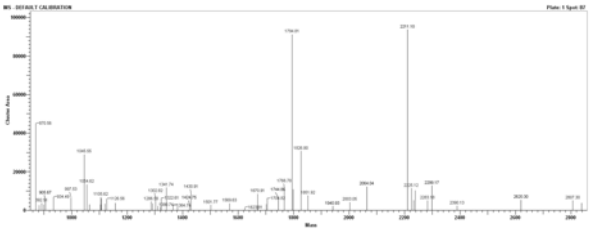


Review: Frohlich,T. and Arnold, G.J. (2006) Proteome research based on modern liquid chromatography - tandem mass spectrometry: separation, identification and quantification. J. Neural Transm. 113, 973-994.

nano-HPLC (MDLC)



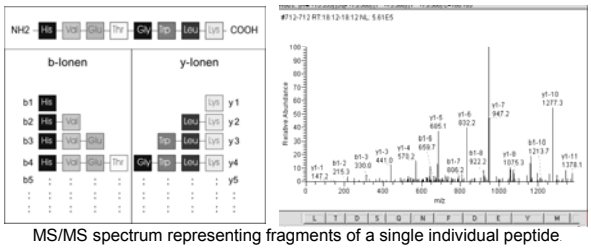
Protein Identification by Tandem-MS (MS-MS)



MS spectrum containing representing a mixture of independant peptides

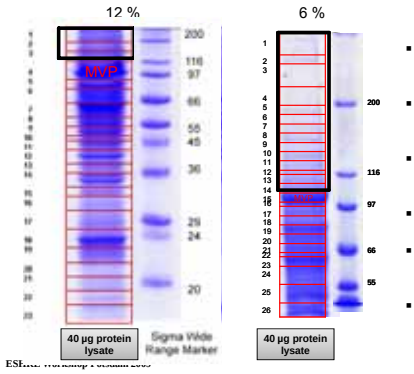
Protein Identification by Tandem-MS (MS-MS)

By collision of a peptide ion with a gas molecule within the mass spectrometer, one of the peptide bond breaks, generating two fragments. The resulting fragment masses are measured in the mass spectrometer, showing a pattern with characteristic mass differences. From these MS-MS spectra, amino acid sequence information can be deduced.



MS/MS spectrum representing fragments of a single individual peptide

Proteome Profiling of Bovine Oocytes



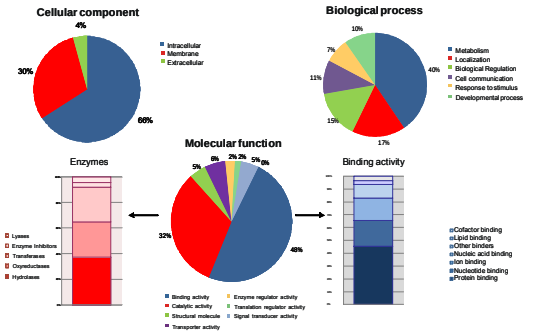
- In gel digestion of individual bands
- Purification of the tryptic peptides
- Nano-LC-MS/MS separation of peptides
- MS/MS analyses
- Mascot identification of peptides and proteins
- Statistical data analsis using „Scaffold“

Scaffold analysis [FDR < 1 %]

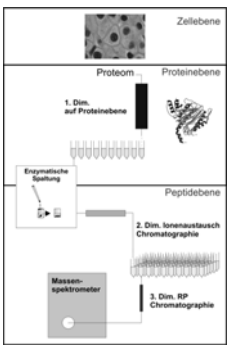
- Identification of 1127 proteins from 12 % gel
- Identification of additional **94** proteins from 6 % gel

				6% Gel	12% Gel
398	unc-51-like kinase 3	IPI00720374	53 kDa		100%
399	Reticulon-4-interacting protein 1	IPI00716010	44 kDa		99%
400	similar to guanine deaminase	IPI00716555	51 kDa		99%
401	WD repeat-containing protein 40A	IPI00696065	51 kDa		99%
402	Myosin-Id	IPI00713054	116 kDa		99%
403	Mitochondrial import inner memb...	IPI00705384	40 kDa	5%	100%
404	similar to Cullin-3	IPI00712202	89 kDa	5%	100%
1042	Eukaryotic translation initiation ...	IPI00718447	105 kDa	100%	100%
1043	similar to KIAA0639 protein isof...	IPI00825744	217 kDa	100%	100%
1044	157 kDa protein	IPI00842466	157 kDa	100%	
1045	AP-3 complex subunit delta-1	IPI00685602	136 kDa	100%	81%
1046	Cyclin-T1	IPI00713491	81 kDa	100%	
1047	similar to E3 ubiquitin-protein li...	IPI00698929	101 kDa	100%	

Gene Ontology of Oocyte Proteins [n = 1221]



Quantitative LC-MS/MS Proteomics



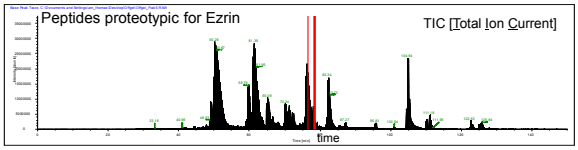
Metabolic labeling of in cell culture or organisms, e.g., SILAC

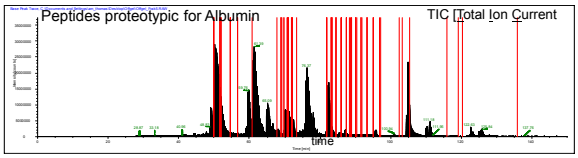
Labeling on the protein level
e.g., ICAT, ICPL

Labeling on the peptide level
e.g., iTRAQ, ^{18}O

Alternative strategy:
Label-free quantification

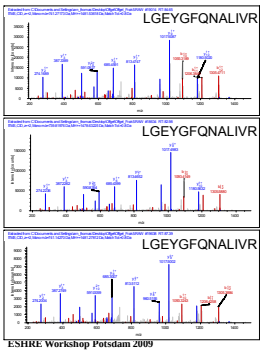
Quantification of LC-MS/MS data by „Peptide Count“



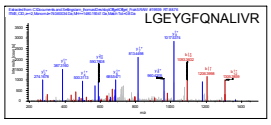


Quantification of LC-MS/MS data by „Spectral Count“

Stage 1



Stage 2



Relative abundance of Prot. X (stage 1 vs stage 2)

$$= \frac{\text{number of MS/MS spectra all proteotypic peps for Prot. X in stage 1}}{\text{number of MS/MS spectra all proteotypic peps for Prot. X in stage 2}}$$

= 3.0

In complex samples:
RSC = log2[(n2 + f)/(n1 + f)] + log2[(t1 - n1 + f)/(t2 - n2 + f)]
n1, n2 - spectral counts for sample 1 and 2
t1, t2 – total spectral count (sampling depth) for samples 1 and 2
f – correction factor 1.25 (Beissbarth et al – Bioinformatics 2004)

Sensitivity determination: nano-LC-MS/MS from 0.9 µg protein (10 oocytes)

Sample preparation:

- **Lysis of 10 denuded bovine oocytes**
- Solubilisation of proteins in 8M Urea in 50 mM NH_4HCO_3
- Dilution to 1M Urea
- Reduction by 5 mM DTT (55°C, 30 min)
- Alkylation by 10 mM Iodacetamid (RT, 15 min)
- Inkubation with 20 ng Trypsin (37°C, o/n)

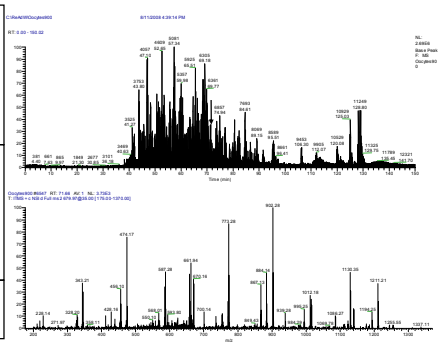
RP Separation:

Column:
C18 PepMap 100, 3µm, 75µm i.d. 15 cm, (LC Packings)
Flow:
240 nl / min
Solvent A (0.1% FA)
Solvent B (87% FA)
Gradient:
80 min; 0% -> 30 % B
30 min 30% -> 60% B
15 min 100% B

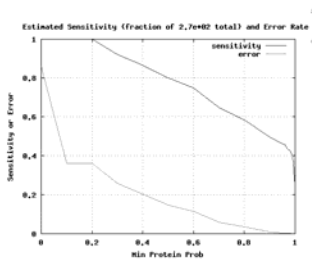
Mass spectrometry:

Thermo LTQ linear Ion Trap

1 "Survey Scan" followed by 3 data-dependent MS/MS Scans.



Sensitivity Determination (statistical validation by the Trans-Proteomic Pipeline TPP)



sensitivity — fraction of all correct proteins with probs >= min_prob

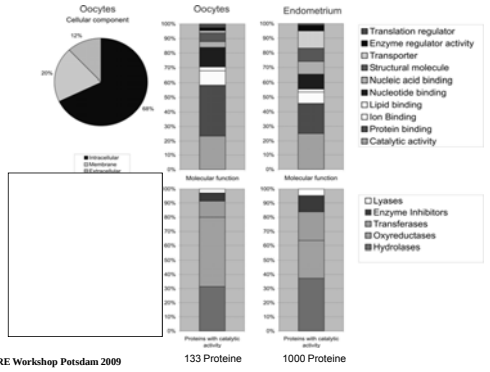
error — fraction of all proteins with probs >= min_prob that are incorrect

min_prob	sens	err	# corr	# incorr
1.00	0.249	0.000	66	0
0.99	0.396	0.001	105	0
0.98	0.425	0.002	113	0
0.97	0.436	0.002	116	0
0.96	0.458	0.004	121	0
0.95	0.462	0.004	122	0
0.90	0.497	0.010	132	1
0.80	0.586	0.035	155	6
0.70	0.646	0.059	171	11
0.60	0.748	0.115	198	26
0.50	0.800	0.148	212	37
0.40	0.867	0.202	230	58
0.30	0.922	0.259	244	85
0.20	1.000	0.361	265	150
0.10	1.000	0.361	265	150
0.00	1.000	0.860	265	1628

Identified from 10 oocytes equivalents: 307 peptides; 133 proteins (FDR < 1%)
Identified from 1 bovine equivalent: 81 peptides 44 proteins (FDR < 1%)

Keller A, Eng J, Zhang N, Li XJ, Aebersold R: **A uniform proteomics MS/MS analysis platform utilizing open XML file formats.** *Mol Syst Biol* 2005, **1**:2005.0017.

Proteins identified from 10 Oocytes represent a variety of functions



Conclusion

- **Proteomic approaches based on DIGE saturation labeling facilitate quantification of 1000 – 2000 proteins in a reasonable time period from 500 ng protein per gel**
- **In 2D Gel based approaches, protein isoforms and their relative abundancy changes can be quantified**
- **Using LC-MS/MS, up to 200 proteins can be identified from 10 bovine oocytes using latest instrumentation**
- **„Spectral counting" facilitates protein quantification from small pools of oocytes or early embryos**

Thanks to

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