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TWO-DIMENSIONAL ELECTROPHORESIS GEL IMAGES SCAN FOR DECOMPOSITION AND DEPLETION ANALYSIS

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Two-dimensional Electrophoresis Gel Images Scan (2dEGIS) implements a mix of computational methods for processing two-dimensional electrophoresis gel images. For advancing the analysis in case-control sample studies, a multi-component decomposition-approximation approach is presented, based on: (1) A global scan aimed to detect discriminative patterns with just a few components; (2) A more localized image scan through aggregated components; (3) The exploration of specific regions with maximal localization power. The tool 2dEGIS represents a novel unifying instrument for the computational analysis of gel images.

Keywords: 2DGel electrophoresis; dimensionality reduction; feature detection.

1. Introduction

2D electrophoresis (2DE) is designed to separate proteins according to their difference in charge and molecular weight. A cost-effective strategy aims to identify a limited number of proteins to be transferred for further analysis to other proteomic techniques, such as mass spectrometry for protein identification purposes.

Statistical methods lead to an automated 2DE gels processing [Wilkins *et al.* (1997)], in particular to detect differential concentration in case-control groups. The support offered by image analysis to 2DE experiments is targeted to a characterization of features that discriminate between diseased and normal samples.

Various quantitative methods [Marengo *et al.* (2005); Hastie *et al.* (2001)] separate the biological from the noise sources, the experimental aberrations and the computational biases. In our setting, biological sources refer to proteins' concentrations present in relatively high or low abundance.

2dEGIS analysis of the observed image uses a few coarse-grid components, together with more localized and aggregated structures, and then enables multiscale

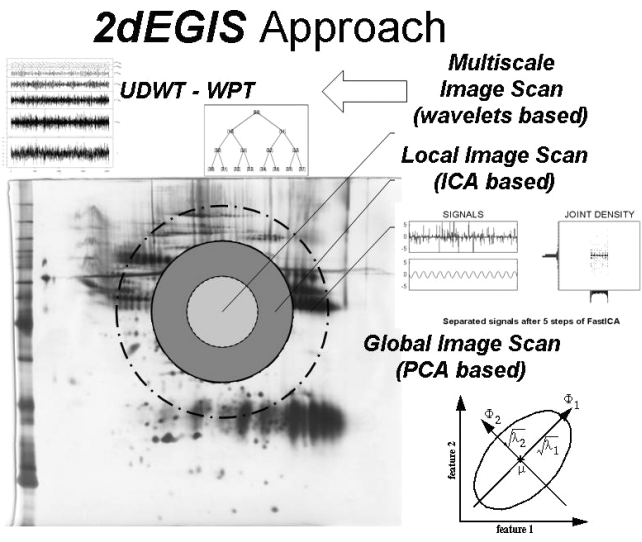

Fig. 1. 2dEGIS approach: global, local and multiscale image scan analysis.

power for specific selection of image regions where biological and non-biological information appear strongly convoluted. Figure 1 offers a sketch of the approach.

2. Methods

2.1. *Sample preparation and 2DE details*

Proteins from bovine serum were separated by charge on a 18 cm nonlinear pH 3–10 IPG (GE Healthcare), then according to size by SDS-PAGE on 10% polyacrylamide gels. Gels were stained with silver nitrate, scanned in a GS800 calibrated densitometer (Bio-Rad) and converted into electronic files for further processing. More details are provided in the Appendix A.

Serum was collected from healthy and sick individuals. The serum was immediately frozen at −20°C until analysis was performed. After protein quantification with 2D-Quant Kit (GE Healthcare), 100 μg of serum proteins were brought up in IEF Buffer (8 M urea, 4% CHAPS, 1% DTT, 15 mM TRIS and 2% Ampholine of pH 3.5–10) to a final volume of 100 μL.

The Immobiline Dry strip (pH 3–10, length 18 cm, GE-Healthcare) was rehydrated with 350 μl buffer containing 8 M urea, 4% CHAPS, 65 mM DTT, 1% Ampholine and 0.002% bromophenol blue[16] for 14 h at room temperature. 100 μg of sample protein was loaded by cathodic cup loading. IEF was performed using Ettan IPGphor II IEF System (GE Healthcare) with a total of 140 kVh. Strips were equilibrated in a solution containing 6 M urea, 30% glycerol, 2% SDS and 50 mM Tris–HCl (pH 8.8) adding 1% w/v DTT in the first step, and 2.5% w/v iodoacetamide in the second step. For the second dimension, proteins were separated by SDS-PAGE on 10% polyacrylamide gels using Ettan Dalt six (GE-Healthcare)

according to following procedure: 30 min at a constant current of 12 mA followed by 24 mA per gel until the bromophenol blue front reached the bottom of the gel.

Subsequently gels were stained with MS modified silver staining. All images were acquired using a ImageScanner III (GE Healthcare) at 600 dpi resolution. Gel images were imported both into Progenesis SameSpots (v3.33.3383; Nonlinear Dynamics, Newcastle, UK) and ImageMaster 2D Platinum v6.0.1 software (GE Healthcare) for analysis. All imported images were processed with Progenesis SameSpots to check image quality (saturation, dimension). The aligned images were then automatically analyzed using the 2D analysis module for spot detection, background subtraction, normalization, and spot matching, and all spots were manually reviewed and validated to ensure proper detection and matching.

As an example, Fig. 2 shows sample images under healthy and diseased conditions. Apart from a different number and location of spots, the densest areas show high-abundance proteins that may mask other proteins of lower abundance but possibly superior interest.

2.2. *Data analysis*

In 2DE Gels, a quite convoluted feature map appears with the relevant biological information mixed to various noise sources. Many proteins have to be separated for then proceeding with their identification, and achieving a final map. Statistical and machine learning instruments can be useful to discriminate between samples by the detection power with which they perform feature selection and pattern recognition.

Also, noise should be isolated or its impact mitigated, such that when features and patterns can be distinguished more clearly, then both disease and control samples can gain classification accuracy.

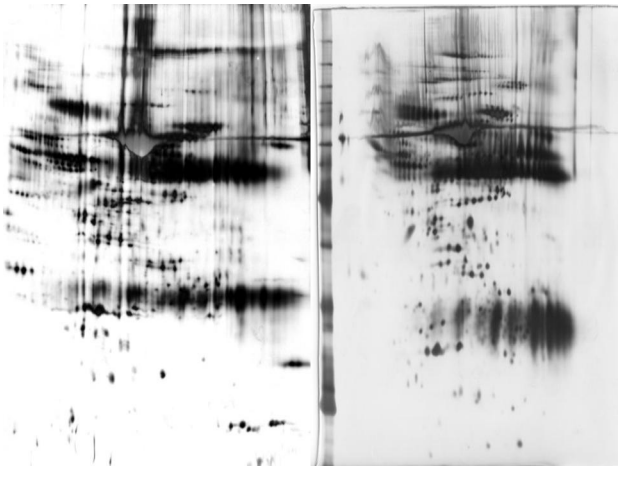

Fig. 2. Sample images: health (left) & disease (right) status.

Specialized literature already presents a good set of standard techniques and established algorithmic tools for the applications in image processing (rich references for image processing are Nixon and Aguado (2002); Gonzales and Woods (2002); for pattern recognition see Theodoridis and Koutroumbas (2003); for statistical learning theory and computational approaches see Hastie *et al.* (2001).

Such methods can be applied in our study context too, with the available 2DE gels. The problem with 2DE images is that they are quite hard to process by numerical algorithms running on fast computers, especially when optimization procedures have to be performed.

Designing a statistical model under some parametric assumptions would involve estimating parameters through some criteria, but always at the risk of overfitting and model misspecification. Leaving the model flexible in the functional forms which are specified, in a non-parametric statistical fashion, would make even more cumbersome the computational load, in addition to a loss of precision in variance estimation relatively to unknown parameters.

Therefore, we believe that more heuristic approaches using statistics and machine learning methods, can be usefully pursued for the 2DE image analysis tasks. Among such methods, we have considered that blind source separation and multiscale techniques offer an interesting approach due to the presence of at least three properties:

(a) *Data adaptivity*, regardless their complex nature;
(b) *Denoising power*, which allows to filter out disturbances and interferences;

Dimensionality reduction, which reduces the problem to examining just a few components or regions of the feature or coefficient spaces, respectively, and assuming that both may represent the most important data characteristics.

In our context, the relevance of the spots in the gels has to be contrasted with the degree of interference that is present; thus, even if one looks at the measured protein abundances, the most interesting proteins have often a lower abundance and are masked by highly abundant proteins of lesser interest.

The rationale for choosing among methods that treat images is usually dependent on the kind of available images, first of all. Therefore, we decided to focus on methods effective in removing the heterogeneous interferences that are involved in gel images, and recovering the underlying information.

Notably, our choice of methods does not exclude the importance of other excellent techniques based on different principles, for instance Morphological reconstructions based on suitable operators [see seminal work by Serra (1982 and 1988); Serra and Salembier (1993); Beucher (2001)] or Empirical Mode Decomposition (EMD) (see [Huang *et al.* (1998) for seminal work]).

The latter method, in particular, is a data-adaptive approach not needing *ad hoc* distributional or analytical assumptions, and capable of expanding the data into signal-dependent basis functions iteratively estimated via the so-called sifting procedure.

Other studies have appeared on bidimensional EMD for image analysis [see Nunes and Delechelle (2009), Linderhed (2009) and their references, and then extensions in Bhuiyan *et al.* (2009), Wu *et al.* (2009), just to mention a few recent examples] that stimulate further comparative evaluations in the future.

In particular, the EMD strategy is based on breaking the signal or the image into a set of intrinsic mode functions that embed inherent data properties, while with images crucial references become the fitting surfaces (from radial basis functions, thin-plate splines, Delaunay triangulation, etc.).

In general, the EMD presents, fairly high computational costs mostly dependent on the pre-selected fitters, and can also find difficulties in handling noise [concerning denoising, the work by Wu *et al.* (2009) seems very promising]. Specifically for our application, this approach may represent a very good alternative and even a potential empirical refinement to projective techniques based on pre-selected basis functions. However, we leave the comparative and integrative analysis in 2DE context for future developments.

2.3. *Blind source separation*

Image features [Nixon and Aguado (2002)] are typically represented by patterns that recur across the samples. The latter are differentially characterized by features observed under disease and normal status. However, due to the presence of noise and artifacts, 2DE images may present features with biological relevance or not.

Currently, a consensus over the best performing methods that produce and separate such features does not exist. We suggest a novel hybrid methodological approach composed by a mix of instruments which are designed to perform effective 2DE gel image analysis through Blind Source Separation (BSS) techniques [Jutten *et al.* (1988)].

The approach first describes a global image view based on Principal Component Analysis (PCA) [Joliffe (1986)], then explores the local power of Independent Component Analysis (ICA) [Cardoso (1989); Cardoso and Souloumiac (1993); Comon (1994)], and finally, applies wavelets [Daubechies (1992); Meyer (1993); Mallat (1999)] in order to exploit their multiscale and denoising power.

We present these methods, and briefly sketch their well-known algorithmic features, but first emphasize the issue of dimensionality reduction which is crucial in many image analysis applications.

2.4. *Dimensionality reduction*

The underlying strategy pursued by 2dEGIS (some code implementation details are reported in Appendix B) is performing a reduction of redundancy present in the 2DE gel images, in particular by implementing image transform methods able to change the statistical dependence structure. For instance, BSS techniques recover information sources from the observed convoluted noisy images by concentrating on

just a few dimensions that capture the maximal sample co-variation in a projected space.

The employed algorithms may not require statistical assumptions. Retrieval and elucidation of the latent biological components which are mixed together with noise and other non-biological sources, represent hard tasks in the inverse problem addressed by BSS: identifying both the unknown components and the mixing mechanisms that combine them. Once achieved a good recovery performance, discriminative features can be more easily detected.

The tools here presented deal with noise through de-noising, as they select from data eigendecomposition only a few eigenvalues (the largest ones) that are retained relevant for the analysis because summarizing the main sample characteristics.

Thus, it is possible to identify and rank the importance of linear combinations of sample information with regard to the overall covariance structure, and since the smallest eigenvalues correspond to the noisiest modes, by removing them one can mitigate the impact of noise.

Three methods are now introduced in more detail, and their main characteristics and limitations are described, together with their performances.

3. Results

3.1. *Principal components*

PCA represents an orthogonal linear transformation of objects in a new coordinate system where optimality is preserved in least square terms. In particular, each coordinate embeds in decreasing order a certain degree of variability, which is usually represented by the eigenvalue spectrum.

The data can be factorized as $X = A\,S$, with Gaussian and unit variance S sources, and the general mixing matrix A can be decomposed according to the Singular Value Decomposition in $A = U\,\Sigma\,V'$, where U and V are matrices with orthonormal columns and Σ is diagonal. The eigenvectors form the columns of U, and represent the principal components of the data, while the scaling factors in Σ are eigenvalues arranged in decreasing order.

The identified components are second-order independent (or decorrelated) structures that change the variables of interest from p- to m-dimensional (with $m \ll p$) while transforming the data so to maximize the variance. Orthogonality implies that the decomposition is performing ideally under Gaussianity, and only approximately under non-Gaussianity. Therefore, other conditions must hold in order to find statistically independent structures, and in turn other methods have to be employed.

3.2. *Independent components*

In order to overcome the limitations of PCA, ICA has been proposed. ICA is based on different principles compared to PCA [see for instance Hyvarinen, A. and Oja

(1997); Hyvarinen (1999); Hyvarinen *et al.* (2001)], and retrieves the latent factors which underlie the observed data. If these factors exist, they are non-Gaussian (except at most one) and statistically independent (i.e. a stronger condition than being decorrelated).

Starting from the same data representation, the goal of ICA is finding a demixing matrix W which recovers the independent sources S = W X. Thus, W = (1/Σ) U', where the U' operator projects the data into the source space, and the 1/Σ scales the projections to have unit variance. Note that it holds the following: W = O(1/Σ) U', for any orthogonal O, such that also decorrelated Gaussian sources can be recovered as well as non-Gaussian ones. But in case that PCA is implemented, then O = I.

ICA works in a non-orthogonal coordinate system where the data structure is explored by a non-linear optimization search operated by algorithms targeted to diagonalize contrast functions dependent on high-order cumulants, and to maximize non-Gaussianity or information theoretical criteria (entropy). Several algorithms are currently available.

ICA usually finds sparse structures, when they exist or may be found (as an algorithm can fail to identify the hidden sources), and works non orthogonally through a projection where to a whitening step a rotation step is added, which in turn allows to explore the data structure via a non-linear optimization search.

For instance, the algorithm of [Cardoso and Souloumiac (1993)], i.e. Joint Approximate Diagonalization of Eigenmatrices (for real signals) (*JadeR*), after whitening and projecting the data onto the signal subspace, shifts from data to statistics provided by cumulant matrices (which encode the full set of fourth order cumulants) and requiring joint diagonalization.

Then, a permutation (from the most energetic component) and a normalization (to unit variance) of the extracted source signals together allow for a sorted unmixing of the observations, i.e. in order of decreasing energy. The steps are sketched in Table 1.

Also *fastICA* [Hyvarinen and Oja (1997); Hyvarinen (1999)] is among the most popular methods. By implementing both the algorithms, the former is quite fast and direct in its empirical approach, while the latter can be used in a pre-processing phase to check components' sensitivity to a range of distributional characteristics.

3.3. *Image recovery*

A more accurate recovery of the protein map underlying the observed 2D gels is the goal of the analysis, and involves both image decomposition and reconstruction tasks.

In Fig. 3, we can observe how PCA reconstructs the image by using a variable number of PCs. The PCA decomposition decomposes the image at a global level, and considers the variability as the main element to control. With thirty components the image reconstruction power is quite strong. Note that due to the presence of

Table 1. ICA steps in JadeR format.

JADER *Blind separation of real signals using JADE.*

Based on ***Jacobi algorithm.***

a. **Initialization**: whitening matrix D applied to data X, obtain Z;

b. **Statistics**: estimate a maximal set of cumulant matrices *CuM(Z)*;

c. **Contrast Optimization:** find rotation R that maximally diagonalize the cumulant matrices, i.e. $R^* = argmin \sum_i off\_diag\{R'[CuMi(z)]R\}$;

d. **Separation**: estimate V = R * D exp(-1) and S = V exp(-1) X = R*'Z.

Matlab usage: $\gg$ ***W = jader(X)***;
or
$\gg$ ***W = jader(X,m)***, depending on "m" components.

Notes:

(1) If X is nxT data matrix (n sensors, T samples), then
W = jader(X)
is $n \times n$ separating matrix such that **S = W*X** is nxT matrix of estimated source signals.

(2) If we consider a restriction on the n. of components, then
W = jader(X,m)
then W is mxn such that only m sources are extracted by restricting the operation of *jader* to the m first principal components.

(3) The rows of W are ordered such that the columns of **pinv(B)** are in order of decreasing norm. Thus, the 'most energetically significant' components are those appearing as first in the rows of **S = B*X**.

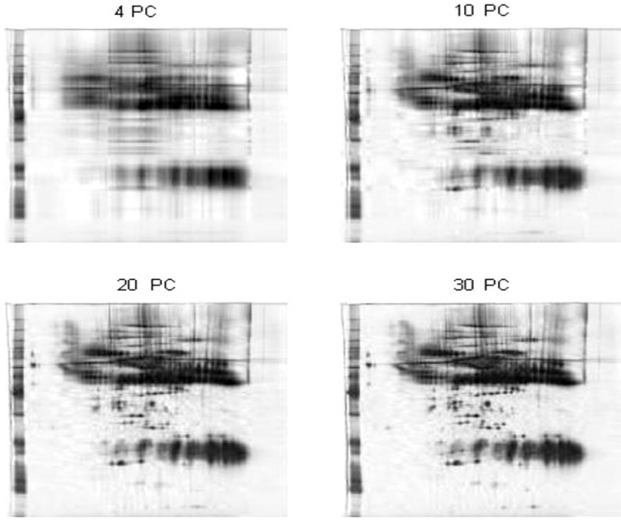

Fig. 3. BSS for case-control comparisons. PCA-based incremental reconstructions with 4, 10, 20 and 30 PCs. Global image sequence appears more precise with growing number of PCs.

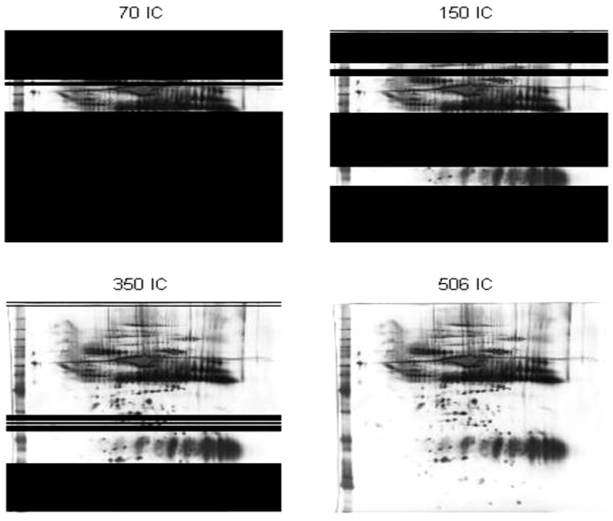

Fig. 4. BSS for case-control comparisons. ICA-based incremental reconstructions with 70, 150, 350 and 506 ICs. Sections of the image appear, apart from the final one (bottom right), due to localization power of ICA.

dense areas, the method is force to use more components to retrieve the interesting spots.

In Fig. 4, ICA is applied and evidence is shown through a sequence of slices emphasizing the localization power enabled by the technique, that step by step finally gets to the global picture reconstructed by many components.

Thus, the local details are suitably reproduced, and the protein map becomes visible at a finer grid in particular regions of the image.

However, in order to reconstruct the whole map, and thus fill in all the regions of the image, many components interacting together would thus be needed. To overcome this limitation and measure the information content of each identified component, we have sorted them according to an entropic criterion.

We have considered that Shannon's entropy is an important measure for evaluating structures and patterns in our data. In practice, the lower the entropy (uncertainty) the more structure is accounted for.

Thus, the first few components are the most relevant (as from low entropy), and identify image regions that are smooth and regular, due probably to the presence of large spots. Vice versa, high-entropy components identify regions where the isolated spots are likely informative at the biological level, but also convoluted with non-biological sources (background, noise and other artifacts).

Overall, ICA reconstructs the image more effectively by aggregating components corresponding specifically to localized image regions. Thus, this method complements PCA which offers only a global decomposition and works optimally under Gaussianity.

3.4. *Tool features*

The tool has required apache, php and javascript (front end), MySQL (db) and R (computing). It has been tested in Linux, and works under Firefox web browser (also under Windows).

By accessing the given link, the tool presents several images that can be produced according to various parameters to be selected. The applet works with pre-installed images for demonstration purposes. In order to have a more complete menu of functions (i.e. insert and modify image options), we turn the reader to the email contact address.

Otherwise, the user can apply the functions to the pre-installed images, then digit the name of one of the three samples ("*um20*", "*az_dis*", "*pr_sane*") as an '**img-find'** entry, and finally mark the corresponding box of the selected sample. Only one sample at the time can be selected.

3.5. *Multiscale components*

The depletion of the high-abundance proteins is a pre-requisite for detecting the low-abundance biomarkers [Bjorhall *et al.* (2005); Yocum *et al.* (2005)]. Since several biochemical methods that bind a large number of proteins often result in potential losses, and mixtures of antibodies in a chromatography column are highly specific but expensive and requiring dedicate instrumentation, we have designed a computational depletion strategy in support of the results obtained by the available experimental depletion kits dealing with human serum proteomes.

2dEGIS then implements denoising to suppress small fine-scale coefficients according to various criteria, and reduces *de facto* the overall redundancy. In particular, quantile thresholding has been used to eliminate a fixed percentage p of entries and thus leave the smallest (in absolute value) p percent of entries to be set to zero.

The depletion power in 2dEGIS has been achieved through discrete wavelet-transform (DWT) and their derived denoisers [Soggiu *et al.* (2009)]. In particular, both the undecimated DWT (UDWT) and the wavelet packet transform (WPT) have been employed as decompositions resulting in very promising results.

In summary, the UDWT deals with complex non-orthogonal data settings by a class of redundant wavelets (i.e. by not applying scale-by-scale decimation of wavelet coefficients), and requires a pseudo-inverse transform to recover the original data.

The second type of wavelet decomposition, i.e. WPT, represents a generalization that combines the Fourier transform frequency resolution power with the DWT time resolution power, and leads to a family of orthonormal transform bases, which includes the DWT.

More in detail, the UDWT gives an increased amount of information about the transformed signal compared to the DWT. It is also called Stationary wavelet transform (SWT), and allows the signal to be never sub-sampled, while instead the filters are up-sampled at each level of decomposition.

Equivalently, applying high and low pass filters to the data at each level produces two sequences at the next level that without decimation retain the same length of the original sequence from which they are computed. Translation-invariance is thus a property achieved by removing both the down-samplers and the up-samplers in the DWT, and by up-sampling the filter coefficients by a factor of $2^{(j-1)}$ in the jth level (or resolution) of the algorithm.

Accordingly, the UDWT is an inherently redundant scheme as the output of each level contains the same number of samples (and correspondingly coefficients) as the input. However, the additional information is very useful, and an improved discrimination between the noisy data and the underlying signals can be achieved in image denoising.

Developed by R.A. Coifman, the WPT generalizes the time-frequency analysis of the DWT, generates a tree structure and can be regarded as any one of a collection of orthonormal transforms, each of which can be readily computed using a very simple modification of the pyramid algorithm for the DWT.

The WPT building block functions $WPT_{j,o,k(t)}$ are very localized in time and more flexible than wavelets in adapting to data types, in particular those consisting of oscillatory patterns. An oscillatory (frequency) index o appears together with resolution j and translation shift k, such that:

$$WPT_{j,o,k(t)} = 2\exp(-j/2)WPTo[2\exp(-j)t - k] \tag{1}$$

Unlike the case of each level calculated by passing the previous approximation coefficients through both low and high pass filters, now the detail and the approximation coefficients are both decomposed. Therefore, with$n$ levels of decomposition there are 2^n different sets of coefficients, but due to the down-sampling process the overall number of coefficients remains the same and no redundancy appears in the system.

The best basis algorithm [Coifman and Wickerhauser (1992)] is applied to the WPT tree structure to deliver the optimal selection of scales. The algorithm adapts the transform to best match the data features, and finds the WPT that minimizes an additive cost function:

$$C = \sum_{j,o} C(WPT_{j,o}) \tag{2}$$

To calculate the best basis, the tree is traversed and each node is marked with its cost value. The best basis set selected is thus different relatively to a particular cost function, and the usual choice (in our case too) is according to the Shannon Entropy commonly used in signal compression and statistical estimation problems:

$$Cse = \sum_{k} [WPT^2 j, o, k] \log[WPT^2 j, o, k] \tag{3}$$

for normalized WPT coefficients.Other cost functions can be implemented too, for instance to deal with tree coefficients thresholding.

For the function depletion that can be accessed by the tool, the following are the requested steps:

(1) *Mark the sample of interest* (*and only that one!*)
(2) *Select the function* "*depletion*"
(3) *Choose image resolution level* (64-128-256-512)
(4) *Click on the image region where the corresponding box should zoom in*
(5) *Perform the desired decomposition* (*UDWT — WPT*), *and if UDWT is chosen also* "*thresh*" *function is available* (*see help instructions*).

4. Concluding Remarks

The methods implemented in 2dEGIS deal with distinct aspects of 2dE gel image information processing. The dimensionality reduction has indicated the following characterizing aspects:

- Denoising power in PCA and ICA delivers better detection of various image features, which can then be incrementally reconstructed;
- Sparsity of PCA and ICA suggests that only a limited number of components is relevant for capturing the image information content;

The sparsity of protein images suggests that just a few components might be relevant for pattern recognition purposes. In particular, PCA refers to a coarse-grained image scan, whose limitations are bypassed by exploiting with ICA the finer details of each specific image region. Therefore, the two techniques are to a certain extent complementary.

Wavelets then provide an effective computational depletion tool, which emphasizes the denoising power at multiple scales. Overall, both coarse and fine details are considered by wavelet methods, which thus appear extremely useful.

Acknowledgments

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