TECHNICAL BRIEF

High performance CCD camera system for digitalisation of 2D DIGE gels

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An essential step in 2D DIGE-based analysis of differential proteome profiles is the accurate and sensitive digitalisation of 2D DIGE gels. The performance progress of commercially available charge-coupled device (CCD) camera-based systems combined with light emitting diodes (LED) opens up a new possibility for this type of digitalisation. Here, we assessed the performance of a CCD camera system (Intas Advanced 2D Imager) as alternative to a traditionally employed, high-end laser scanner system (Typhoon 9400) for digitalisation of differential protein profiles from three different environmental bacteria. Overall, the performance of the CCD camera system was comparable to the laser scanner, as evident from very similar protein abundance changes (irrespective of spot position and volume), as well as from linear range and limit of detection.

Received: September 24, 2015
Revised: April 20, 2016
Accepted: May 31, 2016

Keywords:
2D DIGE / CCD camera / Laser scanner / Intas Advanced 2D Imager / Technology / Typhoon

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Proteomic research within the past two decades has revealed 2D DIGE as gold standard for gel-based, high confidence analysis of protein abundance changes (e.g. [1, 2]). Key requirement for capturing the broad dynamic range and context-specific changes in protein abundances is the precise and sensitive digitalisation of 2D DIGE gels. This has been achieved by laser scanners employing confocal optics (Fig. 1A), with the Typhoon™ three-mode laser scanner (GE Healthcare, Munich, Germany) representing the benchmark instrument for 2D DIGE gel image digitalisation during the past 15 years. Gel-based differential proteomics could benefit largely from commercially available and sensitive, 2D DIGE-suited CCD camera systems (Fig. 1B) that would allow for strongly reduced image acquisition times as well as instrument maintenance costs, resulting from the simple and robust design.

In this study, we tested the prototype of a CCD camera system (Intas Advanced 2D Imager; Intas Science Imaging Instruments GmbH, Göttingen, Germany) (Fig. 1B) and compared its performance to the Typhoon 9400 laser scanner. Experiments involved the proteomically well-characterised, environmental bacteria "*Aromatoleum aromaticum*" EbN1 [3], *Desulfobacula toluolica* Tol2 [4], and *Phaeobacter inhibens* DSM 17395 [5]. For each bacterial strain, four replicate 2D DIGE

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Abbreviations: CCD, charge-coupled device; **LED**, light emitting diode

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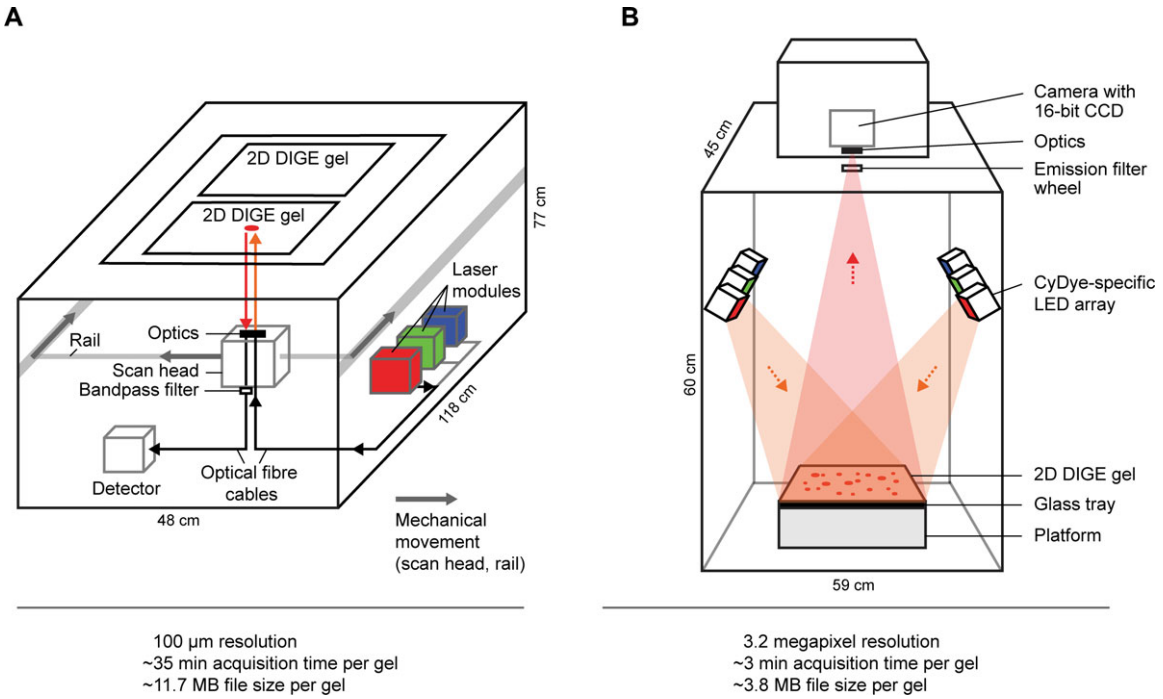

Figure 1. Schematic representation of the compared digitalisation systems. (A) Typhoon laser scanner. For excitation, CyDye-specific laser beams are individually delivered from the respective laser source via optical fibre cables through the scan head (positioned under the gels), and optically focused into the gel plane. Fluorescent light emitted from the gel passes through dye-specific bandpass filters and reaches the detector via another optical fibre cable. The described excitation/emission process occurs repeatedly at high spatial resolution (typically 100 µm). (B) CCD camera system. Two oppositely positioned LED arrays with identical dye-specific filters, forming one LED module, directly and evenly illuminate the complete area of the 2D DIGE gel (21 × 27 cm size) at an optimised angle (Supporting Information Fig. S1). The emitted light from the entire gel passes through a dye-specific emission filter in front of the camera optics. A 16-bit CCD captures, amplifies and converts the light signal via an electric current into 65 536 digital grey scale values.

gels of 21 × 27 cm size were digitalised with both imaging systems to comparatively evaluate instrument performances based on protein spot detection, spot volumes and abundance changes (average ratios). In addition, 1DE gels were used to determine the spectral cross-contamination in the emission of the employed fluorophores (CyDyes™; GE Healthcare), as well as the limit and linear range of detection.

Cultivation of bacterial strains was performed as previously described [4–6]. Preparation of soluble proteins, determination of protein concentrations and all experimental procedures of 2D DIGE (minimal labelling with 200 pmol CyDye per 50 µg protein), digitalisation with the laser scanner and image analysis with the DeCyder™ 2D software (version 7.0; GE Healthcare) were performed as previously described in detail [1]. Protein extracts from cells grown with 4-ethylphenol (strain EbN1), toluene (strain Tol2), or casamino acids (strain DSM 17395) represent three different test states and were each labelled with Cy3. Protein extracts from each bacterial strain grown with fumarate served as reference state and were independently labelled with Cy5. Three strain-specific internal standards were prepared from equal amounts of the corresponding test and reference state and were each labelled with Cy2. An individual gel contained 50 µg each of labelled reference and test state, and of the internal standard. For each strain, four parallel gels were prepared with protein samples from four individual cultures to account for biological variation [7]. Isoelectric focusing (IEF) was conducted using 24 cm-long IPG strips (GE Healthcare) with a nonlinear pH range from 3 to 11 (for strains EbN1 and Tol2) or 3–5.6 (for strain DSM 17395) in a Protean i12 system (Bio-Rad, Munich, Germany). The IEF program used was as follows: 50 V for 13 h, 200 V for 1 h, 1000 V for 1 h, gradual gradient to 10 000 V within 2 h, and 10 000 V for 6 h. Separation of proteins according to molecular mass was done by SDS-PAGE (12.5% acrylamide, v/v) using an EttanDalt*twelve* (GE Healthcare) system. 2D DIGE gels were digitalised directly after electrophoresis remaining sandwiched between two thoroughly cleaned and dry low-fluorescence glass plates (GE Healthcare). First, 2D DIGE gels were digitalised with the CCD camera system and then immediately with the laser scanner; gels in standby position were stored at 4°C in the dark.

In case of the CCD camera system, individual gels were precisely positioned on a black glass tray (CAS 65997-17-3), residing on a size-matched, elevated aluminium platform (Fig. 1B). All interior surfaces of the steel cabinet are powder-coated with a non-fluorescent, highly light-absorbing material. The optical field and focus plane were manually adjusted

Table 1. Results of the DeCyder 2D-based image analysis

	Individual workspaces in DeCyder 2D analysis					
	"A. aromaticum" EbN1		*D. toluolica* Tol2		*P. inhibens* DSM 17395	
	Laser scanner	CCD camera	Laser scanner	CCD camera	Laser scanner	CCD camera
No. of detected spots[a)]						
By automatic detection	1888 ± 165	1660 ± 89	1885 ± 7	1607 ± 62	1871 ± 66	1616 ± 107
After manual refinement	1895 ± 146	1701 ± 70	1879 ± 10	1624 ± 51	1863 ± 62	1631 ± 101
No. of matched spots	1053	970	1067	993	1014	914
No. of congruent spots analysed per bacterial strain (793 in total)[b)]	269		296		228	
Average ratio ≥1.5	148	131	128	113	100	108
Average ratio <1.5	82	97	101	114	63	82
Average ratio ≤−1.5	39	41	67	69	65	38

2D DIGE gels from three different environmental bacteria were digitalised with the CCD camera system as well as laser scanner, and the acquired images were analysed in independent workspaces.
[a)] Four 2D DIGE gels (biological replicates) were analysed per bacterial strain. Manual refinement was mandatory to correct for differences in spot detection and matching errors generated by DeCyder 2D (for examples see Supporting Information Fig. S8).
[b)] Selected number of congruent protein spots that were unambiguously matched in the two different workspaces per bacterial strain.

at the camera (HR3200; Intas) equipped with a true 16-bit full frame CCD image sensor (KAF 3200 ME; ON Semiconductor, Phoenix, AZ, USA). The ChemoStar software (version 0.3.34; Intas) was used to select the dye-specific active LED module-excitation filter pair (Intas) and the corresponding emission filter (Intas), adjust the exposure time and capture the images. Each fluorophore-specific LED module contains two LED arrays, which are positioned on opposite sides and equipped with five high-power LEDs each. Any position bias due to the spatial configuration of fluorophore-specific LED modules was tested and could be excluded (Supporting Information Fig. S1). Specific excitation was achieved with the following filters: 470 nm (bandpass 30 nm) for Cy2, 525 nm (bandpass 20 nm) for Cy3, and 628 nm (bandpass 35 nm) for Cy5. The emission filters were 535 nm (bandpass 43 nm) for Cy2, 607 nm (bandpass 36 nm) for Cy3 and 716 nm (bandpass 40 nm) for Cy5. The exposure time was manually adjusted to achieve grey scale values just below saturation for the most abundant protein spots. Experimental exposure times ranged from 8 to 19 s for Cy2, 38–60 s for Cy3 and 7–80 s for Cy5 for the three bacterial strains investigated. Variance in exposure times for the three CyDyes was due to differences in the light output of the light sources, different transmission factor and opening-width of the fluorescence filters, as well as non-homogenous quantum efficiency of the camera at different wavelengths. Replicate 2D DIGE gels were recorded for each bacterial strain with the same or highly similar settings. Each gel image (65 536 digital grey scale values per colour channel) was saved as independent TIFF file (size ~3.8 MB).

For the Typhoon 9400 laser scanner, the relevant detection parameters were as follows. The lasers were blue (488 nm) for Cy2, green (532 nm) for Cy3, and red (633 nm) for Cy5. The emission filters were 520 nm (bandpass 40 nm) for Cy2, 580 nm (bandpass 30 nm) for Cy3, and 670 nm (bandpass 30 nm) for Cy5. The focal plane was fixed within the gel (at +3 mm). Preliminary scans with a pixel size of 1000 μm resolution were performed for every laser and gel to adjust the PMT voltage (475–580 V) in order to avoid signal saturation in the main scans performed at 100 μm resolution. Signal intensities of each gel image were measured with ImageQuant software (version 5.2; GE Healthcare). Each gel image was saved as independent GEL file (size ~11.7 MB).

Spectral cross-contamination of fluorophore emission with the CCD camera system was determined on the basis of 1DE-separated protein bands and 2DE-separated protein spots (see Supporting Information Table S1 and Fig. S2 for results and experimental details). Irrespective of the protein sample and separation method (1DE or 2DE), spectral cross-contamination of other colour channels (blue, green or red) by non-specific fluorophore emission signals was low (0.5–2.3% in 1DE gels; Supporting Information Table S1), with the highest cross-contamination observed for the Cy3-specific signal in the Cy2 channel (Supporting Information Table S1, Fig. S2). The linear range and limit of detection of the CCD camera system were determined with fluorophore-labelled proteins in 1DE gels. Both properties are comparable to the laser scanner, with the CCD camera system covering a linear range of at least four orders of magnitude (see Supporting Information Fig. S3 for details) and detecting <1.0 ng of CyDye-labelled protein (see Supporting Information Fig. S4 for details).

Strain-specific sets of four 2D DIGE gels were analysed with DeCyder 2D software in two independent workspaces: one for the laser scanner, the other for the CCD camera system. Following spot detection, spots with a volume of <30,000 (non-proteinaceous signals) were excluded from analysis. Then, spot matching was manually controlled and, if necessary, corrected to ensure optimal spot calling per workspace (see Table 1). Finally, congruent spots in the two workspaces (per strain) had to be manually assigned, as the software output labelled them with different spot numbers. This

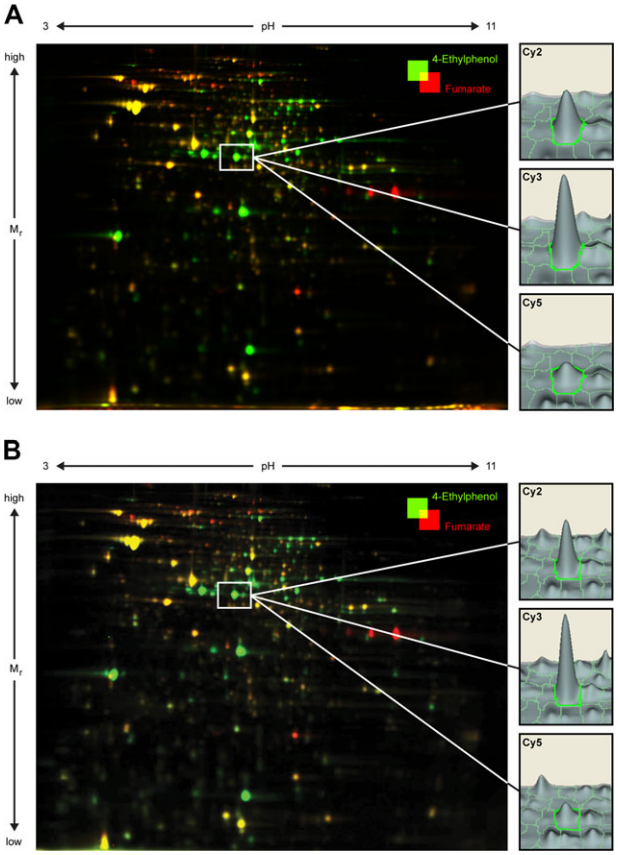

Figure 2. Representative false-colour 2DE gel images (overlay of Cy3 and Cy5 signals at 96 dpi resolution) prepared with protein extracts from "*A. aromaticum*" EbN1. The same 2D DIGE gel was digitalised with the (A) laser scanner and (B) CCD camera system. The right panel displays comparative 3D views of a selected protein spot in the DeCyder 2D software (for Cy2, Cy3 and Cy5 images). Slight differences in 3D spot views of the same spot between (A) and (B) could not be avoided due to the automatic scaling of the spot environment by DeCyder 2D. Respective gel images for strains Tol2 and DSM 17395 are provided in Supporting Information Figs. S5 and S6. Comparative 3D views of low abundant protein spots (i.e. having <5% of the volume observed for the most abundant spot) from all three bacterial strains are presented in Supporting Information Fig. S7.

procedure allowed direct comparison of congruent spots, i.e. shape, boundary, volume and average ratio.

The DeCyder 2D-based image analysis revealed highly similar spot patterns and 3D spot representations between the two compared digitalisation systems (Fig. 2; Supporting Information Figs. S5–S7). For strain EbN1, 1701 ± 70 protein spots were detected in images recorded with the CCD camera, 970 of which were matched in all gels of the workspace (Table 1). In comparison, upon digitalisation with the laser scanner, in the same set of images 1895 ± 146 protein spots were detected, from which 1053 were matched in all gels. A similar number of spots was detected for strains Tol2 and DSM 17395. Again, more spots were detected and matched in laser scanner-digitalised images (Table 1). Overall, the CCD camera system recovered between 86–90% of the protein spots detected in images acquired by the laser scanner. Inspection of spots exclusively detected with the laser scanner suggested that the higher number of spots may derive from the DeCyder 2D inherent procedure of spot detection. In this case, spot detection typically resulted in multiple fragmentations of larger spots or their shoulders (Supporting Information Fig. S8), thus increasing the total number of spots. On the contrary, resolution of the CCD camera system was lower, and single spots were only rarely divided into multiple ones. In addition, two or more individual neighbouring protein spots were more often merged automatically into a single one (Supporting Information Fig. S8). Automatic spot detection could therefore be biased due to the different resolution and sensitivity of the applied digitalisation systems (Fig. 1).

The biologically meaningful output of any 2D DIGE analysis is the average ratio, representing a normalised and statistically confident change in protein abundance between the test and reference state. Analysis of 793 protein spots congruent in both digitalisation systems (Table 1) revealed similar spot volumes (Supporting Information Fig. S9) and derived average ratios (Fig. 3). For 676 protein spots (out of the 793; black dots in Fig. 3), the average ratio was similar for both digitalisation systems, whereas for 117 of them disparate values were observed. From these 117 protein spots, the average ratio of 62 deviated only marginally ($<|0.3|$), whereas 55 showed also larger deviations ($>|0.3|$). The latter fraction included small and large protein spots, as well as ones with unchanged ($<|1.5|$; 14 spots) and significantly changed ($\geq|1.5|$; 41 spots) abundances. The positions of protein spots with deviating average ratios were scattered across the whole gel and differed for the three bacterial strains (Fig. 3), thus excluding position effects due to suboptimal illumination by the CCD camera system. All protein spots with differences in the average ratio were again manually re-assessed, revealing that in most cases automatically generated spot boundaries were not identical for the same spot (e.g., more narrow or wide, shoulders from neighbouring spots included or not) (Supporting Information Fig. S8).

In conclusion, this study demonstrates CCD camera-based digitalisation as a promising alternative to the long established laser scanner systems. Bearing in mind that purchase and maintenance costs of the former are considerably lower and costs for fluorescent dyes are strongly decreasing, one may envision a renaissance of 2D DIGE in the coming years. In particular, hypothesis-driven life science laboratories interested in targetedly applying proteomics may now get access to affordable and robust set-ups to benefit from the advantages of the 2D DIGE approach, originally conceived by Unlü et al. [8]. Casually spoken, the described recent methodological development may contribute to "bringing 2D DIGE to the people".

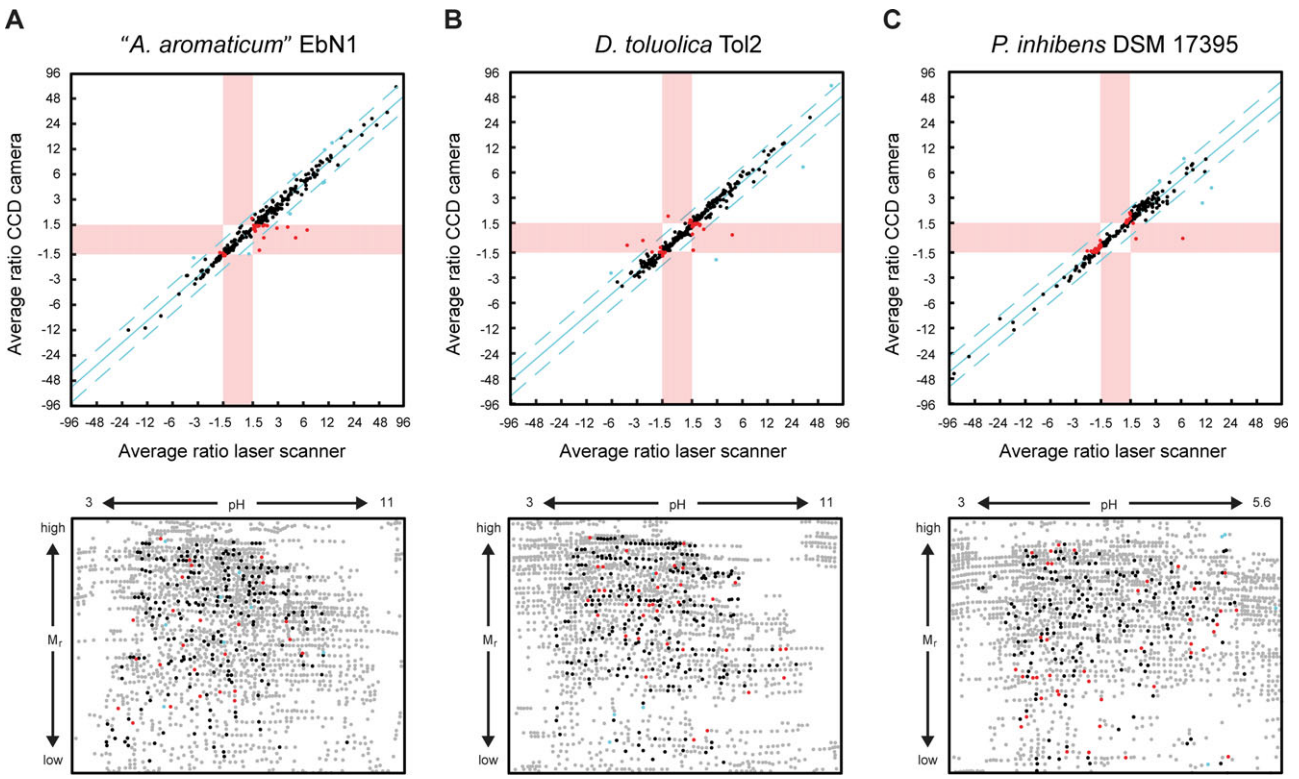

Figure 3. Comparison of 2D DIGE results (average ratio) obtained with the laser scanner or CCD camera for (A) "*A. aromaticum*" EbN1, (B) *D. toluolica* Tol2, and (C) *P. inhibens* DSM 17395. *Upper panel*: Scatter plots comparing the congruence between the average ratios for identical spots digitalised by the laser scanner or CCD camera. The regression line (solid) and 95% confidence intervals (dashed) are displayed in blue. Colour coding of dots: black, protein spots with similar fold changes (676); blue, protein spots located outside the 95% confidence interval (16); red, protein spots with divergent abundance changes (101), i.e. significant (≥|1.5|) in one system and insignificant (<|1.5|) in the other system. The red area indicates this threshold of significance for protein abundance changes [7]. *Lower panel*: Position of congruent (black, blue and red dots) and other detected protein spots (grey dots) in the 2D DIGE gel (synthetic). Comparison of corresponding normalised spot volumes (raw data) for all analysed protein spots is presented in Supporting Information Fig. S9.

The authors are grateful to Christina Hinrichs (Oldenburg) for cultivation and sample preparation. This study was supported by the Deutsche Forschungsgemeinschaft (SPP 1319; SFB TRR 51).

The authors have declared no conflict of interest.

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