What's the Difference? 2D DIGE Image Analysis by DeCyder™ versus SameSpots™

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Abstract

The efficiency and reproducibility of two-dimensional difference gel electrophoresis (2D DIGE) depends on several crucial steps: (i) adequate number of replicate gels, (ii) accurate image acquisition, and (iii) statistically confident protein abundance analysis. The latter is inherently determined by the image analysis system. Available software solutions apply different strategies for consecutive image alignment and protein spot detection. While DeCyder™ performs spot detection on single gels prior to the alignment of spot maps, SameSpots™ completes image alignment in advance of spot detection. In this study, the performances of DeCyder™ and SameSpots™ were compared considering all protein spots detected in 2D DIGE resolved proteomes of three different environmental bacteria with minimal user interference. Proteome map-based analysis by SameSpots™ allows for fast and reproducible abundance change determination, avoid-

ing time-consuming, manual spot matching. The different raw spot volumes, determined by the two software solutions, did not affect calculated abundance changes. Due to a slight factorial difference, minor abundance changes were very similar, while larger differences in the case of major abundance changes did not impact biological interpretation in the studied cases. Overall, affordable fluorescent dyes in combination with fast CCD camera-based image acquisition and user-friendly image analysis still qualify 2D DIGE as a valuable tool for quantitative proteomics.

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Introduction

The introduction of two-dimensional gel electrophoresis (isoelectric focusing combined with SDS-PAGE; 2DE) around 40 years ago by O'Farrell [1975] allowed for the high-resolution separation of complex protein mixtures based on isoelectric point and molecular size. Although the development of immobilized pH gradients for first-dimension separation greatly enhanced the repro-

ducibility of 2DE, gel-to-gel variation still required the use of technical replicates to encompass experimental variation [Görg et al., 2009]. Even though diverse post-electrophoretic fluorescence staining techniques (e.g., SYPRO® ruby or flamingo) already allowed 2DE with high sensitivity and a broad dynamic range, the challenge of gel-to-gel variation remained. In this respect, the concept of two-dimensional difference gel electrophoresis (2D DIGE), introduced in 1997, represented a major advance for quantitative 2DE [Ünlü et al., 1997]. 2D DIGE is based on the application of three mass- and charge-matched fluorescent cyanine-dyes for pre-electrophoretic protein labelling allowing for the separation of three different protein samples within a single gel. Thereby, gel-to-gel variation is avoided since exactly the same electrophoretic parameters are employed to the three samples. Furthermore, the use of an internal standard not only facilitated normalization of biological replicate samples, but also allowed for multiplexing due to the possibility of matching spots across multiple gels and, hence, samples [Gade et al., 2003; Beckett, 2012]. Since its introduction, 2D DIGE has been applied to all kinds of organisms and tissues [for overview see Cramer and Westermeyer, 2012].

Following electrophoretic separation, gel images are visualized and digitalized originally using a 3-laser fluorescence scanner [Gade et al., 2003; Vormbrock et al., 2012] or, as recently introduced, specific CCD camera systems [e.g. Strijkstra et al., 2016]. The digital gel images are then processed by special software to determine quantitative differences in protein abundance. Several software solutions are commercially available, with DeCyder™ (GE Healthcare), Delta2D™ (Decodon), and SameSpots™ (TotalLab) being widely used. Although determining a similar parameter (i.e., abundance change), the way of raw-image processing, spot detection, and normalization differs between these software solutions. A “traditional” approach is applied by DeCyder™ using a co-detection algorithm for spot detection that combines information of all three, co-separated gel images to define a spot. Spot detection is performed for each gel individually, prior to the quantification and normalization of spot volumes and final spot matching to a master gel across the entire gel-set of an experiment (Fig. 1). In contrast, Delta2D™ and SameSpots™ correct for electrophoretic migration differences in spot positions between gels by image warping, i.e., positional correction of gel images combining global and local image transforms prior to spot detection, generating a single fusion image (proteome map). This proteome map contains the information for

all spots detected (no empty values) in the experimental gel set and is the basis for generation of a consensus spot pattern valid for all gels [Berth et al., 2007]. This workflow avoids the (time-consuming) necessity of reviewing the correctness of spot matching as in the case of DeCyder™. Similar to DeCyder™, normalization is performed prior to abundance profile analysis. A detailed comparison of the DeCyder™ and SameSpots™ workflow is provided in Figure 1.

So far, only a few studies have examined the influence of these different software approaches on the obtained quantitative data [Karp et al., 2008; Kang et al., 2009; Silva et al., 2010], albeit mostly focusing on a small subgroup of proteins (≤ 50) for detailed analysis. In this study, the influence of two state-of-the-art 2D DIGE image analysis software solutions, DeCyder™ and SameSpots™, on protein abundance profiles of three bacterial proteomes was investigated. For this purpose, all detected protein spots (730–4,183) of three environmental, proteomically well-studied model bacteria, i.e., *Desulfobacula toluolica* Tol2, “*Aromatoleum aromaticum*” EbN1, and *Phaeobacter inhibens* DSM 17395, were considered.

Results and Discussion

Dataset

To assess the influence of 2D DIGE image analysis on determined abundance differences, protein profiles of three proteogenomically well-studied environmental bacteria were analyzed: marine, sulfate-reducing delta-proteobacterium *D. toluolica* Tol2 [Wöhlbrand et al., 2013], terrestrial, denitrifying betaproteobacterium “*A. aromaticum*” EbN1 [Rabus et al., 2014], and marine, aerobic alphaproteobacterium *P. inhibens* DSM 17395 [Drüppel et al., 2014]. The gel set of each organism contained four gels in which protein extracts of four biological replicate samples were separated to account for biological variation [Zech et al., 2011]. While the test state varied for each bacterium (*D. toluolica* Tol2: toluene; “*A. aromaticum*” EbN1: 4-ethylphenol; *P. inhibens* DSM 17395: casamino acids), cells grown with fumarate served as the reference state in all cases. The internal standard was composed of equal amounts of all test and reference state samples per bacterium [Strijkstra et al., 2016]. This experimental setup yielded on average a total of 3,786–4,183 and 730–915 spots detected by DeCyder™ and SameSpots™, respectively, all of which were considered for the subsequent analyses (Table 1).

Fig. 1. Comparative workflow of discrete steps during 2D DIGE analyses by SameSpots™ and DeCyder™. Overview of consecutive steps of the 2D DIGE analysis by SameSpots™ (left) and DeCyder™ (right) with equivalent sections connected and color coded according to function: yellow, image upload and project creation;

green, spot detection and matching; blue, spot exclusion during detection; purple, spot review; red, statistical analysis and report. For DeCyder™, the different software modules are distinguished. PTM, posttranslational modification.

Spot Detection and Master Gel Assignment

Prior to spot detection, the DeCyder™ software requires the user to specify an estimated number of spots. The DeCyder™ manual recommends setting the number of estimated spots to 2,500–10,000, for which reason four different settings were tested: (i) 1,500, (ii) 3,000, (iii) 5,000, and (iv) 10,000. Estimation of 1,500 spots yielded on average approximately 1,200 detected spots per gel, independent of the sample (online supplementary Fig. S1; see www.karger.com/doi/10.1159/000494083 for all online suppl. material). With 3,000 estimated spots, the

number of detected spots increased to approximately 2,600, and with estimation of 5,000 or 10,000 spots the maximum of approximately 4,000 spots per gel and sample were detected. Notably, comparing the spot coordinates of the spot map estimating 1,500 spots with those estimating more, 86% of the spots did not change in position (one-pixel tolerance) and revealed a difference in the raw spot volume of only 8% (data not shown). The number of detected spots directly influences the DeCyder™ data analysis, since the gel with the most spots detected is selected as the master gel that is subsequently used for

Table 1. Comparison of the number of overall detected spots as well as shared or uniquely detected ones by DeCyder™ and SameSpots™ for the three studied bacteria (spot numbers are average values of 4 biological replicates)

	<i>D. toluolica</i> Tol2		“ <i>A. aromaticum</i> ” EbN1		<i>P. inhibens</i> DSM 17395	
	DeCyder™	SameSpots™	DeCyder™	SameSpots™	DeCyder™	SameSpots™
Detected ¹	4,083	903	4,183	915	3,786	730
Matched/present in all gels	1,927	903	1,851	915	1,757	730
Uniquely detected	3,364	184	3,517	249	3,221	165
Similarly detected		719		666		565
Increased abundance	164	108	121	91	207	94
Decreased abundance	121	86	27	50	74	84
Unchanged abundance	3,798	709	4,035	774	3,505	552
Similar increased abundance		82		38		68
Similar decreased abundance		53		9		31
Similar unchanged abundance		484		523		353
Differently assigned ²	67	33	23	73	56	57

¹ Based on 5,000 estimated spots in the case of DeCyder™.

² Spots with significantly changed abundance determined only by the respective software.

spot matching of all other images of a gel set. The assigned master gel remained constant with $\geq 3,000$ estimated spots, except for the *P. inhibens* DSM 17395 sample ($\geq 5,000$). Since no user influence on spot detection is possible in SameSpots™, and user-defined parameters were reported to influence the degree of statistical confidence of DeCyder™ analysis [Gade et al., 2003], an estimated spot number of 5,000 was used for DeCyder™ spot detection to minimize user bias.

Applying these settings, on average 4,000 spots per sample and gel were detected by the DeCyder™ software, while SameSpots™ detected only 730–915 (Table 1; Fig. 2). Notably, only <50% (on average 1,845 spots) of the DeCyder™-detected spots were present in all gels of the respective gel set (online suppl. Fig. S1), which was twice as many as compared to SameSpots™, where all spots are present in all gels due to the warping routine of the algorithm. Furthermore, 25% of the SameSpots™-assigned spots (in the case of *P. inhibens* DSM 17395 even 30%) were interpreted by DeCyder™ as two or more spots (up to 9) when comparing spot boundaries (online suppl. Fig. S2), indicating that these numbers are falsely high. This assumption is supported by detection of only 25% of the DeCyder™ spots in all gels of an *Erwinia carotovora* proteome analysis applying the SameSpots™ software [Karp et al., 2008]. Notably, in the present study, 73–80% of all SameSpots™ spots were also assigned by the DeCyder™ software (including all “clearly visible” spots; Fig. 2), indicating a similar spot data set for analysis. Previously reported merging of apparently different

spots to one spot (i.e., omitted splitting of overlapping spots) in the case of SameSpots™ [Karp et al., 2008; Morris et al., 2010] could not be confirmed in this study, most likely due to the more advanced version of the SameSpots™ software (2006 vs. 2017).

Differences in Protein Abundance Profiles

Similar criteria were applied to classify a spot to be significantly changed in abundance for both software solutions (i.e., a fold-change $\geq |1.5|$ and an ANOVA *p* value <0.05). To allow for direct comparison of different parameters of corresponding DeCyder™ and SameSpots™ spots, respectively, each DeCyder™ spot was manually matched to its corresponding SameSpots™ spot equivalent. Here, the spot intensity maximum (indicated by DeCyder™) was used as the decisive parameter and the spot with the largest spot volume (internal standard) was chosen in the case of multiple DeCyder™ spots per SameSpots™ spot (online suppl. Fig. S2A).

For all three studied bacterial species, more spots were assigned to be significantly increased in abundance by DeCyder™ as compared to SameSpots™ (121–207 vs. 91–108, respectively; Fig. 2). Out of these, 38–82 were similarly assigned by both software solutions. It should be noted that assignment of multiple spots by DeCyder™ within the spot boundaries of a single SameSpots™ spot (online suppl. Fig. S2A) may artificially increase the respective number of DeCyder™ spots, given they fulfill the respective criteria (i.e., significantly increased abundance in this case). However, for 17–25% of the DeCyder™

Fig. 2. Total number of detected spots and their corresponding abundance characteristics assigned by SameSpots™ and DeCyder™ for *D. toluolica* Tol2, *A. aromaticum* EbN1, and *P. inhibens* DSM 17395. For each bacterium and software, the number of detected spots is given in gray, increased abundance in green, decreased abundance in red, and unchanged abundance in orange (significantly changed abundance: ANOVA p value $\leq 1 \times 10^{-4}$ and

fold change ≥ 1.5 or ≤ -1.5). The number of spots of the reduced dataset similarly assigned by both software solutions is indicated in lighter shading. The number of spots present in both software solutions, but not fulfilling the significance criteria (i.e., ANOVA p value or abundance change) in the respective software equivalent is indicated by hatching (white).

Fig. 3. Inter-software correlation of spot attributes: abundance change and raw volume dot plot. Correlation analysis was performed with the reduced data set (i.e., one DeCyder™ spot is assigned to the equivalent SameSpots™ spot) for the change in abundance (a, c, e) and the raw volume (b, d, f) for *D. toluolica* Tol2, *A. aromaticum* EbN1, and *P. inhibens* DSM 17395. For the abundance change analysis (left column), the 95% interval (assuming a

1:1 relation) is indicated in light green. Spots not meeting this 95% criterion are highlighted in red. The coefficients of determination and the line of best fit are given for the total dataset (including outliers, red) and those meeting the 95% criterion (blue). For the raw volume plots (right column), spots not meeting the 95% criterion in the abundance change analysis are also highlighted in red.

(For figure see next page.)

D. toluolica Tol2 *"A. aromaticum"* EbN1 *P. inhibens* DSM 17395

spots with increased abundance, no significant abundance change or a decreased abundance was assigned by SameSpots™ for the respective spot. Contrariwise, 14–45% of the SameSpots™ spots with increased abundance were not similarly assigned by DeCyder™. In the case of spots with decreased abundance, the number was lower for DeCyder™ than for SameSpots™ (27–74 vs. 50–84), except for *D. toluolica* Tol2 (121 vs. 86), which had similar assignment for 9–53 spots (Fig. 2). Out of these spots with decreased abundance, 5–25% (DeCyder™) and 21–64% (SameSpots™), respectively, were differently assigned. Overall, a rather large number of spots (353–523) was similarly assigned to be not significantly changed in abundance, accounting for 10–13% (DeCyder™) and 64–68% (SameSpots™) of the nonregulated spots, respectively. The comparably low share in the case of DeCyder™ is mainly due to the >4.5-fold higher number of total detected spots, most of which do not have a SameSpots™ equivalent, as well as spot fractionation, i.e., assignment of multiple DeCyder™ spots per SameSpots™ spot. In general, spots that were assigned to be significantly changed in abundance by only one of the two tested software types either just did not meet the abundance change or the ANOVA threshold (seldom both) in the case of the other image analysis system.

Software Impact on Abundance Changes

Differences in determined abundance changes were analyzed using a reduced dataset containing only spots present in both gel sets, i.e., 719 for *D. toluolica* Tol2, 666 for “*A. aromaticum*” EbN1, and 565 for *P. inhibens* DSM 17395, covering 73–80% of all SameSpots™-detected spots. For each protein spot, its SameSpots™ abundance change was plotted versus the DeCyder™ equivalent (Fig. 3a, c, e). Linear regression analysis revealed that the SameSpots™ values were 0.55- to 0.72-fold lower as compared to DeCyder™ (coefficient of determination 0.69–0.92). However, considering only spots meeting the 95% confidence interval (assuming a 1:1 relation), the deviation was reduced to 0.69- to 0.90-fold (coefficient of determination 0.71–0.93), although only 20–31 spots were not considered (out of 565–719). Hence, SameSpots™ apparently calculates an 8–30% smaller change in abundance as compared to DeCyder™ and thus larger fold change differences in the case of a large abundance changes. This finding contrasts with the reported large difference in DeCyder™- and SameSpots™-calculated abundance changes of three selected spots [Kang et al., 2009], but is in line with determined subtle differences of 50 spots in the case of the *E. carotovora* proteome [Karp et al., 2008].

Spot fractionation by DeCyder™, i.e., assignment of 2 or more spots per SameSpots™ spot, may indicate generally larger spot volumes by the latter. However, most spots of the reduced dataset revealed a larger spot volume assigned by DeCyder™ (61–83%; Fig. 3b, d, f), although SameSpots™ assigned a pronouncedly larger volume to some spots (most prominent in the case of *D. toluolica* Tol2). Notably, spots with large differences in calculated abundance changes revealed only average differences with respect to spot volume (red dots in Fig. 3).

Both software solutions apply normalized spot volumes for the calculation of abundance changes. While the procedure is not described in detail for DeCyder™, the normalization procedure of SameSpots™ is replicable. To assess the influence of normalization on abundance change calculation, a custom algorithm was constructed reflecting the SameSpots™ procedure. Comparing the custom calculated normalization factor with that of the SameSpots™ software revealed an average difference of ≤0.01. Hence, the custom calculation may be used to represent the SameSpots™ normalization procedure. By applying the raw spot volumes determined by DeCyder™ in this calculation, the obtained abundance changes were similar to those calculated with SameSpots™ raw volumes (median deviation: *D. toluolica* Tol2: 0.01; “*A. aromaticum*” EbN1: 0.02; *P. inhibens* DSM 17395: 0.08), demonstrating that the differences in software-determined spot volumes do not affect the calculated abundance change.

Software Influence on Biological Interpretation

In general, due to the apparently factorial differences in calculated abundance changes, both software solutions determine highly similar fold changes in the case of proteins with small changes in abundance. For example, a similar increase in abundance was determined for two proteins (out of seven identified) involved in anaerobic degradation of toluene by *D. toluolica* Tol2, i.e., 3.6-fold and 1.9-fold in the case of 2-(hydroxy[phenyl]methyl)-succinyl-CoA dehydrogenase (BbsC) and phenylitaconyl-CoA hydratase (BbsH), respectively (online suppl. Fig. S3). However, minor deviations in the case of proteins with larger fold changes (e.g., 27.7-fold vs. 22.2-fold in the case of benzylsuccinate synthase subunit A [BssA] for DeCyder™ and SameSpots™, respectively), should not be paid too much attention, since the determined fold change is still pronounced, and the relative change in abundance is the same for other test states of the experiments. Hence, an overall similar set of significantly changed proteins will be detected by both software solutions, allowing for a similar biological interpretation, as

exemplified for the toluene degradation proteins of *D. toluolica* Tol2 (online suppl. Fig. S3) and previously reported for a smaller set of proteins [Karp et al., 2008]. It is noteworthy that the impact of sample generation, handling, and preparation, as well as the electrophoresis and image acquisition (i.e., digitalization system), is presumably more pronounced than that of the analysis software [Silva et al., 2010; Zech et al., 2011; Strijkstra et al., 2016].

Conclusions

Although the image upload and project generation is comparable for DeCyder™ and SameSpots™, handling of the latter is more intuitive for the user due to fewer instances of user intervention (with respect to possibility and necessity) as well as its nonmodular structure allowing for a clear and coherent workflow, as reported previously [Silva et al., 2010]. Furthermore, markedly less time has to be invested for quantitative analysis since the warping technique combined with image alignment prior to coherent spot detection supersedes the time-consuming spot matching recommended in the case of DeCyder™. However, the procedure of manual spot boundary changes and addition of not automatically detected spots is easier in the case of DeCyder™, with respect to spot review (2D and 3D) and manipulation. The discovered small, factorial difference in calculated abundance changes is most likely evoked by the DeCyder™ normalization procedure, possibly involving other, unknown implemented processes.

Overall, even 40 years after the invention of 2DE and 20 years since 2D DIGE, the latter represents an affordable and valuable tool for high-resolution, quantitative differential proteomics. This is because of: (i) reduced costs of fluorescent dyes (due to meanwhile multiple vendors), (ii) time-saving and easy to use advanced CCD camera systems, as well as (iii) intuitive workflow and fast image analysis by warped, proteome map-based software, allowing for (iv) determination of highly similar, reproducible, and user-independent protein abundance changes.

Materials and Methods

2D DIGE and Image Acquisition

Inter-software comparison was performed using three distinct 2D DIGE gel sets of three different environmental bacteria: *D. toluolica* Tol2, “*A. aromaticum*” EbN1, and *P. inhibens* DSM 17395. 2D DIGE was performed using 24-cm IPG-strips and image acquisition was performed by a charge-coupled camera system as described in detail by Strijkstra et al. [2016].

General Software Comparison

In this study, image analysis was performed using DeCyder™ version 7.2 (GE Healthcare, Munich, Germany) and SameSpots™ version 5.0.1.0 (TotalLab, Newcastle upon Tyne, UK). Comparison of the different steps from image analysis to spot abundance data calculation (Fig. 1) was based on the information provided in the user manual of DeCyder™ (version 7.0) and the SameSpots™ DIGE tutorial, company homepage, and personal communication (May 2017).

Image Processing

The strain-specific gel sets were imported into the respective software and processed with settings set to defaults or automatic procession. User interference was kept to a minimum. However, it was necessary for the user to provide an estimated number of spots with the DeCyder™ software. For each gel set, the estimated number of spots was set to 5,000 in the Batch processor module. Spot normalization and quantitation was not altered manually in either of the software types. The following information was collected from both software solutions: gel images with spot boundaries, software inherent spot number, spot coordinates, normalization factor, spots excluded from the normalization process, raw and normalized spot volume, fold change, and one-way ANOVA *p* values.

Inter-Software Spot Map Matching

For each gel set, the internal standard gel image of the DeCyder™ master gel (automatically determined) with indicated spot boundaries was employed to create an overlay with the analogous spot map of SameSpots™ (online suppl. Fig. S2). Based on this overlay image, spots present in both spot maps were determined and matched, applying the “center of mass” denoted by DeCyder™ as the decisive parameter. The latter is defined by the DeCyder™ software as the optimal picking location for a respective protein spot.

The DeCyder™ software does not request the manual checking of the spot matching across all spot maps of a gel set. However, it is recommended for spots with assigned decreased or increased abundance. Hence, a second list of DeCyder™ protein spots with corresponding SameSpots™ spot equivalents was created after manual spot confirmation in DeCyder™. Three types of DeCyder™ spots were chosen for manual confirmation: (i) spots with a SameSpots™ equivalent, (ii) spots with increased or decreased abundance (in either software), and (iii) spots with their “center of mass” close to, but not inside the respective SameSpots™ spot boundary. In contrast to DeCyder™, the SameSpots™ software does not request any spot confirmation or correction. Therefore, the same SameSpots™ data set was used for both inter-software spot matching lists.

In several cases, more than one DeCyder™ spot could be assigned to a distinct SameSpots™ spot. To allow for direct comparison of distinct spot attributes per spot, the DeCyder™ spot with the highest raw spot volume of the internal standard (mean of the gel set) was assigned.

Comparison of Spot Statistics

The two different software packages were compared based on the raw spot volume as well as on computed values, i.e., normalized spot volume, fold change (SameSpots™), or the average ratio (DeCyder™), respectively, and one-way ANOVA *p* values. While

decreased abundance is indicated by the DeCyder™ average ratio as a negative value, the SameSpots™ fold change is generally positive. To allow for direct comparison of decreased abundances, respective SameSpots™ spots were targeted in hierarchical grouping of the dendrogram in the software and converted to the corresponding negative value. Spots significantly changed in abundance had a fold change ≥ 1.5 or ≤ -1.5 and an ANOVA p value $\leq 1 \times 10^{-4}$.

Effect of Normalization

The two software packages apply different algorithms to normalize the raw volume data of the test and reference state, respectively, to the internal standard. SameSpots™ uses an iterated median and median absolute deviation approach to calculate upper and lower limits for the log volume ratio.

To model the calculation of the limits by the SameSpots™ software, the median and its deviation can be calculated from the log volume ratio of a gel pair (test state and internal standard or reference state and internal standard, respectively). Subsequently, this step is iterated on the log volume ratio of all spots below the negative median deviation, yielding a new positive and negative median deviation. The latter can then be used as estimation of the lower limit calculation by the SameSpots™ software. The upper limit can be modelled in the same way; however, here the positive median deviation of the spots initially above the positive median deviation is used for the calculation of the normalization factor.

Ten to the power of the mean of the log volume ratio of all spots inside these limits is then used as the normalization factor. The

normalized volume may then be calculated by dividing the raw volume of the test state and reference state, respectively, by the raw volume of the internal standard and subsequent multiplication with the normalization factor. Finally, the abundance change is determined by division of the normalized volume of the test state by the reference state. In a case where the normalized volume is larger in the reference state, the latter is divided by the test state.

This normalization procedure was reconstructed in the R project software and RStudio (version 1.1.383). Unfortunately, a detailed description of the normalization procedure of DeCyder™ is not publicly available.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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