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## The whereabouts of 2D gels in quantitative proteomics

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Running head: Quantitative 2D-PAGE

Abstract:

Two-dimensional gel electrophoresis has been instrumental in the development of proteomics. Although it is no longer the exclusive scheme used for proteomics, its unique features make it a still highly valuable tool, especially when multiple quantitative comparisons of samples must be made, and even for large samples series. However, quantitative proteomics using two-dimensional gels is critically dependent on the performances of the protein detection methods used after the electrophoretic separations. This chapter therefore examines critically the various detection methods, (radioactivity, dyes, fluorescence and silver) as well as the data analysis issues that must be taken into account when quantitative comparative analysis of two-dimensional gels is performed.

**ii. Key Words:** 2D-PAGE, fluorescence dyes, image analysis, organic dyes, polyacrylamide gels, quantification, radioisotopes, silver staining

## 1. Introduction

Since its introduction in the mid-seventies [1, 2], 2D electrophoresis has always been used as a quantitative technique of protein analysis, and it is fair to say that such quantitative analyses (e.g. in [3-8] ) have preceded the real onset of proteomics, hallmarked by the first protein identification techniques, at that time based on Edman sequencing [9-13]. In the current proteomics landscape, completely dominated by tandem mass spectrometry [14, 15], and where 2D gel-based proteomics is now perceived for only niche applications [16], it represents an exception in the sense that this is a proteomics setup where:

- i) protein quantification is not made in a mass spectrometer (and there has been only very limited attempts to break this rule [17] )
- ii) the on-gel quantification step is often used as a screening process to select a limited set of protein spots of interest, that are then further characterized by mass spectrometry, thereby limiting the mass spectrometry analysis time.

These two cardinal features put an enormous pressure on the performances that the on-gel protein detection methods must show, as it is quite clear that what is not detected is never analyzed and thus completely ignored. Thus, the on-gel protein detection methods must be very sensitive, but also linear in response (to be able to detect accurately abundance variations), homogeneous (so that all classes of proteins are detected) and of course fully compatible with mass spectrometry (to ensure easy and accurate protein characterization).

Although these constraints have been quite clear to the community for numerous years, and have led to the implementation of many protein detection schemes, an often overlooked problem is the performance of the 2D electrophoresis itself. In other words, what is the quantitative yield of 2D electrophoresis and how homogeneous this yield is for various classes of proteins. There are very few papers dealing with this issue, but one paper [18] showed that the yield of 2D electrophoresis was rather moderate (20-40%), which is an often overlooked parameter when the overall efficiency of the system is considered. Furthermore, work on membrane proteins [19, 20] has strongly suggested

that such losses are not homogeneous and may be much greater for poorly soluble proteins such as membrane proteins.

Of course, the overall yield of the process will also depend on the efficiency of the protein extraction during the sample preparation process, but this process is so variable from one experimental model to another that it is really beyond the scope of this chapter. Furthermore, this chapter will deal mainly with the quantification issues in 2D gel-based proteomics. Other important issues, such as the scope of gel-based proteomics, the interest of protein electrophoresis in modern proteomics and how electrophoresis systems can be modulated to improve their performances, have been reviewed elsewhere [21-23].

## 2. The protein detection methods

Over the years, numerous on-gel protein detection methods have been used, each having its advantages and drawbacks in the sensitivity/linearity/homogeneity/compatibility multiple criteria selection guide. Over the numerous years of use of 2D electrophoresis, some techniques have almost disappeared, while others are now standards in 2D gel-based proteomics. Many different protein detection schemes have been devised over the several decades of use of 2D gels [24], and only the most important ones will be reviewed in this chapter.

### 2.1. Protein detection via radioisotopes

This is an example of a technique that has subsided now, although it played a key role in the early days of proteomics, before the name was even coined.

Due to its exquisite sensitivity [25, 26] and linearity [27], protein detection via radioisotopes has been associated with almost all the early success stories of 2D electrophoresis, from the determination of protein numbers in cells [28], to the first identification of a protein from 2D gels, i.e. PCNA [29][30], to single cell proteomics [31] or to phosphoprotein studies [32]. These exquisite sensitivity and linearity have even increased with new detection technologies such as phosphor screens [33]. However, except for special purposes where it is almost irreplaceable [18, 34], detection via

radioisotopes has almost disappeared from modern proteomics. In addition to the fact that not all samples are easily accessible to this type of detection (e.g. human samples), increasing safety and regulatory issues hastened the decline of radioactivity in proteomics.

## 2.2. Protein detection via organic dyes

In this mode of detection, the name of the game is to bind as many dye molecules per protein molecules as possible, in order to create a light absorption signal that is detectable. Of course, this process must be as reproducible as possible, and the molar extinction coefficient of the dye also plays a major role in the signal intensity.

For all these reasons, and despite a few attempts to use other dyes [35], colloidal Coomassie Blue, as introduced in 1988 by V. Neuhoff [36], reigns supreme in this subfield. However, its rather limited sensitivity requires high protein loads to be able to detect a decent number of spots, which results in turn in increased sample consumption and decreased separation performances due to protein precipitation, leading to various artifacts such as streaks, most often in the isoelectric focusing dimension.

## 2.3. Protein detection via silver staining

To alleviate the sensitivity problem mentioned above, while keeping the ease of use and low costs associated with methods dealing with visible light absorbance, silver staining was introduced a few years after 2D electrophoresis [37]. However, it is fair to say that the early days of silver staining were troublesome, with erratic backgrounds and sensitivities, and this was due to the complex chemical mechanisms prevailing in silver staining [38]. However, decisive progress was made at the end of the 80's [39], and silver staining is now a reliable technique allying high sensitivity, good reproducibility, low cost and ease of use [40]. This is further discussed in another chapter of this book (Chapter 6).

The main drawbacks of silver staining in modern proteomics are its limited dynamic range (see chapter 6) but also its weak compatibility with mass spectrometry [41, 42], although very high performances have been claimed [43]. It has been shown that the

formaldehyde used in image development was the main culprit [44], and formaldehyde-free protocols have been developed that offer much increased compatibility with mass spectrometry [45] (see also chapter). In addition, the considerable increase of the sensitivity of the mass spectrometers over the years have decreased the severity of the problem.

### 2.3. Protein detection via fluorescence

To alleviate the problems shown by silver staining and Coomassie blue, protein detection by fluorescence has been developed and has shown great expansion over the past few years. Opposite to the strict mechanisms that prevail in visible staining, either with Coomassie Blue or with silver, protein detection via fluorescence can be achieved via multiple mechanisms, thereby offering great versatility to this technique.

The first and oldest mechanism is covalent binding, quite often of probes that are not fluorescent but become so when the covalent binding takes place [46, 47]. While the performances of such probes were not very impressive, and thus of limited use, a quantum leap was achieved when probes with much higher light absorption and emission characteristics were used. Furthermore, with the development of laser scanners, use of a set of closely related probes could be designed to achieve multiplexing [48], resulting in the very popular DIGE technique [49] (see also Chapter 7 in this book).

While this system has shown exquisite performances, it must be kept in mind that only a few fluorescent molecules are bound per protein molecule, resulting in an overall low signal intensity for many proteins. This is not a problem for pure detection, as the enormous contrast allow to use the full power of laser scanners, but this becomes a problem in some instances, e.g. spot excision for mass spectrometry, where more primitive illumination devices are used, e.g. UV tables.

Thus, another popular mode of protein detection via fluorescence uses non covalent binding, which takes place after migration and therefore does not interfere with protein

migration, and which can also take place at much more numerous sites on the proteins, thereby resulting in a much higher signal, although the free fluorescent molecule remaining in the gels decreases the contrast, compared to covalent binding. Although other candidates have been recently proposed [50,51], two molecules dominate this field, namely epicocconone [52], and ruthenium based organometallic complexes [53], [54,55]. These molecules offer a detection sensitivity that is very close to that obtained with silver staining, with a much better linearity and a much better compatibility with mass spectrometry. However, in this latter aspect, they do not perform as well as Coomassie Blue [42].

While these two modes of detection (covalent and non-covalent binding) are light emission counterparts of modes that have been used in visible detection (light absorption) [56], there is a third detection mode that is specific to fluorescence, which is the use of environment-sensitive probes. These molecules that are used for protein detection do not fluoresce in polar environments such as water, but do fluoresce in less polar environments such as protein-SDS complexes. Several molecules have been shown to achieve protein detection in this general scheme. Protocols using protein fixing and denaturing conditions have been devised with some probes of the styryl class [57], [58], while completely non-denaturing conditions could be used for other probes such as Nile Red [59], carbazolyl vinyl dyes [60] and more recently carbocyanines [61].

While the fixing schemes offer no real advantage over the classical non covalent probes, the nondenaturing schemes offer distinct advantages such as speed, blotting ability [60] [61] and more importantly a very good sequence coverage in subsequent mass spectrometry [61].

Last but certainly not least, fluorescence can be used to detect specific motifs on gel-separated proteins, such as sugars [62] or phosphate groups [63], thereby offering a very wide palette of detection schemes with wide versatility.

### 3. The data analysis issues

In most instances where 2D gel-based proteomics is used, the production of the gel image by any of the protein detection methods listed above is not the end of the story, and it is very uncommon that all detected protein spots are excised for protein identification by mass spectrometry. Most often, comparative image analysis is used to determine a few spots whose expression profile within the complete experiment matches biologically relevant events. This image analysis process can be split in four major steps. First data acquisition [4], then spot detection and quantification [5], then gel matching [6] (although gel matching can be carried out prior to spot detection in some analysis systems) and finally data analysis [7]. It must be stressed that this image analysis process has been used very early after the introduction of 2D gel electrophoresis [3, 64, 65], quite often with sophisticated data analysis tools [7, 8, 66, 67], long before the word proteomics even existed.

Although very cumbersome at these early times, image analysis has dramatically progressed over the years, greatly helped by the considerable increase in computer power. However, image analysis is very dependent on the quality of the experimental data, and especially on their reproducibility. In this respect, the generalization of immobilized pH gradient has had a major impact by bringing a level of positional reproducibility that could never be achieved with conventional isoelectric focusing with ampholytes [68-70]. However, image reproducibility is a complex process, and even with the use of immobilized pH gradients, reproducibility is maximized by parallelizing the gels in dedicated instruments [71]. Fortunately enough, such parallel electrophoresis instruments had been developed during the early days of 2D electrophoresis [72, 73], when the inter-run variability was very high.

Even though the reproducibility of 2D gel-based proteomics is much higher than for other setups, as shown by higher requirements [74] and stricter practices [75], there is an important concern that has emerged over the past few years, i.e. the problem of false positives. Although false positives can have an experimental origin [76], a certain proportion is due to the statistical processes used to determine modulated spots, and thus to the problem of multiple testing [77-79]. Although purely statistical tools such as false discovery estimates have been proposed to address this concern [77-79], these tools are not completely well-adapted to the analysis of 2D gel images [80].



In this game of quantitative image analysis to determine spots that show changes in the biological process of interest and thus deserve further studies there is another experimental parameter that plays a key role besides separation reproducibility, namely sample variability and especially biological variability, i.e. from one biological sample to another, before any technical variability introduced by the protein extraction process. This variability grows along with two parameters. One is the plasticity of the proteome, so that variability is often greater in cultured prokaryotic cells than in mammalian ones, as a consequence of the much shorter generation times. The second factor is of course the physiological and genetic heterogeneity, so that in vitro systems are less variable than in vivo systems on inbred laboratory animals, which are in turn less variable than samples obtained in conditions where neither the precise physiological state nor the genetic background can be controlled (typically human samples). In some of the latter cases, the biological variability is so high that it becomes very difficult to find any protein spots showing a statistically significant variation in the experimental process. In such cases, it is tempting to pool samples within the same experimental group, in order to average out the biological variability and facilitate the discovery of modulated proteins. However, it must be kept in mind that interindividual variations are an important part of the problems that do exist in clinics. Thus, this factor cannot be evacuated easily, and pooling must be carefully understood and controlled to achieve correct results [81, 82].

#### 4. The protein unicity issue

In the early days of protein identification from 2D gel spots, e.g. by Edman sequencing or low sensitivity mass spectrometry, the situation was simple: at most one protein was detected per spot. With the increase of sensitivity of the mass spectrometers, several proteins are more and more often identified from one 2D gel spots. This has been perceived as a problem by some scientists [83], but not by others [84]. Data obtained under the worst conditions, i.e. with sub-milligram protein loads and a high sensitivity mass spectrometer, have shown that in most of the cases, the protein identified with the most peptides in one spot accounts for more than 75% of the mass spectrometry signal, so that a "winner-takes-all" protein assignment scheme is legitimate in most cases. In the rare cases where there is still ambiguity from the mass spectrometry signal, an

additional separation of the spot(s) of interest can be carried out to settle the matter [85]

## 5. Conclusions

At the beginning of the 21st century, 2D gel-based proteomics is often depicted as an outdated technique, on the basis of its poor ability to analyze membrane proteins [86] and of its moderate analysis depth [87], [88]. However, in the landscape of proteomic techniques, it still offers unique advantages that make it stand apart (and ahead) of the other proteomic setups for many applications. For example, it is the simplest technique that is able to resolve complete proteins with their trail and combination of post-translational modifications, including protein fragmentation, and this unique ability should be highly valued with our increased knowledge on the importance of post-translational modifications on protein activity. This ability has been used in a variety of studies (reviewed in [22]). Thus, 2D gel-based proteomics has still a lot to offer to the researchers who will be able to use its strengths.

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