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The IVD Directive

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Understanding and evaluating fluorescent microarray imaging instruments

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Design choices of instrument subsystems affect detection limits, field uniformity, and reproducibility.

Microarray technology has greatly contributed to research in many areas including growth, aging, disease onset and progression, drug response, and healing in both plants and animals. Researchers worldwide continue to refine this technology with their sights set on gaining regulatory approval of microarray tools for clinical diagnostic use. What is especially critical is that the microarray imaging systems should exhibit the highest performance standards to facilitate the interpretation of the vast amounts of data that microarrays generate.

The basic requirements of any fluorescent microarray imaging system are to excite the fluorescent dyes on the sample, collect emitted light, and generate digital images of the fluorescent signal. Two general methods are used to acquire such images: laser excitation with a photomultiplier tube (PMT) detector, and filtered white-light excitation with a charge-coupled device (CCD) detector.[1–4] In addition, the laser-based systems can use either a confocal or nonconfocal optical path. While both of these platforms result in digital images of the microarray sample, the design choices made in building such instruments can significantly affect the overall performance of the system.

Detection limits, field uniformity, and reproducibility are the most important indicators of microarray imaging accuracy, especially in developing diagnostics where there is little room for uncertainty. The design of instrument subsystems for light delivery, light collection, and signal processing all affect the performance of these indicators.

ILLUSTRATIONS COURTESY AXON INSTRUMENTS INC.

A digital rendering of the GenePix Personal 4100A microarray scanner by Axon Instruments Inc. (Union City, CA) and the internal fluorescent imaging system.

Fluorescent Microarray Image Acquisition

Excitation Light Delivery. In a laser-based system, a single-wavelength laser beam of a few microns in diameter is scanned back and forth across a sample, exciting an area representing a single pixel at a time. Emission light travels back through the excitation lens and is collected by the PMT. The PMT amplifies the signal from each photon, which is then converted into a digital value used to create an image representing the signal intensity at each pixel position.

In a white-light system, a broad-

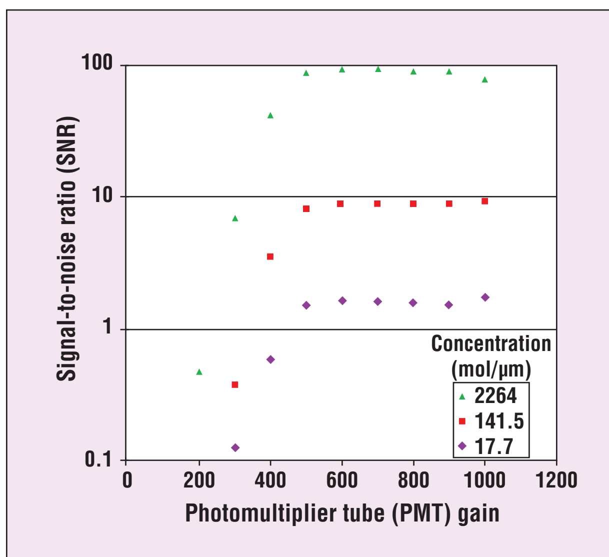

Figure 1. A sample containing spots of different concentrations of Cy3-dCTP was scanned on a GenePix 4000B microarray scanner by Axon Instruments Inc. (Union City, CA) at multiple PMT gain settings ranging from 200–1000. SNRs were calculated for three dye concentrations exhibiting low, medium, and high intensity signals.

spectrum white-light source such as a xenon or mercury lamp provides the excitation light. The excitation wavelength is selected by filtering the white light into a narrower wavelength range. The lamp illuminates a large area of the sample, and the fluorescent emission from the entire field of view is collected by a stationary CCD array. An imaging aperture is opened for varying times to allow the CCD to collect enough light from the sample to create a representative image. The signal intensity at each pixel position on the CCD array is then converted into a digital image.

Laser illumination concentrates high-power monochromatic light in a small spot at the sample surface. The higher power density delivers more light to the fluorescent molecule, therefore much less time is required to excite the dye than with filtered white light. As the laser beam scans the sample, it "dwells" on each pixel position for several microseconds. In contrast, a white-light source illuminates the sample for seconds or minutes while the CCD integrates the emission signal during the entire exposure time.

Emission Light Collection. Two important detector characteristics that contribute to overall system performance are linear range and quantum efficiency. Linear range indicates the range of input signal intensities over which the detector can accurately measure change, such that a given degree of change in input signal generates the same degree of change in output signal. The linear range of a PMT is generally specified as the difference in current between the noise floor and the highest gain setting. For example, the Hamamatsu R3896 by Hamamatsu Corp. (Bridgewater, NJ) that is used in the GenePix microarray scanners by Axon Instruments Inc. (Union City, CA) has a range of 0.01–100 µA, or four orders of magnitude. The signal output from a PMT is converted to a 16-bit digital value, which means the intensity values can be divided into 2^{16} = 65,535 steps, covering about 4.5 orders of magnitude. The magnitude of signal output from a PMT is adjusted by changing the voltage applied to it, which determines the amplification (gain) of the signal as electrons pass through the PMT.[4]

PMTs have an optimum working linear range over which the signal response is most accurate. If the gain is set below this range, the probability that each impinging photon will be converted to an electron is diminished. Above this range, noise begins to interfere with accurate signal measurement. For the Hamamatsu R3896, the optimum working range is from 500 to 1000 V. If the PMT gain is set anywhere within this range, the signal-to-noise ratio for high-, medium-, and low-intensity spots does not change (see Figure 1). A user can scan different samples at different voltages and know that any differences are due to biological effects, not scan parameters. For example, the PMT in the GenePix scanners can measure signal changes over about four orders of magnitude at any given voltage (see Figure 2).

The linear range of a CCD detector is specified as the ratio of the capacity of each well on the CCD array to the readout noise level (i.e., random error due to fluctuations in each pixel measurement). For example, a commonly used CCD array might have a well depth of 20,000 electrons (e^-) and readout noise of 10 e^-, for a linear range of 2000, or about three orders of magnitude.

Most CCDs used in life sciences imaging have 12-bit digital resolution—the signal values can be divided into 2^{12} = 4096 steps, or about 3.5 orders of magnitude. The signal intensity range of a CCD is adjusted by changing the exposure time. Like a PMT, a CCD array is also linear with increasing integration time. However, dark current, or signal generated by random electrons flowing through the device in the absence of light, increases proportionally with exposure and may increase the background signal.[5]

It is important to note that although a CCD camera has a narrower linear range than a PMT, the linear range of fluorescent dye molecules remaining on a biological sample following all the processing steps is rarely more than 2.5 orders of magnitude. For most biological applications, the linear range of either

Instrument performance is dictated by how well all of the elements are integrated into a complete working system. The electronic circuits, alignment of the optical elements, and behavior of the moving parts all affect the quality of the data.

system should be suitable. To take advantage of the maximum range on a given image, the instrument settings for either system should be set such that the brightest spots are not saturating the detector. If a system demonstrates linear performance over a broad range of settings, users can choose a single setting for all microarray samples of a given type, and thus eliminate one source of variation to identify more easily other sources of experimental error and true biological differences.

Another important characteristic of a detector is quantum efficiency (QE), a measure of the electronic signal the device emits relative to the incoming photon signal it receives. As a standalone component, most CCDs used in microarray imaging systems have about twofold greater QE than standard PMTs. However, the limitations of 12-bit digital resolution in CCDs and other design parameters can outweigh the benefits of greater QE. For example, a CCD array that is large enough to collect a single image the size of a microscope slide would be extremely expensive. CCD imaging systems generally capture multiple images of the sample, which are then stitched together to create a single image. Imprecise stitching, photobleaching due to multiple exposures of the overlapping regions, and other artifacts can interfere with accurate quantitation. An alternative to excessive stitching might be to use a camera-type lens to reduce a relatively large area of the microarray onto a smaller CCD surface. However, in all optical systems, if the detector is smaller than the source, losses in light collection efficiency are inevitable.

Confocal versus Nonconfocal Optics. Laser-based microarray scanners can use either a confocal or nonconfocal optical pathway design. Confocal optics were originally developed to image thin sections of a thick sample, such as cells or tissue (see Figure 3).[5] Confocal optics create a very narrow depth of focus to reject signal from beyond that narrow focal plane. Repeated scanning at different depths creates multiple high-quality optical sections that can be reconstructed into a 3-D image of the thick sample. In contrast, on microarrays, most of the background comes from nonspecific binding to the slide surface, which is in the same plane of focus as the sample. Confocal imaging offers no advantages for microarray scanning. The very narrow depth of focus in confocal systems can make them particularly sensitive to slight variations in the flatness of the slide or the slide holder.

Evaluating Performance Characteristics of Microarray Fluorescence Imaging Systems

Although choosing individual components is critical, it is important to recognize that instrument performance is dictated by how well all of the elements are integrated into a complete working system. The electronic circuits, alignment of the optical elements, and behavior of the moving parts all affect the quality of the data. Performance characterization should always be done by comparing and quantifying the scanned images, and not by simply reviewing the specifications of individual components.

Scanner Calibration. An analytical instrument such as a microarray scanner must perform consistently so that different results can be compared over time. In addition, multiple instruments of the same type must produce data that can be compared so that the scientific results can be validated. Reproducibility is especially critical for microarray imagers because the upstream chemical and biological processing steps generate significant variation. In fact, experimental reproducibility remains a major barrier to FDA approval for microarray diagnostic tests. Sources of experimental error and true biological variation can be more easily isolated on a scanner that contributes minimal additional variation. However, mechanical, optical, and electronic components have a finite lifetime, so performance will change as the instrument ages. A robust calibration procedure can ensure reproducibility both over time and among multiple scanners.

Scanner calibration requires matching the performance of the instrument to a known standard. Such a standard might consist of a fluorescent material that absorbs and emits light corresponding to the excitation and emission bands available in the instrument. The sample must be photo-stable such that it gives the same signal output after repeated long-time use. For example, GenePix scanners are benchmarked at the factory to produce a specified signal output from a stable standard using defined scan settings. The test standard is shipped with the instrument so that users can follow the calibration routine periodically over time to re-tune the instrument to the benchmark levels. Multiple instruments can be tuned in the same way, such that they all produce the benchmark signal levels, and therefore produce the same

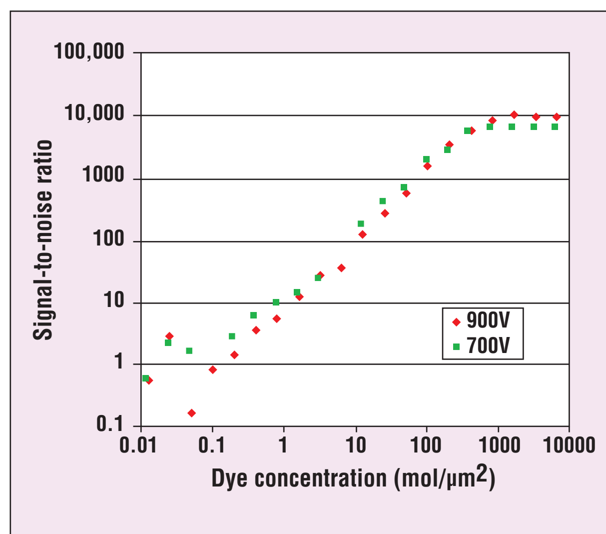

Figure 2. Linear range of the GenePix 4000B at a single PMT gain setting. Cy3 and Cy5 reactive dyes were diluted in a two-fold series in water and deposited on a microscope slide. Dye concentration in the starting dilution was measured on a spectrofluorometer. The sample was scanned with the green channel at 700V and the red channel at 900V, and then quantified.

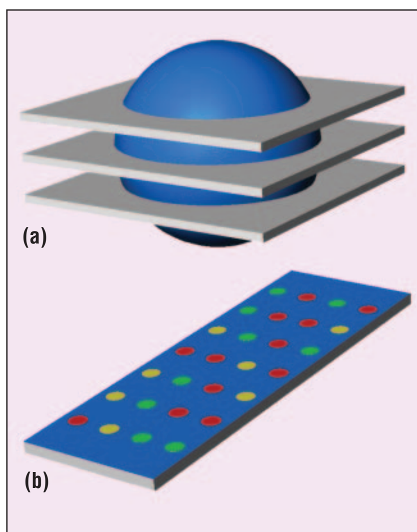

Figure 3. A comparison of confocal imaging for thick samples (a) versus microarrays (b). Confocal optics do not benefit microarray imaging because the primary source of background (shown in blue) is in the same plane of focus as the sample.

signal output from the same sample.

Detection Limit. One of the most common misconceptions about fluorescence imaging is that a brighter image is a better image. Different types and brands of detectors, variations in electronic signal processing, analog-to-digital convertor resolution, and other design differences will produce different absolute intensity values for a given feature. In addition, color mapping, contrast settings, and even monitor settings can influence the apparent brightness of different images.

Detection limit specifies how little signal a system can quantify accurately, not just the minimum signal that might be visible on the monitor. The most reliable measurement for detection limit is the signal-to-noise ratio (SNR).[6] SNR quantifies how well a system can resolve the signal of interest from the background noise. For imaging applications, SNR is calculated as:

$$\mathrm{SNR} = \frac{(\text{Signal} - \text{Background})}{(\text{Standard Deviation of Background})}$$

As an analogy, consider looking for a 2-m tall scarecrow (signal) in a cornfield (background). If all the cornstalks are 1 m tall, then the average background is lower than the signal, and the standard deviation (noise) is also low, so the scarecrow will be clearly visible. If all the cornstalks are 2 m tall, then the average background is the same as the signal, and although the noise is still low, the scarecrow will not be visible. Finally, if the cornstalks range in height equally distributed from 0.5 to 3 m tall, the average background is 1.75 m. The background is lower than the signal, but the variation in height (i.e., the noise) will make it very difficult to see the scarecrow.

Thus, for imaging applications, both the average background level and the standard deviation of the background must be included in the calculation. A criterion for the minimum signal that can be accurately quantified has been established in many signal detection disciplines as the sample value for which SNR = 3.[6,7] Below this point, features may be visible by eye, but the ability to quantify them accurately diminishes significantly.

It is extremely difficult to make an absolute standard to quantify detection limits for fluorescent dyes, simply because many dyes are sensitive to environmental conditions, especially light and age. To make such a standard, users must quantify on a spectrofluorometer the exact molar amount of dye in the sample to be spotted, and also know the volume of solution that adheres to the slide when the particular protocols are in use. There can be no postspotting washes or other treatment that might compromise the initial quantitation. Even with such careful preparation, the slightest decay in signal may cause the sample to fade below the detection limit.

However, in practice, any microarray with values near the detection limit can be used for simple scanner comparisons. To conduct such tests, two or more identical samples should be scanned on two separate instruments, then swapped and scanned again to normalize for any deleterious effects (e.g., photobleaching) from the first scan. SNR comparisons of several identical features with low signal levels can be used to assess differences in detection limit, even if absolute dye concentrations are not available for those features.

Field Uniformity. A uniform imaging field ensures that data from different areas on a microarray slide can be accurately compared. A primary factor that determines field uniformity is the slide itself. Most standard microscope slides are specified to about 40 µm flatness over the entire surface, which means the slide might have hills and valleys as high and as low as 40 µm. These surface variations can translate to quantitative variations, especially in a confocal system in which the depth of field may be only 5–10 µm. In such a system, the sample surface will come in and out of focus as it is scanned. A nonconfocal system with a larger depth of field accommodates for slide surface variations, ensuring accurate light collection over the entire scanned area. Optically flat slides for microarrays are commercially available. The additional investment in these slides may pay off in terms of producing more reproducible results and fewer repeated experiments.

Instrument subsystems such as the surface that supports the slide during imaging, the components that control the motion of the sample and/or the excitation source, and the illumination and collection optics can all affect field uniformity. In both laser and white-light systems, all of these components must

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be precisely specified and aligned to ensure uniform illumination at all points on the sample surface.

The ideal standard to evaluate field uniformity would be an optically flat slide that is uniformly coated with a thin film of a stable fluorescent material. However, in order to characterize properly the uniformity profile of an instrument, the test standard itself would have to be more uniform than the instrument in question so as not to contribute its own nonuniformity to the measurement. While thin-film coating technology is not new, a standard with the required uniform, stable, fluorescent signal in a microscope slide format has yet to be identified.

A suitable substitute test can be done with any fluorescent microarray. Simply scan the array in one orientation, rotate it 180°, scan it again, and compare the signal intensities of identical spots in each scan. A consistently lower signal in the second scan indicates photobleaching. A third scan in either orientation can be used to quantify the photobleaching and subtract its contribution from the uniformity analysis. This rotation test is the most reliable measure of field uniformity using currently available tools. However, it is limited to variations that are asymmetrical with respect to the rotation. For example, a uniform hill or valley in the middle of the slide might go undetected.

Reproducibility. Meaningful biological conclusions cannot be drawn from a single microarray experiment. Whether users have resources to create unlimited arrays of their own design, or purchase prefabricated arrays, reproducible results reduce the number of costly experimental repeats required to formulate statistically and biologically significant conclusions. To ensure reproducible results, users must first observe the recommended warm-up times and operating conditions for their instruments. In particular, both lasers and white-light sources need time to stabilize. In addition, electronics and moving parts can be susceptible to temperature and humidity extremes.

The reproducibility of signal output from a microarray imaging system can be tested by scanning a stable fluorescent standard at identical scanner settings over time (see Figure 4) The best insurance against inevitable long-term signal drift is applying a calibration procedure such as that described above. Short-term scan-to-scan reproducibility can also be tested with any reasonably stable fluorescent sample, although any signal degradation due to photobleaching must be measured and subtracted.

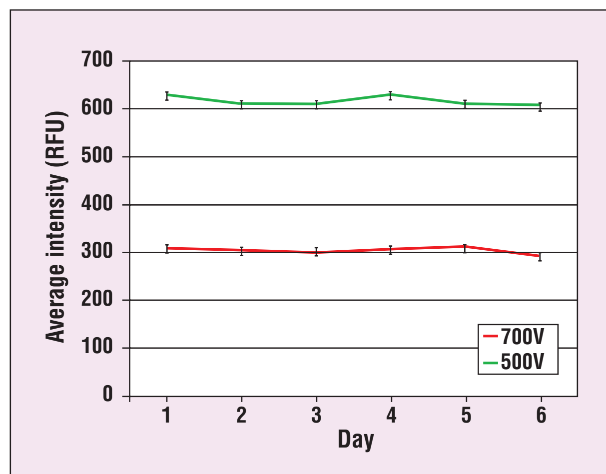


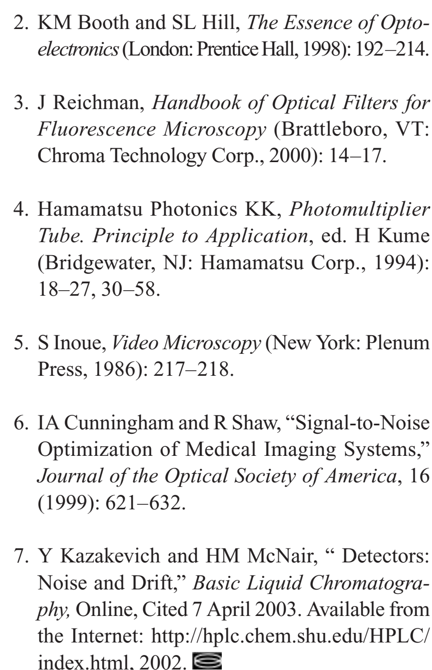
Figure 4. Reproducibility test on the GenePix 4000B. A stable fluorescent plastic material was scanned repeatedly four times per day over six days (red channel 700 V, green channel 500 V). The intensity of the signal from a fixed region on the slide was calculated in relative fluorescence units (RFU). Variance among all scans was 1.6% in the green channel and 2.1% in the red channel. Error bars cover two standard devations from the mean of four measurements.

Conclusion

Specifications of individual components enable manufacturers to define the performance levels they seek. However, the performance of any instrument is determined not by those specifications, but by their integration into a complete functional system. In microarray imaging systems, the quality of the components, the design of the optics and electronics, and the alignment of all elements determine the ultimate image quality, quantitative accuracy, and reproducibility. Careful quantitation of images from identical samples is the only way to accurately compare different imaging systems. The microarray community currently lacks robust quantitative test standards to evaluate key performance parameters, and must make do with limited substitutes. Industry leaders are working in collaboration with the National Institute of Standards and Technology (NIST) to design and develop fluorescent microarray test standards. These NIST-traceable tools will enable manufacturers to achieve new levels of excellence in microarray scanner design.

References

1. RJ Bookman and FT Horrington, "Sampling Characteristics of CCD Video Cameras" in *Optical Microscopy: Emerging Methods and Applications*, ed. B Herman and JJ Lemasters (San Diego: Academic Press, 1993): 115–131.


2. KM Booth and SL Hill, *The Essence of Optoelectronics* (London: Prentice Hall, 1998): 192–214.

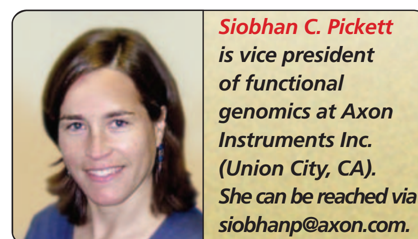
3. J Reichman, *Handbook of Optical Filters for Fluorescence Microscopy* (Brattleboro, VT: Chroma Technology Corp., 2000): 14–17.

4. Hamamatsu Photonics KK, *Photomultiplier Tube. Principle to Application*, ed. H Kume (Bridgewater, NJ: Hamamatsu Corp., 1994): 18–27, 30–58.

5. S Inoue, *Video Microscopy* (New York: Plenum Press, 1986): 217–218.

6. IA Cunningham and R Shaw, "Signal-to-Noise Optimization of Medical Imaging Systems," *Journal of the Optical Society of America*, 16 (1999): 621–632.

7. Y Kazakevich and HM McNair, " Detectors: Noise and Drift," *Basic Liquid Chromatography,* Online, Cited 7 April 2003. Available from the Internet: http://hplc.chem.shu.edu/HPLC/index.html, 2002.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