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When 2D is not enough, go for an extra dimension

Thierry Rabilloud 1,2,3

1: CNRS, Laboratory of Chemistry and Biology of Metals (LCBM), UMR 5249, Grenoble, France  
2: Univ. Grenoble Alpes, LCBM, Grenoble, France  
3: CEA, iRTSV/LCBM, Grenoble France

Postal address

Pro-MD team, UMR CNRS-CEA-UJF 5249, iRTSV/LCBM, CEA Grenoble, 17 rue des martyrs, 38054 Grenoble CEDEX 9, France

email: thierry.rabilloud@cea.fr

### Abstract

The use of an extra SDS separation in a different buffer system provide a technique for deconvoluting 2D gel spots made of several proteins [Colignon et al. *Proteomics*, 2013, 13: xxx-yyyy]. This technique keeps the quantitative analysis of the protein amounts and combines it with a strongly improved identification process by mass spectrometry, removing identification ambiguities in most cases. In some favorable cases, post-translational variants can be separated by this procedure. This versatile and easy to use technique is anticipated to be a very valuable addition to the toolbox used in 2D gel-based proteomics.

### Main text

Because of its robustness, capacity to handle large sample series, easy interface with many other biochemical techniques and above all its unique ability to analyse complete proteins, 2D gel electrophoresis is still a relevant approach in many proteomic studies [1, 2]. In most cases, 2D gel electrophoresis is used as a first quantitative screening process to select the spots which abundance change upon the biological phenomenon of interest. It is then necessary to identify the proteins present in these modulated spots to obtain a quantitative biochemical view of the molecular events at play in the biological phenomenon of interest.

For many years, i.e. from 1990 to 2005, this has been a straightforward process, as the protein analysis techniques, namely Edman's sequencing and then mass spectrometry, always rendered one protein per electrophoretic 2D spot. However, with the ever increasing sensitivity of mass spectrometers, this equation is less and less true with the proportion of singulets (one protein per spot) going down, from 70% in 2005 [3] to 50% in 2013 [4]. This does not disqualify 2D gel-based proteomics per se, as previously stated [5], as long as it is possible to correlate with

good confidence the variation in a spot volume with the variation of one protein. Using a reduction ad absurdum, all the historical data using low sensitivity methods able to identify a moderately complex mixture of proteins, whether Edman's sequencing [6, 7], or tandem mass spectrometry [8, 9] point to the fact that almost all 2D spots are made of a major protein, which explains the quantitative variations in staining, and of minor components that do not play any role in the staining variation but are real components of the protein spot.

This fact should not come as a surprise, when integrating on the one hand the resolving power of 2D gels and on the other hand the dynamic range of the proteome and the number of different protein species present in any complex biological sample.

Thus, the name of the game is to identify this major component in the protein spots. As shown in figure 1 and table 1, this is sometimes quite straightforward (spots 1 to 3) and sometimes almost impossible (spots 4 to 8) from the simple MS/MS output. Several approaches can be designed to circumvent this major problem. The simplest one is to analyse less and less protein in the spots, so that the mass spectrometer will find only the most abundant component. This can be achieved by analyzing small silver-stained spots, with the major risk that many spots will no longer show any identification.

A much more powerful and elegant approach is to combine the peptide by peptide quantitative analysis of SILAC with the ability of 2D gels to resolve complete proteins, as exemplified by a recent work on HeLa cells [4]. However, not all biological systems are easily amenable to SILAC labelling, which is in addition a costly procedure.

All in all, the ideal solution would be to be able to deconvolute the 2D spots into their individual proteic components in a quantitative way. This would allow to check which component(s) account for the quantitative variation in staining while making the mass spectrometric identification unambiguous again. This is exactly what is achieved by the third dimension electrophoresis described by Colignon et al. [10], in which the 2D spots are excised and re-electrophoresed on a different gel system to resolve them into individual components.

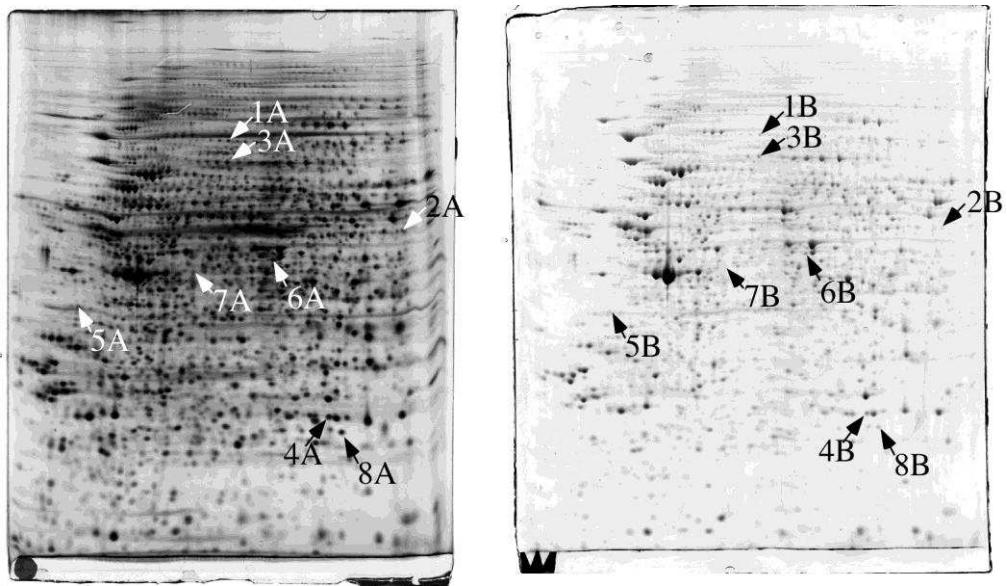
Three-dimensional electrophoresis has been described in the past, but in most cases the third dimension is carried before the conventional IEF-SDS separation [11, 12] and not after it as in the Colignon paper. Consequently, these approaches need to know upfront how to separate the proteins, which is seldom the case in most proteomic studies. Perhaps the most impressive 3D electrophoresis is the gel cube [13], which uses IEF as one separation dimension and two different SDS systems in the other two. While theoretically equivalent to the Colignon setup, the gel cube is much more cumbersome to handle and clearly not as straightforward as an approach which can be carried out on minigels and on only the spots that need it. The only approach described in the literature that can be compared with the Colignon setup is the one described by Vanfleteren [14], but it used a very specialized electrophoretic system which may not be applicable to all proteins.

The few examples shown in the Colignon paper demonstrate both the power and the limitation of the method. As the third dimension is also a SDS electrophoresis, some proteins that comigrate in 2D gels will still comigrate in the third dimension, leading to multiple identifications in mass spectrometry. Despite this intrinsic limitation, the technique works surprisingly well in its ability to deconvolute 2D gels spots, and the well-known versatility of SDS electrophoresis calls for a very wide scope of application, with very few proteins intractable to the third dimension. In addition, it

seems that the third dimension may be sometimes able to separate post-translational variants, which further adds to the attractivity of the technique.

In summary, this technique is easy to implement, cheap, and is likely to bring in most cases the extra separation that is more and more needed to interface safely 2D gels with the more and more sensitive mass spectrometers.

Footnote: the author has declared no conflict of interest



**Figure 1:** comparison of silver-stained and Coomassie blue-stained gels.  
A whole cell extract of RAW264 murine macrophage cell line was separated by 2D gel electrophoresis (linear pH gradient ranging from 4 to 8 in the first dimension, 10% T gel in the second dimension).

Left panel: one hundred micrograms of proteins loaded on the first dimension gel, detection by silver staining

Right panel: five hundred micrograms of proteins loaded on the first dimension gel, detection by colloidal Coomassie Blue

Equivalent spots were excised on the two gels, digested with trypsin, and the resulting peptides analyzed by tandem mass spectrometry on an ion trap instrument. The resulting data are presented on table 1.

Table 1: identification data for the spots excised from the gels shown in Figure 1

Spots 1 to 3 illustrate easy cases where no ambiguity is encountered, whereas spots 4 to 8 illustrate difficult cases where no straightforward identification can be made when supra-optimal amounts of proteins are present in the spots.

spot Nb.	protein name	Swissprot accession number	MW	Nb. Unique peptides	sequence coverage
1A	Mitofilin	Q8CAQ8	83901,1	2	2,64%
1B	Mitofilin ATP synthase subunit alpha	Q8CAQ8 Q03265	83901,1 59754,1	33 1	52,40% 2,71%
2A	ATP synthase subunit alpha	Q03265	59754,1	8	15,90%
2B	ATP synthase subunit alpha Glutamate dehydrogenase 1 Irg1 Serbp1 Ripk3 Ugp2	Q03265 P26443 P54987 Q9CY58 Q9QZL0 Q91ZJ5	59754,1 61417,4 53759,6 44754,6 53336,4 56925,9	28 2 1 1 1 1	54,60% 3,76% 2,05% 3,93% 2,88% 2,36%
3A	Moesin	P26041	67821,8	2	2,60%
3B	Moesin Glycine--tRNA ligase Beta-glucuronidase CTP synthase 1 PDI A4	P26041 Q9CZD3 P12265 P70698 P08003	67768,8 81879,1 74195,7 66690,7 71984,4	27 15 3 3 3	40,20% 19,50% 4,63% 4,74% 5,17%
4A	Hprt1	P00493	24571,3	3	16,50%
4B	Hprt1 Triosephosphate isomerase Flavin reductase (NADPH) GTP-binding nuclear protein dtd1 GSH S-transferase Mu 5 Pcmt1	P00493 P17751 Q923D2 P62827 Q9DD18 P48774 P23506	24571,3 32191,3 22196,7 24427,3 23232,3 26636,5 24641,9	5 4 2 2 1 1 1	27,10% 19,40% 11,70% 11,10% 7,18% 4,02% 4,85%
5A	V-type H+ ATPase sub. d 1	P51863	40302,8	2	6,84%
5B	V-type H+ ATPase sub. d 1 Nucleophosmin Sgta Elongation factor 1-delta Phospholipid scramblase 1	P51863 Q61937 Q8BJU0 P57776 Q9JJ00	40302,8 32560,3 34157,8 31293,4 35913,5	6 4 3 2 2	23,60% 22,90% 11,10% 9,25% 7,01%

	Adprhl2	Q8CG72	39414,3	1	3,24%
6A	eIF 4A-III	Q91VC3	46842,4	8	19,50%
6B	eIF 4A-III Adss2 Clp1 Cathepsin D C-terminal-binding protein 1 Elongation factor 1-gamma EF-Tu mitochondrial	Q91VC3 P46664 Q99LI9 P18242 O88712 Q9D8N0 Q8BFR5	46842,4 50140,8 47629,1 44955 47744,7 50061,3 49399,2	8 4 2 1 1 1 1	27,50% 11,00% 4,71% 4,39% 2,49% 2,97% 2,65%
7A	Protein NDRG1	Q62433	43008,2	2	7,61%
7B	Protein NDRG1 TAR DNA-binding protein 43 Coronin-1A Creatine kinase B-type Aldehyde dehydrogenase BRCA1-A complex subunit BRE Cathepsin D Eif3G Plastin-2	Q62433 Q921F2 O89053 Q04447 P47738 Q8K3W0 P18242 Q9Z1D1 Q61233	43008,2 44547,5 50988,9 42714,1 56537,6 43560,1 44955 35639 70151,9	3 3 2 2 1 1 1 1 1	11,90% 9,90% 8,03% 7,35% 4,43% 2,61% 4,39% 4,06% 2,39%
8A	Psma2	P49722	25926,9	1	5,98%
8B	Psma2 40S ribosomal protein SA GAPDH	P49722 P14206 P16858	25926,9 32885,3 35828,1	2 2 1	12,00% 9,49% 4,20%

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