the FEBS Journal

Computational processing and error reduction strategies for standardized quantitative data in biological networks

Marcel Schilling[1,*], Thomas Maiwald[2,*], Sebastian Bohl[1], Markus Kollmann[2], Clemens Kreutz[2], Jens Timmer[2] and Ursula Klingmüller[1]

1 German Cancer Research Center, Heidelberg, Germany
2 Freiburg Center for Data Analysis and Modeling, University of Freiburg, Germany

Keywords
data processing; error reduction; normalization; quantitative immunoblotting; signaling pathways

Correspondence
U. Klingmüller, German Cancer Research Center, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany
Fax: +49 6221 424488
Tel: +49 6221 424481
E-mail: u.klingmueller@dkfz.de

*Authors who contributed equally to the work presented in this article.

(Received 8 September 2005, revised 25 October 2005, accepted 27 October 2005)

doi:10.1111/j.1742-4658.2005.05037.x

High-quality quantitative data generated under standardized conditions is critical for understanding dynamic cellular processes. We report strategies for error reduction, and algorithms for automated data processing and for establishing the widely used techniques of immunoprecipitation and immunoblotting as highly precise methods for the quantification of protein levels and modifications. To determine the stoichiometry of cellular components and to ensure comparability of experiments, relative signals are converted to absolute values. A major source for errors in blotting techniques are inhomogeneities of the gel and the transfer procedure leading to correlated errors. These correlations are prevented by randomized gel loading, which significantly reduces standard deviations. Further error reduction is achieved by using housekeeping proteins as normalizers or by adding purified proteins in immunoprecipitations as calibrators in combination with criteria-based normalization. Additionally, we developed a computational tool for automated normalization, validation and integration of data derived from multiple immunoblots. In this way, large sets of quantitative data for dynamic pathway modeling can be generated, enabling the identification of systems properties and the prediction of targets for efficient intervention.

Systems biology holds great promise for the targeted development of therapies and more cost-effective drug development. By combining experimental data with mathematical modeling of the dynamic behavior of complex biological networks [1,2], systems biology aims to identify systems properties and to predict perturbation-sensitive targets. However, the major limitation at present is the lack of reliable quantitative data. To determine, test and validate the quantitative accuracy of models, and to capture the characteristic dynamic behavior of systems, techniques that quantitatively and selectively measure biochemical reactions within the cell must be developed [3]. Additionally, a comprehensive set of quantitative and time-resolved data is required to conduct a systems-level analysis [4]. Recent reports show that by analyzing quantitative data generated using fluorescence microscopy [5], electrophoretic mobility shift assays [6] or immunoblotting [7,8], new biological insights can be obtained. However, before this approach can be used for biomedical applications, standardized procedures for data acquisition, reliable normalization methods and generally applicable algorithms for data processing have to be developed.

Cellular responses are regulated by complex signaling networks, and subtle changes in protein concentration

Abbreviations
CCD, charge-coupled device; ECL, enhanced chemiluminescence; Epo, erythropoietin; EpoR, erythropoietin receptor; GST, glutathione *S*-transferase; HA, hemaglutinin-tagged; HRP, horseradish peroxidase; Hsc70, cellular heat shock cognate protein 70; IL-6, interleukin-6; IP, immunoprecipitation; MAP kinase, mitogen-activated protein kinase; PDI, protein disulfide isomerase; PVDF, poly(vinylidene difluoride); STAT, signal transducer and activator of transcription.

or protein modification can trigger the onset of diseases. For the analysis of proteins in complex mixtures, one of the most widely used techniques is immunoblotting, which is based on electrophoresis and transfer to a membrane. The presence of specific proteins on the membrane is detected by antibodies in combination with the utilization of chemiluminescent substrates and exposure to X-ray films. However, because the linear range of X-ray films is very limited, quantification by charged-coupled device (CCD) camera detection is preferable [9]. For rare proteins (such as certain signaling components), prepurification by immunoprecipitation (IP) is required prior to immunoblotting, potentially increasing the overall error owing to additional steps involved in the procedure. To date, only relative values that are difficult to compare between independent experiments have been generated by immunoblotting. Thus, reliable algorithms for error reduction and data processing are required to employ immunoblotting for the generation of high-quality quantitative data.

Another problem in normalization of data from different sources arises from the fact that signaling pathways have been primarily studied in the context of propagatable cell lines. However, as such cell lines have lost restrictive growth control mechanisms, it is of great importance to analyze the behavior of signaling pathways in primary cells. As material that can be isolated from animals or patients is very limited, it is of pressing importance that existing data be combined and compared. Mammalian cells grow either in suspension or attached to a support. Suspension cells are primarily cells of hematopoietic origin and are particularly suited for biochemical studies on cell populations with high temporal resolution because they permit bulk stimulation and rapid sampling. For biochemical studies in adherent cells, separate stimulations are required for each time-point, potentially resulting in a higher sample-to-sample variation. Even more difficult is the analysis of proteins in patient samples. To eliminate errors introduced by the measurement process and to ensure comparability of results, we have developed robust normalization procedures for biochemical data.

We use the erythropoietin receptor (EpoR)-induced activation of ERK1 in the hematopoietic suspension cell line, BaF3-hemaglutinin-tagged (HA)-EpoR, and the interleukin-6 (IL-6)-induced activation of the signal transducer and activator of transcription (STAT)3 in adherent primary hepatocytes, as model systems to establish a robust procedure for error reduction and to develop reliable algorithms for data processing, facilitating the generation of high-quality data by quantitative immunoblotting.

Results

Standardized generation of absolute values

The reliable generation of large data sets depends on the strategies used to achieve comparable results among individual experiments. To achieve this, we convert the relative signals, which are usually generated by immunoblotting, to absolute numbers, such as molecules per cell. As an example, the abundance of the mitogen-activated protein (MAP)-kinase family members, ERK1 and ERK2, in cytoplasmic lysates from BaF3-HA-EpoR cells, was determined by analyzing, in parallel, a serial dilution of purified recombinant ERK2 protein (Fig. 1A, upper panel). The CCD camera-based quantification of recombinant ERK2 was plotted against the number of molecules loaded on the gel. As demonstrated by a linear regression passing through the origin (Fig. 1A, lower panel) and extensive additional studies (see the Supplementary material) the detection was linearly proportional to protein concentration over at least two orders of magnitude. By using a linear regression model (detailed in the Supplementary material) relative signals of endogenous ERK1 and ERK2 were converted to molecules per cell, indicating that in the cytoplasm of an BaF3-HA-EpoR cell, 107 000 ERK1 molecules and 318 000 ERK2 molecules are present. This determination requires the recombinant and the endogenous proteins to be analyzed on the same immunoblot and to share the same antibody epitope. As the CCD camera-based detection is proportional to the number of epitopes, it can even be applied to proteins of different molecular mass, such as isoforms or partial fusion proteins, thus permitting the concomitant determination of multiple signaling components. In addition to ensuring comparability of independent experiments, absolute values can be used to determine the stoichiometry of cellular components, critical for obtaining insights into the quantitative behavior of biological networks.

Error determination of the measurement process

To estimate the inherent noise of data generated by the immunoblotting technique, error determinations were performed. A serial dilution of purified recombinant ERK2 protein was analyzed eight times by immunoblotting using an anti-ERK immunoglobulin (Fig. 1B, upper panel) and quantified by CCD camera-based detection. The estimated error was calculated as the standard deviation of the CCD camera-based measurements. Plotting signal strength vs. estimated error revealed that the expected error behavior of a

conventional CCD camera-based photon counting process cannot be recovered. The systematic error inherent in this technique can phenomenologically be described by a sublinear function. Within our measurement range, ≈20% error for each data point is estimated, whereas for weaker signals this percentage is increased (Fig. 1B, lower panel). This noise consists of two different contributions: pipetting errors, which are constant within a lane but uncorrelated from lane to lane; and blotting errors, which are highly correlated from lane to lane. Pipetting errors arise from differences in cell number, gel loading and antibody detection, while blotting errors are caused by inhomogeneities of the gel or the blot.

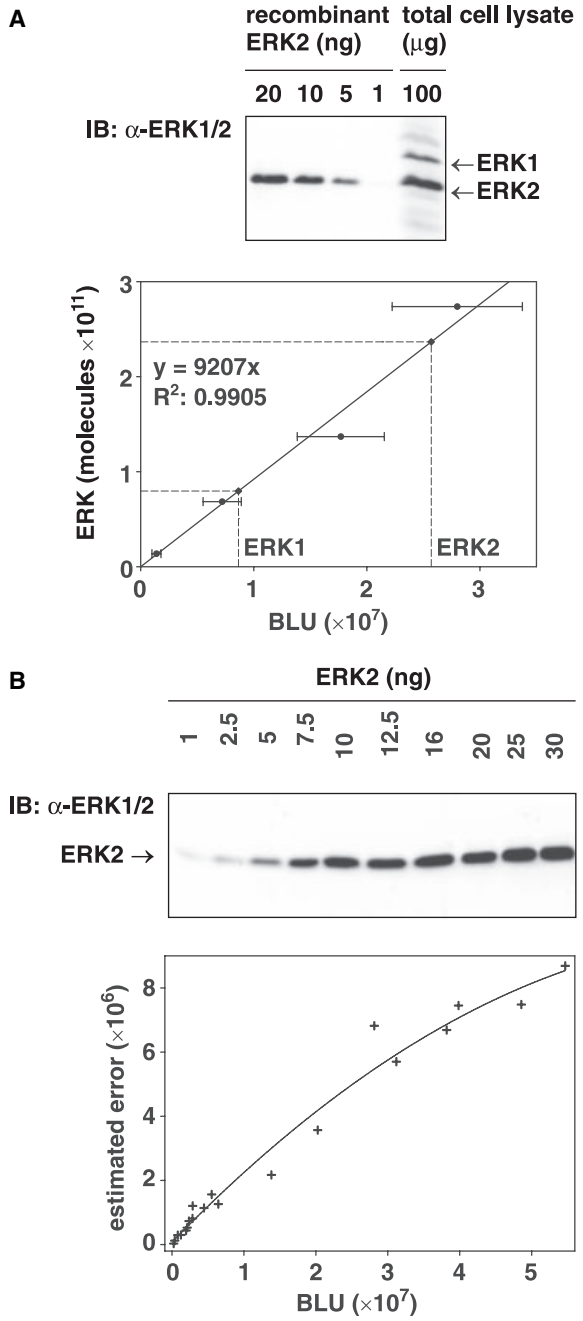

Fig. 1. Conversion of relative values to absolute protein concentrations and error estimation of quantitative immunoblotting. (A) A dilution series of recombinant ERK2 protein, as well as 100 μg of total cellular lysate prepared from BaF3-HA-EpoR cells, were analyzed by quantitative immunoblotting with anti-ERK immunoglobulin. The biomedical light unit (BLU) values of the dilution series were plotted against the number of molecules loaded onto the gel [amount (g)/MW_{ERK2} (g·mol^{-1}) × N_A (molecules·mol^{-1})] and a linear regression through the origin was applied. The slope was used for converting the signals of the total cellular lysate to molecules per cell. Error bars represent estimated errors of the total ERK2 dilution series, as determined in (B). (B) A dilution series of purified ERK2 was separated eight times by SDS/PAGE (10% acrylamide) and transferred to a membrane that was probed with anti-ERK immunoglobulin and subsequently developed with enhanced chemiluminescence (ECL) or ECL advance substrate. The estimated error of the quantified signals was calculated as the standard deviation of the data. To determine the noise inherent in this technique, the signal strength was plotted vs. estimated error and was described by a sublinear function showing a 20% error for each data point within our measurement range.

Eliminating correlated errors by randomized sample loading

To determine steps predominantly contributing to the error obtained by quantitative immunoblotting analysis, we monitored a time-course of erythropoietin (Epo)-induced activation of ERK1 in BaF3-HA-EpoR cells. Identical samples of cytoplasmic lysates were loaded, in a randomized manner, onto two gels, transferred to membranes (blot 1 and blot 2) and analyzed by three repetitive cycles of ERK immunoglobulin reprobing and application of the chemiluminescent substrate (Fig. 2A). Quantification of the signals (Fig. 2B, upper panel) showed that the data obtained by the two blots differed significantly. To reduce the effects of uncorrelated errors, we employed a cubic spline, the smoothness of which is determined by generalized cross-validation. It has been shown previously that time-course behavior can be estimated from noisy data by smoothing splines [10–12]. We emphasize that a sufficiently dense grid of time-points is necessary to keep the bias of this method small. Smoothing of the data is performed to average over the errors contributed by pipetting, electrophoresis and transfer, and other sources of noise.

Surprisingly, uncorrelated errors resulting from antibody detection and reprobing had little effect on the results, as the splines smoothing the data obtained by

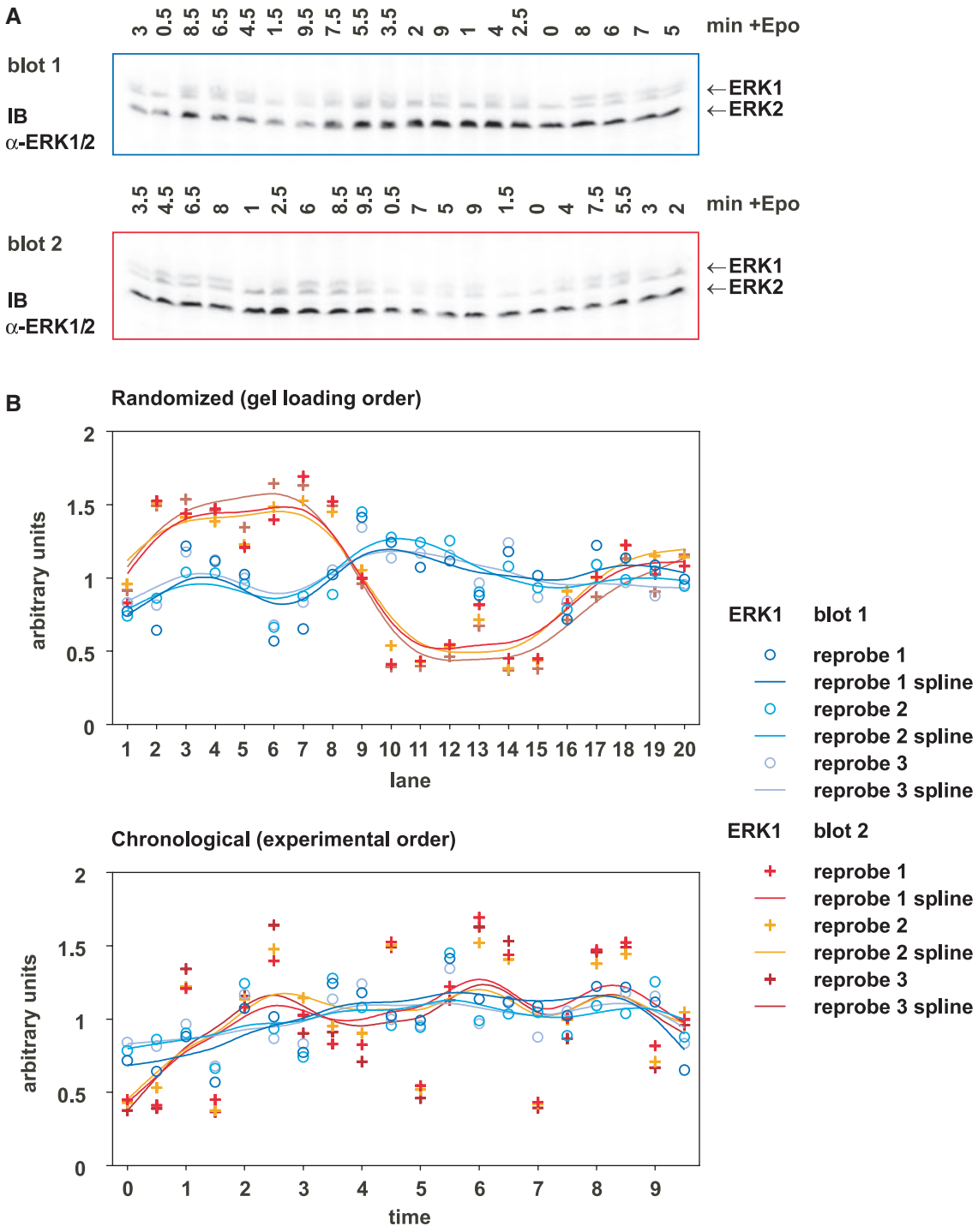

Fig. 2. Randomized sample loading ensures uncorrelated errors. (A) BaF3-HA-EpoR cells were starved and stimulated with 50 units·mL^{-1} erythropoietin (Epo) for 9.5 min, with samples of 1×10^7 cells taken every 30 s. Cells were lysed, and 75 μg of the total cellular lysate at each time-point was separated by two 17.5% SDS polyacrylamide gels using two distinct randomized sample loading orders. Each immunoblot was analyzed by three repetitive cycles of detection with anti-ERK immunoglobulin and subsequent removal of the antibodies by treatment with β-mercaptoethanol and SDS. The obtained signals for ERK1 were quantified by LumiImager analysis. (B) The data show strongly correlated errors when arranged in gel loading order, which are specific for a particular blot but are not affected by reprobing procedures. By arranging the data in chronological order, these correlations are eliminated and the data can be smoothed by spline approximations, as indicated by solid lines. Randomization reduced the standard deviation of the smoothing splines by a factor of 14.

successive reprobing of the same blot were nearly identical. However, the analysis revealed that the data obtained for neighboring lanes was strongly correlated. The apparently different results obtained for identical samples showed that the blotting error leads to aberrant dynamic behavior. Detailed analysis of large data sets revealed a strong correlation between neighboring lanes in immunoblotting analysis, resulting in substantial systematic errors. To separate this spatial correlation from true temporal dynamics in time-course data, we developed standard operating procedures for randomized sample loading, separating consecutive timepoints by a minimum number of lanes. This loading scheme was varied from experiment to experiment to minimize gel border effects. The procedure thereby ensures uncorrelated errors (Fig. 2B, lower panel) and thus facilitates the detection of true dynamic behavior. In this case, randomization reduced the standard deviation of the smoothing splines from 18.6% to 1.4% and thus significantly improves the data quality.

Data correction using normalizers

To reduce the effect of the blotting error and improve the data quality, we used endogenous proteins as normalizers. The time-course of Epo-induced phosphorylation of ERK1 was detected by immunoblotting using a phosphospecific anti-pERK immunoglobulin (Fig. 3A). Subsequently, the antibody was removed and the blot was reprobed, first with an anti-ERK immunoglobulin to determine the total amount of ERK1 in the cytoplasmic lysates and, second, with a mixture of antibodies against endogenous proteins. These proteins, which we termed normalizers, are highly expressed, their levels are not changed during the course of the experiment and antibodies are available that permit efficient detection. As shown in Fig. 3A, the blotting error is strongly influenced by the position of a protein within a blot, as evidenced by the analysis of βActin (42 kDa), protein disulfide isomerase (PDI; 58 kDa), and heat shock cognate protein 70 (Hsc70; 73 kDa) covering the entire separation range of the polyacrylamide gel. Therefore, the signal of a normalizer of similar molecular mass to the protein of interest has to be used to distinguish blotting error from the true protein concentration. The levels of pERK1 and ERK1 were normalized with a smoothing spline applied to the βActin signal. As shown in Fig. 3B, this procedure enabled us to correct for blotting errors in our signals. As expected, the normalized data shows a constant concentration of ERK1 over the entire observation time. By employing purified ERK2 as standard, relative signals for ERK1 were converted to molecules per cell and the proportion of phosphorylated ERK1 was determined by analyzing the fraction of protein that was detected by the anti-ERK immunoglobulin at a higher position in the blot. This ensures the comparability of normalized data derived from independent experiments.

Recombinant proteins as calibrators for IP

For certain proteins, immunoblotting is not capable of generating quantitative data. This problem can be caused by antibodies with weak affinity to the protein, cross-reaction with other proteins resulting in a high background, or by the use of generic phosphotyrosine antibodies. In such cases, the protein of interest has to be prepurified by IP, prior to electrophoresis.

As normalizers are not captured by the antibodies used for the IP, we have established a method to correct for blotting errors as well as inaccuracies in the multistep IP procedure, and to normalize the results obtained. We generated proteins (which we termed calibrators) that share the same epitope as the protein of interest, but differ in molecular mass. Adding a defined amount of calibrator to the lysate prior to IP permits normalization of the results obtained by CCD camera-based detection. We fused the protein domain containing the epitope of the antibody used for IP to a affinity tag for purification (Fig. 4A). Using only part of the protein, calibrators of large proteins or transmembrane proteins could easily be expressed in *Escherichia coli* and purified using affinity beads. We determined the concentration of the calibrators by analyzing a BSA dilution series and the calibrator in a Coomassie Blue-stained gel and quantifying the signals. To define the optimal amount of calibrator that should be added to the IP while still avoiding saturation of the antibodies, increasing concentrations of the calibrator, glutathione *S*-transferase-tagged (GST)-EpoR, were added to lysates of BaF3-HA-EpoR cells prior to IP (Fig. 4B). Plotting the concentration of calibrator added to the lysates vs. signals for HA-EpoR and GST-EpoR showed that the calibrator signal increased linearly in a range between 2.5 and 100 ng. This suggested that the use of a calibrator not only permits quantitative data generation, but also conversion of relative values to absolute protein concentrations. The addition of the calibrator had no effect on the signal for the HA-EpoR up to concentrations of 500 ng of GST-EpoR, indicating that the antibody was in large excess compared with HA-EpoR. Using this data, we calculated that 40 ng of GST-EpoR should be added to lysates to obtain comparable signals for HA-EpoR and the calibrator (Fig. 4C).

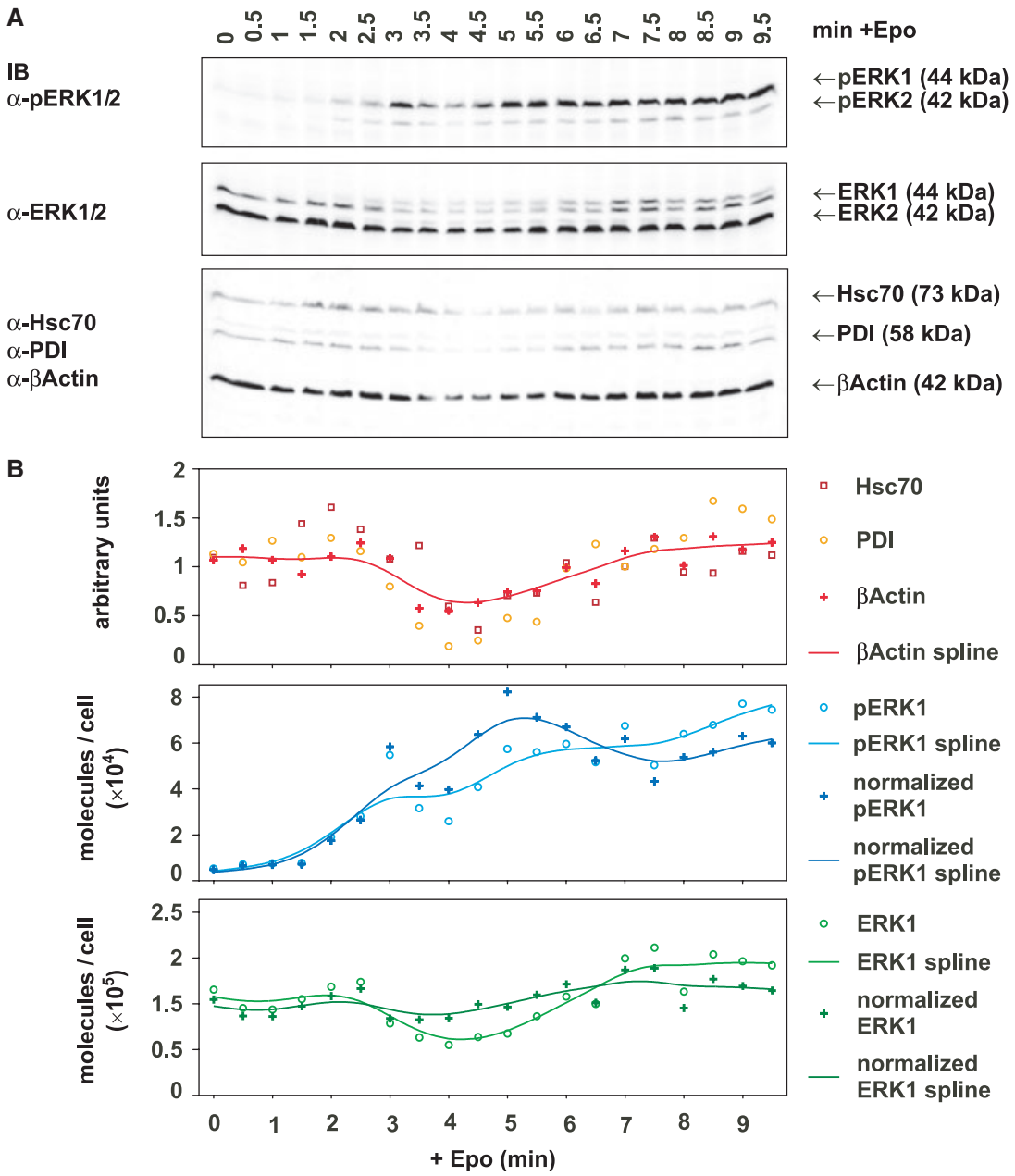

Fig. 3. Correction of phosphorylated and total ERK1 signals using normalizers. (A) BaF3-HA-EpoR cells were starved and stimulated with 50 units·mL^{-1} erythropoietin (Epo) for 9.5 min, with samples of 1×10^7 cells taken every 30 s. Cells were lysed and 75 μg of total cellular lysate at each time-point was separated by electrophoresis on a 17.5% SDS polyacrylamide gel. The immunoblot was analyzed with anti-pERK immunoglobulin, and then reprobed, first with anti-ERK immunoglobulin and second with an anti-heat shock cognate protein 70 (Hsc70)/anti-protein disulfide isomerase (PDI)/anti-(βActin) immunoglobulin mixture. All signals were quantified by LumiImager analysis. (B) The βActin signal was spline-smoothed and used to normalize pERK1 and ERK1 signals, having similar molecular masses. pERK1 and ERK1 signals were converted to number of molecules per cell using the protein standard depicted in Fig. 1. Smoothing spline curves through original and normalized data are shown as solid lines.

Using calibrators for error reduction

The impact of calibrators on data quality is exemplified by an EpoR time-course experiment with randomized gel loading. We stimulated BaF3-HA-EpoR cells with Epo for up to 10 min and added 40 ng of GST-EpoR to each cytoplasmic lysate to control for errors during the IP procedure (Fig. 5A). In

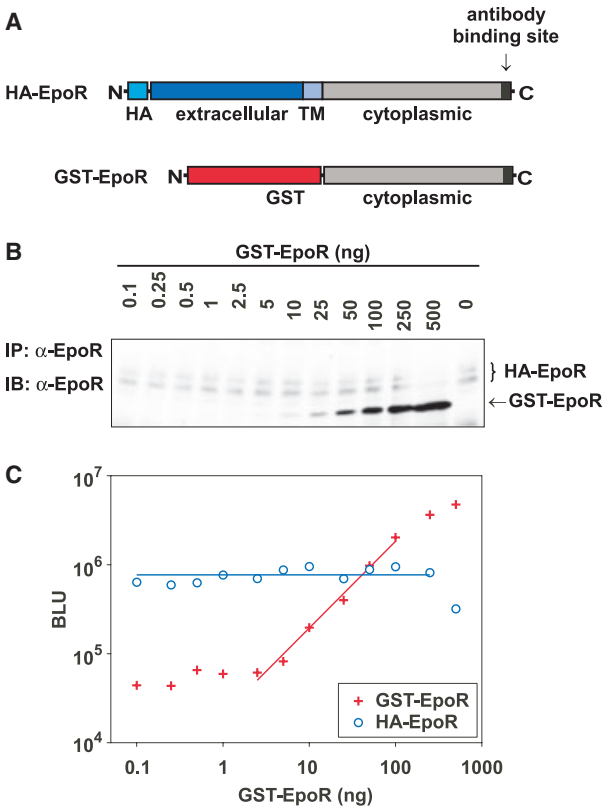

Fig. 4. Titration of the glutathione *S*-transferase tagged-erythropoietin receptor (GST-EpoR) calibrator in immunoprecipitation. (A) The domain structure of hemaglutinin-tagged HA-EpoR is schematically depicted and the binding epitope for the anti-EpoR immunoglobulin is indicated. The calibrator, GST-EpoR, consists of the protein domain containing the antibody-binding site fused to an affinity tag for purification. (B) BaF3-HA-EpoR cells were starved, stimulated with 50 units·mL^{-1} erythropoietin (Epo) for 5 min and lysed. Increasing amounts of recombinant GST-EpoR were added to the lysates and both the GST-EpoR calibrator and the HA-EpoR were immunoprecipitated with anti-EpoR immunoglobulin. The samples were separated on a 10% SDS polyacrylamide gel. The immunoblot was analyzed with anti-EpoR immunoglobulin and quantified by LumiImager analysis. (C) Concentrations of the calibrator were plotted vs. the signals obtained for the HA-EpoR and the GST-EpoR calibrator. A red line depicts the linear relationship between the calibrator concentration added to the lysate and the detected signal within a range of 2.5–100 ng of calibrator addition. The blue line depicting the average signal of the HA-EpoR intersects at 40 ng of GST-EpoR, indicating comparable signals for the calibrator and the HA-EpoR.

addition, the calibrator was used to correct for blotting errors, thereby significantly improving data quality. However, correction steps can be detrimental to the data if a calibrator yields noisy signals or is exposed to different gel/transfer inhomogenieties as the protein of interest owing to a large difference in molecular mass. We therefore developed criteria for automated data correction in IP experiments, as described in the Supplementary material. One necessary condition for these criteria is randomized sample loading. As shown in the Supplementary material, by combining randomized sample loading with calibrators, the standard deviation of immunoblotting data can be improved by more than twofold. The corrected data (Fig. 5B) show the expected behavior of a continuous increase in phosphorylated HA-EpoR and a constant level of total HA-EpoR for 10 min after stimulation with Epo.

Computational data processing using GELINSPECTOR

For automated data processing and to permit data merging of samples analyzed on separate blots, we developed the computer algorithm GELINSPECTOR. This algorithm calculates smoothing splines for the normalizers or calibrators and normalizes blotting data using these splines. Furthermore, the program verifies the normalization, integrates multiple data sets and visualizes the results. To validate our approach, we investigated the effect of our algorithm on time-course data generated from primary hepatocytes. We combined sample randomization with criteria-mediated error reduction using Calnexin and Hsc70 as normalizers. By loading time-points alternating on two gels, the number of data points that could be analyzed together was increased beyond the capacity of a single gel (Fig. 6A). Applying GELINSPECTOR enabled us to normalize the signals and significantly decrease the standard deviation from a smoothing spline, resulting in time-course data with a high temporal resolution (Fig. 6B). The high reproducibility of the time-course dynamics for phosphorylated and total cytoplasmic STAT3 obtained by immunoblotting of cytoplasmic lysates, as well as immunoprecipitates (data not shown), demonstrated that our automated computational data processing is robust and reliably applicable for both methods. These tools facilitate the standardized and automated generation of quantitative data and permit the cost-effective assembly of large, high-quality data sets.

Discussion

Quantitative data generation is becoming increasingly important for obtaining insight into the dynamic behavior of complex biological networks, to elucidate systems properties and to predict targets for biomedical applications. We show that by randomized sample loading and computational data processing, including criteria-based normalization, high-quality quantitative data can reliably be generated by immunoblotting, a

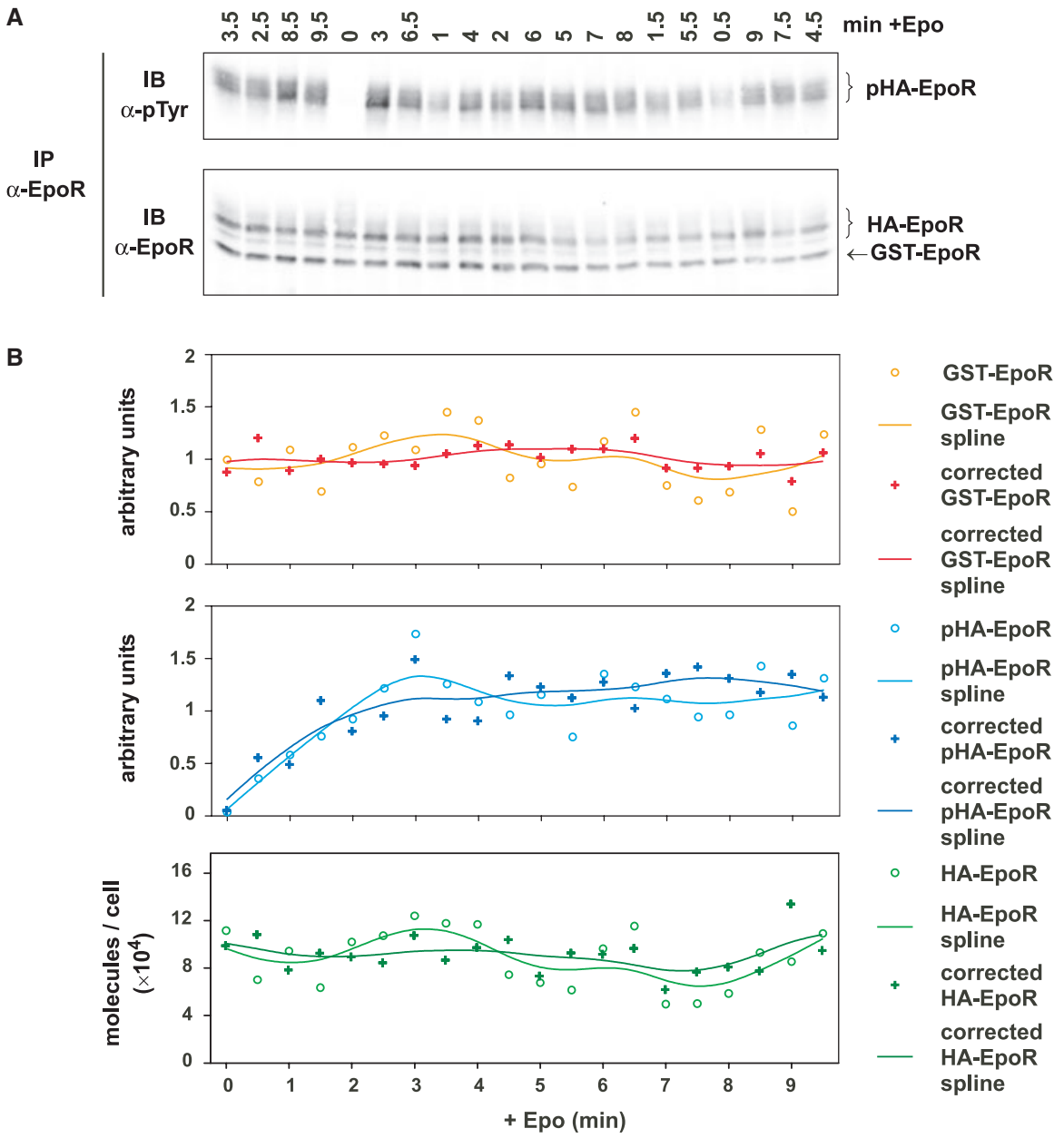

Fig. 5. Correction of hemagglutinin-tagged-erythropoietin receptor (HA-EpoR) signals with the glutathione *S*-transferase (GST)-EpoR calibrator. (A) BaF3-HA-EpoR cells were starved and stimulated with 50 units·mL^{-1} erythropoietin (Epo) for the indicated time. A total of 1×10^7 cells was lysed and 40 ng of GST-EpoR was added to each lysate. Immunoprecipitation was performed using anti-EpoR immunoglobulin, followed by separation on a 10% SDS polyacrylamide gel with randomized sample loading. The immunoblot was analyzed with anti-pTyr and anti-EpoR immunoglobulin and quantified by LumiImager analysis. (B) Time after Epo stimulation was plotted against the signals of HA-EpoR and the calibrator GST-EpoR. A spline smoothing the calibrator signal was used to correct pEpoR signals, whereas the EpoR signal was corrected and converted to molecules per cell. Splines are depicted as solid lines.

widely applied technique. By systematically determining steps contributing to the variability of the experimental data, we identified gel and transfer inhomogeneities as the major source for correlated errors. These correlations could be eliminated by randomized sample loading, and error reduction was achieved by the use of normalizers or calibrators in combination with computational data processing. By converting relative signals to absolute values, comparable results can be obtained from independent

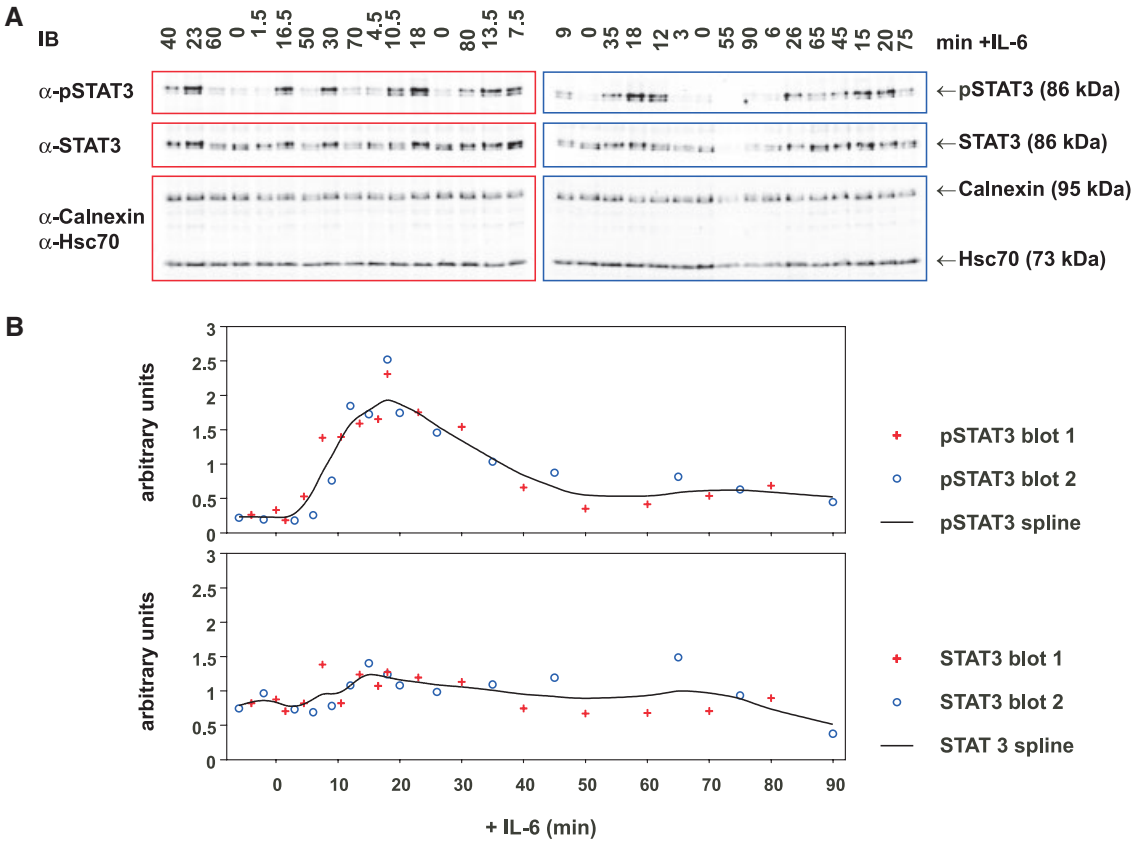

Fig. 6. Quantitative data generation of primary hepatocytes using the computer algorithm GELINSPECTOR. (A) Primary mouse hepatocytes were prepared from mouse livers. A total of 2×10^6 cells for each time-point was cultured on collagen-coated dishes and starved. Interleukin-6 (IL-6) was added (40 ng·mL^{-1}) and the cells were lysed at the indicated time-points. Cytoplasmic lysates were separated by two 10% SDS polyacrylamide gels. Sample loading was randomized with every second time-point on the second gel. Quantitative immunoblotting was performed with anti-phosphorylated signal transducer and activator of transcription 3 (pSTAT3), anti-signal transducer and activator of transcription (STAT3), and an anti-Calnexin/anti heat shock cognate protein 70 (Hsc70) mixture. (B) Immunoblotting data were automatically processed by GELINSPECTOR using Calnexin/Hsc70 signals as normalizers, and the data points were spline-smoothed, as indicated by solid lines.

experiments and used for the assembly of large sets of quantitative data.

Randomized sample analysis is a general strategy to prevent correlated errors, for example in double-blind comparative clinical studies [13] and in the design of DNA microarray experiments [14]. Here, we use this approach to separate spatial blotting effects from real changes in protein levels (i.e. their true dynamic behavior). By simulations of typical time-course experiments, we demonstrated that randomization reduces the standard deviation of immunoblotting data by more than twofold (see the Supplementary material for simulations). Sample randomization is thus a simple procedure that significantly improves data quality without increasing experimental efforts.

To reduce errors inherent in blotting techniques, such as inhomogeneities in the gel as well as transfer, normalizers are used that are present at a similar position in the blot as the molecule of interest and which are detectable with a strong constant signal. We identified several housekeeping proteins of different molecular mass that can be reliably used as normalizers. The normalization procedure cannot be applied if a normalizer differs too much in molecular mass from the protein of interest because it is exposed to different gel/transfer inhomogenieties and therefore does not permit an adequate estimation to be made of the blotting error. To ensure accuracy of data normalization, we applied spline approximation and developed data processing criteria. The resulting computer algorithm, GELINSPECTOR, compares the standard deviation of both the normalized and the unprocessed data to a first estimate of the values. Only if the normalized values are closer to the estimate, is normalization by computational data processing accurate and results in significantly improved data quality.

In the case of grouped data, such as mutant to wild-type comparisons used in diagnostic approaches, the first estimate is the mean value of the same sample loaded in replicates. In other cases, where a continuous dependency exists (such as in time-course or in dose–response experiments), the first estimate is a regression curve if the functional relationship is known or a smoothing spline if unknown. These functions are implemented in GELINSPECTOR.

Our method is not only applicable to proteins concentrated by IP, but also, as we show in Fig. 6A, for the detection of proteins in total cellular lysates of primary hepatocytes. Furthermore, our data processing procedures permit the quantification of low abundance proteins, or modifications, as demonstrated for the Epo-induced phosphorylation of ERK1/2 (Fig. 3A). The EpoR, a member of the hematopoietic cytokine receptor family, activates the MAP kinase signaling cascade to a much lesser extent than receptor tyrosine kinases, such as the epidermal growth factor receptor or the platelet derived growth factor receptor.

Similarly to normalizers, calibrators added in IP experiments permit criteria-based data normalization. Importantly, calibrators, in addition, facilitate the conversion of relative signals to absolute values, such as molecules per cell. For the analysis of cellular lysates, this can be achieved by coloading known amounts of recombinant proteins onto the gel, which are detected by the same antibody as the protein of interest. Using microscopic techniques, the volume of a cell can be estimated, allowing conversion of molecules per cell to protein concentrations. The generation of absolute values provides additional information regarding absolute protein concentrations that cannot only be used to compare signals derived from independent immunoblot experiments, but also to identify the amount of a given protein in a single cell and to determine the stoichiometry of cellular components [15].

The proposed methods can be applied to other blotting techniques, such as northern and Southern blotting analysis, as inhomogeneities in gel and transfer are likely to cause correlated errors in all blotting data. Similarly, correlations can be eliminated by randomization and the errors can be reduced by criteria-based normalization.

Recently developed strategies for quantitative determination of protein levels and modifications include mass spectrometry techniques based on isotope-coded affinity tags [16] and isotope-coded protein labels [17]. By labeling different samples with distinct isotopes, relative changes can be quantified using mass spectrometry. It is even possible to determine absolute values by the addition of synthesized peptides of known quantities as standards. However, these methods are still very expensive, technically demanding and have the disadvantages of requiring large amounts of cellular material.

By developing quantitative immunoblotting as a robust and reliable technique for quantitative data acquisition under standardized conditions, we establish an easy to handle and cost-effective alternative that permits the assembly of large data sets with high temporal resolution. This provides an important tool for diagnostic purposes and the targeted development of novel therapeutic applications.

Experimental procedures

Cell lines and primary cell cultures

The retroviral expression vector, pMOWS, containing HA-EpoR cDNA, was introduced into BaF3 cells by retroviral transduction. Cell lines stably expressing HA-EpoR (BaF3-HA-EpoR) were selected and maintained in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) in the presence of puromycin.

Primary hepatocytes were isolated from male Black-6 mice (6–8 weeks old) (Charles River, Wilmington, MA, USA). Livers were perfused with Hanks buffer supplemented with collagenase II (Biochrom, Berlin, Germany). Experiments were carried out in accordance with the German Animal Welfare Act of 12 April 2002 and the European Council Directive of 24 November 1986. Intact liver capsules were transferred into Williams' medium (Biochrom) supplemented with fetal bovine serum, insulin, L-glutamine and dexamethasone. Hepatocytes were removed from the capsules, enriched by centrifugation and cultured on collagen I-coated dishes (BD Biosciences, Franklin Lakes, NJ, USA) in Williams' medium E (Biochrom) supplemented with L-glutamine and dexamethasone.

Expression, purification and quantification of recombinant proteins

Unphosphorylated purified ERK2 was purchased from Cell Signaling Technologies (Beverly, MA, USA). The cytoplasmic domain of the EpoR was cloned into pGEX-2T (Amersham Biosciences, Piscataway, NJ, USA) and expressed in *E. coli* BL21 CodonPlus-RIL bacteria (Stratagene, La Jolla, CA, USA). Proteins were extracted by lysozyme lysis and sonication. Glutathione agarose beads (Sigma-Aldrich, St Louis, MO, USA) were added to lysates and proteins were eluted by the addition of reduced glutathione (Sigma-Aldrich). For the quantification of purchased and purified proteins, dilution series of purified BSA (Sigma-Aldrich) and the recombinant proteins were separated by 10% SDS/PAGE and stained with Coomassie Brilliant Blue.

The gel was documented using the trans-illumination mode of a LumiImager (Roche Diagnostics, Mannheim, Germany). Proteins were quantified using LUMIANALYST software (Roche Diagnostics).

Time-course experiments

BaF3-HA-EpoR cells were starved for 5 h in RPMI 1640 (Invitrogen) supplemented with 1 $mg{\cdot}mL^{-1}$ BSA (Sigma-Aldrich) and then stimulated with 50 $units{\cdot}mL^{-1}$ Epo (Cilag-Jansen, Bad Homburg, Germany). For each time-point, 10^7 cells were taken from the pool of cells and lysed by the addition of $2 \times$ Nonidet P-40 lysis buffer, thereby terminating the reaction.

A total of 2×10^6 primary hepatocytes were cultured for 24 h after plating on collagen I-coated 60 mm dishes (BD Biosciences) in Williams' medium E (Biochrom) supplemented with L-glutamine and dexamethasone. Cells were starved for 5 h in Williams' medium E supplemented with L-glutamine. Each dish was stimulated with 40 $ng{\cdot}mL^{-1}$ IL-6 in Williams' medium E containing L-glutamine. The medium was removed from the cells, $1 \times$ Nonidet P-40 lysis buffer was added, and cells were collected using a cell scraper.

Immunoprecipitation and quantitative immunoblotting

For IP, cytosolic lysates were incubated with anti-EpoR immunoglobulin (Santa Cruz, La Jolla, CA, USA) or anti-STAT3 immunoglobulin (Cell Signaling Technologies). Immunoprecipitated proteins and total cellular lysates were separated by SDS/PAGE and transferred to poly(vinylidene difluoride) (PVDF) or nitrocellulose membranes. Proteins were immobilized with Ponceau S solution (Sigma-Aldrich) followed by immunoblotting analysis using the anti-phosphotyrosine mAb, 4G10 (Upstate Biotechnology, Lake Placid, NY, USA), the anti-(tyrosine phosphorylated STAT3) immunoglobulin or the anti-(double phosphorylated p44/42 MAP kinase) immunoglobulin (both Cell Signaling Technologies). Antibodies were removed by treating the blots with β-mercaptoethanol and SDS, as described previously [18]. Reprobes were performed using anti-EpoR (Santa Cruz), anti-STAT3 or anti-(p44/42 MAP kinase) (both Cell Signaling Technologies) immunoglobulins. For normalization, antibodies against βActin (Sigma-Aldrich), PDI, Hsc70 and Calnexin (all Stressgen, Victoria, Canada) were used. Secondary horseradish peroxidase (HRP)-coupled antibodies (anti-rabbit HRP, anti-mouse HRP, protein A HRP) were purchased from Amersham Biosciences. Immunoblots against phosphorylated EpoR and total EpoR were incubated with enhanced chemiluminescence (ECL) substrate (Amersham Biosciences) for 1 min, and exposed for 10 min on a LumiImager (Roche Diagnostics). All other immunoblots were incubated with ECL Advance substrate (Amersham Biosciences) for 2 min, and exposed for 1 min on a LumiImager (Roche Diagnostics). For quantifications, LUMIANALYST software (Roche Diagnostics) was used.

Spline approximation and signal normalization

Smoothing splines were applied to the noisy data to estimate the actual values. Their smoothness was determined by generalized cross-validation, minimizing the mean square error between the estimated time-course and the data [10,12]. Splines were used for criteria-mediated error reduction by GELINSPECTOR, as described in the Supplementary material.

Computational data processing by GELINSPECTOR

The computer algorithm GELINSPECTOR requires MATLAB 6.5 and the freely available statistics environment R1.9 or above. It visualizes the blotting error in a gel domain, rearranges randomized gel loadings into chronological order, applies the presented criteria-mediated normalization procedure, and merges data deriving from one experiment measured on several gels. Choices to calculate a first estimate include constant, sigmoidal and spline functions. Splines are calculated using the mgcv library [11] from R or the MATLAB spline toolbox. After specifying proteins of interest, normalizers, calibrators, the measurement files and some global options (such as the preferred figure format pdf, eps, jpg or png), GELINSPECTOR works completely automatically. Figures of all important steps, and files with the processed data, are created. GELINSPECTOR is available from the authors.

Acknowledgements

We thank Stefan Rose-John for the generous gift of IL-6, Sabine McNelly for technical help with hepatocyte preparation and Jan G. Hengstler for the development of standard operating procedures for the preparation of primary hepatocytes. We also thank Nils Blüthgen and Peter J. Nickel for helpful suggestions and Ute Baumann for excellent technical assistance. We are grateful to Jennifer Reed for critically reading the manuscript.

This work was supported by the funding priority 'Systems of Life – Systems Biology' of the German Federal Ministry of Education and Research (BMBF).

References

1 Kholodenko BN, Demin OV, Moehren G & Hoek JB (1999) Quantification of short term signaling by the epidermal growth factor receptor. *J Biol Chem* **274**, 30169–30181.

2 Schoeberl B, Eichler-Jonsson C, Gilles ED & Muller G (2002) Computational modeling of the dynamics of the MAP kinase cascade activated by surface and internalized EGF receptors. *Nat Biotechnol* **20**, 370–375.
3 Bhalla US, Ram PT & Iyengar R (2002) MAP kinase phosphatase as a locus of flexibility in a mitogen-activated protein kinase signaling network. *Science* **297**, 1018–1023.
4 Kitano H (2002) Systems biology: a brief overview. *Science* **295**, 1662–1664.
5 Nelson DE, Ihekwaba AE, Elliott M, Johnson JR, Gibney CA, Foreman BE, Nelson G, See V, Horton CA, Spiller DG *et al.* (2004) Oscillations in NF-kappaB signaling control the dynamics of gene expression. *Science* **306**, 704–708.
6 Hoffmann A, Levchenko A, Scott ML & Baltimore D (2002) The IkappaB-NF-kappaB signaling module: temporal control and selective gene activation. *Science* **298**, 1241–1245.
7 Swameye I, Muller TG, Timmer J, Sandra O & Klingmuller U (2003) Identification of nucleocytoplasmic cycling as a remote sensor in cellular signaling by data-based modeling. *Proc Natl Acad Sci USA* **100**, 1028–1033.
8 Bentele M, Lavrik I, Ulrich M, Stosser S, Heermann DW, Kalthoff H, Krammer PH & Eils R (2004) Mathematical modeling reveals threshold mechanism in CD95-induced apoptosis. *J Cell Biol* **166**, 839–851.
9 Feather-Henigan K, Hersey S, Johnson A, Milosevich GM & Hines K (1999) Immunoblot imaging with a cooled CCD camera and chemiluminescent substrates. *Am Biotechnol Lab* **17**, 44–46.
10 Craven P & Wahba G (1979) Smoothing noisy data with spline functions. *Numer Math* **31**, 377.
11 Wood SN (2003) Thin plate regression splines. *J R Statist Soc B* **65**, 95–114.
12 Green P & Silverman B (1994) *Nonparametric Regression and Generalized Linear Models*. Chapman & Hall, London.
13 Hill AB (1951) The clinical trial. *Br Med Bull* **7**, 278–282.
14 Kerr MK (2003) Design considerations for efficient and effective microarray studies. *Biometrics* **59**, 822–828.
15 Li M & Hazelbauer GL (2004) Cellular stoichiometry of the components of the chemotaxis signaling complex. *J Bacteriol* **186**, 3687–3694.
16 Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH & Aebersold R (1999) Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat Biotechnol* **17**, 994–999.
17 Schmidt A, Kellermann J & Lottspeich F (2005) A novel strategy for quantitative proteomics using isotope-coded protein labels. *Proteomics* **5**, 4–15.
18 Klingmuller U, Lorenz U, Cantley LC, Neel BG & Lodish HF (1995) Specific recruitment of SH-PTP1 to the erythropoietin receptor causes inactivation of JAK2 and termination of proliferative signals. *Cell* **80**, 729–738.

Supplementary material

The following supplementary material is available for this article online:

Doc. S1. Computational processing and error reduction strategies for standardized quantitative data in biological networks.

This material is available as part of the online article from http://www.blackwell-synergy.com