IMAGING

Flat Fielding

with VersaDoc™ Imaging Systems

- **VersaDoc systems correct inherent nonuniformity when imaging samples**
- **Unique flat fielding technology compensates for nonuniformities of both light source and lens**
- **Superior dynamic correction method works for both UV and white light imaging**
- **Variability is 30-fold less than competitor's system, with a 1.1% coefficient of variation**

The VersaDoc Imaging Systems Eliminate Nonuniformities with Patent-Pending Flat Fielding Technology

Introduction

Bio-Rad has developed a method of dynamic correction for image nonuniformity that is superior to traditional static image correction. Several sources of nonuniformity are inherent in all imaging systems. These include the detector, the lens, and the light source. By careful design and manufacturing, the sources of nonuniformity can be minimized but not eliminated. To further improve the ability of an imaging system to generate an accurate and quantifiable image, software can be used to correct for the physical limitations of the instrument.

Lenses are typically positioned over the center of the sample to be imaged and therefore must capture light that originates at different distances from the lens. This positioning leads to different light intensities collected from the center to the periphery of the image. The differences in intensity are most pronounced when the field of view is large. Intensity differences can be compensated for mathematically but the correction values change with the aperture, focal plane, and zoom position of the lens. Therefore, using a single correction factor is an impractical solution and a dynamic means of generating a correction factor is preferable.

Lens correction does not account for illumination variables. Light sources emit light of variable intensity along the length of the bulb, and the intensity also decreases with distance from the bulb. Reflections from within or around the light source are further sources of variability. A scanning light source, such as a flat-bed scanner, can correct for this problem along the scanning direction, but nonuniform intensity along the axis of the bulb is still present.

Bio-Rad has introduced the family of VersaDoc imaging systems, which has addressed the issues of nonuniformity in digital image acquisition. The design of these systems minimizes introduction of nonuniformity through the lens by employing high-efficiency F-mount lenses. The transmitted light source has a unique design that utilizes only reflected light from the bulbs in order to generate a particularly uniform field of illumination. Finally, to remove the remaining hardware-related nonuniformity, the VersaDoc imaging systems use a reference illumination plate and acquisition software to mathematically adjust the acquired sample image to generate a virtually flat field of intensity.

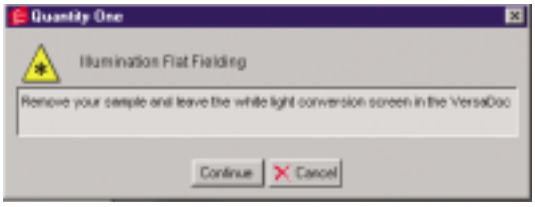


Fig. 1. VersaDoc acquisition window in Quantity One software.

Illumination Flat Fielding Technology Corrects Lens and Light Source Nonuniformities

The VersaDoc imaging systems use a dynamic correction file to account for image nonuniformity rather than using a static correction factor, which is typically employed. The Bio-Rad method corrects for both lens and light source nonuniformity since the correction factor is generated with lens and lighting conditions identical to the sample image. After capturing an image of a sample, the acquisition window in Quantity One® software prompts the researcher to place a reference plate on the transilluminator in order to acquire a reference image (Figure 1). The reference plate is a material that serves as an approximation of a uniform field of light emitted from a typical sample matrix. An image generated from the reference plate using the current light source

BIO-RAD

and lens settings in the VersaDoc instrument will record any nonuniformities caused by the system. Software analyzes the reference image and quantifies any deviation from the theoretical homogeneous light intensity for every pixel. Any nonuniformity caused by the physical limitations of the instrument is then corrected in each pixel of the sample image to produce a flat field.

Methods

Imaging was done on VersaDoc Model 1000 and 3000 imaging systems (catalog #170-8010 and 170-8030) and an Alpha Innotech FluorChem 8000 system. Data were analyzed with Quantity One software version 4.2.3 (catalog #170-8601) and Microsoft Excel 97.

Results

The Fluorescent Reference Plate Emits a Uniform Field of Light Intensity

Illumination flat fielding of an image assumes that the reference image is generated from a known material that emits a uniform field of light. The assumptions were verified for a VersaDoc imaging system by analyzing the uniformity of images from the fluorescent reference plates in two orientations that differed by 90º. Both images showed virtually identical uniformity at each point in the image. A similar experiment performed with the white light conversion screen, which is placed on the UV transilluminator to convert UV to visible light, resulted in low variability as well (data not shown). These results indicate that the fluorescent reference plate and white light conversion screen generate a uniform light intensity regardless of orientation, and therefore are excellent reference materials to use for illumination flat fielding that approximates experimental samples.

The reference plate should be handled and stored with care.

The VersaDoc Imaging System Has Superior Uniformity Compared to the Competition

The nonuniformity of the VersaDoc imaging system is low even before flat field correction, with a coefficient of variance (CV) of approximately 11% (Figure 2A). The low nonuniformity is a result of the high-quality F-mount optics and the unique design of the transmitted light source. In contrast, the image nonuniformity of the Alpha Innotech FluorChem 8000 system, a representative competitor system, is far greater than that of the VersaDoc, with a CV of 32% over the entire image area (Figure 2B).

The variability in the FluorChem system is most pronounced along the horizontal axis (Figure 2C, pink line), showing a dramatic reduction in intensity with increasing distance from the center of the image. At the most extreme, the intensity deviates by 80% from the average. Without using illumination flat fielding, the VersaDoc also shows some variability along this axis, but deviates from the average by only 10% at most (Figure 2C, blue line). Both systems show better uniformity along the vertical axis, but again the VersaDoc exhibits smaller deviations from the average (Figure 2D).

Illumination Flat Fielding Improves Image Uniformity 10-Fold

A 10-fold reduction in the coefficient of variance, to approximately 1%, occurs after applying Bio-Rad's illumination flat fielding algorithm to the platen image (Figure 3A). This is greater than a 30-fold reduction in variability compared to the same image acquired by the Alpha Innotech imaging system (Figure 3B).

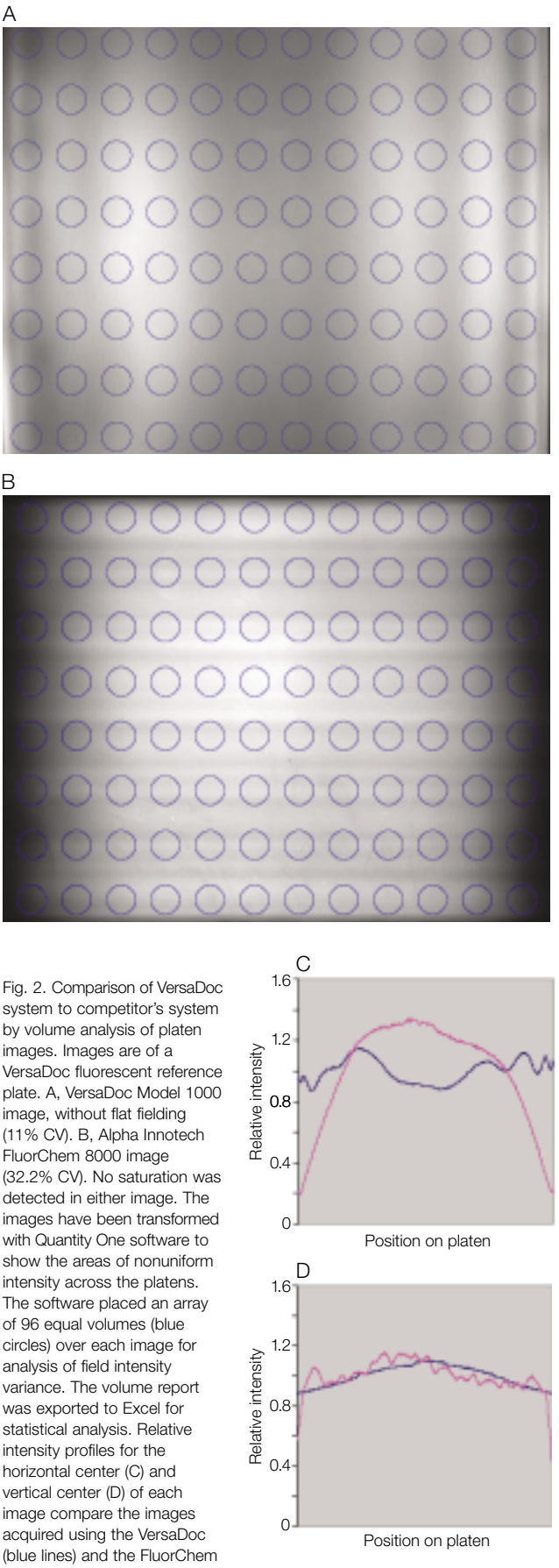
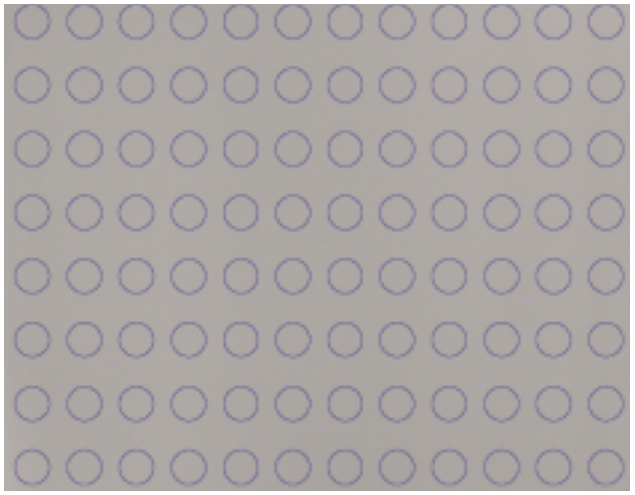
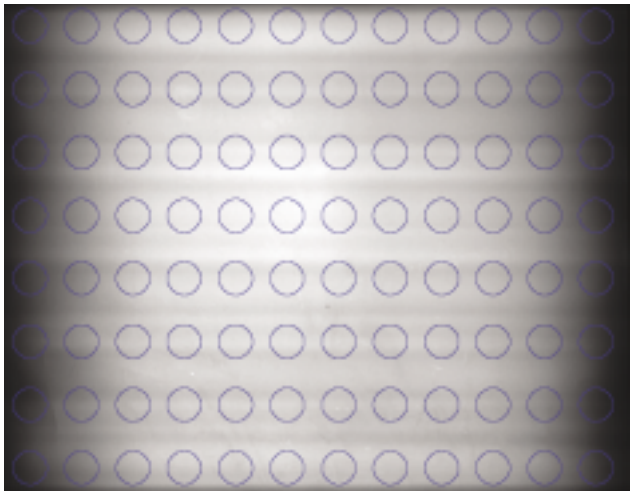


Fig. 2. Comparison of VersaDoc system to competitor's system by volume analysis of platen images. Images are of a VersaDoc fluorescent reference plate. A, VersaDoc Model 1000 image, without flat fielding (11% CV). B, Alpha Innotech FluorChem 8000 image (32.2% CV). No saturation was detected in either image. The images have been transformed with Quantity One software to show the areas of nonuniform intensity across the platens. The software placed an array of 96 equal volumes (blue circles) over each image for analysis of field intensity variance. The volume report was exported to Excel for statistical analysis. Relative intensity profiles for the horizontal center (C) and vertical center (D) of each image compare the images acquired using the VersaDoc (blue lines) and the FluorChem (pink lines).

A



B



C

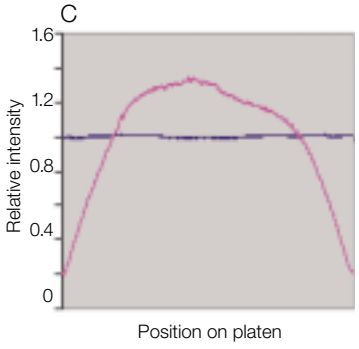


Fig. 3. Comparison of VersaDoc system with flat fielding correction to competitor's system by volume analysis of platen images. A, VersaDoc imaging system; the VersaDoc achieves a 1.1% CV with illumination flat fielding. The reference plate imaged in Figure 2B was left in place and imaged a second time using the flat fielding option. The same 96-volume array was copied from the initial uncorrected image to maintain exact pixel registration. B, reacquisition of the same image with the Alpha Innotech FluorChem imaging system, with a 32% CV. C, a horizontal lane profile across the center of the images, which compares the image intensities acquired using the VersaDoc (blue line) and the FluorChem (pink line).

Comparing the horizontal intensity profile in Figure 3C to the corresponding profile in Figure 2C shows the extent of improvement by the illumination flat fielding algorithm. The entire length of the profile is now virtually homogeneous.

Images Are Visibly Improved by Illumination Flat Fielding

Not only does a VersaDoc with image correction give better quantitative analysis, it also generates a better visual image. This is best demonstrated using a large area of the platen, which shows the extremes of the imaging system hardware nonuniformities. As seen in Figure 4, areas that would have displayed nonuniform background and sample staining due to illumination and lens limitations without correction become very uniform when illumination flat fielding is applied to the image.

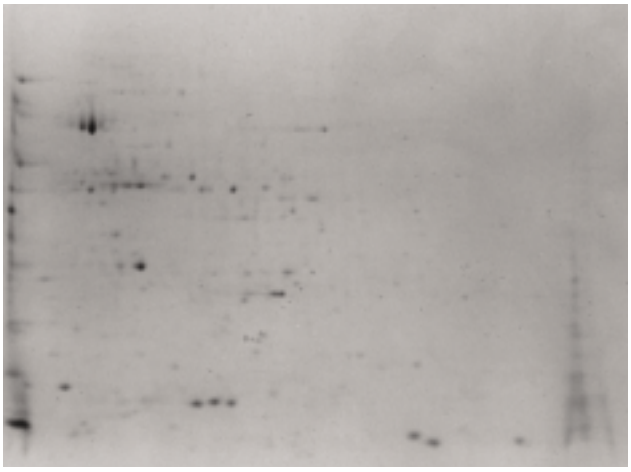


Fig. 4. VersaDoc Model 3000 white light image of a 20 cm 2-D gel using illumination flat fielding. *Pseudomonas putida* extract was focused on a pH 4–7 ReadyStrip™ IPG strip, then separated on a 20 x 20 cm Tris-HCl 8–16% gradient polyacrylamide gel. The gel was stained with Bio-Safe™ Coomassie stain. The destained wet gel was imaged on the white light conversion screen. Images were transformed to display the intensity levels for data distributed over 256 gray levels. Sample courtesy of Michael Kertesz, University of Manchester, UK.

Conclusion

Illumination flat fielding is a mathematical adjustment of the intensity of each pixel in an image to accurately represent the data as it would appear if imaged using optimal hardware. In reality, no hardware is perfect, so use of a dynamic correction factor allows quantitative data to be more accurately recorded by digital images.

Both the fluorescent reference plate and the white light conversion screen used to generate a reference image for illumination flat fielding emit uniform light regardless of orientation on the platen. However, these materials must be undamaged to avoid introduction of error into the corrected image. Even though illumination flat fielding can compensate for intensity variations across a given image, it cannot compensate for differences in intensities between two independent images, regardless of whether the same lens settings and light source are used. Therefore, each gel being imaged needs to have internal standards for reference to allow quantitation between images.

With Bio-Rad's patent-pending illumination flat fielding technology, you can remove a significant variable in the use of digital image acquisition to quantitate biological samples. Direct comparison of data generated from a FluorChem imaging system and a VersaDoc show that variability of image intensity, already improved about 3-fold with VersaDoc hardware, is improved approximately 30-fold when illumination flat fielding is used.

The tested FluorChem system is typical of most available systems that utilize C-mount lenses, which exhibit a high amount of nonuniformity. Therefore, the results shown here are expected to approximate competitor systems in general. By utilizing an F-mount lens, a uniquely designed transmitted light source, and dynamic flat field correction, VersaDoc imaging systems generate an image that is quantitatively and visually better than the competition.

Ordering Information

Catalog #	Description
170-8010	VersaDoc Imaging System Model 1000, PC
170-8011	VersaDoc Imaging System Model 1000, Mac
170-8030	VersaDoc Imaging System Model 3000, PC
170-8031	VersaDoc Imaging System Model 3000, Mac
170-8050	VersaDoc Imaging System Model 5000, PC
170-8051	VersaDoc Imaging System Model 5000, Mac

Coomassie is a trademark of Imperial Chemical Industries, PLC. Excel is a trademark of Microsoft Corp. Mac is a trademark of Apple Computer.

The ProteomeWorks System™

The ProteomeWorks system is the global alliance between Bio-Rad Laboratories, Inc. and Micromass-Waters, Ltd., dedicated to furthering proteomics research.

BIO-RAD

Bio-Rad Laboratories, Inc.

Life Science Group

Web site *www.bio-rad.com* ***USA*** *(800) 4BIORAD* ***Australia*** *02 9914 2800* ***Austria*** *(01)-877 89 01* ***Belgium*** *09-385 55 11* ***Brazil*** *55 21 507 6191* ***Canada*** *(905) 712-2771* ***China*** *(86-21) 63052255* ***Czech Republic*** *(420) 2-4141 0532* ***Denmark*** *45 44 52-1000* ***Finland*** *358 (0)9 804 2200* ***France*** *01 47 95 69 65* ***Germany*** *089 318 84-177* ***Hong Kong*** *852-2789-3300* ***India*** *(91-124) 6398112/113/114, 6450092/93* ***Israel*** *03 951 4127* ***Italy*** *39 02 216091* ***Japan*** *03-5811-6270* ***Korea*** *82-2-3473-4460* ***Latin America*** *305-894-5950* ***Mexico*** *52 5 534 2552 to 54* ***The Netherlands*** *0318-540666* ***New Zealand*** *64-9-4152280* ***Norway*** *47-23-38-41-30* ***Poland*** *(48) 22-8126 672* ***Portugal*** *351-21-472-7700* ***Russia*** *7 095 721 1404* ***Singapore*** *65-2729877* ***South Africa*** *00 27 11 4428508* ***Spain*** *590 5200* ***Sweden*** *46 (0)8-55 51 27 00* ***Switzerland*** *061 717-9555* ***United Kingdom*** *0800-181134*

Bulletin 2753 US/EG Rev A *01-719 0502 Sig 0402*