Research

Sources of nonlinearity in cDNA microarray expression measurements

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Abstract

Background: A key assumption in the analysis of microarray data is that the quantified signal intensities are linearly related to the expression levels of the corresponding genes. To test this assumption, we experimentally examined the relationship between signal and expression for the two types of microarrays we most commonly encounter: radioactively labeled cDNAs on nylon membranes and fluorescently labeled cDNAs on glass slides.

Results: We uncovered two sources of nonlinearity. The first, which led to discrepancies in analysis affecting the fluorescent signals, was signal quenching associated with excessive dye concentrations. The second, affecting the radioactive signals, was a nonlinear transformation of the raw data introduced by the scanner. Correction for this transformation was made by some, but not all, image-quantification software packages.

Conclusions: The second type of nonlinearity is more troublesome, because it could not have been predicted *a priori*. Both types of nonlinearities were detected by simple dilution series, which we recommend as a quality-control step.

Background

DNA microarray technology allows the simultaneous analyses of thousands of genes [1-3]. There are two major platforms for cDNA microarrays: membrane-based arrays (porous surfaces like nylon) and chemically coated glass-based arrays. In both cases, thousands of cDNA fragments are robotically deposited on the substrate. The nylon membrane microarrays are hybridized with ^{32}P or ^{33}P-labeled cDNA targets, and microarrays on glass are hybridized with fluorescent dye-labeled cDNA targets. After hybridization, the radioactive or fluorescent signal intensities are measured using a phosphorimager or laser scanner, respectively. The signal intensities are surrogates for the expression levels of the genes in the samples under testing and are used to make biological inferences.

A key assumption in the analysis of microarray data is that the quantified signal intensities are linearly related to the expression of the corresponding genes in the target sample. We experimentally examined this relationship. Our investigations

uncovered two sources of nonlinearity: signal quenching and a nonlinear (square-root) transformation of the raw data introduced by the scanner. Users presented with the same image but using different software packages may arrive at quite different conclusions about levels of differential expression. In both cases, the nonlinearities were revealed by serial dilution experiments. Given the lack of an absolute scale for microarray measurements, we recommend serial dilution experiments as a quality-control step.

Results

Measurement of fluorescent signals from glass-based microarrays

To assess response linearity on glass slides, we designed two dilution experiments. In the first, a set of serially diluted (factor of two) Cy3-labeled oligonucleotide samples ranging from 0.4-0.003 μg/μl was arrayed on a slide in a 2 x 5 grid of 8 x 8 patches. Each row within a patch was a serial dilution; each patch contained eight replicates of the dilution. The slide was scanned with a laser scanner, and the image obtained (Figure 1a) was analyzed using the ArrayVision quantification software. If spot intensity is linearly related to the amount of labeled cDNA, then a plot of the log (base 2) background-corrected signal intensity as a function of the serial dilution steps should have a slope of -1.0. However, at this concentration range, a slope of -0.46 was observed (Figure 1b). This means that when the concentration was halved, the intensity was consistently decreased by some factor less than one half. The total drop (across eight dilution levels) in concentration was 128-fold; the total drop in observed intensity was roughly nine-fold. This result is not surprising because fluorescence quenching is known to play a major role when the fluorescent material is present at such high concentrations. Quenching occurs when large numbers of fluorophores are highly concentrated so that photons emitted by one molecule can be reabsorbed by another molecule, thus artificially decreasing the detected signal [4].

A second experiment was designed using both Cy3- and Cy5-labeled oligonucleotides spotted in a much lower concentration range: from 0.01 to 0.000078 μg/ml. Signals in this range were detected using scanning parameters similar to those we normally use for hybridization experiments. The two images are shown in Figure 2. The images (with ten separate patches and eight replicate sets in each patch representing 80 replicates) were quantified with ArrayVision and plotted against the dilution steps. The average slope of the mean line for the Cy5-labeled oligonucleotide was -0.96 and -0.87 for Cy3-labeled oligonucleotide (Figure 3). A linear relationship is seen for both Cy3 and Cy5 in this concentration range, and the slopes are close to the expected value of -1.0. The lack of perfect correlation to the actual signal intensity may be a result of quenching, and Cy3 may have more quenching effect than Cy5.

Measurement of radioactive signals on a membrane array

To assess response linearity on nylon, we carried out a dilution experiment where a serially diluted known amount of ^{32}P-ATP was spotted on a nylon membrane. After being exposed to a phosphorimager screen and measured by a STORM PhosphorImager, a GEL image file (Figure 4a) was produced and the signals analyzed using the ImageQuant analysis software (Molecular Dynamics, Sunnyvale, CA). As expected, the signals were linearly related to the powers of ½ that is, to 1, ½, ¼, and so on (Figure 5a). This result indicates that the ImageQuant software gives accurate readings of the signals from a GEL image file. However, ImageQuant was not designed to handle high-density microarray images that contain closely spaced spots. Thus, microarray images produced by the STORM PhosphorImager are often quantified using other software. We requantified our image file using two commercial software packages: GLEAMS and ArrayVision.

The membrane had been scanned by the STORM PhosphorImager at a 45° angle (not by design). Because neither GLEAMS nor ArrayVision cope well with microarray images at this angle, we loaded the GEL file into ImageJ [5], an image-editing program available from the National Institutes of Health (NIH). We rotated and cropped the image, and saved it as a Tagged Image Format File (TIFF) (Figure 4b) which was loaded into both commercial software packages. The results from both packages indicated that the signal intensities were proportional to the square root of the true concentrations (Figure 5), in disagreement with both theory and the ImageQuant results. In fact, the pixel-by-pixel intensity data are square-root-transformed before being saved as a GEL file. When an image-editing program (such as ImageJ) processes these data, tags describing this transformation are not preserved in the resulting TIFF file.

To determine whether this square-root transformation could affect the results of a microarray experiment, we performed a hybridization experiment using a Research Genetics GF200 GeneFilter. Messenger RNA extracted from a GA-10 Burkitt lymphoma cell line was radioactively labeled with ^{33}P during reverse transcription, hybridized to the GF 200 GeneFilter, and exposed to a Molecular Dynamics STORM PhosphorImager. The image was saved as a GEL file, which was loaded directly into both GLEAMS and ArrayVision, without transforming it to a TIFF file. Each package quantified the mean intensity and the local background intensity at each spot and the results were compared graphically (Figure 6). In each subpanel, the horizontal axis is the intensity reported by ArrayVision and the vertical axis is the result reported by GLEAMS. The most striking feature of Figure 6 is that the most reliably measured spots - the spots where both software packages identify a gene that is expressed at high level - are not linearly related. The nonlinear relationship between the results was estimated by trial

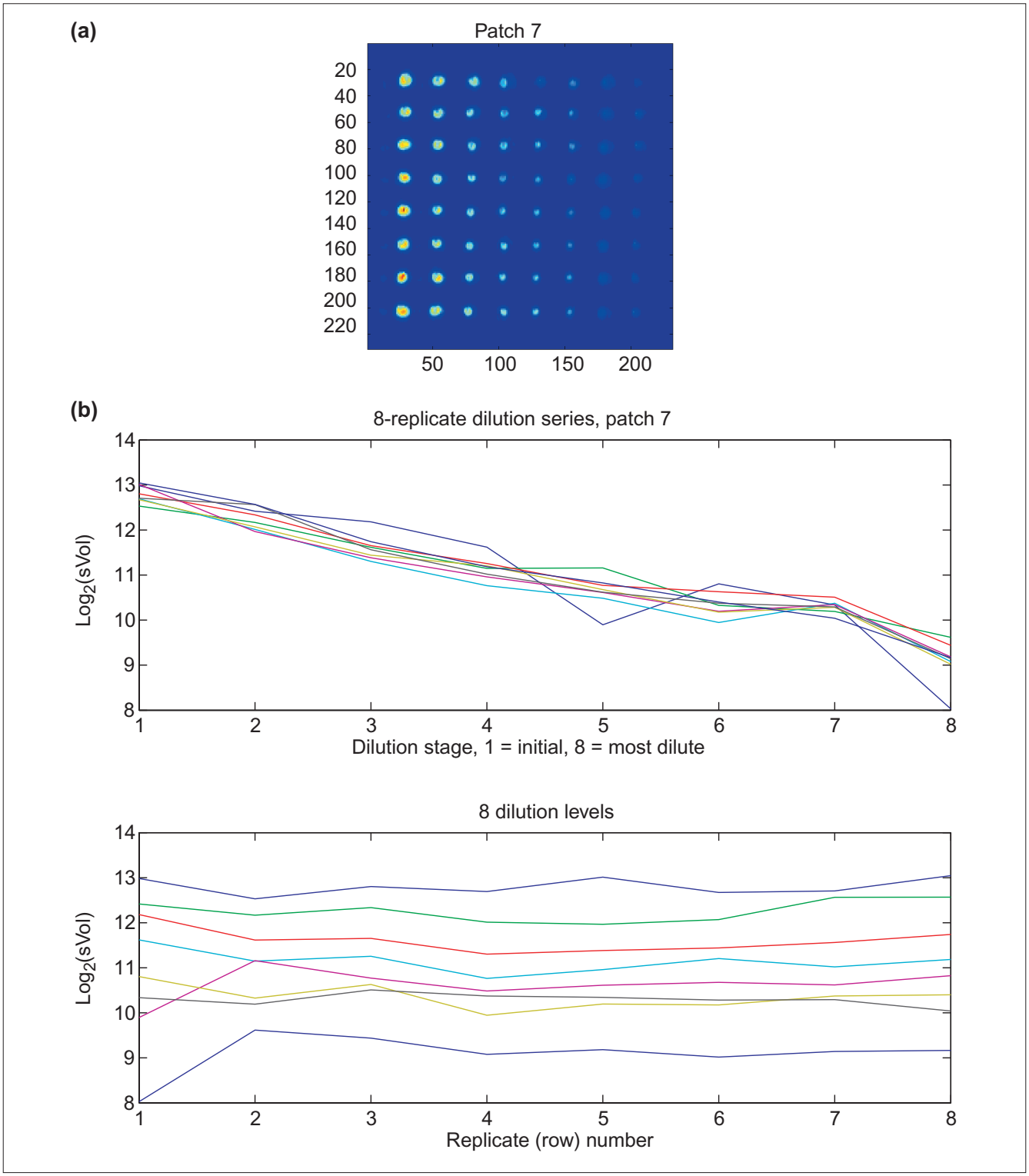

Figure 1
Dilution experiment with fluorescently labeled samples. **(a)** A patch from the first dilution experiment with Cy3-labeled oligonucleotides on glass, showing eight replicates of a serial dilution. **(b)** Log_2 background-corrected intensity values for the spots in the patch, going from left to right (dilution stages) and from top to bottom (replicates). The linear decrease shows that intensity is dropping as a power of concentration, but the slope suggests that this power is not what is expected. Note the consistency across replicates. sVol is the background-corrected volume, where volume is the density of each spot multiplied by its area, and density represents the average of all the pixels in the spot.

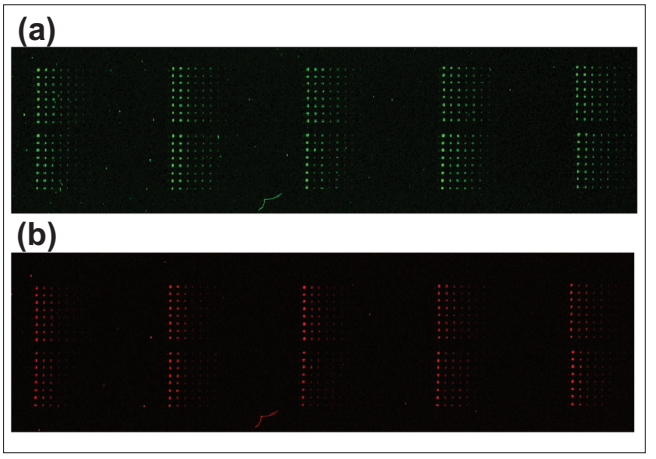

Figure 2
Fluorescence dilution experiment at a lower concentration range. The second dilution experiment on glass, with much lower concentrations of dye used. **(a)** Cy3; **(b)** Cy5. The array design is a 2 x 5 grid of 8 x 8 patches. Each row in a patch is a serial dilution; thus the serial dilution has been repeated 80 times on this glass slide. The spots are about 400 μm apart and about 200 μm in diameter. The Cy3 image was scanned at a gain of 45 and the Cy5 image at a gain of 50, as commonly done for the Cy3 and Cy5 images in our laboratory.

and error to be $y = 15\sqrt{x}$. The results reported by the two software packages followed the same curve on ten additional GEL image files (data not shown).

Discussion

This study was conducted to assess the response linearity of measurements from cDNA microarray experiments using the two most frequently used systems. The study was performed not only because of the general need for quality control, but also because of the complexity of the process of acquiring data from microarrays. Images and data are often transferred between different computer programs, and many instruments used for microarray research are new and insufficiently tested. Thus, it is rather optimistic to take the numbers generated from a series of machines and software at face value. Simple dilution experiments revealed problems that have implications for the biological interpretation of gene expression data produced from microarray experiments.

Our experiments on glass provided an assessment of the degree of signal quenching for the two fluorescent/glass microarrays. In dilute solutions fluorescence intensity is linearly proportional to the concentration with all other parameters being constant. However, in a sample with absorbance exceeding 0.05 at the emission wavelength, the relationship becomes nonlinear and the measurements are distorted (by self absorption, inner filter effect, quenching) [4,6]. Fluorescence properties of such labeled DNA probes have been studied [7,8].

Our experiments on membranes provide instances where different microarray-specific image-analysis programs were applied to the same images and produced divergent results. In each instance, at least one of the software packages produced results that were linearly related to the square root of the results produced by another package. The significance of this finding for the biological interpretation of gene expressions is very clear. Where users of software package 1 might detect, for example, a four-fold change in gene expression, users of software package 2 would see only a two-fold change. If two-fold change is set as a threshold, the same

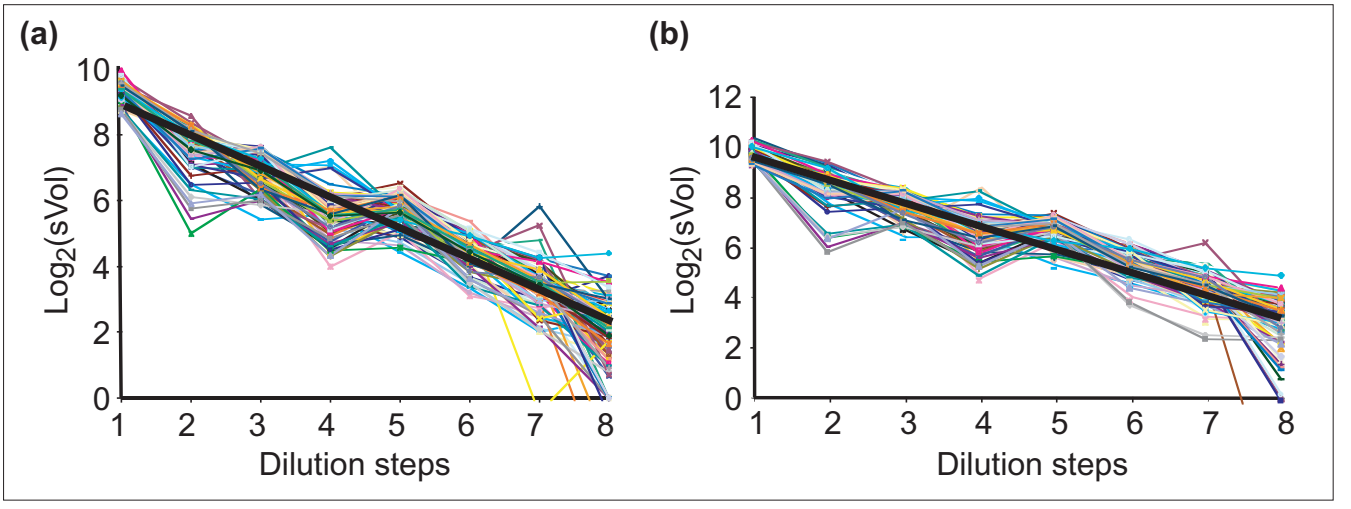

Figure 3
Log intensities plot as a function of concentration. Background-corrected log-intensities with the lower dye concentrations. **(a)** Cy3; **(b)** Cy5. In both cases, the slopes are much closer to the expected value of -1.

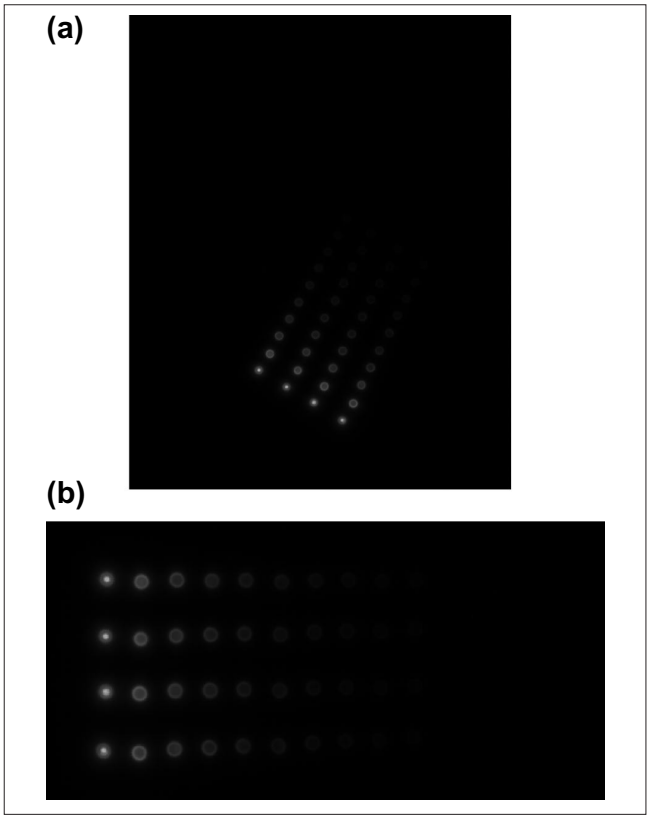

Figure 4
Dilution experiment with radiolabeled samples. TIFF images of the radioactively labeled dilution experiment **(a)** before and **(b)** after rotation and cropping.

data can be viewed as significant or insignificant, depending on which software package is used.

The explanation for the divergent results in our experiments is simple: the hardware (scanner) applied a mathematical transformation to the data before writing them to the image file. The nature of this transformation was not communicated to the software (image-quantifying program) that analyzed the data. Consequently, the software assumed (incorrectly) that the values in the file were linearly related to the original intensity levels.

In our case, the STORM PhosphorImager produced a GEL file. This file contained numerical values for each pixel, which need to be squared to exhibit the proper linear relationship. The problem lies with the fact that the internal structure of a GEL image file is essentially identical to that of a TIFF image file, so any program that can read a TIFF file can read a GEL file and even manipulate the contents as if it were a TIFF file. But, if the file is then saved as a TIFF file, its GEL file origins are lost. This leads to two scenarios for bad data. In the first scenario, a GEL file is loaded into two software packages. Software package 1 recognizes that a GEL file includes a nonlinear transformation and corrects for it. Software package 2 treats the GEL file as a TIFF file and does not correct for nonlinearity. The results from the two packages therefore disagree. In the second scenario, the GEL file is saved as a TIFF file after editing. Software package 1, which correctly dealt with a GEL file, now sees a TIFF file and applies no transformation because none is generally needed for TIFF files. Software package 2 sees a TIFF file and deals

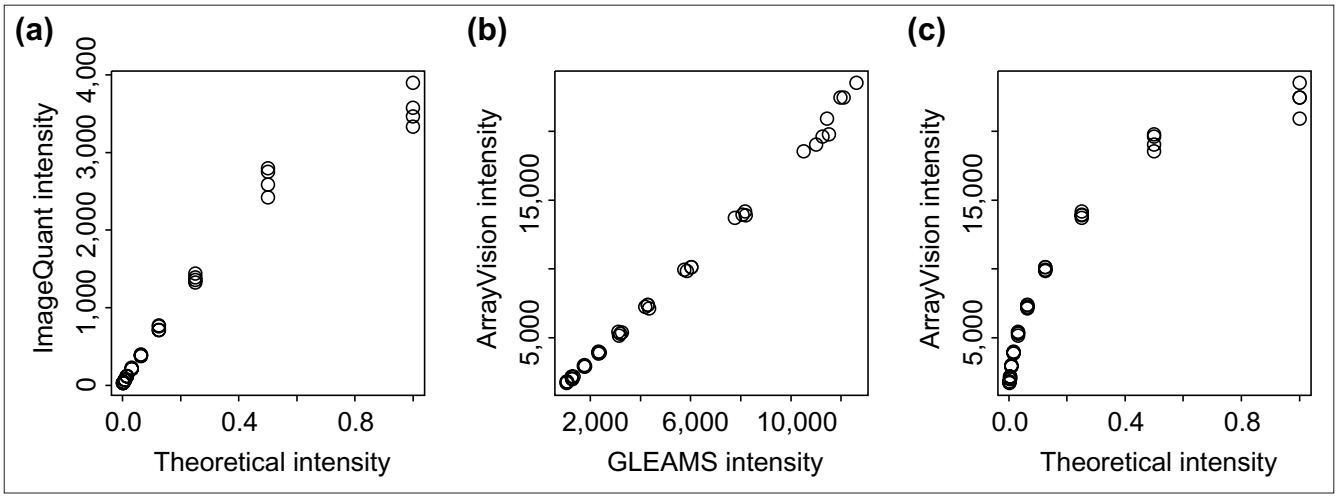

Figure 5
Comparison of software packages for image analysis. Each panel shows theoretical intensity levels (as fractions of the initial concentration in the dilution series) or measured intensity values (in arbitrary units) for the radioactively labeled dilution experiment. ImageQuant was used on the original GEL file; GLEAMS and ArrayVision were used on the TIFF file produced from the GEL file by rotation and cropping. **(a)** ImageQuant values are linearly related to the theoretical values. **(b)** The values reported by GLEAMS and ArrayVision are linearly related. **(c)** The values reported by ArrayVision are not linearly related to the theoretical values. In this case, both ArrayVision and GLEAMS provide measurements related to the square root of the theoretical values.

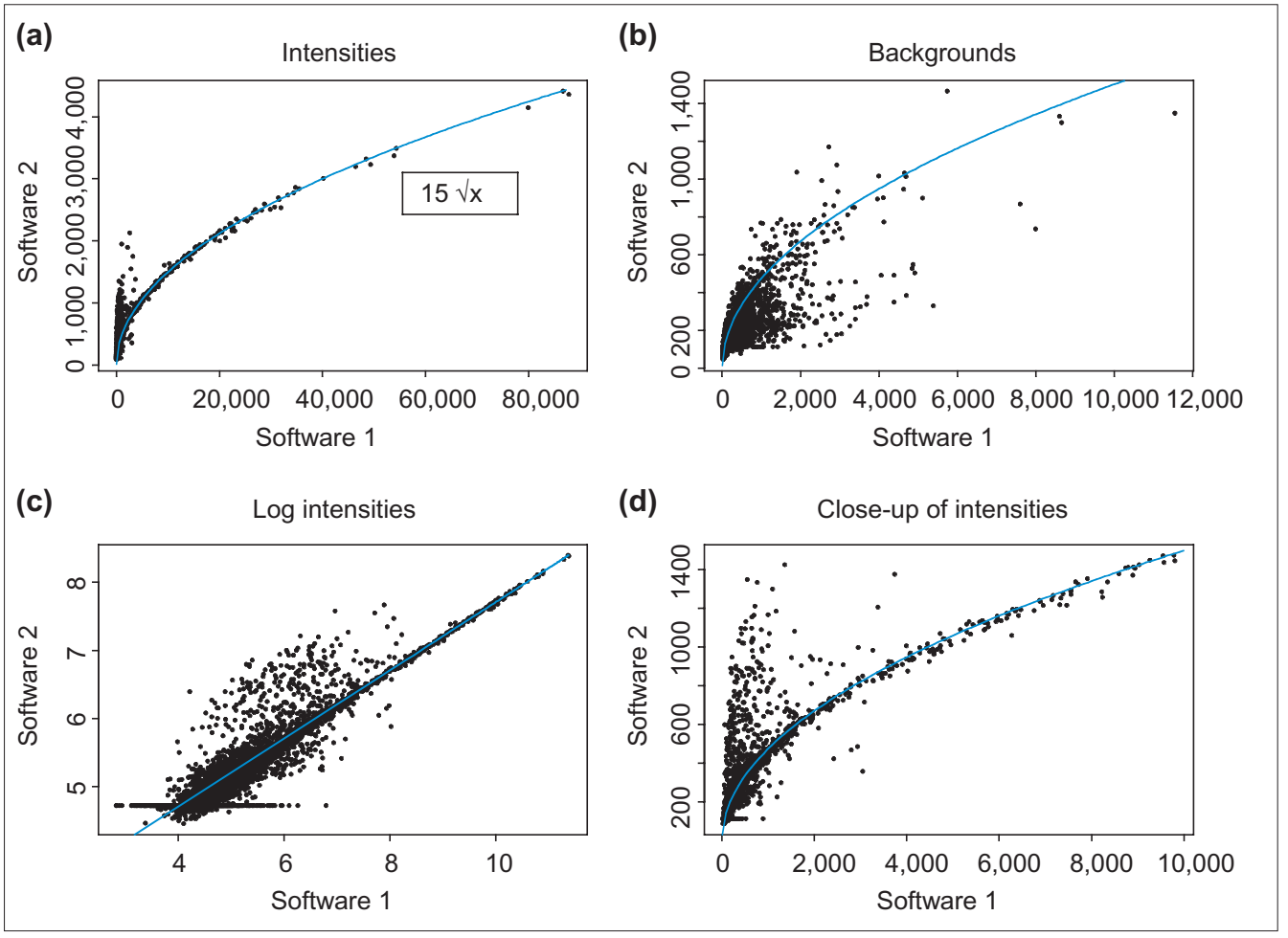

Figure 6
Comparison of signal intensity and background intensity. **(a-d)** Intensity and background values produced by software packages 1 and 2 (ArrayVision and GLEAMS respectively) were applied to the GEL file produced by the follow-up experiment with radioactive labeling. ArrayVision finds the appropriate transformation; GLEAMS does not.

with it as before. The results from the two packages now agree, but both are wrong because we have removed the information the packages need to perform correctly.

It is worth pointing out another common instance where a square-root transformation is applied to microarray data. In a two-color fluorescence experiment, the microarray is scanned twice, at different wavelengths corresponding to the different dyes used in the assay. Each scan is saved as a separate 16-bit grayscale image. It is possible to combine the two grayscale images into a single 24-bit color image, sometimes called a false-color image. One simply imports the first image into the red channel and the second image into the green channel. However, a 24-bit full color image allocates only 8 bits to each channel. In order to pack a 16-bit number representing the scanned intensity into an 8-bit space, some information must be discarded. For instance, the software operating the GenePix 4000A Microarray Scanner (Axon Instruments, Foster City, CA) provides four packing options (note that the Axon manual says that packing is a bad idea if investigators want to get numbers from the image later). The default option is to perform a square-root operation. The remaining options preserve linearity, but truncate the data, either by preserving low values, preserving high values or preserving middle values. Although it is tempting to discard the two grayscale images and save only the full-color image, doing so would unavoidably discard essential aspects of the data.

The primary data produced by a microarray experiment is the original scanned image, which is stored as a computer file. Any processing of this image file has the potential to change, lose or otherwise corrupt data. We have seen that square-root transformations are incorporated in some programs. All general-purpose image-editing programs provide multitudes of additional transformations that can be used to

brighten, sharpen or smooth images. Even though the square-root transformation appears to be the only transformation in common use among current generations of scanners, it is conceivable that other transformations may be introduced in the future.

In summary, when designing a protocol for a set of microarray experiments, researchers should perform dilution series as one of their standard calibration experiments. Processing of the array through the scanner and quantification software that will be used in the experiments can confirm that the reported results are linearly related to the known input values.

Methods

Fluorescent labeling

For the experiments on glass, cyanine 3-labeled (Cy3), cyanine 5-labeled (Cy5) [7] and unlabeled 30mer oligonucleotides were synthesized (Synthegen, Houston, TX). Plain glass slides from Fisher Scientific were coated with polylysine according to the published procedure [9]. An arrayer from Genomic Solutions (Ann Arbor, MI) was used to spot the oligonucleotides onto the treated glass slides. A 48-pin head from Genomic Solutions was used to create an array design of a 2 x 5 grid of 8 x 8 patches with a spot spacing of about 400 μm.

The slides were scanned on a GeneTac LS IV laser scanner (Genomic Solutions) with laser energy sources for measuring Cy3 and Cy5 fluorophore. Data from the dual-lasers are collected as separate TIFF files for each of the two lasers.

The images were processed using the analysis software program ArrayVision, version 5.1 (Imaging Research, Inc., St Catherine's, Ontario, Canada) and GLEAMS version 2.0 (NuTec Sciences, Houston, TX). Background-corrected intensity was determined for each element of each array.

Radioactive labeling

For the experiments on membranes, 1 μl ^{32}P- α-dATP stock solution (NEN Life Science Products, Inc., Boston, MA) was first diluted 100 times, then 5 μl of this mixture was diluted two-fold by adding 5 μl water. This process was repeated to generate a serial dilution. Next, 1 μl of each diluted sample was spotted onto a nylon membrane. After hybridization, the nylon membrane was exposed to the STORM PhosphorImager from Molecular Dynamics (Sunnyvale, CA), which produced a GEL image file. ImageQuant analysis software (Molecular Dynamics) was used to quantify the images.

For the follow-up experiment, a GF200 Human GeneFilter microarray was purchased from Research Genetics (Huntsville, AL). Total RNA was isolated from a GA-10 Burkitt lymphoma cell line (a kind gift of Aaron Rapaport, University of Maryland). Ten μg total RNA were reverse-transcribed and ^{33}P-labeled following the standard procedure. The labeled cDNAs were hybridized to the GeneFilter.

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References

1. Lockhart DJ, Winzler EA: **Genomics, gene expression and DNA arrays.** *Nature* 2000, **405:**827-836.
2. DeRisi JL, Iyer VR: **Genomics and array technology.** *Curr Opin Oncol* 1999, **11:**76-79.
3. Khan J, Saal LH, Bittner ML, Chen Y, Trent JM, Meltzer PS: **Expression profiling in cancer using cDNA microarrays.** *Electrophoresis* 1999, **20:**223-229.
4. Kubista M: **Experimental correction for the inner-filter effect in fluorescence spectra.** *Analyst* 1994, **119:**417.
5. **ImageJ** [http://rsb.info.nih.gov/ij/]
6. Cantor CR, Schimmel PR: *Biophysical Chemistry: Techniques for the Study of Biological Stucture and Function.* San Francisco: W.H. Freeman and Co; 1980.
7. Mujumdar RB, Ernst LA, Mujumdar SR, Lewis CJ, Waggoner AS: **Cyanine dye labeling reagents: sulfoindocyanine succinimidyl esters.** *Bioconjug Chem* 1993, **4:**105-111.
8. Randolph JB, Waggoner AS: **Stability, specificity and fluorescence brightness of multiply-labeled fluorescent DNA probes.** *Nucleic Acids Res* 1997, **25:**2923-2929.
9. Schena M, Shalon D, Davis RW, Brown PO: **Quantitative monitoring of gene expression patterns with a complementary DNA microarray.** *Science* 1995, **270:**467-470.