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MINIREVIEW ARTICLE

Operator- and software-related post-experimental variability and source of error in 2-DE analysis

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Abstract In the field of proteomics, several approaches have been developed for separating proteins and analyzing their differential relative abundance. One of the oldest, yet still widely used, is 2-DE. Despite the continuous advance of new methods, which are less demanding from a technical standpoint, 2-DE is still compelling and has a lot of potential for improvement. The overall variability which affects 2-DE includes biological, experimental, and post-experimental (software-related) variance. It is important to highlight how much of the total variability of this technique is due to post-experimental variability, which, so far, has been largely neglected. In this short review, we have focused on this topic and explained that post-experimental variability and source of error can be further divided into those which are software-dependent and those which are operator-dependent. We discuss these issues in detail, offering suggestions for reducing errors that may affect the quality of results, summarizing the advantages and drawbacks of each approach.

Keywords 2-DE · Post-experimental variability · Pixel-based method · Spot-based method

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Introduction

Two-dimensional gel electrophoresis (2-DE) is a valuable method for the separation and comparison of complex protein mixtures, providing information regarding the variation of their relative quantities and the possibility to study post-translational modifications. 2-DE is widely used by the proteomic community and, at best, this technique may allow separating up to 10,000 protein spots on a unique gel (Klose and Kobalz 1995).

However, this method still suffers from some drawbacks: the separation method requires a lot of manual work and expertise and it is difficult to obtain highly reproducible 2-D gels even from the same sample. The overall variability which affects 2-DE includes biological, experimental, and post-experimental (software-related) variance. Several studies have been designed to optimize sample processing (Polaskova et al. 2010; Canas et al. 2007; Lopez 2007), to reduce experimental variability and to increase 2-D gel reproducibility. With the same aim, other authors focused their attention on some experimental factors that may cause false significant differences in spot intensities, such as using: (1) different gel casters to prepare second dimension gels (Fuxius et al. 2008); (2) IPG strips from different packages (Taylor and Coorssen 2006; Fuxius et al. 2008); (3) different focusing systems (Choe and Lee 2000) and (4) different electrophoresis chambers (Zhan and Desiderio 2003).

On the other hand, a significant fraction of the overall variability is due to post-experimental analysis (Silva et al. 2010; Wheelock and Buckpitt 2005). Some recent publications have focused their attention specifically on this topic in the 2-DE analysis process (Millioni et al. 2010a, b; Silva et al. 2010; Berth et al. 2007; Clark and Gutstein 2008; Stessl et al. 2009), but the importance of reducing it still seems to be rather underestimated.

Table 1 shows the list of all currently available software packages for 2-DE analysis. Depending on their workflow, these programs can be divided into two main categories (Fig. 1):

1. Spot detection first: it is the classical approach, where the image information is first reduced into a set of spot centers, boundaries and volumes for each image. Spot matching and subsequent statistical analysis are based on the spot geometry and volume data.
2. Image warping first: the alignment step is based on the whole image information and is performed prior to spot detection. The following steps involve the creation of a fusion image of all warped images, spot detection in the fusion image, and the push of the spot borders from the fusion image to all individual warped images.

Redfin Solo, Same Spots, and Delta2D belong to this latter category and are also known as 4th generation analysis software.

Since variability greatly influences the outcome of quantitative analysis, it is important to discern among the different sources of variability and their individual contribution to the global variability. So, we propose to further divide the post-experimental variability into two categories: operator-related and software-related. In this review, we will discuss in detail these types of variability, offering suggestions for reducing errors that may affect the quality of results and summarizing the advantages and disadvantages of different approaches.

Operator-related variability

In a recent study (Silva et al. 2010), an expert user and a novice user performed the analysis of the same set of 2-DE gels using the software, Same Spot. The expert user was the person involved in the development of Same Spot, while the novice user had experience in 2-DE analysis with other types of analysis software. Interestingly, the novice user achieved a slightly lower overall variance than the expert one. This result is suggestive of a good evaluation for Same Spot, which is 4th generation software. In fact, the aim of the highly automated workflow of 4th generation software is to reduce software-induced variance and subjectivity; hence, using Same Spot, users can achieve the same results, even if their experience with the specific program is different. Certainly, this interesting result would have more weight if the test had been carried out by more than one operator per group.

Apart from this isolated case, most of the currently used software packages for 2-D gel analysis are time-consuming and only minimally automated (Clark and Gutstein 2008). So, even if a software package may be accurate and precise if used by an expert operator, on the other hand it may be imprecise, inaccurate, and unreliable when used by a less experienced practitioner.

Here below, we discuss the points that we feel crucial for a proper analysis of 2-DE gels and for which the odds of good execution lie only in operator skill, experience, and patience.

Starting with good images

Gel images can be captured by using transmittance scanners (with an optical density of 4), charge-coupled device cameras, or laser imaging devices, depending on the protein labeling or staining techniques applied to enable spot visualization. A trivial, but very serious error could be the improper setting of image digitalization parameters.

Table 1 Current commercial software packages for 2-DE gel image analysis

Software	Company
DeCyder 2-D Differential Analysis	Amersham Biosciences GE Healthcare http://www.gelifesciences.com
Delta2D*	Decodon http://www.decodon.com
Dymension	Syngene http://www.2dymension.com
GELLAB II	National Cancer Institute
GELLAB II+	Scanalytics, Incorporated
Image Master 2-D Platinum	Amersham Biosciences GE Healthcare http://www.gelifesciences.com
Investigator HT Analyzer	PerkinElmer http://www.perkinelmer.com
Melanie	Geneva Bioinformatics http://www.genebio.com
PDQuest 2-D Analysis Software	Bio-Rad Laboratories http://www.bio-rad.com
Phoretix 2-D Advanced	Nonlinear Dynamics http://www.nonlinear.com
Progenesis SameSpots*	Nonlinear Dynamics http://www.nonlinear.com
Proteomweaver	Bio-Rad Laboratories http://www.bio-rad.com
Redfin*	Ludesi http://www.ludesi.com/

* Indicate software packages of 4th generation

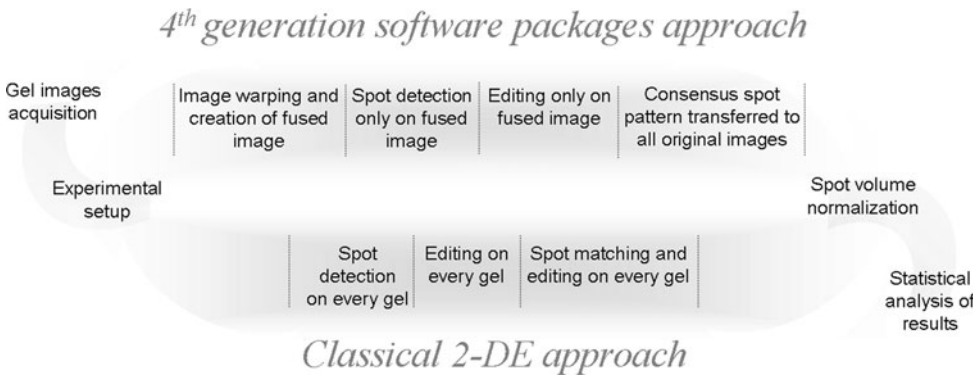

Fig. 1 Principal steps of classical and 4th generation software analysis workflow

Generally, it is recommended to acquire gel images into TIFF files with 16 bit of grayscale depth at 300 dpi resolution.

Cropping the gel

The cropping of the gel image is normally used to exclude gel areas that are not of interest for the matching. This can improve matching quality and reduce calculation time. Areas excluded are usually at the gel edges, where the background is less uniform. Typically, gel image cropping is performed manually. However, Wheelock and Buckpitt (2005) stated that neither Phoretix 2D Expression nor PDQuest 7.2 could repeatedly quantify protein spots in the same 2-DE gel image in a reproducible manner unless the manual image cropping was done with the utmost care by the operator. It was shown that only a slight shift of image boundaries caused a variation of 4–10% CV in protein quantitation, even if relative spot volumes were obtained by normalizing the spot volume over all the spots in the gel. To address this problem, Damodaran and Rabin (2007) proposed an objective software-based method to uniformly crop 2-DE gel images and consequently reduce the variability that arises from the manual cropping. Their method is based on the use of an image management tool, e.g. Picture Manager Software which is readily available as a part of Microsoft Office Suite, which provides a cropping tool with pixel values along the *XY* axes. Identical cropping can be obtained using the same cropping tool for all the gels; thus, eliminating error in this step. To our knowledge, the cropping operation made available by Picture Manager Software could also be done with several of the software packages that are provided with the scanners. Apart from these exceptions, a good rule of thumb is reducing to a minimum the use of image elaboration software which is not specifically designed for 2-DE image analysis. Otherwise, there is a risk of losing important information, such as the removal of gray levels.

Searching for false positive spots

The main responsibility of the operator is to check the quality of spot detection for each spot in each gel. The aim is to minimize the number of false positive spots (due to the presence of artifacts such as bubbles, imperfections in the gel, or dye residues), the presence of which is largely important in determining the quality and accuracy of subsequent gel matching (Millioni et al. 2010a; Raman et al. 2002; Rosengren et al. 2003). Operator interventions greatly reduce the incidence of these errors. This is time-consuming but necessary work, since the presence of false positive spots is the main cause of bad subsequent matching. In any case, a scrupulous operator must also check the quality of matching done by the software, without taking anything for granted. In this sense, the skill and the patience of the operator in finding and deleting false positive spots among all the detected spots and in manually checking the spot matching among gels will greatly influence the quality of the final data.

False negative spots: What to do?

It is often necessary for operators to intervene manually to correct mistakes in spot segmentation. Operator subjectivity and ability can make the difference between success and failure, weakening the analysis. Particularly, it may be necessary to manually draw the border of real, but undetected or badly detected spots (the so-called false negative spots). We estimated the error in spot quantification after manual spot segmentation, which was performed by different operators, using two different software packages (Melanie 7.05 and Proteomweaver 3.0.1.0) (Millioni et al. 2010b). Our results clearly showed that this operation was associated with high inter- and intra-variability as well as an ever-present overestimation of subsequent spot intensity, especially when spots were weak, with up to sixfold difference. This error was independent of the software package used. For comparative studies, we suggest making

a separate analysis of spots which have been manually segmented by imposing a requirement for at least a three-fold difference in spot intensity in addition to the use of statistical tests.

In this context, we found a valuable feature in Delta2D: when the automatic spot detection has detected a spot, each manual attempt to segment such a spot (after the spot has been cancelled) brings back the original spot, with the automatic segmentation. This is useful, e.g. when detected spots are erroneously cancelled by the user and it is not possible to return to the original work without losing all the operations that have been done in the meantime.

Software-related variability

Some studies highlight discrepancies among results when different commercially available image tools are used for the gel analysis, especially in terms of comparability of the obtained outcome when the same gel images are used. It has been reported that different 2-D gel analysis software packages deliver distinctly different results (Millioni et al. 2010a; Raman et al. 2002; Rosengren et al. 2003; Wheelock and Buckpitt 2005; Clark and Gutstein 2008), and this may be due to the algorithms used for all analysis operations. At the beginning of the long pipeline of gel analysis, denoising and background subtraction operations have critical effects on the quality of subsequent operations such as spot detection, modeling, and volume estimation. Noise suppression methods employed in commercial software packages are based on the application of spatial filters (Appel et al. 1997; Rogers et al. 2003b). These filters strongly influence spot quantification in both spot- and pixel-based software packages, since they tend to erode spot edges and to alter the intensity values of spot pixels (Cannistraci et al. 2009; Tsakanikas and Manolakos 2009).

Improving denoising performance can have a positive impact on subsequent operations, such as spot volume estimation and accurate relative protein abundance quantification. Interestingly, a novel nonlinear filter (median-modified Wiener filter, MMWF) was recently developed and compared with other well-established denoising techniques (Cannistraci et al. 2009). MMWF was evaluated as the best filter for global denoising, its best setting remaining unaffected by the type of noise. In addition, while the median filter eroded the edge of isolated spots and filled the space between closer spots, MMWF preserves the morphology of spots.

Also background subtraction is generally considered to be an essential feature of 2-DE analysis due to the variable background produced by most staining protocols. However, applying a background subtraction algorithm (among the tested, there were background subtraction algorithms named “mode of non-spot”, “lowest on boundary”, and “average on boundary”, “floating ball”, “median”, “weighted mean”, “power mean”, “contra mean”, “adaptive”, “no smoothing”) tended to increase the variance (Silva et al. 2010; Kreil et al. 2004; Wheelock and Buckpitt 2005).

The effects of a background subtraction algorithm should therefore be evaluated in cases when its use is considered to be essential. Interestingly, regardless of the different kind of background subtraction performed, the use of 4th generation software packages has the potential of reducing variance arising from this operation. In fact, spot detection is performed first in the fusion image and then the spot borders from the fusion image are transferred to all individual warped images. Obtaining identical spot boundaries across all gel images also leads to a normalization of the background subtraction, since the area for background subtraction remains constant, thus reducing inter-gel variability (Silva et al. 2010).

After image pre-processing operations, the spot characterization which follows includes spot shape and volume quantification, which is a fully automated operation, based on artificial image models for objective evaluation. In 2-DE analysis, the most commonly used spot model is the bivariate Gaussian distribution (Rogers et al. 2003a). However, it has been estimated that in any 2-DE map there are at least six orders of magnitude difference between the least and the most abundant proteins (Lefkovits et al. 2000), and the Gaussian formulation is more appropriate for representing smaller spots than larger ones (Bettens 1999). Protein spots are in fact formed by a diffusion process which is only adequately represented by a Gaussian distribution when the spot area is small (Bettens 1999), while for larger spots the area assumes different irregular (e.g. tear drop shape) or flat-top shapes. On the other hand, some studies are in agreement declaring that the primary contributors to process error are the less intense rather than the more intense spots (Millioni et al. 2010a, b; Smales et al. 2003). Even if most of the software packages use the Gaussian formulation to describe spot appearance, it is of essential importance that software packages recognize all the possible spot shapes that can be represented in a typical 2-DE gel. Hence, significant variation among results, depending on the program used, could be due to different capabilities of the software packages in image pre-processing operations, in recognizing different spot shapes, and in accurately calculating their volume. This kind of post-experimental variability is independent of operator skill and is solely due to the software package used. For example, the spot-based Gaussian fitting algorithm utilized in PDQuest (Bio-Rad) was found to introduce about two-fold higher levels of software-related variance when compared to the pixel-based spot detection algorithm used in

Progenesis (CV median 8 vs. 4%) (Wheelock and Buckpitt 2005).

In most of the published papers on software-related variability in 2-DE analysis, and with particular regard to the evaluation of these programs, software packages have been tested in different ways, using different gel images as well as different operators, thus making it difficult to arrive at an accurate and definitive evaluation of a program's quality. Moreover, data obtained even a few years ago cannot actually be considered compelling because of the software's continuous updating and evolution. For example, Stessl et al. (2009) showed significant variations among results not only in 2-DE analysis, depending on the software package, but also on the version of the same software package!

It is really difficult to make a general comparison of software for 2-DE analysis. To our knowledge, a set of gels available to all authors for carrying out the comparative evaluation of software packages for the analysis of 2-D gels does not exist. An attempt was made by Raman et al. (2002), but the proposed test set has some limits so it should not be considered as a "gold standard". In fact, the provided images of real gels have only 8 bit depth, but current good practices in analyzing 2-D gel use always 16 bit images. The same authors also created a set of artificial images to test the quantitative analysis, since an ideal quantitative test could be performed on spots of a known volume to see which program calculates the value closest to the real one. This test set included artificial spots with different quantities, using a Gaussian model with a constant height and a varying diffusion coefficient. However, this constant height is not at all a valid spot model and only the Gaussian shape was taken into consideration.

On the other hand, most recent publications (Millioni et al. 2010a; Silva et al. 2010; Dowsey et al. 2006; Grove et al. 2006; Berth et al. 2007; Clark and Gutstein 2008) are in agreement in concluding that 4th generation pixel-based software workflow (Fig. 1) has some advantages over spot-based software, as explained in the following sections.

Spot-based versus pixel-based methods and analysis time

In spot-based software packages, spot detection is the first step and must be applied to each single gel to find all protein spots, drawing their boundaries. Next, the program tries to match spots detected on individual gels among all the different gels of the experiment. Then, volumes are computed and normalized for each spot on each gel. It is widely demonstrated that, to obtain reliable results, automated matching requires extensive manual interventions, resulting in the introduction of a large degree of subjectivity (Millioni et al. 2010a; Raman et al. 2002; Rosengren et al. 2003; Kreil et al. 2004; Fodor et al. 2005; Biron et al. 2006; Clark and Gutstein 2008).

In the pixel-based software packages of the 4th generation, the first step is the warping process, an image transformation that removes gel variances by aligning spot positions across gel images, leaving intact the spots' gray values, which are used for quantitation. This is an area-based method performed directly on the raw data, and thus is independent of the spot detection results. Rather than detecting spots on each individual gel and then drawing vectors to match cognate spots, spots are first warped to each other. After warping, the next step is the creation of a fusion gel that includes every spot existing on any image of the analysis. Spot detection and editing are performed only on the fusion image and then all information is transferred to each replicate, as a "spot mask" (Luhn et al. 2003; Berth et al. 2007).

So, this workflow has the benefit of rendering unnecessary the time-consuming spot detection and editing for each gel since these steps are carried out only on the fusion gel. Moreover, manual interventions needed to correct pixel-based warping are fewer than those required for spot-based matching (Millioni et al. 2010a). Hence, the 4th generation software has a shorter overall analysis time (about 50%) with respect to the spot-based method (Millioni et al. 2010a; Clark and Gutstein 2008). Moreover, alignment prior to spot detection allows the algorithms to use multiple sources of information, including smears, tail, etc., which has been shown to improve analysis (Dowsey et al. 2006).

The problem of missing data when using spot-based software packages

The workflow of spot-based software packages has an important flaw. With this approach, the operator has to resolve matching conflicts manually by inspecting all images involved, but this process takes a lot of time and does not resolve all the ambiguities. The remaining spot-matching ambiguities are reported as a blank entry in the final analysis table. These missing values could mean either that there was really no spot on the gel or that the software was unable to detect it or alternatively that the correct match was not found. In any case, the profiles for spot abundance contain gaps that reduce the statistical confidence of the quantitative analysis (Albrecht et al. 2010). The number of missing values in a match set can be large (Ahmad et al. 2006; Grove et al. 2006): 70–90% of all spots in one experiment show missing values, meaning that nearly all spots are present in at least one gel and absent in at least one other gel. This risk of missing data is eliminated with the 4th generation software (Silva et al. 2010; Ahmad et al. 2006; Berth et al. 2007): spots are detected

only in the fusion gel and then transferred as a "mask" to all gels that were previously warped, without the possibility of losing data. This represents an important improvement in 2-D gel analysis.

Experiment size

It has been shown, using both PDQuest and Progenesis, that the percent of correct automatic spot matches and the quality of software package performance decrease as experiment size increases (Clark and Gutstein 2008). The reason for this problem is still unclear. However, it might be considered intuitive that a high number of replicate gels would not necessarily produce better results, because of the errors in each individual gel. Moreover, it has been reported that, in the case of the spot-based approach, the number of missing values escalates as the number of gels compared and analyzed increases (Grove et al. 2006). Hence more operator manual interventions will be required in direct proportion to the number of gels studied. Since the performance of spot-matching algorithms worsens when more gels are added to the analysis (Rye et al. 2008), a possible solution would be the use of a 4th generation program, where the spot matching is substituted by the image warping and the creation of the fusion gel image.

Statistical analysis

To efficiently analyze the large amount of data generated by 2-DE, there is a need for analysis tools, not only for image analysis, for the statistical selection of significantly changed spots (variable selection). Different kinds of results may be generated depending on the statistical tools made available by the different programs for 2-DE analysis (Maurer et al. 2005; Meunier et al. 2005).

For variable selection, a wide variety of different approaches exists (Smit et al. 2008). In general, the variable selection methods aim to detect the significant variables either one by one with a univariate approach (e.g. ANOVA) or by including the information of all the variables in a multivariate approach (e.g. Partial Least Squares with jackknife, Cross Model Validation, Power-PLS and CovProc) (Jessen et al. 2002).

Therefore, with respect to the univariate analysis, the multivariate approach has the additional benefit of providing information about possible relationships between the samples and variables, allowing focusing the attention on proteins that are involved in the same biochemical pathway (Jessen et al. 2002). Regarding this, DeCyder software packages (GE Healthcare), thanks to multivariate statistics tools, enable the combined analysis of different data sets, aiding in the biological interpretation of results by matching with data from protein databases.

Finally, it should always be taken into account that some of the significantly changed proteins reported are due to natural variation. Hence, to reduce the probability of these false positives, the selection of variable spots should be performed by imposing a requirement for at least a 1.5-fold difference in spot intensity in addition to the use of statistical criteria. Severe criteria would, therefore, be important in improving the result quality reducing the false positive rate and operator-related errors.

Conclusions

Two-dimensional gel analysis has undergone continuous improvements over the years, mostly aimed at minimizing technical variability. This goal culminated with the DIGE approach, which allows multiple protein samples to be separated on the same 2-D gel (Engelen et al. 2010; Westermeier and Scheibe 2008). The quality of post-experimental analysis has also shown significant improvements especially with the introduction of 4th generation software packages that consent to a faster and more objective analysis with respect to previous generation software. Even if technical variability is actually more documented in the literature, post-experimental variability may be sufficient to obscure the biological changes under investigation, and this issue should stimulate more careful attention on how gel analysis is carried out. As manual validation, the operator must select "best-behaved" spots as spots deemed to be correctly detected and matched across all gels and then include only them in the statistical analysis. Similarly, the low-quality 2-D gels, e.g. the less reproducible technical replicates inside a data set, should be excluded from the analysis. Unfortunately, there are still no specific criteria to assess the quality of a single gel or of a set of technical replicates in an objective way. The establishment of such quality criteria would certainly be an important step to further improve the robustness of the data obtained by 2-DE.

Of course, any other further refinements in the development of post-experimental analysis will also have a significant impact on improving both the throughput and objectivity of 2-D gel analysis.

Conflict of interest The authors declare that they have no conflict of interest.

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